Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

In silico and *in vivo* Immunological Characterization of Aquaporin 1 Peptide and Ferritin2 Recombinant Protein of Brown Dog Tick *Rhipicephalus sanguineus* (Acari: Ixodidae) for Vaccine Development

Heba F. Alzan[1,2*], Seham H.M. Hendawy[1,2], Bassma S.M. Elsawy[1,2], Hoda S.M. Abdel-Ghany[1,2], Sobhy Abdel-Shafy[1,2], Tetsuya Tanaka[3,4] and Mona S. Mahmoud[1,2*]

[1] *Parasitology and Animal Diseases Department, Veterinary Research Institute, National Research Centre, 33 El Buhouth St., Dokki, Cairo, 12622, Egypt.*
[2] *Ticks and Tick-Borne Diseases Research Unit, Veterinary Research Institute, National Research Centre, 33 El Buhouth St., Dokki, Cairo, 12622, Egypt.*
[3] *Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, 890-0065, Japan.*
[4] *Laboratory of Animal Microbiology, Graduate School of Agricultural Science, Tohoku University, Sendai, 980-8572, Japan.*

Abstract

THE brown dog tick, *Rhipicephalus sanguineus* (*R. sanguineus*), is a significant vector of pathogens causing canine monocytic ehrlichiosis and babesiosis. While several emerging tick proteins show promise as vaccine targets, few have been rigorously tested. Given the crucial roles of aquaporin 1 (RsAQP1) in blood feeding and ferritin 2 (RsFer2) in hemoglobin processing for tick physiology, these two proteins represent potential targets for disrupting essential biological functions. However, traditional *in vivo* vaccine trials are resource intensive. This study employed an immunoinformatics approach to predict and compare the immunological properties of a synthetic peptide derived from RsAQP1 and a recombinant RsFer2 protein as potential vaccine candidates against *R. sanguineus*. The evaluation considered their potential as individual vaccines and as a cocktail in Baladi (native Egyptian) dogs. *In silico* analysis predicted that both the RsAQP1 peptide and the RsFer2 protein contain several promising B-cell and T-cell epitopes with high antigenicity and immunogenicity scores. The cocktail vaccine was predicted to induce a humoral immune response for the entire duration of the experiment. Consistently, from the animal experiment, all vaccinated dogs developed significant antibody levels and were notably higher in the case of injection with rFer2 protein or in the case of mixed antigens (AQP1&rFer2) vaccination. Western blot analysis indicated the recognition of the native tick antigen by the vaccinated dog serum either in the case of individual or combined vaccination. In conclusion. the favorable *in silico* immunological profiles of the RsAQP1 peptide and rRsFer2 protein, both individual rRsFer2 protein and as a cocktail with RsAQP1 peptide, suggest their potential as effective subunit vaccine candidates against *R. sanguineus*. These findings warrant further *in vivo* tick investigation to validate their protective efficacy in dogs.

Keywords: *Rhipicephalus sanguineus*, aquaporin 1, ferritin 2, recombinant vaccine, Baladi dogs.

Introduction

The brown dog tick *Rhipicephalus sanguineus* (*R. sanguineus*) is one of the most widespread ticks in the world [1–3]. Its main host is dogs and is suspected to act as a vector of dog pathogens such as *Babesia vogeli* and *Ehrlichia canis* [4], but it can also be found in other animals and humans. Moreover, *R. sanguineus* is a vector of many zoonotic disease agents, such as *Coxiella burnetii*, *Ehrlichia canis*, *Rickettsia conorii*, and *Rickettsia rickettsii* [5]. This tick has endophilic (an indoor) behavior in temperate zones, as it could be found on wall cracks, inside houses, and in furniture [4], but in tropical zones, exophilic behavior is more usual [6]. The ecological plasticity and broad vectorial competence of *R. sanguineus* position it as a

---

*Corresponding authors: Heba F. Alzan, E-mail: Hebaalzan@gmail.com, Tel.: 01098578989
and Mona S. Mahmoud, E-mail: Monasaid3000@yahoo.com, Tel.:0106520503

(Received 02 September 2025, accepted 24 October 2025)
DOI: 10.21608/ejvs.2025.419478.3100

paramount vector of pathogens with considerable medical and veterinary implications [7].

Effective tick control not only lessens infestations on animals but also prevents the spread of blood parasites, thereby reducing the economic burden of tick-borne diseases across animals. The use of chemicals is considered the primary measure for control of these ectoparasites. However, the intensive use of these chemicals has drawbacks such as food contamination, environmental pollution, and developing resistance by ectoparasites. Those limitations of current methods underscore the necessity for new approaches to tick control. Eco-friendly acaricides and tick vaccines represent ideal solutions [8].

Currently, there is no commercial vaccine for *R. sanguineus*. While there has been significant research into developing vaccines against ticks, the only commercially available anti-tick vaccines (like Gavac® and formerly TickGARD®) are based on the Bm86 antigen, primarily effective against *Rhipicephalus microplus* (the cattle tick) [8]. These are not specifically designed for or highly effective against *R. sanguineus*. However, there is ongoing research into various candidate antigens (like aquaporins, ferritins, serine protease inhibitors, cement proteins, etc.) for *R. sanguineus* and other tick species [9]. Many studies have shown promising results in experimental settings (e.g., in laboratory animals like mice, rabbits, or dogs), demonstrating some level of reduced tick feeding, engorgement, or reproduction. However, these are still in the research and development phase and have not led to a widely available commercial vaccine for the brown dog tick [10]. Therefore, developing a highly effective and broadly protective vaccine against ticks like *R. sanguineus* is challenging due to factors such as its complex biology and their interactions with hosts, antigenic variability within tick populations, the need for broad-spectrum protection against different tick stages and species [11]and lastly, high cost and complexity of bringing a vaccine to market [12].

Tick osmoregulation, vital for feeding and reproduction, has driven extensive research into aquaporin proteins (water channels) in blood-feeding arthropods. These proteins are considered promising tick vaccine candidates due to their crucial role in water transport [13–15]. For instance, the recombinant protein *Rhipicephalus microplus aquaporin* 1 (RmAQP1) showed 73-76% efficacy as an anti-cattle tick vaccine against *R. microplus*. However, when tested against *R. sanguineus*, RmAQP1 offered low immunoprotection for adult ticks, though it showed some potential against larvae and nymphs. This reduced efficacy is likely because an *R. microplus* protein was used against *R. sanguineus*, suggesting that an aquaporin originating from *R. sanguineus* itself would be more effective [1]. Thus, the current work is based on the use of *R. sanguineus* AQP1 in anti-tick vaccines, including identifying specific immunogenic regions.

Hemoglobin, a major component of a tick's blood meal, presents both benefits and harms, primarily due to its iron content [16, 17]. The breakdown of hemoglobin releases heme and iron, which can generate reactive oxygen species (ROS) and disrupt iron metabolism, crucial for tick survival [16–18]. Iron-binding proteins like ferritins are vital for ticks, acting as antioxidants to counteract oxidative stress during blood feeding and supporting reproduction. For instance, secretory ferritin 2 (HlFER2) from *Haemaphysalis longicornis* controls vitellogenin genes, making it a promising protective antigen against tick infestations [19]. Further evidence from *Hyalomma rufipes* shows that knocking down HrFer2 significantly impacts blood-feeding duration [20], underscoring the critical role of ferritin proteins in tick iron metabolism. Recently, in a rabbit model, *Hyalomma anatolicum* FER2 protein was used as an individual vaccine and in combination with subolesin protein as a cocktail vaccine against *R. sanguineus* tick. The efficacy was 95.9% and 90.9%, respectively, against *R. sanguineus* infestation [10]. These findings support the possibility of using mono-antigenic and cocktail-based vaccines, including *R. sanguineus* Fer2 protein and the immunogenic part of the *R. sanguineus* AQP1 for anti-tick vaccine development against *R. sanguineus*.

Since developing effective anti-tick vaccines in dog trials demand extensive research and costly experimental trials, computational immunology, known as immunoinformatics, represents a crucial prediction tool that was applied. This tool is to identify the immune reaction against a subunit vaccine, either by using a single peptide or protein in the vaccine or a cocktail form. This kind of analysis, combined with an *in vivo* study, will validate what is needed to evaluate potential vaccine candidates [21, 22].

We are conducting a vaccination experiment in Egyptian dogs to determine the protective potential of a synthetic *R. sanguineus* AQP1 peptide and recombinant *R. sanguineus* fer 2 proteins against *R. sanguineus* infestation. A key aspect of this study involves comparing the predicted immune reactions from immunoinformatics (*in silico*) analysis with the actual responses observed in vaccinated animals (*in vivo*). A comprehensive evaluation of the immunoprotection offered by *R. sanguineus* AQP1 and Fer2 against *R. sanguineus* infestations in dogs requires additional tick studies.

Material and Methods

Tick collection and colony establishment

For the establishment of the *R. sanguineus* tick Egyptian strain, engorged adult *R. sanguineus* (three-host tick species) female ticks were collected from an infested dog at a pet animal clinic, in Damanhour, Beheira, Egypt, and were identified according to Walker et al. [23]. To confirm the morphological classification proceeded on *R. sanguineus*, PCR was applied on tick DNA samples using specific primers targeting *cytochrome c oxidase subunit-1* (*CO1*) gene, one of the most useful markers for ticks' taxonomy at species level [24–26]. The primer sequences were CO1-F5'-GGAACAATATATTTAATTTTTGG-3' and CO1-R5'-ATCTATCCCTACTGTAAATATATG-3'. The amplification reactions were performed in 25 µl total volumes, which consist of 12.5 µl of Sigma 2× JumpStart™ REDTaq® ReadyMix™ (Foster City, California, USA), 10 µm of CO1-F and CO1-R, 8.5 µl nuclease free water, in addition to 2 µl of each DNA sample. The negative control was a no-template control (NTC). The PCR conditions to amplify the target DNA sequence were according to Chitimia et al. [27]; Lv et al.[26] and Abdullah et al.[3]. All amplicons were visualized by 1.5 % agarose gel electrophoresis (Invitrogen, Waltham, USA).

