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# Cross-species reactivity of antibodies against *Ixodes persulcatus* ferritin 2 to *Rhipicephalus microplus*

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**Abstract**

**There are several studies that confirm the possibility of developing a vaccine against tick infestations. An immuno-bioinformatics approach was used to identify conserved antigenic regions between *Ixodes persulcatus* and *Rhipicephalus microplus* ferritin 2 (FER2) in order to compose a novel putative vaccine. In addition, *R. microplus* were fed on blood containing antibodies anti-recombinant FER2 from *I. persulcatus* (rIp-FER2). The results revealed that anti-ferritin antibodies led to a decrease in the engorgement weight of the *R. microplus* females. Conservation of the predicted antigenic regions in different tick species suggests that this protein could be useful to develop a vaccine for cross-species protection.**

Key Words: ferritin, tick, vaccine

Ticks are hematophagous ectoparasites that are spread around the world. Besides the physical damage caused to the host by the parasitism, during blood feeding some pathogens (e.g. protozoa, bacteria and viruses) are transmitted to the host[23)]. Therefore, ticks and tick-borne diseases are matters of interest to the veterinary and medical communities[38)]. Also, economic losses caused by ticks are of concern to livestock farmers, veterinarians and consumers, since costs are estimated at billions of dollars annually worldwide[38)]. Currently, application of synthetic chemical acaricides is the mainly strategy to control ticks, but in addition to pollute the environment, resistant tick populations have been selected, contributing to a gradual inefficiency of this method[17,25)]. For this reason, alternative strategies to tick control are required.

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One promising methodology is to vaccinate hosts using tick-derived antigens, as has already been shown for the Bm86 antigen (protecting cattle against *Rhipicephalus microplus* and other tick species infestations)[28,31,39,40,47]. However, Bm86 immunization achieved success in a limited number of related tick species, which restricts the areas where it can be applied. Consequently, an anti-tick vaccine that confer protection against multiple tick species is an interesting alternative[32]. A further desirable aspect of a universal tick vaccine is to be composed of highly conserved proteins among tick species or containing limited differences. Moreover, a cross-protection would be cost-effective, mainly in regions where there are economical limitations to develop an immunological control method against multiple tick species[32].

Ferritins are iron storage proteins that have a role in iron transport, storage and detoxification in ticks, as well as other organisms[13,20,48]. There are two ferritins that are fundamental to blood feeding and reproduction, which are present in all tick life stages[11,13,18,20]: ferritin 1 (FER1) have a role in iron storage; and ferritin 2 (FER2) is implicated in iron transport from gut to other tissues. The intrinsic role of FER2 to iron homeostasis have been shown by RNA interference (RNAi)[11,20] and vaccination[12,18] assays. Antibodies against FER2 and FER2 RNAi were injurious to tick blood feeding, oviposition and fecundity. Since FER2 amino acids sequence is highly conserved, altogether, these factors suggest that FER2 is a potential candidate to a cross-species anti-tick vaccine. Recently, we reported that *Ixodes persulcatus* -derived FER2 (Ip-FER2) showed concentration-dependent iron-binding ability and high amino acid conservation consistent with FER2s of other tick species. Furthermore, vaccination with the recombinant Ip-FER2 elicited a significant reduction in the engorgement weight of *I. persulcatus*[15].

The development of vaccines with cross-protection against different tick species may be particularly useful since a host can be infested by more than one tick species. For instance, in some countries, a cattle host may be infested at the same time by *Rhipicephalus appendiculatus*, *Amblyomma variegatum*, *Rhipicephalus decoloratus*, *Rhipicephalus annulatus* and *R. microplus*[8,30]. Thus, a broad-spectrum tick vaccine could simplify the commercial production[32] and the clinical veterinary practice. Therefore, in this work, the cross-reactivity between anti-rIp-FER2 antibodies and *R. microplus* ferritins was evaluated by *in silico* and *in vivo* studies.

The similarity between deduced amino acid FER2 sequences from *I. persulcatus* (196 amino acids, GenBank KF311110) and *R. microplus* (189 amino acids, GenBank CK190528) were analyzed using Clustal W[44] in the BioEdit software (version 7.1.3.0)[21] (Figure 1A). The antigenic index in tick ferritins was predicted using the Jameson-Wolf algorithm in LASERGENE software (version 7.0.0). Antigenic determinants were calculated by combining existing methods for protein structural predictions[22]. There were six conserved regions between sequences that overlapped with antigenic regions (amino acids 24-27, 60-66, 77-82, 126-131, 153-168 and 176-180 from *R. microplus* ferritin). To determine homology models, SWISS-MODEL algorithms in the web platform[3,4,42,46] was used. Since the crystal structure of tick ferritins are unknown, ferritin from other organisms were examined to be used as a template, using target-template alignments. From the 619 templates found, *Chaetopterus variopedatus* ferritin was selected (Protein Data Bank - 5wpn)[27] to generate tick ferritin model. Three-dimensional visualizations of *in silico* simulations were generated using Jmol (http://www.jmol.org/), and the conserved antigenic regions in common between *I. persulcatus* and *R. microplus* FER2 were highlighted (in dark blue) in *R. microplus* three-dimensional structure (Figure 1B) in order to reproduce the possible binding sites of anti-rIp-FER2 antibodies in *R. microplus* FER2.

