Revista Argentina de Microbiología 56 (2024) 364–367

asociación argentina de microbiología

REVISTA ARGENTINA DE MICROBIOLOGÍA

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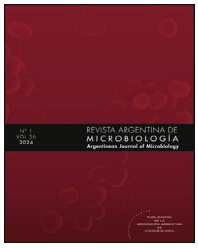

BRIEF REPORT

The potential use of arginine kinase from the brown tick *Rhipicephalus sanguineus* as a biomarker for vector exposure in the surveillance of Rocky Mountain spotted fever

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Received 19 March 2024; accepted 6 August 2024
Available online 2 October 2024

KEYWORDS
Rhipicephalus sanguineus;
Arginine kinase;
Epitopes;
Rocky Mountain spotted fever;
Rickettsia rickettsii

Abstract The brown dog tick (*Rhipicephalus sanguineus*) is the vector of *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (RMSF) in Northern Mexico and Southwestern United States. The immune response to a tick protein in the sera of humans or animals may reveal the zones with a high propensity to acquire RMSF, and vector control strategies may be focused on these zones. Arginine kinase (AK) is a highly antigenic invertebrate protein that may serve as a marker for tick exposure. We used *R. sanguineus* recombinant AK in an indirect ELISA assay with RMSF-positive patient sera. The response to AK was significantly higher against the sera of RMSF patients than the control sera from healthy participants without contact with dogs. To validate the antigenicity of tick AK, we mutated one predicted conformational epitope to alanine residues, which reduced the recognition by RMSF patients' immunoglobulins. This preliminary result opens a perspective towards the development of a complimentary technique

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https://doi.org/10.1016/j.ram.2024.08.001

based on *Rs*AK as an antigen biomarker for vector serological surveillance for *Rickettsia* RMSF prevention.

PALABRAS CLAVE
Rhipicephalus sanguineus;
Arginina quinasa;
Epítopos;
Fiebre manchada de las Montañas Rocosas;
Rickettsia rickettsii

El uso potencial de la arginina quinasa de la garrapata café *Rhipicephalus sanguineus* como biomarcador de exposición a vectores en la vigilancia de la fiebre manchada de las Montañas Rocosas

Resumen En el norte de México y el suroeste de Estados Unidos, la garrapata café del perro (*Rhipicephalus sanguineus*) es el vector de la bacteria *Rickettsia rickettsii*, causante de la fiebre manchada de las Montañas Rocosas (RMSF). La respuesta inmune a una proteína de garrapata puede revelar la exposición al vector, sugiriendo zonas en las que hay mayores posiblidades de contraer RMSF, y puede proporcionar mejores estrategias para el control de estos vectores en dichas zonas. La arginina quinasa (AK) es una proteína altamente antigénica que puede servir como marcador de exposición a garrapatas. Algunos experimentos con *enzyme-linked immunosorbent assay* (ELISA) indirecto empleando AK recombinante de *R. sanguineus* mostraron que la respuesta a AK con sueros de pacientes positivos para RMSF fue significativamente mayor que con sueros control (participantes sanos sin contacto con perros). Para validar la antigenicidad de la AK de la garrapata, se evaluó la misma respuesta a una AK mutante (E1), donde un epítopo conformacional se sustituyó por residuos de alanina, lo que redujo el reconocimiento por parte de las inmunoglobulinas de los pacientes con RMSF. Los resultados de este trabajo sugieren la viabilidad de utilizar arginina quinasa de *R. sanguineus* (RsAK) como biomarcador de antígeno para la vigilancia serológica en la prevención de RMSF.

The brown dog tick *Rhipicephalus sanguineus* is a significant public health concern in Northern Mexico and Southwestern United States as the vector for Rocky Mountain spotted fever (RMSF) disease, *Rickettsia rickettsii*[4,7]. It is well established that vector bites generate a cellular and humoral immune response in the host and antibodies to salivary components have been used as biomarkers of vector exposure[11]. Therefore, identifying a tick protein that produces antibodies in humans or animals can become a biomarker of tick exposure.

The vector bite produces an immune response in the host that can be followed as a marker of exposure for epidemiological surveillance[1]. *R. sanguineus* is a three-host parasite as it requires a blood meal from a host before progressing to the next life stage (larvae to nymph, nymph to adult) or before reproduction as an adult. Dogs are the natural hosts of the brown dog tick, making humans who share spaces with dogs highly susceptible to tick bites and infection with the disease[8].