The picked female tick was incubated at 25±1°C with 75-80% relative humidity in an incubator (Friocell, MMM, Germany) for oviposition at the laboratory of Tick and Tick–borne diseases Research Unit, National Research Centre, Egypt. After hatching, larvae were allowed to feed on healthy, pathogen-free rabbits using a back-capsule technique to get fully fed larvae [2, 28]. After 10 days of incubation, the fully fed larvae molted into unfed nymphs, which allowed them to be fed on another rabbit to get fully engorged nymphs [29]. After molting nymphs to unfed adults, an equal number of unfed male and female adults were allowed to feed on rabbits for 5 days [30] to obtain the fully fed females. All the life cycle steps, and incubation periods are shown in a schematic illustration in Fig. 1. The established colony is kept in the Tick Rearing Facility at the animal house, National Research Centre, Egypt.

Bioinformatic and Immunoinformatic analysis

Genes' identification by genomic search in NCBI and bioinformatics analysis were performed using Transmembrane Hidden Markov Model package 2 (TMHMM2) (http://www.cbs.dtu.dk/services/TMHMM-2.0). Also, antigenicity prediction was carried out (for individual antigen *R. sanguineus* Fer2, AQP1 proteins, and AQP1 synthetic peptide as vaccine candidates) using the immunomedeicine group from the faculty of medicine, Madrid, Spain (http://imed.med.ucm.es/Tools/antigenic.pl) and Bioinformatics EMBOSS explorer (https://www.bioinformatics.nl/cgi-bin/emboss/antigenic).

The immunoinformatic analysis was performed on both tick proteins to predict their immune protection potency as an immune response simulation upon the dog vaccination. The C-IMMSIM online server available at (http://kraken.iac.rm.cnr.it/C-IMMSIM) was utilized to predict host immune response simulation. This *in silico* immune simulation analysis tool is used to validate the immunogenicity and the immune profile response of both *R. sanguineus* AQP1 synthetic peptide and recombinant Fer2 protein individually or in combination as vaccine candidates able to trigger the host immune response. All parameters were set as default except for antigen injections. Three injections were set at 1, 15, and 21 days, where each time step of simulation corresponds to 8 h through the experiment for 7 weeks [31]. Bm86 recombinant protein (the base of the commercial anti-tick vaccine, Gavac vaccine for *R. microplus*) and its homologue *R. sanguineus* Rs 86 were included as controls for better contextualization of the immune response prediction.

Identification of Aquaporin 1 peptide and Ferritin 2 protein in Egyptian R. sanguineus tick

DNA was extracted from different stages of *R. sanguineus*. RNA was extracted from an unfed adult female and kept in 70% ethyl alcohol. The unfed adult female tick sample was homogenized using an auto mill (Tokken, Chiba, Japan). RNA extraction was performed by magLEAD 6gC (Precision System Science Co., Ltd., Chiba, Japan). Single-strand cDNA was prepared by reverse transcription using the ReverTra Ace cDNA Synthesis Kit (Toyobo, Osaka, Japan). PCR was conducted using AmpliTaq Gold 360 Master Mix (Applied Biosystems, Foster City, CA, USA). Primer sequences for gene amplification were RS-AQP1 forward primer: ACGATGTTGGACAGCGTGAA and RS-AQP1 reverse primer: TCTCTAAAAAATTGCCTCAT for the *R. sanguineus aqaporin1* gene. Primer sequences for gene amplification were RsFer2 forward primer: ACCATGCTTCGAGTCGTGCT and RsFer2 reverse primer: CTGTTAGGTTCGCAGTTGCT for the *R. sanguineus ferritin2* gene. The PCR profile was as follows, AQP1: 95°C for 10 min, 40 cycles of the denaturation step at 95°C for 30 seconds (s), an annealing step at 52°C for 60 s, and an extension step at 72°C for 60 s, and then a final extension step at 72°C for 7 min. FER2: 95°C for 10 min, 40 cycles of the denaturation step at 95°C for 30 s, an annealing step at 59°C for 60 s, and an extension step at 72°C for 60 s, and then a final extension step at 72°C for 7 min. The actin gene was used as a control. PCR products were cloned into the T-Vector pMD20 vector (Takara Bio Inc, Shiga, Japan). The purified plasmids were expressed in *Escherichia coli* DH5α cells, grown in Luria-Bertani (LB) broth medium with ampicillin. The resulting plasmids were checked for accurate insertion through the analysis by restriction enzymes, and the target sequences were read using the automated sequencer. The plasmids were purified using the Qiagen Plasmid Mini Kit (Qiagen). Two plasmid clones from each gene [*RsAQP1* gene and *RsFer2* gene] were sent for sequencing. The resulting sequences of the Egyptian *RsAQP1* gene and *RsFer2* gene were then compared with the annotated ones of the Genebank at nucleotide and amino acid levels.

Preparation of R. sanguineus tick Aquaporin 1 (AQP1) peptide and recombinant Ferritin 2 (rFer) protein

One synthetic peptide representing the immunogenic region of Egyptian RsAQP1 protein, containing *in silico* predicted B-cell epitopes, was chosen. RsAQP1 #122-132 peptide- keyhole Limpet Hemocyanin (KLH) conjugation, CGGVRAVTGSNA, was generated (COSMO BIO CO., LTD, Japan). This peptide was conjugated with KLH using the m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) method. A recombinant Egyptian *R. sanguineus* Ferritin 2 protein was produced in Chinese hamster ovary (CHO) cells (GenScript Biotech, Rijswijk, Netherlands).

Vaccine preparation and immunization experiment design

Twelve female dogs (Baladi breed "Egyptian native breed"; weight 20-25 kg; age 16-18 months) were carefully selected for this immunization trial. This specific breed was chosen due to its native genetic background and presumed natural exposure to local tick populations, making it a highly relevant model for evaluating vaccine efficacy in an endemic setting. The use of only female dogs of a similar age and weight range aimed to minimize variability due to sex, developmental stage, and size, thereby reducing confounding factors and increasing the statistical power of the results. Those animals were provided by the veterinary medicine college, Cairo University. All animals were clinically examined for any ectoparasite infestations as well as molecularly screened for pathogenic infections to ensure their health status. The dogs were randomly assigned to four experimental groups of three animals (Fig. 2). Animal groups were labeled according to the injectable vaccine material as the following: A group: received AQP1 peptide and adjuvant (250 µg/ml per dog). The rationale here was to assess the immunogenic potential of RsAQP1 as a standalone vaccine candidate, given aquaporins' critical role in tick osmoregulation and previous promising (though limited) results with aquaporins from other tick species. The adjuvant was included to enhance the immune response. Group B: received rFer2 protein (295 µg/ml per dog). RsFer2 was chosen due to its central role in tick iron metabolism and oxidative stress protection, as well as prior evidence suggesting ferritin's involvement in tick reproduction and blood feeding. This group aimed to evaluate RsFer2 as an individual vaccine target. Group C: received a mix of AQP1 and rFer2 (same antigen concentration). The rationale for this group was to investigate the potential for enhanced or broader protective immunity by combining two distinct antigens targeting different crucial physiological pathways in the tick. A cocktail approach often aims to overcome limitations of single-antigen vaccines and induce a more robust, multi-faceted immune response. Group D: control group, received an identical volume of Phosphate-Buffered Saline (PBS) and Adjuvant up to 1 ml. This control group is essential for establishing baseline responses and ruling out any effects attributable to the adjuvant itself or the injection procedure, ensuring that any observed immune responses or protective effects in the other groups are specifically due to the antigens.

To ensure proper antigen dispersion and maximize exposure to antigen-presenting cells at multiple sites and to minimize local reactions and optimize systemic uptake, the injection was performed as 500 ul on the right side and the other 500 ul on the left side for each animal. All the injections were performed through the intramuscular route. Everything was performed under aseptic conditions during vaccine preparation, and all buffers and tubes were autoclaved. Regarding the adjuvant, 500ul of Complete Freund's Adjuvant (CFA) was used as initial injections, and then Incomplete Freund's Adjuvant (IFA) was replaced in booster shots. Blood samples were collected from each animal before each immunization. Immunization was performed in three consecutive doses. The first interval was two weeks from the second one, while only one week was between the second and the last vaccination. Pre-immune sera were collected from all animals and then collected weekly until the end of the experiment. The experiment design is demonstrated in Fig. 2. After each immunization, all animals were monitored for any adverse reactions through clinical examination, and detection of any systemic or local signs (localized pain, itching, swelling, lumps, fever, etc.).

Determination of dog serum antibody level by indirect ELISA

Serological analyses of antigen-specific antibody response (total IgG) to vaccination, in both immunized and control groups, according to days of serum collection (from day zero to three weeks after the third immunization) were determined by indirect ELISA according to Alzan et al. [32]and Scoles et al. [33]. Briefly, 96-well flat-bottom ELISA plates (Linbro, Flow Laboratories, Connecticut, USA) were coated overnight at 4°C with either AQP1-KLH peptide (concentration 1 µg/well) or rFer2 protein (250 ng/well) in a 50 µl total volume of carbonate buffer, pH 9.6. The plates were washed three times with 0.01 M Phosphate Buffer Saline (PBS) containing 0.05% Tween 20 at pH 7.2 (PBS-T), and then the plates were blocked using 2% dry skim milk (Sigma-Aldrich, Switzerland) in a coating buffer for 1 h at 37°C. After three washes with PBST, the plates were incubated with dog serum samples diluted 1:25 in PBST for 1 h at 37°C, then washed as before. Plates were incubated with Rabbit-anti-canine (whole molecule) peroxidase secondary antibody conjugate (Sigma-Aldrich St. Louis, MO, USA) (1/5000) for 1 h at 37°C and washed three times as before. The color reaction was developed by adding 50 µl/ well of substrate solution containing 0.04% (w/v) OPD (Sigma) at 37°C. The colorimetric reaction was stopped after 10 min with a solution of 0.16 M sulfuric acid, and the optical density (OD) was determined at a wavelength of 450 nm with an ELISA reader (Universal microplate reader, ELX8000 UV; Bio-TEK, United States). Statistical analysis was performed to estimate the differences between the different vaccinated dog groups using a two-tailed Student *t*-test for the antibody response in week 6.