The possible cross-reactivity between antibodies against rIp-FER2 and *R. microplus* ferritins was accessed using capillary feeding

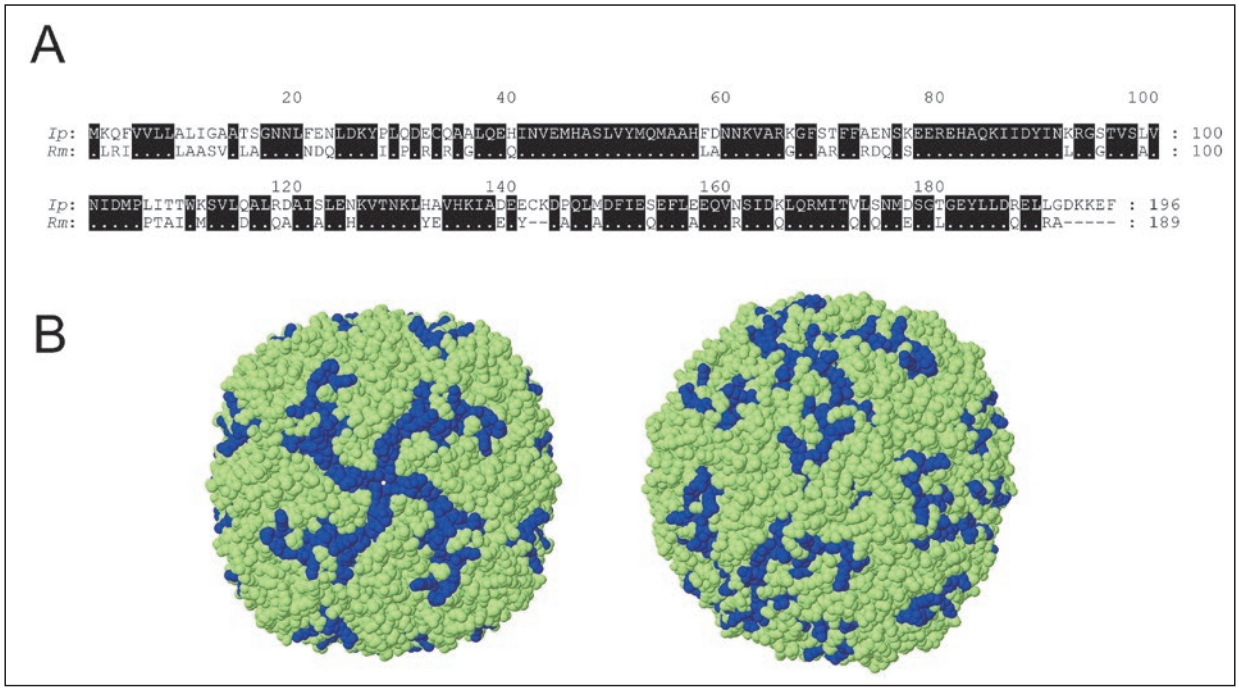


**Fig. 1. Ferritins from *Ixodes persculcatus* and *Rhipicephalus microplus*.** (A) Deduced amino acids sequence alignment from *I. persulcatus* (GenBank KF311110) and *R. microplus* (GenBank CK190528) ferritins. (B) *In silico* tertiary structure of *R. microplus* FER2 with conserved antigenic regions between *I. persulcatus* and *R. microplus* ferritins in dark blue. Structure is displayed in front (left) and perpendicular (right) views, using space-filling style.

assay, which was conducted at the Federal University of Rio Grande do Sul (UFRGS). All procedures were approved by the local experimental ethics committee (Protocol number 38748—CEUA UFRGS). The open reading frame of the Ip-FER2 was previously characterized and cloned in the plasmid pET43c. The rIp-FER2 was expressed in *Escherichia coli* and purified by affinity chromatography[15]. The protein was detected in the soluble fraction, and Western blot analysis revealed a protein of 20-25 kDa recognized by anti-histidine antibodies (data not shown). To prepare anti-rIp-FER2 antibodies, one 6 weeks-old New Zealand rabbit was subcutaneously immunized four times at 14-day intervals with 100 μg of rIp-FER2 emulsified in a Montanide 888/Marcol 52 adjuvant, as described previously[2] with slight modifications. The rabbit blood was collected, and serum separated by centrifugation at 10,000 × g for 10 min at 4°C. The serum specificity was tested using an enzyme-linked immunosorbent assay. Subsequently, IgGs were purified from anti-rIp-FER2 and non-immunized sera in a HiTrap™ Protein G column according to the manufacturer's protocol (GE Healthcare, Buckinghamshire, England, UK).

