Toll-like receptors in fat body and salivary gland tissues in the cattle tick *Rhipicephalus microplus**

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ABSTRACT. Rezende S.deF., Fontenele M.R., Masuda C.A., de Oliveira P.L., Araujo H.M.M., Bittencourt V.R.E.P. & Leite M.de S. **Toll-like receptors in fat body and salivary gland tissues in the cattle tick *Rhipicephalus microplus.*** [Receptores Toll-like em corpo gorduroso e glândula salivar do carrapato bovino *Rhipicephalus microplus*.] *Revista Brasileira de Medicina Veterinária, 38(supl.3):98-102, 2016*. Programa de Pós-Graduação em Ciências Veterinárias, Anexo 1, Instituto de Veterinária, Universidade Federal Rural do Estado do Rio de Janeiro, BR 465, KM 47, Seropédica, RJ 23890-000, Brasil. E-mail: milaneleite@ufrrj.br

Toll-like receptors (TLRs) play an important role in the recognition of pathogen components and subsequent activation of the innate immune response, which then leads to development of immune responses. In arthropods the fat body and salivary glands are important organs in the defense system against invading pathogens. In this study, we identified for the first time the presence of TLRs in fat body and salivary gland tissues of cattle tick *Rhipicephalus microplus.* Our results show that the expression of TLRs in fat body tissue are not found in all cells, but is specific to some cell types, in salivary glands TLRs protein expression occur in acini structure. We suggest that immune pathways are active in both fat body and salivary glands in the tick. The potential use of TLRs as a target for vaccine formulations against is discussed.

KEY WORDS. Innate immune, fat body, salivary gland, cattle tick.

RESUMO. Receptores Toll-like (TLRs) desempenham um importante papel no reconhecimento de componentes de agentes patogênicos e subsequente ativação da resposta imune inata, que, em seguida, leva ao desenvolvimento da resposta imune. Em artrópodes, tais como insetos, o corpo gorduroso é um órgão importante no sistema de defesa contra os agentes patogênicos invasores. Neste estudo, foi identificada pela primeira vez, a presença de TLRs em corpo gorduroso e glândulas salivares de *Rhipicephalus microplus*. Os nossos resultados indicam que a expressão de TLRs em corpo gorduroso não ocorre em todas as células, mas é específico para alguns tipos celulares. Em glândulas salivares

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a marcação ocorre em ácinos, sugerindo que a via imune está ativa neste tecido do carrapato. O uso potencial de TLRs como um alvo para formulações de vacinas contra *R. microplus* é discutido.

PALAVRAS-CHAVE. Imunidade inata, corpo gorduroso, glândula salivar, carrapato bovino.

INTRODUCTION

The tick *Rhipicephalus microplus* is one of the most important bovine ectoparasites in Brazil and in several other countries worldwide. Economic losses to the cattle industry in Brazil are estimated at two billion dollars per year, due to reduced milk and meat production, slower growth of infested animals and disease transmission (Grisi et al. 2002). Studies on tick physiology and development are essential to develop new methods for tick control.

Cattle tick control is mostly based on the use of chemical acaricides. However, their continual application and improper use have many negative side effects, including the development of resistance to chemicals in tick populations, in addition to food and environmental contamination (Beugnet & Chardonnet 1995, Jonsson 1997, De La Fuente et al. 1999). Research on the development, physiology, and biochemistry of ticks, such as studies aiming to understand the innate immune response, is an important strategy for the development of new methods of control parasite.

In arthropods, immune response is elicited by innate reaction against exacerbated microbial growth and proliferation of pathogens. The innate immune response in arthropods relies on activation of Toll and Immune deficiency (Imd) signaling pathways (Lemaitre & Hoffmann 2007). Microbial pathogens are recognized by Toll-like receptors (TLRs) and thus activate the innate immune system, ultimately leading to the secretion of antimicrobial peptides (Bulet et al. 2004, Levashina 2004, Hancok & Sahl 2006). In insects, the fat body is the most important tissue where the activation of immune system takes place, but the function of these pathways has not been well studied in other arthropod groups such as ticks.