Tick protein preparation and Western blot analyses

The Egyptian *R. sanguineus* unfed adult (as a native antigen) was used for antigen preparation according to Galay et al.[18]. Firstly, adult ticks were thoroughly washed with distilled water and then washed with PBS. Ticks were homogenized and suspended in PBS, then subjected to sonication using a 20 kHz Sonicator (Sonics, Vibra cell Ultra sonicator, USA) for 20 min. The homogenate was centrifuged at 12,000×*g* for 30 min in a cooling centrifuge, and the supernatant was collected. The protein concentration was determined according to the method of Lowry et al. [34].

To prove the reactivity of dog sera against the native tick proteins, 50 µg of unfed adult *R. sanguineus* antigen and 10 µg rFer2 were fractionated through 12% SDS-PAGE under reducing conditions according to Laemmli [35], Novex™ Sharp Pre-stained Protein Standard marker was used (Thermo Fisher Scientific, Waltham, MA, USA). The fractionated native and recombinant proteins were blotted on nitrocellulose membrane sheets for 1.5h at 150 V, 120A, and 50W according to Towbin et al.[36]. After blocking with 1% dry skimmed milk in Tris-buffered saline (TBS) for 1 h at room temperature, the membrane was washed three times, 10 min each in TBS. The sheets were incubated overnight with 1:25 primary antibody (dog serum), one representative dog belonging to each experimental group collected at the 5th week post immunization in a diluting buffer (0.5% BSA/ TBS buffer). The control negative was serum from dogs vaccinated with adjuvant only and used at the same dilution. The sheets were washed as mentioned above and incubated for 1 h with anti-canine IgG (whole molecule) peroxidase antibody conjugate (Sigma-Aldrich, USA) diluted at 1:2,000 in a diluting buffer. The immune reactive bands were developed by incubation of the membranes in the substrate solution (1-chloronaphthol (30 mg) (Sigma-Aldrich, USA) dissolved in 11 ml methanol, followed by the addition of TBS up to 50 ml and 30 ul of 30% H_2O_2) for 3–5 min. The reactive bands were visualized using a gel documentation system (Bio-Rad, Hercules, USA).

Since using the AQP1-KLH peptide in western blot analysis as a control positive was difficult, a dot blot was performed to evaluate the reactivity of dog serum immunized with the AQP1-KLH peptide. The AQP1-KLH peptide and unfed *R. sanguineus* adult antigen (10 µl of each with a concentration of 15 µg) were blotted onto a nitrocellulose membrane. The membrane was incubated for one hour at room temperature and then blocked with 5% dry milk in TTBS (50 mM Tris, 0.5 M NaCl, 0.05% Tween-20, pH 7.4) for 1 h. The nitrocellulose strips were incubated with the primary antibody for 1 h at room temperature in TTBS. After washing, the strips were incubated with secondary antibodies for 1 h at room temperature. The strips were washed three times (10 min each) in TTBS on a rocker. Finally, the strips were visualized [33, 37].

Assessment of band intensity measurement in western blot membrane

Regarding the intensity of reactivity against the antibodies in the serum from the mixed animal group, the signal intensity percentage of the two protein bands (AQP1 and Fer2) was measured and analyzed. The parameters as total loading protein (native tick protein) with primary and secondary antibodies, were the same. The measurements were performed in triplicate to calculate the average and the standard deviation. Statistical analysis estimated the differences between the two bands in the same lane in the western blot membrane using the two-tailed Student *t*-test with equal variance.

Results

Tick colony establishment

The PCR result revealed that all expected amplicon size (850bp) of *R. sanguineus CO1* gene was detected in all examined samples (9 samples) as shown in Fig. 3. The authors established and maintained the identified and pathogen-free *R. sanguineus* tick on rabbits' laboratory colonies at the National Research Centre. The different developmental stages of the established colony were illustrated in Fig.s 4, 5, 6, and 7.

In silico sequence analysis for R. sanguineous AQP1 and Fer2 protein

The bioinformatic analysis showed that the *RsAQP1* gene, a water-specific gene, isolated from salivary gland mRNA full length was 1,194 bp with two untranslated regions of 120 bp and 202 bp at the 5' and 3' termini, respectively. A polyadenylation signal, AATAAA, was located 15 bp upstream of the poly(A) tail [15]. Gene and encoding protein characters are shown in Table 1. The *RsFer2* gene, an iron storage protein, is a gene coding sequence conserved among eight hard tick species [38]. The mRNA gene length is 668 bp. The characterization of these genes and encoding proteins are shown in Table 1.

Aquaporin 1 (AQP1) and ferritin 2 (Fer2) are entirely unrelated proteins with distinct structures and physiological functions. Both RsAQP1 and RsFer2 as independent yet complementary vaccine targets due to their respective, well-documented critical roles in tick survival and reproduction. Our *in silico* analysis confirmed the presence of conserved motifs characteristic of functional aquaporins, suggesting its potential as a functional protein. While none of the TM domains were found in the RsFer2 protein (Fig. 8).

Based on antigenicity predictions performed via EMBOSS Explorer, all three antigens so demonstrated antigenic potential. The average scores were 1.0321 for RsFer2, 1.0586 for RsAQP1, and 1.0415 for the RsAQP1 synthetic peptide, indicating their promise for vaccine development.

Immune response simulation

The simulated immune response using both RsAQP1 peptide and rRsFer2 protein as vaccine candidates for 50 days revealed an elicited humoral immune response throughout the vaccine trial schedule in the case of individual rRsFer2 protein or combination with RsAQP1 peptide. The IgG and IgM antibodies reached their highest level in the case of the controls, the Bm86, the commercial anti-tick vaccine “Gavac vaccine for *R. microplus*”, achieved 170,000 arbitrary units on day 30, and its homologue *R. sanguineus* Rs86 reached 200,000 arbitrary units on day 30 (Fig. 9). In the case of the combination of both vaccine candidates, IgG and IgM antibodies reached a robust level of 190,000 arbitrary units on day 30 (Fig. 10A), while it was 170,000 arbitrary units on day 30 in the rRsFer2 individual case (Fig. 10A) which is almost similar to the controls in both cases. In addition, the pronounced level (5×10^5 ng/ml) of IFN-gamma was remarkable throughout the vaccination schedule (Fig. 10B) in individual and dual immune simulations, which was typical of the same level in the case of the control (Fig. 9). Likewise, the Bm86 and Rs86, the danger signal (D) was predicted as 0 level during individual or dual vaccination using rRsFer protein (Fig. 9 and Fig. 10B). In contrast, the D signal was predicted as the highest level reached 10,000 ng/ml upon simulation using individual synthetic AQP1 peptide (Fig. 10B).

Antibody responses in dogs immunized with R. sanguineus AQP1-KLH peptide and rFer2 protein

ELISA results demonstrated that all immunized dogs successfully mounted antigen-specific antibody responses (Fig. 11). Notably, immunization with the AQP1 peptide alone (Group A) consistently elicited lower antibody titers compared to vaccination with the rFer2 protein alone or the combined AQP1-rFer2 cocktail. Specifically, anti-AQP1-KLH antibodies in Group A showed an initial peak (OD 1.9) after the first immunization, remaining stable until week 6 (Fig. 11A). In the cocktail group (Group C), anti-AQP1-KLH antibody levels reached their peak at week 6 (OD 1.8), suggesting a slightly different response pattern in terms of kinetics or magnitude when co-administered. Statistical analysis indicated no significant difference in anti-AQP1-KLH titers between Group A and the cocktail Group C (P=0.74), yet significant differences were observed when Group A was compared to Group B (P=0.04) and the control Group D (P=0.0098).

Conversely, anti-rFer2 protein antibody levels in the single vaccination group (Group B) steadily climbed, reaching a peak OD of 2.23 at week 6 (Fig. 11B). The cocktail group (Group C) also showed a steady increase in anti-rFer2 antibodies, with a peak around OD 2.0 at week 6, slightly less than the single rFer2 vaccination. There was no significant difference in anti-rFer2 titers between Group B and Group C (P=0.17). As expected, Group C's anti-rFer2 response was significantly different from Group A (P=0.005).

Recognition of native tick proteins AQP-1 and Fer2 by vaccinated dog antibodies

In SDS-PAGE analysis, the *R. sanguineus* unfed adult tick native protein and rFer2 were fractionated using 12% gel under a reduced condition. As demonstrated in Fig. 12, the blue arrow indicates the expected size of *R. sanguineus* AQP1 protein (~ 30 kDa). The black arrow indicates the expected size of the native Fer2 protein in *R. sanguineus* unfed adult antigen (~ 22 kDa). To detect the immune reactivity and specificity of the resulting antibodies from the vaccinated dog groups, native *R. sanguineus* unfed adult homogenate was used as the antigen in the western blot against the dog serum. Serum antibodies of dogs vaccinated with AQP1-KLH peptide, either in the case of individual peptide vaccination (G1) or in the case of mixed vaccination (G3) reacted with native proteins of *R. sanguineus* unfed adult (Fig. 13). The reactive band was visualized at the expected size of AQP1 native size (~ 30 kDa). The same was observed in the case of rFer2 protein; the reactive band was observed at a location close to the expected size (~ 20 kDa). Serum from the control group (G4) showed no reactivity with the tick native antigen, as it was expected (Fig. 13).

Band intensity percentage analysis revealed that AQP1 and Fer2 proteins band display a distinct intensity compared to each other (Fig. 14). The AQP1 band intensity percentage was significantly more than the one for Fer2 protein band ($P = 5.97295^{E-06)}$. Band intensity percentage measurement for both proteins is shown in Fig. 14.

Discussion

The brown dog tick (*R. sanguineus*), or kennel tick, is a widespread external parasite. This tick is particularly concerning due to its role in transmitting diseases to dogs, other animals, and even people. In this study, we characterized the immunogenicity of *R. sanguineus* AQP1 peptide and rFer2 protein as single or dual anti-tick vaccine candidates. The antigenic analysis was based on an *in silico* prediction, and an *in vivo* immunological investigation. The results of the immune system simulation support the hypothesis of a dual anti-tick vaccine using rFer2 protein and AQP1 peptide. Antigenicity predictions confirmed their potential as antigens. Importantly, the serological results referring to the presence of reactive antibodies suggest the actual antigenicity of both proteins.