*Rhipicephalus microplus* were obtained from a laboratory colony (Porto Alegre strain, Porto Alegre, Brazil). Ticks were reared on Hereford cattle (*Bos taurus taurus*) brought from a naturally tick-free area and maintained in insulated pens[37]. A calf was infested with 15 days-old larvae and, 21 days later, partially engorged female ticks were removed from the host. These ticks were weighed and placed on plates where they were randomly sorted into three groups (blood, pre-immune, and rIp-FER2 ahead of the artificial feeding experiment. Each group contained 25 females weighing between 30 and 65 mg. The blood group was fed only on calf blood, whereas the pre-immune group was fed on rabbit non-immunized antibodies (100 μg IgG per tick)

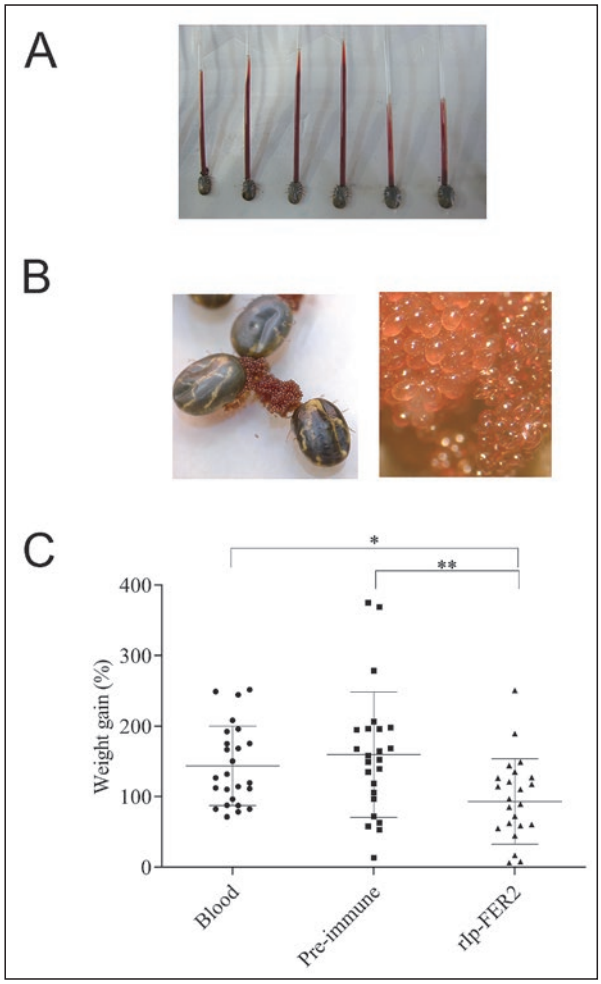


**Fig. 2. Effect of anti-rIp-FER2 antibodies on blood capillary feeding of *Rhipicephalus microplus*.** (A) Partially engorged females were artificially fed using capillaries filled with calf blood including purified antibodies from pre-immune or rIp-FER2 immune sera. (B) Representative image of engorged *R. microplus* females and eggs. (C) The ticks were artificially fed on control bovine blood or blood including antibodies derived from preimmunized or rIp-FER2-immunized animals. Statistical analysis was performed using ordinary one-way ANOVA tests (multiple comparisons) in GraphPad Prism v. 7.00 software. $*P = 0.04$ and $**P = 0.004$.

mixed with calf blood, and the rIp-FER2 group was fed on anti-rIp-FER2 antibodies (100 µg IgG per tick) mixed with calf blood. In pre-immune and rIp-FER2 groups, after ticks had consumed all blood containing 100 µg of antibodies, they fed *ad libitum* on calf blood without the addiction of antibodies. The artificial feeding process was performed using capillaries filled with each group's calf blood[16] (Figure 2A). After 24 h of feeding, ticks were weighed, placed on plates, and incubated (28°C ± 1°C, 85–90% humidity) for 10 days to induce oviposition (Figure 2B). The egg cluster of each female was weighed and placed in a tube to hatch. After incubation under the conditions described above for a further 20 days, the larvae from each female were weighed. Statistical analysis was performed using ordinary one-way ANOVA tests (multiple comparisons) using GraphPad Prism software (version 7.00), and $P < 0.05$ was considered to indicate statistical significance.

