Published in final edited form as:

Exp Parasitol. 2016 May ; 164: 91–96. doi:10.1016/j.exppara.2016.03.002.

Inhibition of the classical pathway of the complement system by saliva of *Amblyomma cajennense* (Acari: Ixodidae)

Paula F. Franco^a, Naylene C.S. Silva^a, Vladimir Fazito do Vale^{a,b}, Jéssica F. Abreu^a, Vânia C. Santos^a, Nelder F. Gontijo^a, Jesus G. Valenzuela^c, Marcos H. Pereira^a, Mauricio R.V. Sant'Anna^a, Alessandra P.S. Gomes^d, and Ricardo N. Araujo^{a,*}

^aLaboratório de Fisiologia de Insetos Hematófagos, Departamento de Parasitologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

^bLaboratório de Simulídeos e Oncocercose, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brazil

^cVector Molecular Biology Section, LMVR, National Institute of Allergy and Infectious Diseases, NIH, Rockville, MD, USA

^dInstituto de Ciências Biológicas e da Saúde, Pontifícia Universidade Católica de Minas Gerais, PUC Minas, MG, Brazil

Abstract

Inhibition of the complement system during and after haematophagy is of utmost importance for tick success in feeding and tick development. The role of such inhibition is to minimise damage to the intestinal epithelium as well as avoiding inflammation and opsonisation of salivary molecules at the bite site. Despite its importance, the salivary anti-complement activity has been characterised only in species belonging to the *Ixodes ricinus* complex which saliva is able to inhibit the alternative and lectin pathways. Little is known about this activity in other species of the Ixodidae family. Thus, the aim of this study was to describe the inhibition of the classical pathway of the complement system by the saliva of *Amblyomma cajennense* at different stages of the haematophagy. The *A. cajennense* saliva and salivary gland extract (SGE) were able to inhibit the complement classical pathway through haemolytic assays with higher activity observed when saliva was used. The anti-complement activity is present in the salivary glands of starving females and also in females throughout the whole feeding process, with significant higher activity soon after tick detachment. The SGE activity from both females fed on mice or horses had no significant correlation ($p > 0.05$) with tick body weight. The pH found in the intestinal lumen of *A. cajennense* was 8.04 ± 0.08 and haemolytic assays performed at pH 8.0 showed activation of the classical pathway similarly to what occurs at pH 7.4. Consequently, inhibition could be necessary to protect the tick enterocytes. Indeed, the inhibition observed by SGE was higher in pH 8.0 in comparison to pH 7.4 reinforcing the role of saliva in protecting the intestinal cells. Further studies

*Corresponding author. ICB-UFMG, Bloco i4 sala 177, Av Antonio Carlos 6627, Pampulha, Belo Horizonte, MG CEP 31270-901, Brazil., rnaraujo@icb.ufmg.br (R.N. Araujo).

Conflicts of interest

The authors declare that they have no competing interest.

should be carried out in order to identify the inhibitor molecule and characterise its inhibition mechanism.

GRAPHICAL ABSTRACT

Keywords

Saliva; Amblyomma cajennense; Anti-complement; Haematophagy; Classical pathway

1. Introduction

The complement system is an important effector mechanism participating in both innate and acquired vertebrate immune responses (Farries and Atkinson, 1991). The inhibition of the host complement system by haematophagous animals seems to be of utmost importance and has been described in distinct and distant phylogenetic species (reviewed by Schroeder et al., 2009). In blood-sucking arthropods, this inhibition is directly linked to success in feeding, development and reproduction (Wikel and Allen, 1977).

The major function of the complement inhibitors seems to be the protection of the intestinal epithelium from the attack of the complement system during and after the blood ingestion (Barros et al., 2009). Complement inhibitors are present in the saliva as well as in the midgut (Barros et al., 2009; Mendes-Sousa et al., 2013). As saliva is ingested with the blood during feeding (Soares et al., 2006) the anti-complement activity present in the gut is likely to be performed by intestinal and salivary molecules. Furthermore, during feeding, the activation of the complement system would lead to opsonisation of salivary molecules by products of the C3 cleavage and the formation of inflammatory anaphylatoxins such as C3a and C5a (Dunkelberger and Song, 2010; Ribeiro and Spielman, 1986). The inhibition of these phenomena is useful to prevent or delay the onset of an immune response against salivary proteins and prevent inflammation at the feeding site.