The first identification of Toll receptor as an activator of the immune response was in a *Drosophila melanogaster* cell line (Rosetto et al. 1995). Toll-like receptors were identified later in diverse vertebrate and invertebrate species (Takeda & Akira 2001). In *Drosophila,* Toll receptors are essential for embryonic development (Stein et al. 1991, Hashimoto et al. 1998, Valanne et al. 2011) and innate immunity (Eleftherianos & Schneider 2011). In these flies, nine Toll-like receptors are encoded in the *Drosophila* genome, including the Toll pathway receptor Toll. The induction of the Toll pathway by fungi or gram-positive bacteria leads to the activation of cellular immunity as well as the systemic production of certain antimicrobial peptides (Valanne et al. 2011).

In the present work, we identified the presence of Toll-like receptors in tissues and cells important to the innate immune response of the tick *R. microplus.* The identification of these proteins with specific antibodies opens a new window for future research on the mechanisms of response to the Toll signaling pathway in ticks. Furthermore, it establishes a potential additional target for the development and trial of new vaccines.

MATERIALS AND METHODS

Ticks

Rhipicephalus microplus was obtained from calves that were artificially infested. The ticks were collected in the stalls floor, and were immersed in a 1% sodium hypochlorite solution for 5 min, rinsed with sterile distilled water and dried with sterile tissue paper for cuticle antisepsis and were kept in Petri dishes, at 27 ± 1 °C and RH ≥ 80% until use.

Western blot

Fat body of fully engorged females, salivary glands of partially engorged females of tick *R. microplus* and *Drosophila melanogaster* larvae (used as control) were homogenized in an ice-cold lysis buffer (Phosphate Buffered Saline - PBS containing 0.01 % Triton-20 and protease inhibitors: 2 mM phenylmethylsulfonyl fluoride - PMSF and complete protease inhibitor cocktail (Roche)). Subsequently, samples were denatured by adding sample buffer (SB 1x: 50 mM Tris pH 6.8, 2% SDS, 0.1% Bromophenol and 10% glycerol) and 100 mM DTT (Dithiothreitol) and centrifuged (13.000 g, 5 min, 4 °C). Supernatants were collected and stored at −80. Prior to denaturation treatment, one aliquot of each homogenate was used to estimate the total protein content performed by a modified Lowry's assay (Markweel et al. 1978). Equal amounts of protein (40 μg) from each homogenate were separated by 10 % polyacrylamid SDS--PAGE and electroblotted onto PVDF membrane (Biorad). In order to saturate the unspecific protein-binding sites, the membranes were incubated for 1 h at room temperature with TBS-T (Tris 50mM, NaCl 0.1M, Tween 0.05%, pH7.2) containing 1.5 % bovine serum albumin. The membranes were then incubated overnight with the primary polyclonal rabbit antibody anti-Toll (d-300) in a 1:500 dilution. After washing with TBS-T, the blots were incubated at room temperature for 2 hours under continuous shaking in the presence of a peroxidase-conjugated secondary anti-rabbit mouse antibody diluted 1:2.000 in TBS-T. After washing, membranes were incu-

bated with the chemoluminescent substrate (Pierce) for 4 min at room temperature, and then exposed to Bio Max light-1 film (Kodak). Toll protein levels were determined by computer-assisted densitometric analysis of the exposed films. To control protein loading, membranes were stripped of antibodies in 200 mM glycine buffer containing 1 % SDS, pH 2.2 at room temperature, and re-probed with anti-aTubulin (DM1α, Sigma, 1:4.000). Results are expressed as the mean ± standard error of the mean. Comparisons with controls were done using the Student's t-test. A difference was considered significant for $p<0.05$.