The *in silico* immune response simulations revealed distinct immunological profiles for each vaccine candidate, either individually or in combination (Fig. 10). Individually, the RsAQP1 peptide was predicted to elicit no antibody isotypes with a high level of danger signal. Although a high “danger signal” was predicted, this suggests activation of cellular immunity and a robust cytokine response (Fig. 10B). Conversely, the individual immune simulation of recombinant RsFer2 protein as a vaccine candidate induced a good level of total antibody isotypes reaching 170,000 arbitrary units with no level of danger signal (Fig. 10A). The strong immunogenicity predicted for RsFer2 is consistent with the success of other tick protein vaccines like Bm86 (Gavac), and its homologue Rs86 (Fig. 9), validating its potential as a vaccine candidate. Interestingly, the immune stimulation of both antigens as a dual anti-tick vaccine elicited the highest antibody isotypes, reaching 190,000 arbitrary units, also with no danger signal level (Fig. 10A and B). This could mean a potential synergistic interaction between the two antigens, rather than causing antigenic competition, where they may enhance the overall humoral immune response [39, 40]. The minimal danger signal predicted for the RsFer2 and cocktail formulations suggests a potentially favorable safety profile with a lower risk of inflammatory side effects *in vivo*. Despite the low predicted antibody production for the RsAQP1 peptide alone, it was included in our *in vivo* experiment for several key reasons. First, its critical role in tick osmoregulation makes it a valuable biological target. Second, the *in silico* model itself predicted a strong cellular response (evidenced by the high danger signal and cytokine production). Many effective vaccines aim to stimulate a combined humoral and cellular response. Finally, previous studies on AQP1(or related AQPs) family proteins in other tick species have shown promise, supporting its inclusion for experimental validation [13, 15, 33].

Baladi dogs were immunized with RsAQP1 peptide and rRsFer2 protein, either individually or in combination. Total IgG was estimated against both vaccine candidates using indirect ELISA. The individual immunization with RsAQP1 peptide elicited a lower level of total specific IgG (Fig. 11A) than the immunization with rRsFer2 protein as a single vaccine candidate (Fig. 11B) compared to the control group, upon using their corresponding antigen in a detection assay. This result affirmed the predicted data of the individual immune simulation of RsAQP1 peptide, which could be returned to failure of RsAQP1 peptide to be recognized by multiple MHC I and/or MHC II alleles [41, 42]. That might be attributed to inefficient antigen processing or inappropriate proteasomal cleavage process, and the nonspecific binding affinity between the peptide and MHC I and II [43]. Consequently, the B cell is not stimulated enough to produce total IgG. In contrast, the immunization with rRsFer2 protein as a single vaccine candidate comes with individual immune simulation prediction of the rRsFer2 protein. The possible explanation could be attributed to the immunogenicity of this antigen and its capability to induce total IgG, as it had multiple B-cell and T-cell epitopes that were immunodominant enough to induce the host immune system [44, 45]. Thus, the dual immunization with both vaccine candidates showed the same consistency and elicited a good level of total specific IgG against rFer2 protein more than AQP1 peptide (Fig. 11A and B), compared to the other group.

A blotting analysis was performed to prove the specificity of the total IgG elicited in all immunized groups. The specificity of the total IgG against each vaccine candidate is revealed in Fig. 13. The native *R. sanguineus* unfed adults showed a reactivity at the specific molecular weight of ~30 kDa for AQP1 and ~20 kDa for rFer2 proteins. Simultaneously, the reactivity against the *R. sanguineus* AQP1-KLH peptide and rFer2 protein in immunized groups (G1, G2, and G3) versus the control group (G4) assuring the specificity of total IgG. In the case of the Fer2 protein, it was noticeable that the molecular mass of native proteins was larger than expected, and that could be possibly due to the formation of common dimers in SUB/AKR with functional implications as well as the glycosylation of Fer2 protein [46, 47]. Moreover, in the other studies, based on the amino acid sequences, the molecular mass of native midgut SUB in the *Rhipicephalus haemaphysaloides* and that of FER2 in *Ornithodoros* (*O*) *erraticus* and *O. moubata* were slightly larger than expected. However, antibodies were specific to their respective epitopes [47, 48]. The interpretation of the attachment of carbohydrate chains to proteins (glycosylation) alters the protein's three-dimensional structure and stability. Moreover, it could mask peptide epitopes or expose a unique epitope to the immune cells. Therefore, it will affect antigenicity or immune recognition. In addition, Dimerization of protein might result in low immunogenicity due to hidden epitopes or might result in better immunogenicity due to multiepitopes displayed with dimer formation. Therefore, the western blot specificity could be affected according to the state of glycosylation and dimer formation.

The relative intensity of the bands for RsAQP1-KLH and RsFer2 means relative antibody response to each protein in the serum, not the expression level of the native proteins in the tick. The higher intensity observed for RsAQP1, therefore, indicates that the antibody response generated against RsAQP1-KLH in the cocktail-vaccinated dogs is proportionally stronger in its recognition of the native protein compared to the antibody response against RsFer2 by the same serum, under the conditions tested (Fig. 14) although it has more antibodies against RsFer2 than RsAQP1-KLH.

Further study regarding the tick fitness parameters and elucidation of immune-protective traits of these elicited total IgG will be investigated as specific and cross-protective vaccine candidates in future work.

Conclusion

The current work was designed to define and characterize *in silico* and *in vivo* immune reaction against AQP1 predicted immunogenic peptide and rFer2 protein upon dog vaccination. Either immune simulation or real animal vaccination results prove lower antibody reaction against *R. sanguineus* AQP1-KLH peptide if compared with the *R. sanguineus* rFer2 protein. Although our results indicated the recognition of the native tick antigen by the vaccinated dog serum either in case of individual or combined vaccination, the involvement of tick application on vaccinated animals is recommended to confirm the resultant data

Acknowledgments

Not applicable.

Funding statement

The research was supported by a joint research grant from the Science and Technology Development Fund [STDF], Egypt (Grant Number 42839) and the Japan Society for the Promotion of Science [JSPS] Bilateral Program, Japan (Grant Number JPJSBP120206002).

Declaration of Conflict of Interest

The authors declare that they have no competing interests.

Ethical of approval

All institutional and national guidelines for the care and use of animals were followed. The protocol was approved by the Egyptian Medical Research Ethics Committee of the National Research Center (No. 20-187, 3/12/2020).

TABLE 1. The predicted features of *RsAQP1* and *RsFer2* genes and their putative proteins.

Gene	Protein length [aa]	Molecular weight [kDa]
RsAQP1	291	30,929
RsFer2	172	19,905

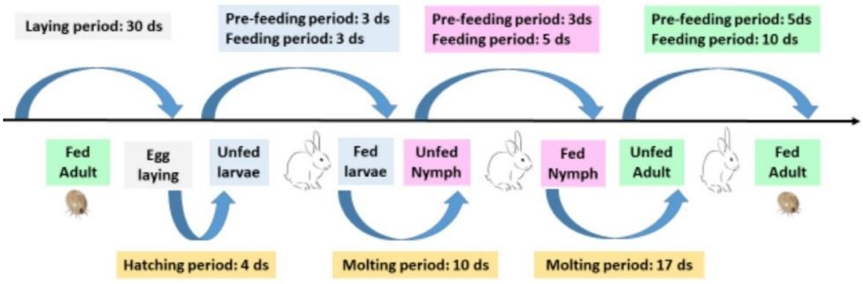


Fig. 1. Schematic illustration of *Rhipicephalus sanguineus* tick life cycle that reared on rabbit *in vitro* [90 Days (ds)].

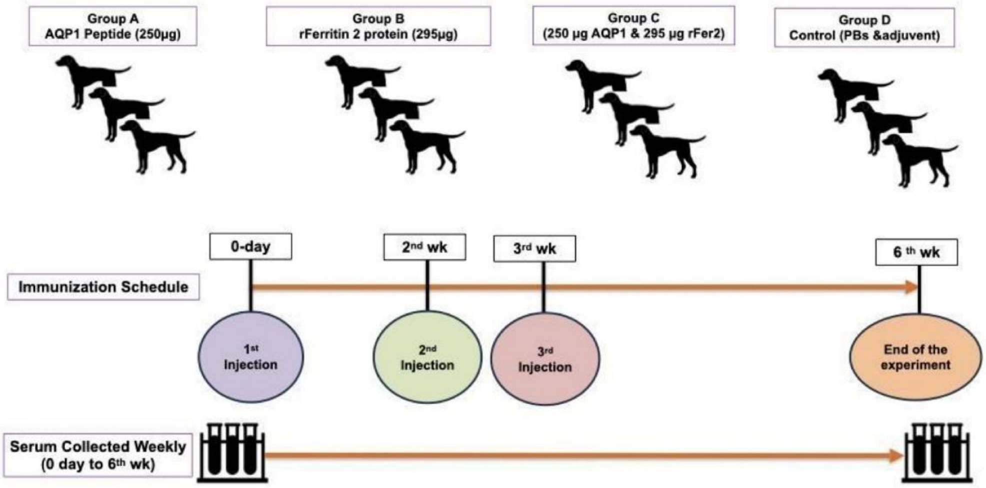


Fig. 2. Schematic representation of dog immunization schedule by AQP1-KLH synthetic peptide and rFer2 protein.

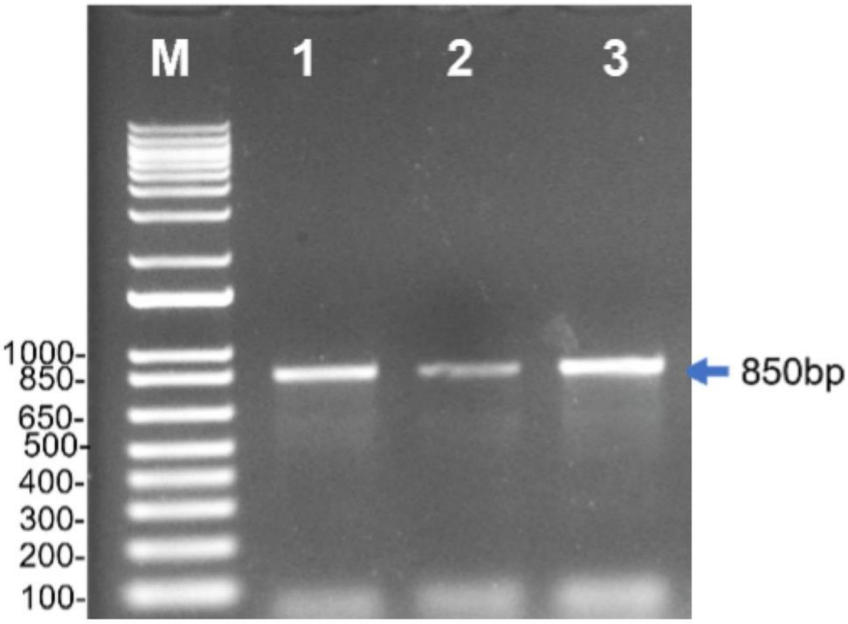


Fig. 3. Molecular identification of tick species (*R. sanguineus*) by PCR using 1.5% agarose gels stained with ethidium bromide. Lane M: 1 Kb Plus DNA Ladder, Lane 1,2,3 fully fed female ticks gave the expected amplicon size (850bp) of *R. sanguineus CO1* gene.

