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Introducing a new anti-*Rhipicephalus (Boophilus) microplus* tick recombinant vaccine candidate using cathepsin and tropomyosin multi-epitope gene

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Abstract

Rhipicephalus (*Boophilus*) spp. are important vectors for *Babesia* and *Anaplasma* species causing severe economic losses in livestock. Chemical compounds are commonly used to control tick infestation; however, acaricides resistance in tick has led to move toward alternative strategies such as vaccination. In this study, we introduced a vaccine candidate, namely CaTro against *Rh. microplus* tick composing of immunogenic B-cell epitopes derived from *Rh. microplus* cathepsin L and tropomyosin proteins. To evaluate this vaccine candidate, firstly the CaTro sequence was inserted into the prokaryotic expression vector and the recombinant protein CaTro was expressed in Bl21 bacteria. Afterward, purification was performed by Ni-NTA affinity chromatography. The quality of purified recombinant CaTro was also analyzed using sodium dodecyl sulfate-gel electrophoresis and western blotting. Moreover, to evaluate the induction of immune response, the rabbits were immunized with purified recombinant protein combined with Freund's adjuvant. The findings of this study revealed molecular weight of expressed protein (CaTro) as 38.00 kDa. Furthermore, anti-CaTro antibody was detected in immunized rabbit's sera through dot blotting; while, there was not any response to the control rabbit's sera. The results suggest that CaTro is a potential candidate to develop an anti- *Rh. microplus* tick.

Introduction

Hard ticks (family: Ixodidae) are considered as most significant pests plaguing livestock and causing severe direct and indirect economic losses. Ticks can transmit different pathogens and babesiosis and anaplasmosis are important tick-borne diseases being very common in ruminants in different parts of the world.[1,2] The hard tick, *Rhipicephalus* (*Boophilus*) spp. are important vectors for *Babesia* and *Anaplasma* species. Both protozoa have significant zoonotic effect and cause high mortality in livestock annually; therefore, performing control against tick infestation is crucial. There are several prevention methods for this purpose.[1,2] At present, the most common control program is using chemical compounds. Due to the emergence of acaricide-resistant tick, other prevention strategies such as vaccination against ticks could be an alternative method.

Many researchers have shown that vaccination against tick infestation with immunogenic proteins induces a protective immune response in the host.[3] At present, two vaccines based on Bm86 antigen against *Rh. microplus* (Gavac and TickGard) are commercially available; but, due to genetic diversity in tick populations in various regions, their effectiveness varies.[4,5] Therefore, the effort to develop effective vaccines is still ongoing.[6] The use of new antigens with less genetic variation may induce a more effective immune response in the host.[6] On the other hand, it seems that use of multi-antigen vaccines can improve protective immune responses.[6]

Cysteine proteases are expressed in a broad variety of organisms. These proteases have an effective role in host immune evasion, molting, feeding and hemoglobin digestion in some parasites. One of the members of cysteine proteases is cathepsin L being found in the midgut and eggs of *Rhipicephalus* ticks and it has been

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suggested that this enzyme has a role in yolk protein degradation during embryogenesis.[7]

Many studies have introduced potential usages to stimulate protective immunity against some of the parasites using muscle molecules as candidates for vaccine production.[8-10]Also, other studies have shown that tropomyosin can induce host immune responses.[8] Tropomyosin is an alpha-helix protein, in parallel with two spiral chains binding to actin filaments in muscle and non-muscle cells. This protein plays an important role in actin activity regulation and immune and allergic reactions and it has been introduced as a candidate for a vaccine despite its allergenicity.[11,12]

Recently, researchers have incorporated immune-genic epitopes of various vaccine candidate genes for producing the high-efficiency vaccine and more sustainability in the host.[13,14] One of the important steps in the design of immunogenicity vaccines is the prediction of the epitopes.[15-16] In several studies, each of tropomyosin[8-10] and cathepsin[17-19] proteins alone was suggested as anti-tick vaccine candidates. In a recent study, the authors have analyzed completely the B-cell epitopes of cathepsin and tropomyosin proteins of *Rh. microplus* by some softwares and online servers; then, they have selected high immunogenic epitopes.[20] Afterward, in the present study, the designed epitopes were cloned and expressed and in order to evaluate of their immunogenicity, purified protein accompanied with Freund's adjuvant was injected into the rabbits.