Antibody Staining

Fat body of fully engorged females and salivary glands of partially engorged females of tick *R. microplus* was dissected in ice-cold PBS and fixed for 40 min in PBS containing 4 % formaldehyde (Merck) at room temperature. Fat bodies were washed in PBT (PBS + Tween 0.01 %) and then blocked for 1 hour with 5 % pre-immune goat serum (Vector) in PBT at room temperature, followed by an overnight incubation with the primary polyclonal antibody rabbit anti-Toll (d-300, Santa Cruz) in a 1:100 dilution and monoclonal mouse anti-pTyr antibody (Cell Signaling) in a 1:400 at 4 °C. Subsequently, fat bodies were washed in PBT and then incubated for 2 hours with fluorescently coupled secondary antibodies (Molecular Probes): Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 647 donkey anti-mouse in a 1:400 dilution at room temperature. Nucleus were stained using DAPI (4 ', 6-diamidino-2-phenylindole) in a 1:500 dilution at room temperature. For imaging, tissues were mounted in 70 % glycerol in water and imaged using a Leica SP5 confocal microscope. Results are expressed as the mean ± standard error of the mean. Comparisons with controls were done using the Student's t-test. A difference was considered significant for $p<0.05$.

RESULTS

Identification of Toll-like proteins by Western blot

Western blot analysis with polyclonal toll antiserum against (d-300) of *D. melanogaster* indicates the presence of Toll-Like receptor in fat body of fully engorged females and salivary glands of partially engorged tick *R. microplus*. In this analysis, we detected double or single protein band migrating at 116 kDa, in fat body and salivary gland of the tick, respectively (Figure 1). Acrude extract of *D. melanogaster* larvae was included as a positive control, showing a 126 kDa band.

Immunolocalization

Fat body and salivary glands from *R. microplus* were dissected and incubated with polyclonal antiserum against toll (d-300) of *D. melanogaster* and subsequently analyzed by confocal microscopy. The results showed Toll protein is diffuse in the cytoplasm or concentrated in intracellular vesicles in salivary glands, partly colocalization with phospho-tyrosine staining (Figure 2A). We also observed the presence of Toll staining in Fat body. In this, Toll is present in all round-shaped trophocytes, distributed at the plasma membrane, and absent from cuboidal-shaped trophocytes (Figure 2B). In some round-shaped trophocytes Toll staining is restricted to intracellular vesicles, partly colocalization with phospho-tyrosine staining (Figure 2C).

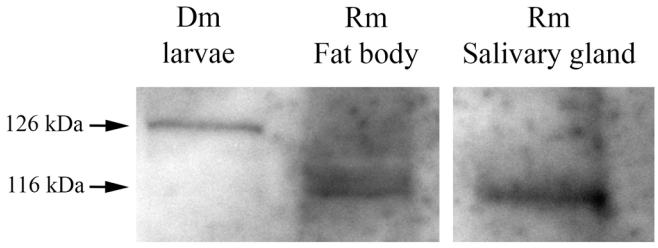

Figure 1. Toll receptor is detected by Western blot analysis of fat body and salivary glands tissues. Polyclonal antibody against Toll (D-300) detects a double or single protein band migrating at 116 kDa, in *R. microplus* (Rm) fat body and salivary gland, respectively. The double band may represent different Toll proteins or pos-tranlational modification of a single protein. Toll from *D. melanogaster* (Dm) larvae migrates as a 126 kDa band.

DISCUSSION

Western blot analysis detected double or single protein band migrating in fat body and salivary gland of the tick, respectively. The double band may represent different Toll proteins or pos-tranlational modification of a single protein. *D. melanogaster* larvae shows a 126 kDa band. Probably isoforms involved in the formation of the dorsal-ventral axis in *Drosophila* (Gerttula et al. 1988).