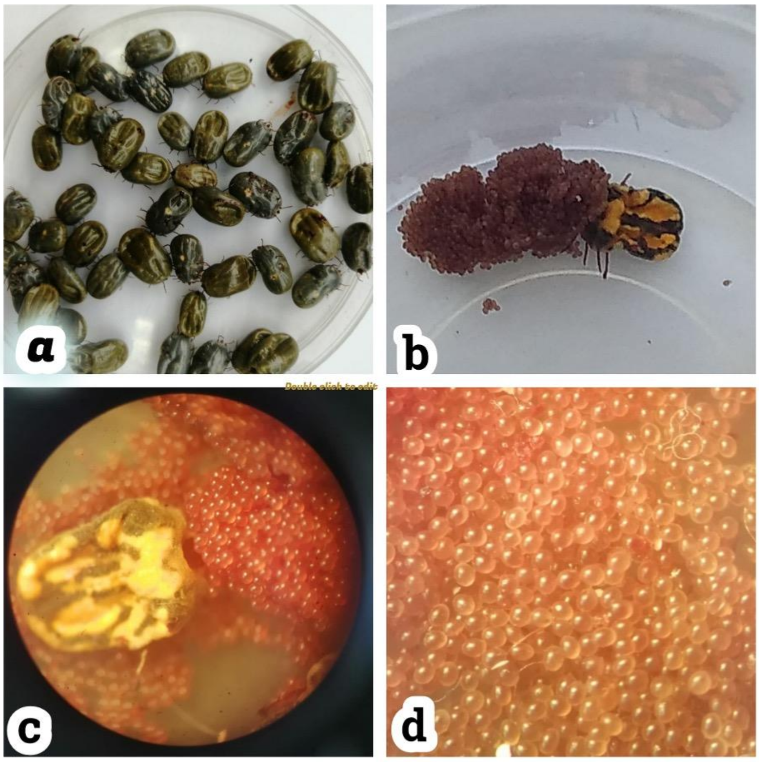


Fig. 4. The oviposition of *R. sanguineus* tick: a) Fully- fed *R. sanguineus* female b) & c) *R. sanguineus* female after egg laying. d) *R. sanguineus* eggs under binocular microscope.

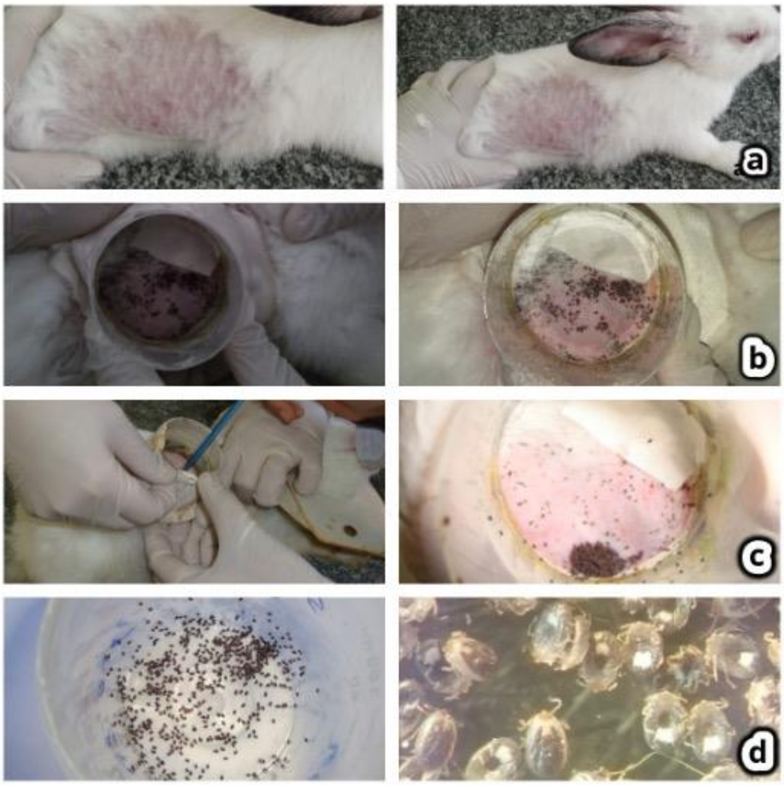


Fig. 5. The rearing of the larval stages *in vitro* on rabbit back capsule. A) Shaved rabbit and application of back capsule. B) Partially fed larvae attached on the shaved rabbit skin. C) Collection of engorged larvae. D) Incubation of fully fed larvae for molting.

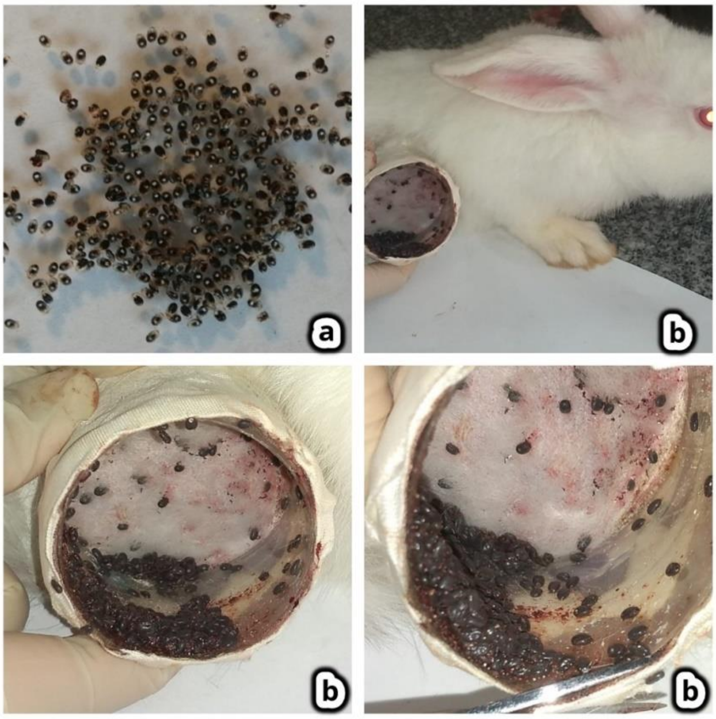


Fig. 6. The rearing of the nymph stages *in vitro* on rabbit using back capsule. A) Molting and emerging of unfed nymph. B) Collection of fully fed nymph after their feeding period on the rabbit back capsule (5 days).

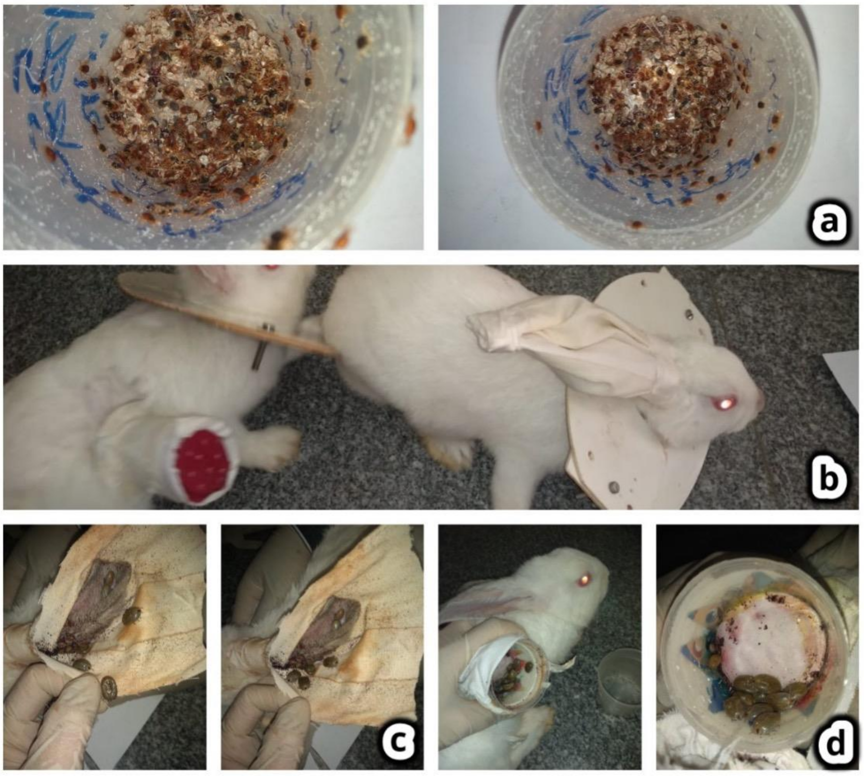
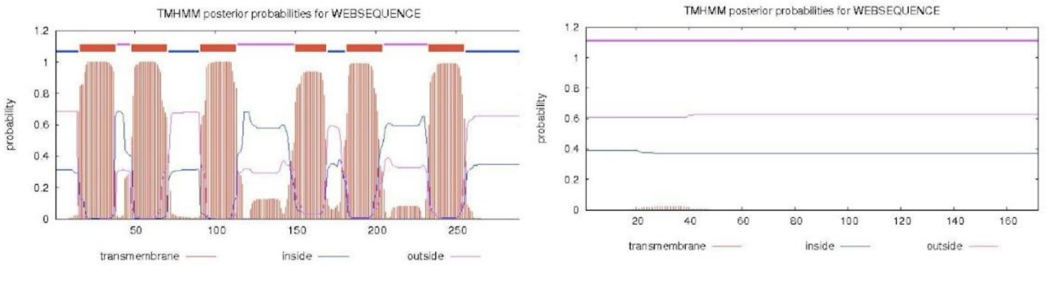


Fig. 7. The rearing of the adult stages *in vitro* on rabbit using back capsule and ear bag. A) Molting and emerging of *R. sanguineus* unfed adults reared on rabbit. B) Rearing of *R. sanguineus* unfed adults on rabbit using back capsule and ear bag. C) Collection of fully fed adult *R. sanguineus* tick from ear bag. D) Collection of fully fed adult *R. sanguineus* tick from back capsule.