The importance of complement inhibition was shown by previous studies. In guinea pigs, complement depletion reduced the resistance to *Dermacentor andersoni* larvae (Wikel and Allen, 1977). *In vitro* studies to uncover the vaccine antigen BM86 mechanism of action against *Rhipicephalus (Boophilus) microplus* demonstrated more damage to the intestinal epithelium in the presence of active complement system (Kemp et al., 1989). The lack of the anti-complement molecule ISAC in the saliva of *Ixodes scapularis* led to a significant body weight reduction on fed ticks (Soares et al., 2005).

Despite its importance among Ixodid ticks, only species of the *Ixodes ricinus* complex had in-depth studies on the presence and activity of anti-complement molecules in their saliva, as

shown for *Ixodes dammini* (Ribeiro, 1987) *I. hexagonus*, *I. uriae* (Lawrie et al., 1999), *I. ricinus* (Couvreur et al., 2008; Daix et al., 2007; Lawrie et al., 2005, 1999) and *I. scapularis* (Tyson et al., 2007, 2008; Valenzuela et al., 2000). Interestingly, the anticomplement inhibition described for most *Ixodes* species were only for the alternative pathway, one exception is the lectin-pathway inhibitor found in *I. scapularis*' salivary glands (Schuijt et al., 2011).

There is a lack of studies in the literature on the inhibition of the complement system by tick saliva from genera other than *Ixodes*. In an isolated work, the AV422 peptide identified in *Amblyomma americanum* was shown to inhibit the formation of terminal complement complexes by the classical pathway (Mulenga et al., 2013). This inhibition of the classical pathway, firstly described for Ixodid ticks, suggests that *Amblyomma* may provide a mechanism of inhibition different from that observed for the genus *Ixodes*.

In order to generate a better understanding on the inhibition of vertebrate complement system by ticks, this study investigated the inhibition of the classical pathway present in the salivary glands of the tick *A. cajennense* at different physiological stages. *A. cajennense* is of great importance in Brazil since it has a wide distribution and low host specificity, especially during immature stages (Estrada-Peña et al., 2014). It also causes economic losses in livestock and is the main vector of the Brazilian Spotted Fever, being strongly associated with humans in Brazil (Galvão et al., 2005; Labruna et al., 2002).

2. Materials and methods

2.1. Experimental ticks

Specimens of *A. cajennense* were obtained from the colony (second generation) kept at the Department of Parasitology UFMG or collected from naturally infested horses kept on the UFMG Experimental Farm, located in the municipality of Pedro Leopoldo, MG, Brazil. Horses were adult males and females (3–10 years old) of mixed breed not treated with acaricides during the last two months. The UFMG colony was originated from ticks collected at this same location.

2.2. Colony maintenance and feeding of the experimental groups

Ticks were kept in an incubator at 28 ± 2 °C and $85 \pm 5\%$ relative humidity. All feedings were performed in Swiss mice using feeding chambers described by Bouchard and Wikel (2005). During all procedures, mice were maintained in appropriate cages ($30 \times 19 \times 13$ cm L x W x H, maximum 5 animals/cage) and kept in a room with controlled temperature (25 ± 2 °C).

To obtain females at different physiological stages, feeding chambers were assembled into seven groups containing six mice each (females aged 6–8 week old) which were used as feeding source for the ticks (one couple with 20–30 days of fasting per mouse). The ticks were examined for attachment (6 h after being placed in contact with the host) and every 2 days when females were removed, weighed and dissected to obtain the salivary gland extract (henceforth called SGE) which was stored at -80 °C. Groups contained ticks with 2, 4, 6, 8

and 10 days of feeding, in addition to the fasting group and one with ticks that spontaneously detached from the host.

All procedures involving animals were in accordance and approved by the Ethics Committee on Animal Experimentation (CETEA/UFGM) under the protocol number 137/2011.

2.3. Saliva collection and preparation of salivary gland extracts (SGE)

Females were washed with distilled water, attached dorsally to a double face tape placed in a piece of cardboard and injected directly on the haemocoel with 3–5 μL of 2% pilocarpine (Sigma) in PBS (pH 7.4). Females were kept in a moistened chamber at 37 °C until the end of salivation (approximately 2 h) and the collected saliva was transferred to 1.5 mL tubes (each tube contained saliva from up to three females) and kept at –80 °C until use.