These results indicate the presence of Toll-like receptors in different tissues of *R. microplus*. This finding is in agreement with the now widely accepted presence of the TLRs as a ubiquitous component of the innate immune system, revealing a high degree of conservation between mammalians, invertebrates and plants, and a preservation of the general organization of the immune signaling pathways during evolution (Ishii et al. 2008).

We also observed the presence of Toll staining in salivary glands. In salivary glands, Toll protein is diffuse in the cytoplasm or concentrated in intracellular vesicles. Partial colocalization with phospho-tyrosine staining suggests these may represent endocytic vesicles. The salivary glands of ixodids are responsible for the elaboration of a substance and play a key role in osmoregulation, ion regula-

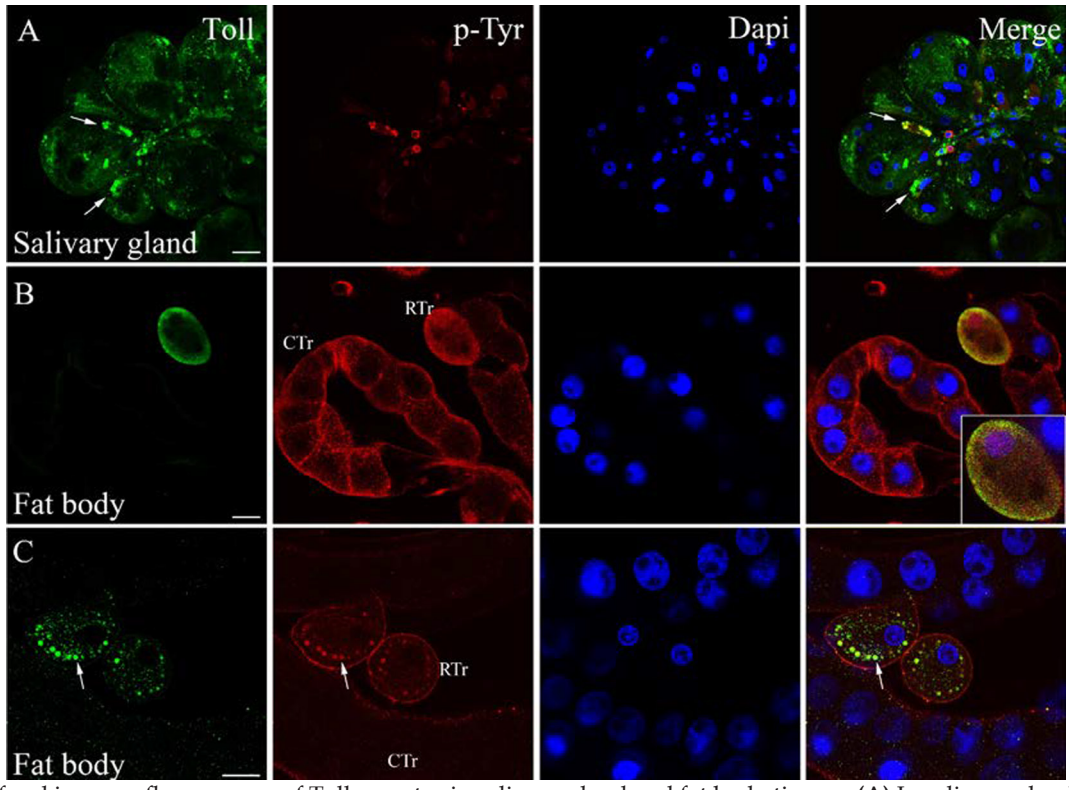

Figure 2. Confocal immunofluorescence of Toll receptor in salivary gland and fat body tissues. **(A)** In salivary glands Toll protein (green) is diffuse in the cytoplasm or concentrated in intracellular vesicles (arrow). Partial colocalization with phospho-tyrosine staining (red) suggests these may represent endocytic vesicles. **(B)** In fat bodies, Toll is present in all round-shaped trophocytes (RTr), distributed at the plasma membrane, and absent from cuboidal-shaped trophocytes (CTr). Insets shows detail of Toll staining in Rtr, with DAPI staining collected at a different focal plane. **(C)** In some RTr Toll staining is restricted to phospho--tyrosine positive intracellular vesicles (arrow). Dapi is used to reveal nuclei (blue). Bar in **(A)** is 40 mm and **(B, C)** is 20 mm.