R. sanguineus AQP1

R. sanguineus Ferretin 2

Fig. 8. The results of trans-membrane domain prediction for RsAQP1 and RsFer2 proteins.

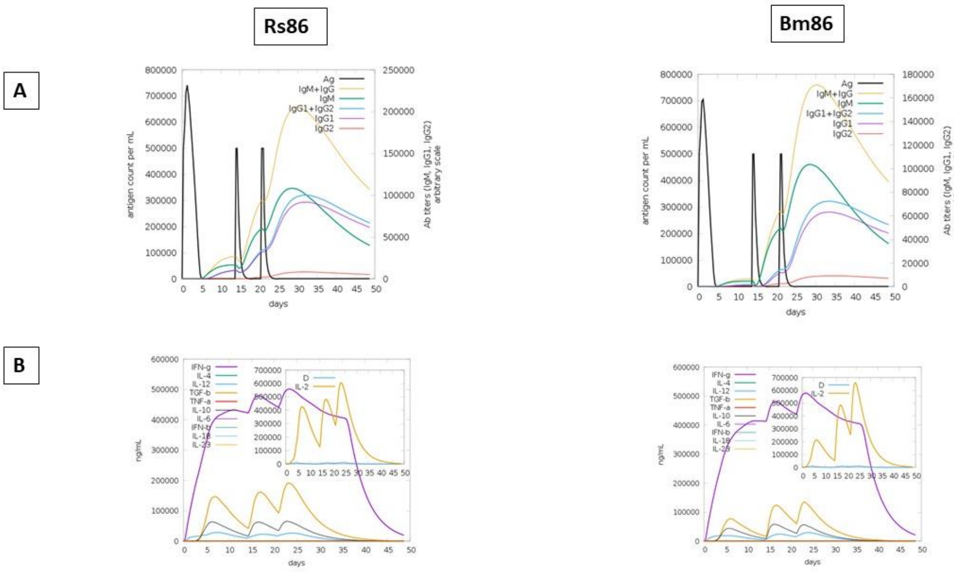


Fig. 9. The immune response prediction for Rs86 and Bm86 proteins as control positive. The levels of antibody isotypes (IgM, IgG1 and IgG2) prediction (A), IFN-gamma predicted level (B).

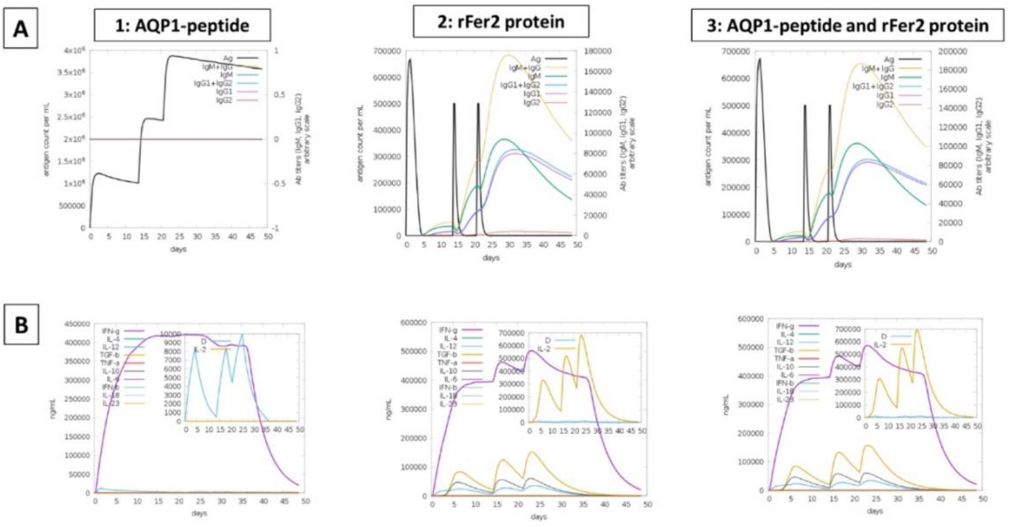


Fig. 10. Demonstration of the immune response prediction for using both RsAQP1 peptide and rRsFer2 protein as vaccine candidates. The levels of antibody isotypes (IgM, IgG1 and IgG2) prediction (A), IFN-gamma predicted level (B).

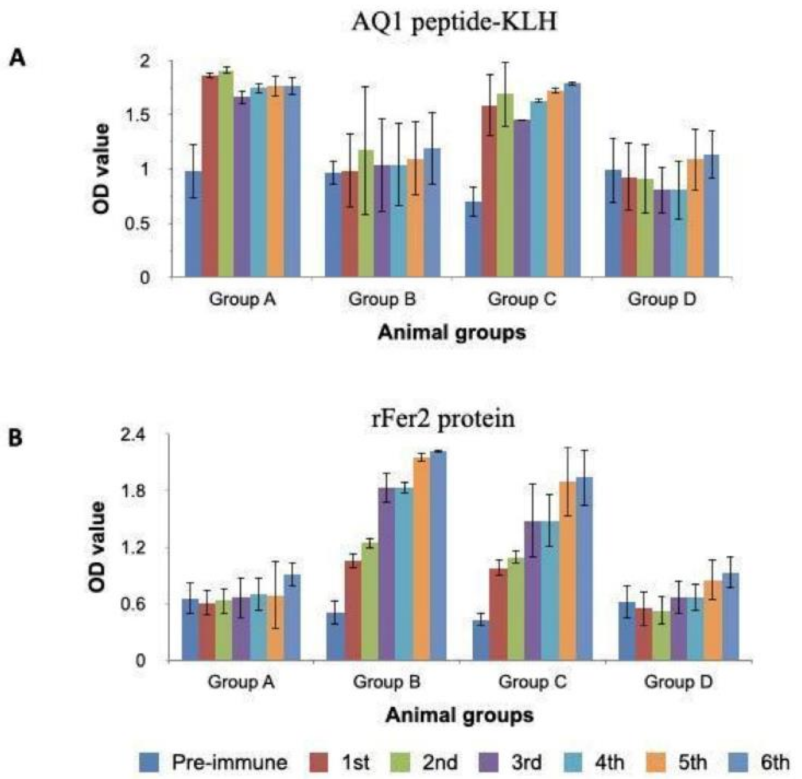


Fig. 11. Detection of anti-*R. sanguineus* AQP1-KLH peptide and rFer2 protein in vaccinated Baladi dogs using indirect ELISA. Optical density (OD) values were obtained in an ELISA analysis (Y axis). Serum was collected from different accinated animal groups (X axis). Error bars represent the standard error of the mean (SEM) of the measured values.

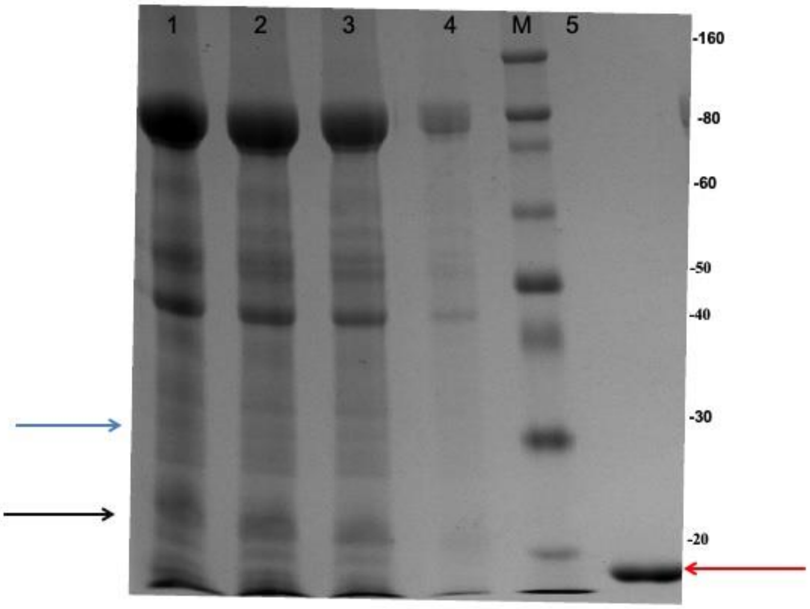


Fig. 12. A Gel electrophoresis 12% of *R. sanguienus* unfed adult and ferritin 2 recombinant protein. Lane M Marker Novex™ Sharp Pre-stained Protein Standard, Lane 1,2,3 and 4 unfed *R. sanguienus* adult native antigen with different concentration (90, 70, 50 and 30ug/lane) respectively. Lane 5 ferritin 2 recombinant protein. Blue arrow indicates the expected size of aquaporin 1 in *R. sanguienus* adult antigen (~30 kDa). Black arrow indicates the expected size of ferritin 2 in *R. sanguienus* adult antigen (~22 kDa). Red arrow indicates the expected size of ferritin 2 recombinant protein~20 KDa (19.9 kDa).