In Sonora State, northwestern Mexico, RMSF is known to have 40–45% mortality[3] in infected patients. RMSF is expanding towards southwestern locations of the USA[9]. Worldwide, many tick-borne diseases are spreading to previously unreported geographical areas.

On the other hand, arginine kinase (AK) is a critical enzyme in energetic invertebrate metabolism with similar functions to creatine kinase in vertebrates[5]. It produces ATP from the metabolite phosphoarginine in invertebrates and is highly antigenic[13,15]. AK has been found in the proteome of insect salivary glands, including the hematophagous bed bug *Cimex lectularius*, a vector for *Trypanosoma cruzi*[15]. Therefore, AK has been suggested as a biomarker for Chagas disease, toxocariasis, and *Psoroptes ovis* infestation.

The *in silico* amino acid sequence analysis shows that AKs have conserved epitopes at residues 92–101[14]. Moreover, the same study identified four potential species-specific epitopes for *R. sanguineus* AK (RsAK). The proteins from a vector can trigger an immune response when the host is bitten by the vector[12].

In this study, we examined the seroreactivity of recombinant (Wt) RsAK and a mutated variant (E1) against the sera of RMSF wild-type patients. The rationale is that *Rickettsia* infection is acquired by a tick bite, introducing salivary proteins into the host's skin.

Gomez-Yanes (2022) used the DiscoTope 2.0 algorithm to identify a tick-specific epitope located within residues 105–109, 240, 241, 334, 337, and 340, which was named the E1 conformational epitope. These residues were mutated to alanine in the *Rs*AK nucleotide sequence, synthesized, and cloned into the pET11a vector for overexpression in *Escherichia coli* by GenScript®. Chemically competent bacterium BL21 DE3 Gold was transformed by standard protocols with the recombinant plasmid. The E1-mutant protein was purified using a cobalt metal affinity column and polished

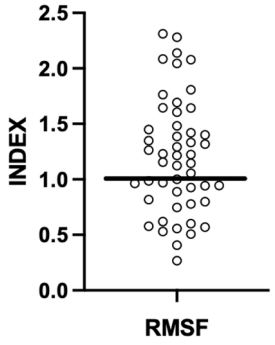

Figure 1 Index values were calculated by indirect ELISA using RMSF patient's sera and control sera (non-exposed subjects).

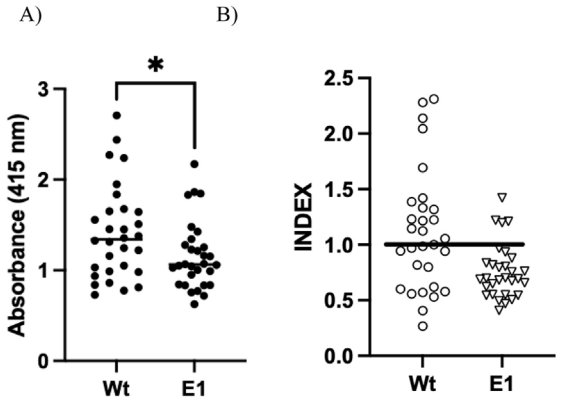

Figure 2 Indirect ELISA experiments. Panel A, a comparison of the response towards the wild-type *Rs*AK or E1-mutant proteins; Panel B, a comparison of the Index value of the wild-type *Rs*AK and E1-mutant.

with a gel filtration column. Recombinant expression and wild-type (Wt) *Rs*AK purification procedures were performed as previously described[6].

For the indirect ELISA experiments, a Falcon® 96-well polystyrene microplate was coated with the antigen: 50 μl of 0.3 μM protein solution and incubated at 4 °C overnight. We used the WT *Rs*AK or the E1 mutant diluted in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). Then, the wells were blocked for 1 h with 0.1% blotting grade blocker from Bio-Rad® and 1% of bovine serum albumin in TBST (0.1 M Tris–HCl, 0.05% Tween 20, 5 mM sodium azide, 0.1%, pH 7.3); and then the plate was washed three times with TBST. As controls, we used 50 blood serum samples from RMSF-positive patients and twenty negative sera.