Materials and Methods

Designing of multi-epitope gene and construction of recombinant plasmid CaTroRh. In a recent study, the polytopic gene was designed using immunoinformatic methods as follows: [20] First, we retrieved the sequence of tropomyosin (Accession Number (AN): O97162) and cathepsin (AN: Q9NHB5) proteins of *Rh. microplus* from UniProtKB database. Next, three-dimensional structure was modeled and discontinuous B-cell epitopes were predicted by IEDB database and then, selected epitopes were linked. After selecting the B-cell epitopes of cathepsin and tropomyosin proteins, due to the need for certain degree of flexibility, the selected B-cell epitopes of both proteins were connected using flexible linker (GGGGS), for which the sequence was as follows: FARIFNGHHGTR KTGGSTFLPPANVNDSSLPKVVDWRKKGAVTPVKDQGQC GSCWAGGGGSKLAMVEADLERAEERAETGETKIVELEEEL RVVGNNLKSLEVSEEKALQKEETYEGGGGSSQEILRTQWE AFKTTHKKSYQSHMEELLRFKIFTENSLIIAKHNAKYAKGL VSY. Finally, we carried out reverse translating of the designed sequences into the DNA and synthesizing recombinant protein namely CaTroRh by Biotech Company, Tehran, Iran.

Expression of recombinant protein CaTroRh in *E. coli BL21.* First, the competent cells were produced from *E. coli BL21* bacteria. The *E. coli BL21* was cultured in Luria Bertani (LB) broth medium. Calcium chloride and cold shock were used to prepare competent bacteria from *E. coli BL21*. Then, restriction enzymes (EcoRI/XhoI) were added to 5´ and 3´ ends of sequences and synthetic DNA fragment was cloned into the pET-32a. Next, 1 µg of the synthesized construct (CaTroRh) by Biotech Company was added to 20.00 µL of competent bacteria of *E. coli BL21* and cultured on LB agar plate containing 2.00 µg mL^{-1} ampicillin. After that, a single colony of bacteria in solid LB medium was cultured in 5.00 mL LB liquid medium overnight; next, 20.00 µL of the cultured medium was transmitted to 200 mL liquid LB medium and incubated at 37.00 ˚C on a shaking incubator until optical density 600 (OD) reached 0.50. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (100 mM; 200 µL) was added and cultured for 3 hr at 37.00 ˚C. Then, the cells were harvested by centrifugation at 8,000 rpm for 6 min at 4.00 ˚C. Afterward, the pellet was lysed by B-7M urea buffer (NaH_2PO_4 0.10 M, Tris HCl 0.10 M and urea 7.00 M) and the induction of competent bacteria was detected by the 12.00% sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE) according to Laemmli.[21]

Purification of recombinant protein CaTroRh. Purification of the recombinant protein CaTroRh by Ni-NTA affinity chromatography was performed according to the manufacturer's instructions (Qiagen, Hilden, Germany). Then, recombinant protein was briefly suspended in 2.00 M urea lysis buffer (NaH_2PO_4 20.00 mM, NaCl 500 mM, imidazole 10.00 mM and pH: 8.00) and in order to destroy the bacterial wall completely, ultrasonication was performed in an ice bath with 0.50 cycle, 40 amplitude and time intervals of 90 sec, two 60 sec and two 20 sec bursts with a 10 sec rest period between each burst. Collected lysate was centrifuged at 14,000 *g* for 15 min to pellet the cellular debris. The supernatant was transferred to a fresh microtube. Twenty-five µL of the lysate was removed for SDS- PAGE and the remaining lysate was placed on ice until use. The lysate was applied slowly onto the prepared purification column containing 2.00 mL resin and bonded for 20 min at room temperature. After washing with binding buffer (NaH_2Po_4 20.00 mM, NaCl 500 mM, imidazole 10.00 mM and pH: 4.50), the protein was eluted twice with buffer A (NaH_2Po_4 20.00 mM, NaCl 500 mM, imidazole 500 mM and pH: 6.30). Protein concentration was calculated by the Warburg and Christian method by absorbance at 280 nm.[22]