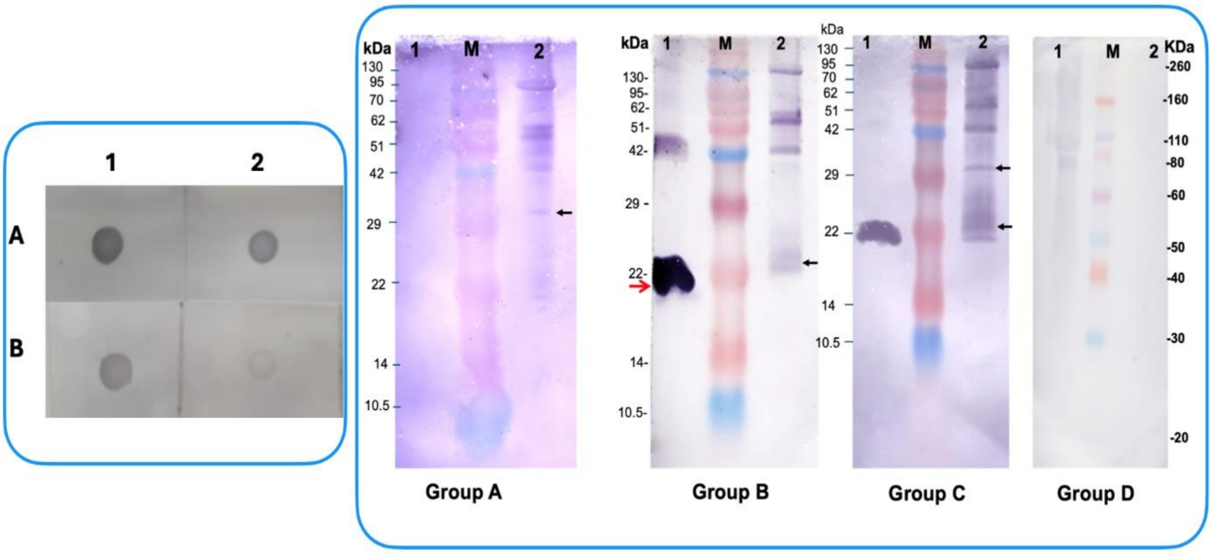


Fig. 13. The left part shows the results of Dot blot analysis of unfed *R. sanguineus* adult antigen and AQP1-KLH peptide. Lane 1: unfed *R. sanguineus* adult antigen, lane 2: *R. sanguineus* AQP1-KLH peptide with concentration 15 µg. Row A: antigens blotted against dog serum (GroupA) vaccinated with AQP1-KLH peptide. Row B: antigens blotted against dog serum (Group D) vaccinated with only adjuvant. The right part demonstrates Western blot analysis of unfed *R. sanguineus* adult antigen Lane1: rFer2 protein, Lane 2: Sera from vaccinated dogs in different groups were used, Lane M: Chromatein Prestained Protein Ladder (Vivantis, Malaysia) for group A, B and C. Novex™ Sharp Pre-stained Protein Standard marker for group D. In group A, black arrow indicates the expected size of native AQ1 in *R. sanguineus* adult antigen, (~30 kDa). In group B, black arrow indicates the expected size of native Ferritin 2 in *R. sanguineus* adult antigen, (~20 kDa). Group C, upper and lower black arrow indicates the expected size of native AQP1 and native ferritin 2 in *R. sanguineus* adult antigen respectively.

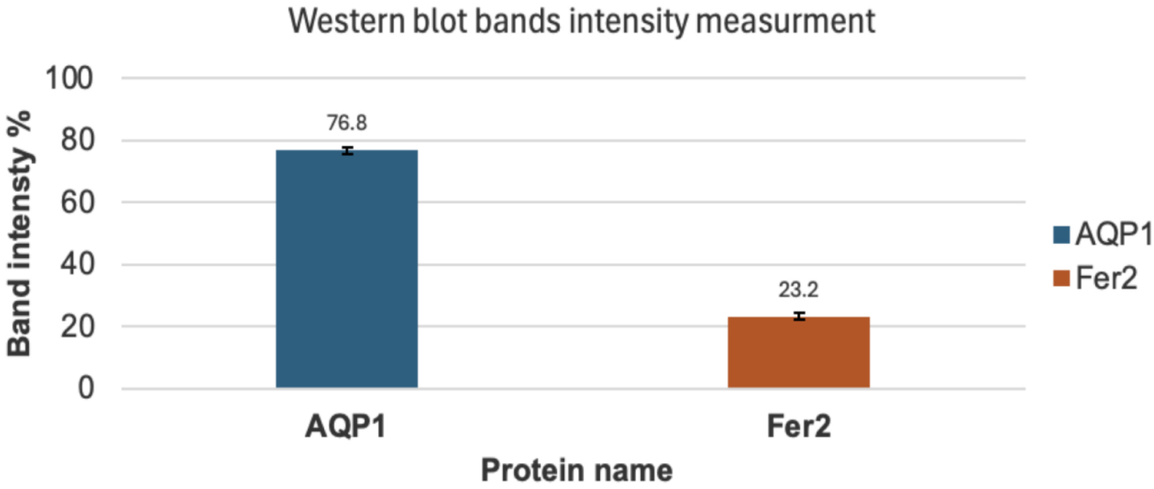


Fig. 14. Image Analysis and Quantitation for Western Blotting shown in chart with band intensity percentage (Y axis) and protein name (X axis).

References

1. Évora, P.M., Sanches, G.S., Guerrero, F.D., León, A.P and de Bechara, G.H.: Immunogenic potential of *Rhipicephalus* (*Boophilus*) *microplus* aquaporin 1 against *Rhipicephalus sanguineus* in domestic dogs. *Revista Brasileira de Parasitologia Veterinária*. **26**, 60–66 (2017)
2. Abdel-Ghany, H., Alzan, H., Hendawy, S., El-Sadawy, H. and Abdel-Shafy, S.: In vitro evaluation of the effect of entomopathogenic nematodes on different developmental stages of the brown dog tick Rhipicephalus sanguineus (Acari: Ixodidae). *Acarologia*,**64**,323–334(2024). https://doi.org/10.24349/1hyi-f1vq
3. Abdullah, H.H.A.M., El-Molla, A., Salib, F.A., Allam, N.A.T., Ghazy, A.A. and Abdel-Shafy, S.: Morphological and molecular identification of the brown dog tick *Rhipicephalus sanguineus* and the camel tick *Hyalomma dromedarii* (Acari: Ixodidae) vectors of Rickettsioses in Egypt. *Vet. World,* **9**, 1087 (2016)
4. Dantas-Torres, F. and Otranto, D.: Rhipicephalus sanguineus (Brown dog tick). *Trends in Parasitology. Trends Parasitol*., **38**, 993–994 (2022). https://doi.org/10.1016/j.pt.2022.08.011
5. Sabadin, G.A., Parizi, L.F., Kiio, I., Xavier, M.A., da Silva Matos, R., Camargo-Mathias, M.I., Githaka, N.W., Nene, V. and da Silva Vaz Jr, I.: Effect of recombinant glutathione S-transferase as vaccine antigen against *Rhipicephalus appendiculatus* and *Rhipicephalus sanguineus* infestation. *Vaccine,* **35**, 6649–6656 (2017)
6. McGarry, J.W.: Travel and disease vector ticks. *Travel Med. Infect. Dis*., **9**, 49–59 (2011)
7. Otranto, D., Dantas-Torres, F. and Breitschwerdt, E.B.: Managing canine vector-borne diseases of zoonotic concern: part one. *Trends Parasitol***.,** **25**, 157–163 (2009)
8. Alzan, H.F., Mahmoud, M.S. and Suarez, C.E.: Current vaccines, experimental immunization trials, and new perspectives to control selected vector borne blood parasites of veterinary importance. *Front Vet Sci*., **11**, 1484787 (2024)
9. Ndekezi, C., Nkamwesiga, J., Ochwo, S., Kimuda, M.P., Mwiine, F.N., Tweyongyere, R., Amanyire, W. and Muhanguzi, D.: Identification of Ixodid tick-specific aquaporin-1 potential anti-tick vaccine epitopes: An in-silico analysis. *Front. Bioeng Biotechnol*., **7**, 236 (2019)
10. Zeb, I., Parizi, L.F., Israr, M., da Silva Vaz Jr, I. and Ali, A.: Cross-species immunoprotective antigens (subolesin, ferritin 2 and P0) provide protection against *Rhipicephalus sanguineus sensu lato*. *Parasit. Vectors,* **17**, 3 (2024)
11. Nepveu-Traversy, M.-E., Fausther-Bovendo, H. and Babuadze, G.: Human tick-borne diseases and advances in anti-tick vaccine approaches: A comprehensive review. *Vaccines* (Basel). **12**, 141 (2024)
12. Mihajlović, J., Hovius, J.W.R., Sprong, H., Bogovič, P., Postma, M.J. and Strle, F.: Cost-effectiveness of a potential anti-tick vaccine with combined protection against Lyme borreliosis and tick-borne encephalitis in Slovenia. *Ticks Tick Borne Dis*., **10**, 63–71 (2019)
13. Guerrero, F.D., Andreotti, R., Bendele, K.G., Cunha, R.C., Miller, R.J., Yeater, K., Pérez and de León, A.A.: *Rhipicephalus* (*Boophilus*) *microplus* aquaporin as an effective vaccine antigen to protect against cattle tick infestations. *Parasit Vectors*, **7**, 1–12 (2014)
14. Hussein, H.E., Scoles, G.A., Ueti, M.W., Suarez, C.E., Adham, F.K., Guerrero, F.D. and Bastos, R.G.: Targeted silencing of the Aquaporin 2 gene of *Rhipicephalus* (*Boophilus*) *microplus* reduces tick fitness. *Parasit. Vectors***, 8**, 1–12 (2015)
15. Ball, A., Campbell, E.M., Jacob, J., Hoppler, S. and Bowman, A.S.: Identification, functional characterization and expression patterns of a water-specific aquaporin in the brown dog tick, *Rhipicephalus sanguineus*. *Insect Biochem Mol Biol*., **39**, 105–112 (2009). https://doi.org/https://doi.org/10.1016/j.ibmb.2008.10.006
16. Hendawy, S.H.M., Alzan, H.F., Abdel-Ghany, H.S.M., Suarez, C.E. and Kamel, G.: Biochemical analysis of *Hyalomma dromedarii* salivary glands and gut tissues using SR-FTIR micro-spectroscopy. *Sci. Rep*., **14**, 8515 (2024)
17. Galay, R.L., Umemiya-Shirafuji, R., Mochizuki, M., Fujisaki, K. and Tanaka, T.: Iron metabolism in hard ticks (Acari: Ixodidae): the antidote to their toxic diet. *Parasitol. Int.,* **64**, 182–189 (2015)
18. Galay, R.L., Miyata, T., Umemiya-Shirafuji, R., Maeda, H., Kusakisako, K., Tsuji, N., Mochizuki, M., Fujisaki, K. and Tanaka, T.: Evaluation and comparison of the potential of two ferritins as anti-tick vaccines against Haemaphysalis longicornis. *Parasit Vectors,* **7**, 1–10 (2014)
19. Galay, R.L., Aung, K.M., Umemiya-Shirafuji, R., Maeda, H., Matsuo, T., Kawaguchi, H., Miyoshi, N., Suzuki, H., Xuan, X. and Mochizuki, M.: Multiple ferritins are vital to successful blood feeding and reproduction of the hard tick *Haemaphysalis longicornis*. *Journal of Experimental Biology*, **216**, 1905–1915 (2013)
20. Gao, Z., Zheng, P., Wang, K., Ji, X., Shi, Y., Song, X., Liu, J., Yu, Z. and Yang, X.: The molecular and functional characterization of ferritins in the hard tick *Hyalomma rufipes*. *Parasit. Vectors,* **15**, 368 (2022)
21. Aguttu, C., Okech, B.A., Mukisa, A. and Lubega, G.W.: Screening and characterization of hypothetical proteins of *Plasmodium falciparum* as novel vaccine candidates in the fight against malaria using reverse vaccinology. *Journal of Genetic Engineering and Biotechnology*. **19**, 103 (2021). https://doi.org/https://doi.org/10.1186/s43141-021-00199-y
22. Rawal, K., Sinha, R., Abbasi, B.A., Chaudhary, A., Nath, S.K., Kumari, P., Preeti, P., Saraf, D., Singh, S. and Mishra, K., Identification of vaccine targets in pathogens and design of a vaccine using computational approaches. *Sci. Rep*., **11,** 17626 (2021)