tion and excretion (Gregson 1967). These glands are also responsible for the production of cement secretions (Cowdry and Danks 1933), anticoagulants (Franscischetti et al. 2009), and hydrolytic (Tatchell 1969) and proteolytic enzymes (Howell et al. 1975).

The acini also produce a plethora of substances that counteract vertebrate host hemostasis, inhibiting platelet aggregation, coagulation and promoting vasodilation (Franscischetti et al. 2009). The salivary glands are also the main transmission route for pathogenic organisms (Nunes et al. 2005), always be in touch with microorganisms and thus increasing the need for an active immune system. In spite of all these important functions, the salivary glands of ticks remain poorly studied with regards to their morphology and histology, especially considering the large number of tick species.

The results showed that confocal immunofluorescence of Toll receptor only specific cell types were stained in the fat body. According with Coons et al. (1990), the fat body in ticks, unlike that of insects, would be formed by only one cell type, the trophocytes. On the other hand, Denardi et al. (2008) have observed the presence of two types of trophocytes in partially engorged females of *A. cajennense*, which were designated round-shaped trophocytes and cuboidal trophocytes according to its morphological characteristics.

Here, we could clearly distinguish these two cell types in the fat body of *R. microplus*, and only cells similar to the round-shaped trophocytes were stained, distributed at the plasma membrane, and absent from cuboidal-shaped trophocytes (Figure 2B). In some round-shaped trophocytes Toll staining is restricted to intracellular vesicles, partly colocalization with phospho-tyrosine staining (Figure 2C). This suggested that these cells are possibly involved in immune response in ticks. Morphometric analysis showed that the cellular and nuclear areas of round-shaped trophocytes are larger than those of cuboidal trophocytes, indicating that the arrangement of the former provides more contact area with the haemolymph (Denardi et al. 2008), thus being more in touch with the invading pathogens. The cell specific expressions of the Toll-like receptor also indicate that the round-shaped trophocytes and the cuboidal trophocytes are indeed different cell types from the same organ. These cell populations present not only distinct morphology but also distinct functions.

CONCLUSION

In this report we show the expression of Toll-like receptors in ticks, displaying expression among important tissues and cells engaged in the innate immune response of the cattle tick *R. microplus*. Confocal microscopy immunolocalization of these receptors also showed a cell-specific staining pattern in the fat-body providing evidence that the function of the round-shaped trophocytes is distinct from the cuboidal trophocytes. Our data open a new window for future mechanistic investigation about toll signaling pathway response in ticks. This report also provides an additional entry point for the trial of novel potential targets for vaccine development against the cattle tick *R. microplus*.

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REFERENCES

Beugnet F. & Chardonnet L. Tick resistence to pyrethroids in New Caledonia. *Vet. Parasitol.,* 56:325-338, 1995.

Bulet P., Stocklin R. & Menin L. Anti-microbial peptides: from invertebrates to vertebrates. *Immunol. Rev.*, 198:169-84, 2004.

Cowdry E.V. & Danks W.B.C. Studies on coast fever II. Behavior of the parasite and the development of distinctive lesions in susceptible animals. *Parasitology,* 5:1–63, 1933.

Coons L.B., Lamoreaux W.J., Rosell-Davis R. & Starr-Apires L. Fine structure of the fat body and nephrocytes in the stages of *Dermacento variabilis*. *Exp. Appl. Acarol.,* 8:125–142, 1990.