Sodium dodecyl sulfate-gel electrophoresis and western blot analyses. The quality of purified recombinant CaTroRh was analyzed on a 12.00% SDS-PAGE with 5.00% stacking gel electrophoresis and western blotting. For western blot analysis, 25.00 µL of purified recombinant protein was resolved on a 12.00%

SDS-PAGE gel. The gel was split into two pieces. One of them was stained with Coomassie brilliant blue and the other was transferred to polyvinylidene difluoride membrane. Non-specific binding sites were blocked by membrane incubation with 3.00% skim milk solutions and washed with Phosphate-buffered saline (PBS)-Tween 20. Then, the membrane was incubated with anti-poly-histidine-Horseradish Peroxidase (HRP; Biotech Co., Isfahan, Iran) at the room temperature for 45 min and washed with PBS-Tween 20. Subsequently, the membrane was incubated with 3,3′-diamino-benzidine tetrahydrochloride (DAB) at the room temperature and after 5 min, positive signals appeared as brown bands. The membrane was immediately washed with distilled water.

Rabbit immunization. At the beginning of the experiment, six white New Zealand white rabbits weighing 3,500 g were randomly divided into two groups of three rabbits as treatment and control. The rabbits of the treatment group were immunized two times at 14-day intervals with 0.50 mL of purified protein CaTro (200 µg mL^{-1} in 1.00 mL PBS; pH: 7.20) each time, with 0.50 mL Freund's complete adjuvant in the first injection and 0.50 mL incomplete adjuvant in the second injection intra-muscularly. At the same time, rabbits of control group were injected with 1.00 mL of the mixture of PBS and Freund's complete adjuvant intra-muscularly. After two weeks, re-injection was performed as in the previous step; but, incomplete adjuvant was used. Two weeks after the last immunization, blood samples of control and treatment groups were obtained to determine the presence of anti-CaTroRh antibodies. The rabbits' sera were separated by centrifugation at 3,000 rpm for 10 min and kept at – 20.00 ˚C until use.

Dot blotting. One µL of recombinant protein was spotted onto nitrocellulose strips and 1.00 µL of PBS was used as a negative control being allowed to dry at room temperature. Then, the strips were incubated with 500 µL of PBS (pH: 7.20) containing 2.00% skim milk on a shaker at room temperature for one hr to block the antigen-free regions on the paper. After washing the nitrocellulose membranes with PBS buffer, 500 µL of the 1: 500 of rabbit diluted sera from the treatment and control groups were added to each membrane for 30 min. The membranes were washed again and 500 µL of dilution of 1/1000 of polyclonal goat anti-rabbit immunoglobulin (Ig)/HRP was added onto each paper. Then, they were placed on a dark cabinet for an hr. The membranes were washed three times again and finally, the DAB H_2O_2 included substrate was immediately poured onto the papers. After observing the brown spots due to the reaction of antigen to antibody, the papers were washed with distilled water and dried afterwards.

Results

Recombinant plasmid induction and its evaluation by SDS-PAGE method. A comparison of the protein pattern in the bacterial lysate containing the recombinant plasmid, bacterial lysing without plasmid and plasmid-free bacteria before and after induction by IPTG revealed the expression of recombinant CaTro protein (Fig. 1).

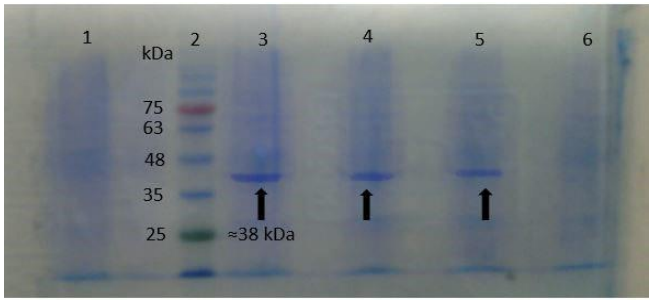

Fig. 1. Sodium dodecyl sulfate-gel electrophoresis results before and after induction of *E. coli BL21*. Lane 1: *E. coli BL21* containing non-induced plasmid; Lane 2: Marker; Lane 3: *E. coli BL21* containing CaTro 1 hr after induction; Lane 4: *E. coli BL21* containing CaTro 2 hr after induction; Lane 5: *E. coli BL21* containing CaTro 3 hr after induction; Lane 6: *E. coli BL21* containing CaTro before induction.