All sera were diluted 1:100, and 50 μl was applied to each blocked and antigen-coated well. After a 1-h incubation, the microplates were washed with TBST, and 50 μl of 1:4000 anti-human IgG conjugated with alkaline phosphatase was added as a secondary antibody. The plate was incubated for 30 min and washed with TBST. The enzymatic reaction was developed for 30 min with an alkaline phosphatase substrate from Bio-Rad®, following the manufacturer's instructions, and the plate was read at 415 nm. Absorbance data were further analyzed in terms of the Index value. The average absorbance of the controls (negative sera) was calculated to establish a threshold value. Then, two standard deviations were added individually for each antigen (Wt or E1 mutant *Rs*AK protein). The Index value was obtained by dividing each data sample by the threshold value. Samples with an Index value of 1 or higher were considered positive. Data were subjected to a two-tailed paired Student's *t*-test ($p < 0.05$), followed by a one-tailed paired Student's *t*-test to assess significant differences in the magnitude of immunodetection of Wt *Rs*AK and E1 mutant by sera positive and negative for RMSF.

To investigate *Rs*AK as a potential marker for the risk of RMSF infection, we conducted an indirect enzyme-linked immunosorbent assay (ELISA) utilizing fifty human sera from confirmed RMSF patients as the antibody source. The average Index value for the patient sera against the WT *Rs*AK was 1.207 ($p < 0.05$) (Fig. 1), and 60% of the patients had an Index value equal to or above one. This result show that most RMSF patients developed an IgG immune response to a tick antigenic protein, similar to the response observed in patients exposed to sand flies or the tick vector in Lyme disease[12]. The Index values below 1 suggest limited tick exposure, likely resulting from one or a few bites by Rickettsia-infected ticks that led to an infection.

Moreover, if *Rs*AK is a *bona fide R. sanguineous* antigen that correlates with vector exposure, the mutation of an epitope should reduce the RMSF patient's blood sera recognition. In Figure 2A, the mean absorbance of thirty RMSF patients using the Wt or E1 antigens in the indirect ELISA assay is significatively different, confirming that *Rickettsia*-infected patients sera recognize the *Rs*AK Wt protein *vs*. the mutated E1 more strongly. The average absorbance for the RMSF patient sera against the Wt was 1.413, which is significatively different ($p < 0.05$) from the average absorbance towards the E1 protein (1.158). When the data is expressed as Index values (Fig. 2B), only four sera have values above 1 for the E1 mutant, strongly supporting the relevance of the E1 epitope as part of the *Rs*AK protein antigenicity.

It is widely accepted that the vector induces an immune response in the host. For example, the sera from outdoor workers showed immunoreactivity towards extracts of the vector *Ixodes dammini* salivary glands. Moreover, those positive to the tick proteins positively correlated with immune reaction against Lyme disease *Borrelia burgdorferi* bacteria ($r = 0.49$, $p = 0.0002$)[12]. To further validate whether *Rs*AK is a *bona fide* antigen, a conformational epitope predicted by DiscoTope was mutated to alanine, effectively reducing the antigenicity of the protein. The response was variable, as reflected in the absorbance variability. For example, flagellin, a *B. burgdorferi* antigen, is recognized by 87% of Lyme disease patients, 75% of syphilis patients, and 43% of non-diseased humans. In the present study, we identified a *Rs*AK conformational epitope spanning residues 105–109, 240, 241, 334, 337, and 340. Before this study, no phosphagen kinases as markers for vector surveillance had yet been proposed. Additionally, this study provides new information on the specific epitopes on arginine kinase for vector parasites such as *R. sanguineous*.

Efforts to combat vector-transmitted diseases are directed toward both the vector and the pathogen. In the case of dog tick life cycles, chemical strategies have been the primary focus for eradication. However, despite well-documented social determinants for RMSF, more data is needed regarding actual vector exposure in populations[2].

The components of tick saliva have been described, with our prior experience with AK as an antigenic protein, we postulated that tick *Rs*AK may be recognized as an immune exposure marker. RMSF patients significatively recognized *Rs*AK as an antigen compared to the healthy blood donor subjects, as has been proposed for other molecules such as serine protease inhibitors[10]. To our knowledge, phosphagen kinases have not yet been proposed as markers for vector surveillance and deserve further consideration. We aim to expand this research to a broader high-risk population and establish a strong correlation between environmental and social determinants and the immune response to *Rs*AK as an antigenic tick biomarker.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

We thank CONAHCYT grant CF-2019-610264 for support, a Ph.D. scholarship to Ana Carolina Gomez-Yanes, and a postdoctoral scholarship to Elena N. Moreno-Cordova. A bioethics protocol has been approved for this research, and proper material transfer agreements were obtained from the Laboratorio Estatal de Salud, Sonora, after a protocol review. We thank Rene Valenzuela for his technical support. The bioethics committee from CIAD had approved using human sera samples (CONBIOÉTICA-26-CEI-001-20200122).

This research was partially supported by the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases (JGV).

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