To obtain the SGE, females were washed with distilled water and their salivary glands were individually dissected in saline (0.9% NaCl). Each pair of glands were transferred to 1.5 mL tubes containing 10 μL of saline, placed in a ultrasonic bath for 40 s and centrifuged at 14,000 g for 5 min. The supernatant was transferred to a new tube and stored at –80 °C until use.

The amount of protein in samples was measured by the method described by Bradford (1976) using bovine serum albumin as standard.

2.4. Inhibition of haemolysis by the classical pathway assay

The assay was performed according to Cavalcante et al. (2003). Briefly, 1 mL of sheep erythrocytes were opsonised with anti-sheep erythrocyte antibodies at 1:1000 diluted in GHB^{2+} buffer (5 mM HEPES, 145 mM NaCl, 0.15 mM CaCl_2 , 0.5 mM MgCl_2 and 0.1% gelatin) at the concentration of $2 \times 10^8 \text{ cells} \times \text{mL}^{-1}$.

In a 1.5 mL tube, 50 μL of human serum diluted 1:60 in GHB^{2+} (pH 7.4) was added to 25 μL of tick sample (SGE or saliva) and 50 μL of a suspension of sheep erythrocytes opsonised with anti-sheep antibodies. The mixture was incubated at 37 °C for 30 min and then added to 500 μL saline at 4 °C. The tubes were centrifuged at 1700 g for 60 s and 200 μL of the supernatant were transferred to 96 well plates and measured in an ELISA reader (Molecular Devices) at 414 nm. Assays were performed in triplicate. In each experiment, three different controls (without samples) were used as follows: total haemolysis - saline was substituted by distilled water; positive control - haemolysis caused by serum without any inhibitor; and negative control – without serum where only spontaneous haemolysis is present. Serum concentration was adjusted in order to obtain at least 90% of the total haemolysis. The results were expressed as percentage of haemolysis inhibition in relation to the positive control. In the assays performed to study the complement system at the physiological pH found in the tick midgut lumen, the GHB^{2+} buffer was set to pH 8.0. In Fig. 4A the results were shown as percentage of haemolysis.

2.5. Measurement of the intestinal pH

The intestinal pH of *A. cajennense* females was measured using H^+ sensitive microelectrodes made from glass capillaries with tips thin enough to be introduced into the

midgut of the ticks (Santos et al., 2008). Two types of microelectrodes were prepared, the reference and the H⁺ sensitive that were connected to a high-impedance apparatus for measurement of the potential difference. The values measured in mV were converted into pH units in comparison to calibration curves made with known pH solutions. The measurements were made in five engorged females, 6–7 h after tick collection.

2.6. Statistical treatment of data

The statistical analysis was performed using the GraphPad Prism program for Windows version 5.01. Data normality was assessed by the Kolmogorov-Smirnov test and the difference between groups was measured by ANOVA followed by Dunnett or Bonferroni (more than two groups) or Student t test (for two groups). The Pearson correlation coefficient was used to evaluate the relationship between the study variables. The level of significance was set at $p < 0.05$.

3. Results and discussion

Saliva and SGE of fully engorged *A. cajennense* females (466–940 mg) inhibit the classical pathway of the human complement system in a dose-dependent manner, with higher levels of inhibition seen for saliva (Fig. 1). Heated SGE had significantly lower ($P < 0.05$) anti-complement activity. Assays using factor B-depleted serum (where no alternative pathway is activated) confirmed that inhibition occurs through the classical pathway. The difference between saliva and SGE was expected, since tick salivary glands have no storage capacity (Alarcon-Chaidez, 2013) and additional components, such as intracellular proteins, dilute the SGE samples (Ribeiro et al., 2004). As the activity is present in SGE, they were used in the remaining experiments.

The presence of the classical pathway inhibitory activity in *A. cajennense* saliva corroborates the data of Mulenga et al. (2013) indicating that *Amblyomma* ticks are able to inhibit the classical pathway. This results remarkably differs from that obtained with *Ixodes* tick saliva which inhibit only the alternative and lectin pathway (Couvreur et al., 2008; Schuijt et al., 2011; Tyson et al., 2008; Valenzuela et al., 2000). These findings indicate different mechanisms of complement inhibition in ticks from different genera.

Experimental infestations in mice showed that no significant change in *A. cajennense* body weight ($p > 0.05$) occurred until the eighth day of feeding (Fig. 2A). These results are consistent with tick feeding phases with little weight variation in the preparatory and slow feeding phases and substantial increase in the rapid feeding phase, which typically comprises the last 12–24 h of feeding (Sonenshine, 1991).