Evaluation of the purified proteins by SDS-PAGE and western blotting. Figure 2A shows the protein band of CaTro with (with 284 amino acids) molecular weight about 38.00 kDa. After that, the gel was divided into two parts by a blade; one part for confirmation of the presence of protein which was transferred to a container having Coomassie brilliant blue and the other part was used for western blotting in order to produce antigen-antibody interaction. Based on the immune reaction of the recombinant protein to antibody conjugate, a brown band was found with 38.00 kDa (Fig. 2B).

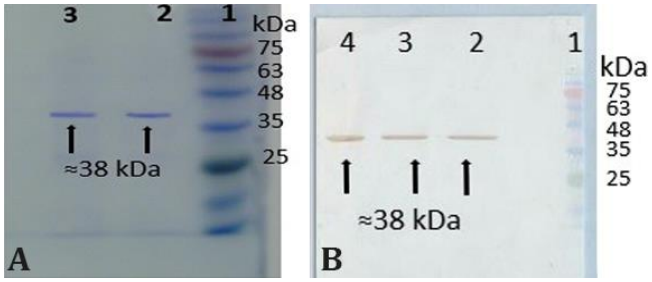

Fig. 2. A) The results of purified protein evaluation using sodium dodecyl sulfate-gel electrophoresis. Lane 1: Marker; Lanes 2 and 3: Purified protein. **B)** Western blotting between the CaTro recombinant protein and anti-His tag conjugate. Lane 1: Marker; Lanes 2, 3 and 4: CaTro protein.

Immune responses of immunized rabbits. As it is shown in Figure 3, the immunized rabbits' sera reacted to CaTro protein; but, there was not any response to the control rabbits' sera.

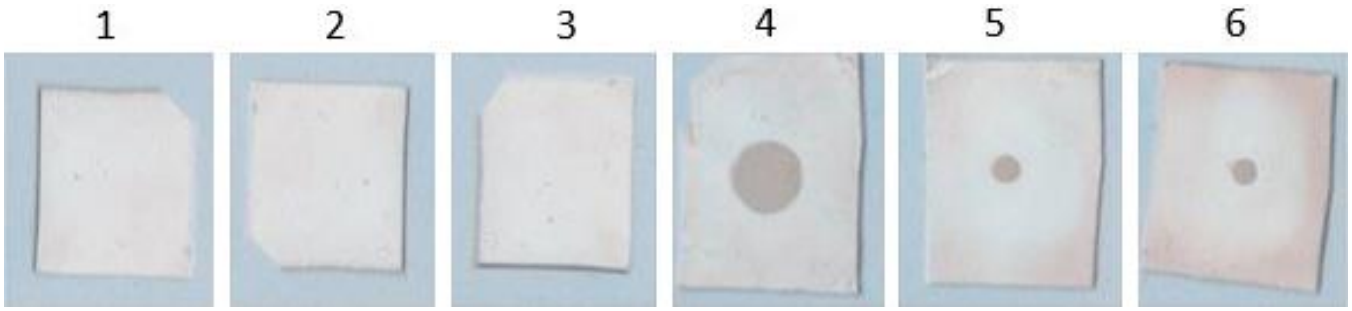

Fig. 3. Dot blotting results of rabbits in the control and treatment groups two weeks after the second injection. 1, 2 and 3: Control group; 4, 5 and 6: Treatment group.