De la Fuente J., Rodríguez M., Montero C., Redondo M., García- García Jc., Méndez L., Serrano E., Valdés M., Enríquez A., Canales M., Ramos E., Boué O., Machado H. & Lleonart R. Vaccination Against ticks (*Boophilus* spp.): the experience with the Bm86-based vaccine Gavac. *Genet. Anal.,* 15:143-8, 1999.

Denardi S.E., Bechara G.H. & Camargo-Mathias M.I. New morphological data on fat bodies of semi-engorged females of *Amblyomma cajennense* (Acari: Ixodidae). *Micron,* 39:875–883, 2008.

Eleftherianos I. & Schneider D. *Drosophila* immunity research on the move. *Fly,* 5:247-254, 2011.

Francischetti I.M., Sa-Nunes A., Mans B.J., Santos I.M. & Ribeiro J.M. The role of saliva in tick feeding. *Front. Biosci.,* 1:2051-88, 2009.

Gerttula S., Yishi J. & Anderson K.V. Zygotic expression and activity of the *Drosophila* Toll gene, a gene required maternally for embryonic dorsal-ventral pattern formation. *Genetic,* 119:123–133, 1988.

Gregson J.D. Observations on the movement of fluids in the vicinity of the mouthparts of naturally feeding *Dermacentor andersoni* stiles. *Parasitology,* 57:1–8, 1967.

Grisi L., Massard C.L., Borja M.G.E. & Pereira J.B. Impacto econômico das principais ectoparasitoses em bovinos no Brasil. *Hora Vet.,* 21:8-10, 2002.

Hancock R.E. & Sahl H.G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol.,* 24:1551-7, 2006.

Hashimoto C., Hudson K.I. & Anderson K.V. The toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a trans-membrane protein. *Cell ,* 52:269-279, 1998.

Howell C.J., Neitz A.N.H. & Potgieter D.J.J. Some taxic, physical and chemical properties of the oral secretion of the tampam, *Ornithodoros savignyi* (1927). *Onderstepoort J. Vet. Res.,* 42:99–102, 1975.

Ishii K.J., Koyama S., Nakagawa A., Coban C. & Akira S. Host innate immune receptors and beyond: making sense of microbial infections. *Cell,* 3:352-63, 2008.

Jonsson N.N. Control of cattle ticks (*Boophilus microplus*) on Queensland dairy farms. *Aust. Vet. J.*, 75:802-807, 1997.

Lemaitre B. & Hoffmann J. The host defense of *Drosophila melanogaster. Annu. Rev. Immunol.* 25:697–743, 2007.

Levashina E.A. Immune responses in *Anopheles gambiae. Insect. Biochem. Mol. Biol.,* 34:673-8, 2004.

Nunes E.T., Bechara G.H., Saito K.C., Denardi S.E. & Camargo-Mathias M.I. Morphological, histological, and ultrastructural characterization of degenerating salivary glands in females of the cattle--tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Acari: Ixodidae). *Micron,* 3:437–447, 2005.

Rosetto M., Engstrom Y., Baldari C.T., Telford J.L. & Hultmark D. Signals from the IL-1 receptor homolog, Toll, can activate an immune response in a *Drosophila* hemocyte cell line. *Biochem. Biophys. Res. Commun.,* 209:111-116, 1995.

Stein D., Roth S., Vogelsang E. & Nusslein-Volhard C. The polarity of the dorsoventral axis in the Drosophila embryo is defined by an extracellular signal. *Cell*, 65:725-735, 1991.

Takeda K. & Akira S. Roles of Toll-like receptors in innate immune responses. *Genes Cells,* 6:733-742, 2001.

Tatchell R.J. The ionic regulatory role of the salivary secretion of the cattle tick, *Boophilus microplus*. *J. Insect. Physiol.,* 15:1421–1430, 1969.

Valanne S., Wang J.H. & Rämet M. The *Drosophila* Toll signaling pathway. *J. Immunol.,* 186:649-656, 2011.