23. Walker, A.R.: Ticks of domestic animals in Africa: a guide to identification of species. Bioscience Reports Edinburgh (2003)
24. Mangold, A.J., Bargues, M.D. and Mas-Coma, S.: Mitochondrial 16S rDNA sequences and phylogenetic relationships of species of *Rhipicephalus* and other tick genera among *Metastriata* (Acari: Ixodidae). *Parasitol. Res*., **84**, 478–484 (1998)
25. Erster, O., Roth, A., Wolkomirsky, R., Leibovich, B. and Shkap, V., Comparative analysis of mitochondrial markers from four species of *Rhipicephalus* (Acari: Ixodidae). *Vet. Parasitol*., **198**, 364–370 (2013)
26. Lv, J., Wu, S., Zhang, Y., Chen, Y., Feng, C., Yuan, X., Jia, G., Deng, J., Wang, C. and Wang, Q.: Assessment of four DNA fragments (COI, 16S rDNA, ITS2, 12S rDNA) for species identification of the *Ixodida* (Acari: Ixodida). *Parasit Vectors,* **7**, 93 (2014)
27. Chitimia, L., Lin, R.-Q., Cosoroaba, I., Wu, X.-Y., Song, H.-Q., Yuan, Z.-G. and Zhu, X.-Q.: Genetic characterization of ticks from southwestern Romania by sequences of mitochondrial cox 1 and nad 5 genes. *Exp. Appl. Acarol*., **52**, 305–311 (2010)
28. Abdel-Shafy, S.: Scanning electron microscopy and comparative morphology of *Hyalomma anatolicum excavatum*, *H. dromedarii* and *H. marginatum marginatum* (Acari: Ixodidae) based on nymphs. *Acarologia,* **48**, 3–18 (2008)
29. Abo Talep, E., Abuowarda, M., Abdelshafy, S., Mahmoud, N.E. and Fahmy, M.: Seasonal Variation and Morphometric Differentiation of Egyptian Strain of *Rhipicephalus sanguineus* (Acari: Ixodidae). *Egyptian Journal of Veterinary Sciences*. **55**, 1109–1118 (2024)
30. Heyne, H., Elliott, E.G.R. and Bezuidenhout, J.D.: Rearing and infection techniques for *Amblyomma* species to be used in heartwater transmission experiments. (1987)
31. Rapin, N., Lund, O., Bernaschi, M. and Castiglione, F.: Computational immunology meets bioinformatics: the use of prediction tools for molecular binding in the simulation of the immune system. *PLoS One*, **5**, e9862 (2010)
32. Alzan, H.F., Bastos, R.G., Ueti, M.W., Laughery, J.M., Rathinasamy, V.A., Cooke, B.M. and Suarez, C.E.: Assessment of *Babesia bovis* 6cys A and 6cys B as components of transmission blocking vaccines for babesiosis. *Parasit Vectors*. **14**, 1–13 (2021)
33. Scoles, G.A., Hussein, H.E., Olds, C.L., Mason, K.L. and Davis, S.K.: Vaccination of cattle with synthetic peptides corresponding to predicted extracellular domains of *Rhipicephalus* (*Boophilus*) *microplus* aquaporin 2 reduced the number of ticks feeding to repletion. *Parasit. Vectors*, **15**, 49 (2022)
34. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Boil. Chem*., **193**, 265–275 (1951)
35. Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685 (1970)
36. Towbin, H., Staehelin, T. and Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of The National Academy Of Sciences*, **76,** 4350–4354 (1979)
37. Heinicke, E., Kumar, U. and Munoz, D.G.: Quantitative dot-blot assay for proteins using enhanced chemiluminescence. *J. Immunol. Methods*. **152**,227–236(1992). https://doi.org/https://doi.org/10.1016/0022-1759(92)90144-I
38. Xu, G., Fang, Q. Q., Keirans, J. E. and Durden, L. A. Ferritin gene coding sequences are conserved among eight hard tick species (Ixodida: Ixodidae). *Annals of the Entomological Society of America*, **97**, 3, 567-573(2004).
39. Ndawula Jr, C. and Tabor, A.E.: Cocktail anti-tick vaccines: The unforeseen constraints and approaches toward enhanced efficacies. *Vaccines* (*Basel*). **8**, 457 (2020)
40. Ndawula Jr, C.: From bench to field: A guide to formulating and evaluating anti-tick vaccines delving beyond efficacy to effectiveness. *Vaccines* (*Basel*), **9**, 1185 (2021)
41. Carvalho, L.H., Sano, G., Hafalla, J.C.R., Morrot, A., De Lafaille, M.A.C. and Zavala, F.: IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat Med*., **8**, 166–170 (2002)
42. Jurado, A., Carballido, J., Griffel, H., Hochkeppel, H.K. and Wetzel, G.D.: The immunomodulatory effects of interferon-gamma on mature B-lymphocyte responses. *Experientia*., **45**, 521–526 (1989)
43. Yang, Y., Wei, Z., Cia, G., Song, X., Pucci, F., Rooman, M., Xue, F. and Hou, Q.: MHCII-peptide presentation: an assessment of the state-of-the-art prediction methods. *Front. Immunol*., **15**, 1293706 (2024)
44. Parizi, L.F., Githaka, N.W., Logullo, C., Zhou, J., Onuma, M., Termignoni, C. and da Silva Vaz Jr, I.: Universal tick vaccines: candidates and remaining challenges. *Animals*, **13**, 2031 (2023)
45. Abbas, M.N., Jmel, M.A., Mekki, I., Dijkgraaf, I. and Kotsyfakis, M., Recent advances in tick antigen discovery and anti-tick vaccine development. *Int. J. Mol. Sci*., **24**, 4969 (2023)
46. Kasaija, P.D., Contreras, M., Kabi, F., Mugerwa, S. and Fuente, J. de la: Vaccination with recombinant subolesin antigens provides cross-tick species protection in Bos indicus and crossbred cattle in Uganda. *Vaccines* (*Basel*), **8**, 319 (2020)
47. Oleaga, A., González-Pérez, S. and Peréz-Sánchez, R.: First molecular and functional characterisation of ferritin 2 proteins from *Ornithodoros argasid* ticks. *Vet. Parasitol.*, **304**, 109684 (2022)
48. Lu, P., Zhou, Y., Yu, Y., Cao, J., Zhang, H., Gong, H., Li, G. and Zhou, J.: RNA interference and the vaccine effect of a subolesin homolog from the tick *Rhipicephalus haemaphysaloides*. *Exp. Appl. Acarol*., **68**, 113–126 (2016)

التوصيف المناعي الحاسوبي والمختبري لببتيد الأكوابورين 1 والبروتين المؤتلف الفيريتين 2 في قرادة الكلب البنية الريبسيفلس سانجوينس لتطوير لقاح

هبة فتحي الظن [1،2]، سهام هنداوي [1،2]، بسمة الصاوي [1،2]، هدى عبد الغني [1،2]، صبحي عبد الشافي [1،2]، تيتسويا تاناكا [3،4] ومنى سعيد محمود [1،2]

[1] قسم الطفيليات وأمراض الحيوان، معهد البحوث البيطرية ، المركز القومي للبحوث، 33 شارع البحوث، الدقي، القاهرة، 12622، مصر.
[2] وحدة أبحاث القراد والأمراض المنقولة بالقراد، معهد البحوث البيطرية ، المركز القومي للبحوث، 33 شارع البحوث، الدقي، القاهرة، 12622، مصر.
[3] معمل الأمراض المعدية، الكلية المشتركة للطب البيطري، جامعة كاغوشيما، كاغوشيما، 890-0065، اليابان.
[4] معمل ميكروبيولوجيا الحيوان، كلية الدراسات العليا للعلوم الزراعية، جامعة توهوكو، سينداي، 980-8572، اليابان.

الملخص

تركز الدراسة على تطوير لقاح ضد قرادة الكلب البنية (*Rhipicephalus sanguineus*) ، وهي ناقل رئيسي لأمراض الكلاب. بدلاً من الطرق التقليدية المكلفة، تم استخدام أساليب مناعية حاسوبية (*Immunoinformatics*) لتحليل بروتينين من القراد – ببتيد RsAQP1 والبروتين المؤتلف – RsFer2 كهدف محتمل للقاح. توقعت التحليلات احتواء كليهما على عناصر قوية (مستضدات)، (Epitopes) يمكنها تحفيز استجابة مناعية. أظهرت التجارب على الكلاب البلدية المصرية أن التطعيم، وخاصة باستخدام RsFer2 وحده أو ممزوجاً معRsAQP1 ، ولّد بنجاح مستويات عالية من الأجسام المضادة تعرفت على بروتينات القراد الطبيعية. وتخلص الدراسة إلى أن هذه المرشحات واعدة لتطوير لقاح وتستحق مزيدًا من الاختبارات لتأكيد فعاليتها الوقائية.

الكلمات الدالة: الأكوابورين1، الفيريتين 2 ، لقاح مؤتلف ، الريبسيفلس سانجوينس (قرادة الكلاب البنية) ، الكلاب البلدي.