Discussion

Acaricide-resistance has led to move towards new tick control strategies such as vaccine production.[23] Nowadays, there are two anti-tick vaccines (TickGard and Gavac) in the world based on the Bm86 antigen extracted from the midgut of *Rh. microplus*. These vaccines have shown low efficiency in some isolates of mentioned tick,[24,25] which could be due to the diversity of these isolates.[25] So far, several immunogenic proteins have been introduced as vaccine candidates for ticks.[26-28]

One of these immunogenic proteins is cathepsin known as a secretory protein in *Rh. microplus* and it has a critical role in vitellin degradation and meal digestion.[29] Cathepsin B-like and cathepsin L-like proteins of *Toxoplasma gondii,*[30] cathepsin L-like cysteine protease in *Rh. microplus,*[19] cathepsin-L3 and B3 in *Fasciola hepatica,*[31] and cathepsin l-like in *Rh.annulatus*[32] have been introduced as vaccine candidates by different researches. Tropomyosin is one of the other functional proteins used for immune responses induction against insect infestations such as tick and mite. This protein has an important role in actin activity regulation and immune and allergic reactions.[20] So far, the mentioned protein also has been used as a vaccine candidate in *Dermanyssus gallinae,*[9] *Psoroptes ovis,*[33] and *Rh. microplus*.[34]

Due to the effectiveness of these proteins, in present project, both proteins (i.e., cathepsin and tropomyosin) as fused vaccine were used. In the recent study, we designed and produced a recombinant protein composed of cathepsin and tropomyosin epitopes.[20] We constructed the CaTro and after transformation of the CaTro into *E. coli BL21* cells, the CaTro-His6 protein was induced with IPTG. To evaluate the protein expression, SDS-PAGE was performed with 12.00% gel pre- and post-induction of the CaTro. The pET-32a is fused with the 109aa Trx•Tag thioredoxin protein to increase the solubility and subsequently to decrease the inclusion body formation.[35] The protein concentration in bacterial lysate was calculated using Warburg and Christian method as 0.20 µg mL^{-1}.[22] The SDS-PAGE results revealed that molecular weight of expressed protein (CaTro) was about 38.00 kDa and cells lysis indicated that the protein was highly expressed. As it was expected, there was no inclusion body verifying suitable expression. The cause of inclusion body formation is usually due to the presence of a stress response similar to heat shock.[35]

The results of dot blotting revealed the existence of anti-CaTro antibody in sera of the immunized rabbits with recombinant CaTro confirming that the mentioned protein is immunogenic.

Up to date, to make the vaccine, many researchers have used a combination of several different genes such as glutathione S-transferase gene, vitelline-degrading cysteine endopeptidase and procathpsin of *Rhipicephalus* (*Boophilus*) yolk[36], B-cell epitopes derived from *Schistosoma mansoni* cathepsin D protein (*Sm*-CatD) flanked by GCN4 helix promoting peptide (a promiscuous T-helper epitope) and a lipid core peptide system.[37]

Huntley *et al.*, have shown that tropomyosin of *P. ovis* produces Ig G and Ig E antibodies in infected sheep.[33] Also, injection of recombinant protein troponin-like P27/30 has induced immune responses against *Haemaphysalis langicornis* in mice and rats.[38] Therefore, it seems that muscular proteins such as tropomyosin can be used as an anti-tick vaccine candidate.

Parizi *et al.*, and Leal *et al.*, have used *Boophilus* yolk cathepsin to protect infected calves against ticks.[36,39] These studies have indicated high immunity response in the calves of treated group compared to the control group after injecting the mentioned protein. Producing an appropriate immunity in infected mice with *F. gigantica* after immunization with cathepsin L1G and cathepsin B2 of this worm was also indicated by Changklungmoa *et al.*, and Chantree *et al.*, respectively.[17,40] On the other hand, to evaluate the effectiveness of this protein, Chantree *et al.*, have used cathepsin B1 and B2 together;[40] they showed that when a mixture of both proteins was used, the burden of infections in immunized mice is much less than when each of these proteins is injected alone into mice. The injection of polytopic construct into a rabbit led to the production of anti-CaTro antibodies. In this study, on the fourteenth day, due to the induction of affinity maturation, half of the initial reminder was injected and on the 28th

day, a blood sample was taken. Since the second week after injection, the responses are switched to class G immunoglobulin (isotype switching),[41] so the responses are predominantly Ig G.

Finally, the results suggest that the fused cathepsin and tropomyosin proteins may be a proper candidate for producing protective immunity against tick infestation requiring experimental tick challenging calves in farms and investigating its efficacy in future researches.

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Conflict of interest

There are no conflicting interests and financial disclosure associated with this article.

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