The feeding period and weight gain of *A. cajennense* females fed on different hosts may vary considerably. In this study, ticks finished feeding between 7 and 12 days and weighted 503–841 mg (mean 667 mg), these findings being relatively in line with previous works using different host as horses (8–10 days with 601.9 mg mean weight) (Sanavria and Prata, 1996), cattle (7–12 days body weight not estimated) (Hooker et al., 1912) and naive rabbits, the most common host used in the laboratory, with 4–36 days (mean 7.3–8.5 days) and 284.5–384 mg mean weight (Pinter et al., 2002); 10.7 days with 348.3 mg mean body

weight (Almeida et al., 2008), 8–30 days (weight not measured) (Cunha, 1978) and 362–676 mg body weight (feeding period not measured) (Nunes, 2009).

The development of the tick salivary glands were related to the body weight only during feeding. The SGE had increased protein content from day 8 with significantly higher amounts of protein after 10 days of attachment ($P < 0.05$) (Fig. 2B), which suggests that this is the time of greatest *A. cajennense* salivary activity when feeding on mice. Previous works have shown that, after the start of feeding, the size of the glands increases significantly, reaching up to 25 times its initial size (McSwain et al., 1982; Sauer et al., 1995). The decrease in SGE protein content after detachment is also in accordance with the physiology of the salivary gland and may be caused by the degeneration of the glands triggered by the detachment from the host (Harris and Kaufman, 1981; Lindsay and Kaufman, 1988).

The assessment of the classical pathway inhibition by *A. cajennense* salivary glands showed that the SGE from fasting ticks already have a background anti-complement activity that remains relatively similar throughout the whole feeding process. Such findings indicate that the anti-complement activity is important for the tick from the insertion of its mouthparts into the host's skin until the end of feeding. The presence of complement inhibition in unfed females and throughout feeding was also observed for *I. ricinus* salivary glands for the inhibition of the alternative pathway (Daix et al., 2007; Lawrie et al., 1999). However, Lawrie et al. (1999) and Daix et al. (2007) observed a progressive increase in the anti-complement activity (or anti-complement molecules) throughout the feeding process, a fact that was not observed for *A. cajennense*. The increased anti-complement activity in detached ticks (Fig 2C) is an unexpected finding, suggesting this is a molecule that resists salivary gland degradation induced by tick detachment. In addition, this molecule probably has a continuous production (at least for some time) after tick detachment and may accumulate in the salivary gland, therefore being detected at higher levels in the SGE from ticks after feeding.

The correlation between female body weight and the anti-complement activity was not significant for both females fed on mice ($r = 0.331$; $p = 0.074$) (Fig. 3A) or horses (Pearson, $r = -0.187$, $p = 0.185$) (Fig. 3B). Moreover, the mean haemolysis inhibition obtained from 7.5 μg of SGE samples was similar between females fed on mice ($29 \pm 14\%$) or horses ($33 \pm 12\%$) ($p = 0.229$). These results indicate that the expression of complement inhibitors from *A. cajennense* is consistently stable and do not rely upon the physiological status of the tick during feeding or host species. Collectively, these results show that SGE from *A. cajennense* females collected in different hosts or with different body weights (partially or fully engorged) could be used in experiments to measure their classical pathway inhibition of the complement system without generating significant bias on the results. Such results corroborate previous studies that showed some change in gene expression between fully and partially engorged females or when fed on different hosts, whereas other genes may remain with similar levels of expression (Garcia et al., 2014; Tirloni et al., 2014).

All anti-complement assays were performed at pH 7.4, which is the pH found in blood and tissues of *A. cajennense* mammalian hosts. The inhibition of the classical pathway obtained from *A. cajennense* saliva and SGE at pH 7.4 indicates that saliva is able to inhibit the

classical pathway at the bite site. To evaluate the possible anti-complement action in protecting the tick midgut epithelium, the pH in the intestinal lumen of engorged females collected from naturally infected horses, weighing 480–970 mg ($n = 5$) was measured and showed a pH of 8.04 ± 0.08 , indicating that the host's blood undergoes a mild alkalisation within the midgut of the tick.

Haemolytic assays carried out in the absence of inhibitors showed that alkalisation does not affect the activation of the complement classical pathway, since there is similar haemolysis ($p > 0.05$) at pH 8.0 and 7.4 (Fig. 4A), which confirms that the complement system is active in the newly ingested blood stored in the midgut lumen of these arthropods.