Interestingly, as shown in Figure 2C, the weight gain of female ticks fed on blood including anti-rIp-FER2 antibodies was significantly reduced (41.7 ± 29.8 mg, 89.1 ± 62.7%) compared with the controls from blood group (70.5 ± 29.9 mg, 143.8 ± 56.2%) and pre-immune group (76.4 ± 48.5 mg, 153.1 ± 93.2%). There was no significant difference in the weight gain of ticks fed on control blood and those fed on blood including antibodies derived from non-immunized animals. Unfortunately, there were no differences in the egg weight and hatching rate among any of the blood-fed tick groups (data not shown).

The protection provided by immunization using the commercialized Bm86 vaccines against *R. microplus* infestation ranges from 51–91%[34,38], a consequence of Bm86 amino acids sequence heterogeneity within *R. microplus* populations[14]. Such variation reflects not only the different susceptibility among *R. microplus* populations, but also host features (nutrition and breed). Indeed, cross-protection induced by Bm86 and its homologue protein were evaluated in infestations by *Rhipicephalus* genus (*R. annulatus, R. decoloratus, R. appendiculatus, R. haemaphysaloides* and *R. sanguineus*), and by *Hyalomma anatolicum* and *H. dromedarii*[1,5,7,9,24,31,35,36,43]. From these experiments, the most successful results were obtained in infestations by *R. annulatus*, a protection level above 99%[9,36].

The homologues tick subolesin (SUB) and mosquito akirin (AKR) have been shown as possible antigens to develop a vaccine that encompasses both arthropods[10]. However, vaccination experiments have shown that SUB antigen alone has a higher efficacy among vectors (*Ixodes ricinus*, *Aedes albopictus* and *Phlebotomus perniciosus*) than SUB/AKR chimeras[29]. On the other hand, immunization using SUB/AKR chimera (Q38) reduced rabbit infestations by *I. ricinus* (99%) and *Dermacentor reticulatus* (46%) larvae[6]. Recently, Trentelman and colleagues[45] used a mixture of bovine antibodies against Bm86 and SUB in artificial feeding experiments with *Rhipicephalus australis* larvae and observed a synergistic effect that lead to circa 63% reduction in feeding. It was suggested that while antibodies against Bm86 would bind to gut epithelium, antibodies against SUB would bind to salivary glands' acini and rectal sac epithelium. Further, SUB antigens from *Rhipicephalus appendiculatus*, *R. decoloratus* and *A. variegatum* were used to immunize *Bos indicus* breed and *B. indicus/B. taurus* crossbreed cattle[24]. After host infestation with these tick species, cross-protection was observed, mainly affecting early stages of *R. appendiculatus* and *A. variegatum*. Analyzes show that immunization with *R. appendiculatus* SUB was more successful, since it was not only effective against cross-species but also against the species itself.

Another potential antigen to be used in a cross-protective vaccine is glutathione S-transferase (GST). It was shown that immunization using GST from *Haemaphysalis longicornis* confers 57% and 67% protection against *R microplus* and *R. appendiculatus* infestations, respectively[33,41]. The importance of the degree of similarity between sequences among tick species was shown using GST as model. On the surface of GST homodimers from *R. appendiculatus*, *R. decoloratus*, *Amblyomma variegatum*, *H. longicornis* and, in a less extent, *R. microplus*, there are conserved B-cell epitopes. Anti-sera from these peptides cross-reacted to non-homologous tick GSTs, corroborating with the idea that consensus epitopes are a critical step in a cross-protective anti-tick vaccine[30].

The potential of FER2 as a vaccine target was first reported by Hajdusek and colleagues[18]. Rabbit immunization experiments using *I. ricinus* FER2 were 98% effective in reducing infestations by this same tick. On the other hand, immunizing cattle with *R. microplus* FER2 lead to a 64% efficacy against this tick infestations and a cross-protection of 72% against *R. appendiculatus*. A lower efficacy (49%) was observed when *H. longicornis* FER2 was used to immunize rabbits, which were then infested with this same tick species[12]. The other tick ferritin, FER1, has also been used in vaccination experiments, but the results have been less successful[12,19].

*Rhipicephalus microplus* causes major losses in the livestock industry due to a reduction in productivity and to the damage caused to host skin during infestation[23]. In addition, besides the presence of a variety of tick species worldwide, multiple tick species infestation in the same host are reported in many countries[8,30]. Consequently, it is essential to develop or to improve vaccines targeting a cross-protection between different tick species, since a broad-spectrum vaccine may simplify the commercial production[32].

The current work suggests that cross-reactive antibodies induced by rIp-FER2 immunization could be useful against *R. microplus* infestations. Although further experiments are required to attest this proposition, the reduced weight gain of *R. microplus* females fed on blood containing antibodies against rIp-FER2 are in consonance with other FER2 immunization data.

#### Conflict of interest statement

The authors declare that they have no competing interests.

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