The action of the complement inhibitors within the midgut of the ticks was accessed by haemolytic assays performed at both pH 8.0 and pH 7.4. The results showed that the alkalisation of the intestinal content favours the action of the salivary complement inhibitors, since the percentage of the classical pathway inhibition was significantly higher ($p < 0.05$) in most SGE samples (from 3.75 to 15 μg) at pH 8.0 compared to pH 7.4 (Fig. 4B).

These results are in line to those observed by Barros et al. (2009) for complement activation in human serum and by Mendes-Sousa et al. (2013) for complement activation in dog, chicken and guinea pig serum which also demonstrated that the alkalisation of the pH does not affect the activation of the classical pathway. On the other hand, these studies showed that the alkalisation reduces the alternative pathway activation, suggesting an additional mechanism of alternative pathway inhibition in the newly ingested blood meal.

Taken together, these results suggest that the inhibition of the classical pathway in the intestine of *Amblyomma* ticks could have an important role in midgut protection during and after blood ingestion. As showed by Hlatshwayo et al. (2004), serum antibodies from rabbits exposed to *A. cajennense* and *A. hebraeum* unspecifically recognise antigens from several organs of the tick, including the midgut epithelium and basement membrane. Those antibodies may be responsible for the local activation of the classical pathway that will cause damage to the midgut epithelium and may also carry knock-on effects on other physiological processes especially blood metabolism and reproduction (Gonsioroski et al., 2012). The increased salivary gland and the final phase of feeding will produce a higher amount of saliva (with inhibitors) in order to deal with the higher amount of blood that is arriving at the midgut environment.

As seen in triatomine bugs (Barros et al., 2009), the anti-complement activity in the midgut may be aided by inhibitors expressed locally in the gut and secreted into the lumen or even operate as transmembrane proteins.

Acknowledgements

The authors would like to thank Mrs Marcia Gomes for her technical assistance and the anonymous reviewers for invaluable suggestions that improved the manuscript. The work was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais and Conselho Nacional de Desenvolvimento Científico e Tecnológico.

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HIGHLIGHTS

- Saliva and salivary gland extracts of *A. cajennense* inhibit the classical pathway.
- Salivary anti-complement activity is present in fasting ticks.
- Salivary anti-complement activity is similar in ticks from different body weights.
- Salivary anti-complement activity is similar in ticks fed on mice and horses.
- Salivary anti-complement activity is more efficient in pH 8.0 than in pH 7.4

Fig. 1.

Inhibition of the classical pathway of the human complement system by saliva and salivary gland extract (SGE) of fully engorged *A. cajennense* females. Heated SGE: SGE was heated for 3 min before the assays. SGE (fB depleted serum): human serum used in assays was replaced by human serum depleted for factor B (Complement Technology, Inc.). Data is shown as mean $\pm$ standard deviation ($n = 3$). ^a indicates SGE is statistically different from saliva and ^b indicates Heated SGE is statistically different from Saliva and SGE (ANOVA, Bonferroni; $p < 0.05$).

Fig. 2.

Body weight (A), salivary gland extract (SGE) protein content (B) and inhibition of the classical pathway by the SGE (C) of *A. cajennense* females at different periods during feeding activity on mice, starving and after detachment of the host. Data is shown as mean $\pm$ standard deviation (n = 6). AF = after feeding (spontaneous detachment). Asterisks indicate statistical difference from starving ticks (ANOVA, Dunnett; *p < 0.05, ***p < 0.0001). Samples used in C had 7.5 µg of proteins.

Fig. 3. Correlation between body weight and the inhibition of the classical pathway by salivary gland extracts of *A. cajennense* females fed on mice under laboratory conditions ($n = 36$) (A) and on horses from endemic area ($n = 52$) (B). r = Pearson correlation coefficient. All samples used had 7.5 μ g of proteins.

Fig. 4. Haemolytic activity (A) and inhibition promoted by the salivary gland extract of *A. cajennense* (B) in the classical pathway of human complement at different pHs. Data is shown as mean $\pm$ standard deviation ($n = 3$). Asterisks indicate statistical difference between results in pH 7.4 and 8.0 (Figure A–T Test, $p > 0.05$; Figure B–ANOVA, Bonferroni; * $p < 0.05$, ** $p < 0.01$). All samples used in B had 7.5 μ g of proteins.