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Expression and determination of immunization dose of recombinant tropomyosin protein of *Hyalomma anatolicum* for the development of anti-tick vaccine

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ABSTRACT

Present investigation was carried out to standardize the immunization dose of one recombinant antigen in rat model before conducting large animal experimentation. Tropomyosin (TPM), a muscle associated and highly conserved protein found in all species of invertebrates, was cloned, expressed in prokaryotic system and the downstream process was standardized. SDS-PAGE analysis showed a distinct band of approximately 51 kDa. Western blot analysis using specific sera gave a strong reaction of approximately the same size as that of SDS-PAGE. For standardization of immunization dose, the rTPM at three different dosages viz., 150, 300, 450 µg was used to immunize wister rats and the antibody response was titrated by ELISA. Applying ANOVA, highly significant difference in anti-rTPM titre was recorded between the animals injected with 300 µg total dose (TD) and other dosages selected for the study. The significantly high antibody tire at 1:25600 dilution observed in animals immunized with 300 µg TD was selected for further study on *in vivo* immunization of calves and experimental challenge by the tick stages.

Key words: Degenerate primers *Hyalomma anatolicum*, pRham expression vector, Tropomyosin, Vaccine target

Ticks are obligate hematophagous ectoparasites of livestock and humans, acts as vectors of a number of animal diseases and second to mosquitoes as vectors of human diseases globally (de la Fuente *et al.* 2008, Galay *et al.* 2014). Ticks and Tick-borne (TTBDs) diseases gained significance due to their economic impact in terms of mortality and morbidity on livestock industry and due to their public health importance. Presently, many emerging infectious diseases arise from zoonotic pathogens, and most of them are transmitted by ticks (Ghosh and Nagar 2014, Manjunathachar *et al.* 2014). *Hyalomma anatolicum* is one of the most common tick species in Asian countries, acts as vector for many human and animal diseases such as Crimean-Congo hemorrhagic fever and tropical bovine theileriosis, respectively (Mourya *et al*. 2012, Yadav *et al*. 2014).

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Currently, the tick control method is focused on application of acaricides (Ghosh *et al.* 2007). However, this has several disadvantages viz. adverse effect on public health and ecosystem. Moreover, ticks are developing resistance to most of the available acaricides and the cost of development of new group of acaricides is becoming non-economical (Graf *et al*. 2004, Rodriguez-vivas *et al*. 2011). Henceforth, it is necessary to search an alternative, eco-friendly and cost effective method for tick management. Immunization of hosts is considered as one of the promising components in integrated tick management (ITM) system in which frequency of use of insecticides can be minimized significantly (de la Fuente and Kocan 2006) and may prolong the life of developed chemical acaricides (Guerrero *et al*. 2012).

Ticks are solely depending on host blood meal for their survival and reproduction. Muscle associated proteins play a significant role in blood feeding process (Tian *et al*. 2015). Tropomyosin (TPM), an actin regulator protein, belongs to a family of highly conserved proteins found in all species of vertebrates and in invertebrates, binds to major groove of the actin filament which provides stability and flexibility to the filaments required for proper contraction of muscles, necessary for physiological process and thus targeted as probable vaccine candidate (Behrmann *et al*. 2012, Ranjbar *et al*. 2015). However, till date only one *in vivo* experimental trial using rTPM has been conducted in rabbit model (Tian *et al*. 2015). The functional characterization of *H. anatolicum* TPM has recently been done through RNA interference (RNAi) and was found to be involved with the vital physiological functions (Ghosh *et al*. 2016-unpublished data). However, before conducting very costly *in vivo* immunization and challenge study in normal host, it is mandatory to standardize the delivery system and immunization dose for achieving maximum antibody titre. Accordingly, the present investigation was aimed to generate recombinant TPM and to work out the immunization dose in rat model as a prerequisite for large animal experimentation.

MATERIALS AND METHODS

Reference tick: The reference homogenous *T. annulata* free *H. anatolicum* (IVRI line II; registration no. NBAII/IVRI/HA/1/1998) was used for RNA isolation.

RNA extraction, RT-PCR, cloning and sequencing of Tropomyosin gene: Total RNA was isolated from 100 mg partially fed females of *H. anatolicum* IVRI line II using Trizol™ following the manufacturer's protocol with minor modifications. The purity of the RNA was quantified by NanoDrop® spectrophotometer. The cDNA synthesis was carried out from total RNA using Revertaid® first strand cDNA synthesis kit (Thermo Fisher Scientific, USA) containing moloney murine leukemia virus (MMLV) reverse transcriptase.

For specific amplification of the gene, the nucleotide sequences of various tick species were aligned and degenerate PCR primers were designed based on conserved motifs of ticks. The sense primer (TPM1) 5'-ATGGA(G/T)GCCATCAAGAA(G/A)AA(G/A)ATGCAG-3' and the antisense primer (TPM2) 5'-GAGCAGCGGTRGAAA CAACGGC-3' were designed to amplify approximately a 962 bp product. The primer pair was optimized for the PCR amplification of TPM (Tropomyosin) gene of *H. anatolicum*. In a 50 µl reaction, targeted gene was amplified from cDNA prepared from reference tick line at optimized PCR condition and the amplicons were electrophoresed in 1% agarose gel. The corresponding nucleotide band was purified from the gel with GeneJET™ gel extraction Kit (Fermentas, USA) and cloned into TA cloning vector (pTZ57R/T) using InsTAclone™ PCR Cloning Kit (Fermentas, USA) and colony PCR positive clones were sequenced by outsourcing at Department of Biochemistry, DNA sequencing facility at UDSC, University of Delhi South Campus, New Delhi.

Expression and purification of recombinant tropomyosin: The predicted protein coding sequence of TPM was expressed using Lucigen's *E. cloni* 10 G chemically competent cells with pRham^{TM} expression vector (Lucigen). For enzyme free cloning, the pre-processed pRham vector is co-transformed with insert DNA having ends complementary to the vector. Briefly, forward primer (TPM3) and reverse primers (TPM4) were designed having complementary ends to the vector sequence. After verification of PCR product through agarose gel electrophoresis, the corresponding nucleotide band was purified from the gel with GeneJET™ gel extraction kit (Fermentas, USA) and was mixed with the 25 ng of pRham^{TM} expression vector and transformed directly into chemically competent *E. cloni* 10 G cells. Positive clones were selected by colony PCR using pRham^{TM} forward and pETite reverse primers provided with the kit. For expression, 5–10 transformed colonies were grown individually at 37°C in 10 ml LB broth medium (Himedia, India) supplemented with Kanamycin. The recombinant TPM was induced with 20% L-rhamnose and the culture was grown additional for 12 h at 37°C. The expression was checked by SDS-PAGE and the colonies showing good production of recombinant protein were selected for scale up production and purification. The selected clones were grown and induced as above and centrifuged.

To purify the expressed protein, the cell pellets were individually resuspended in lysis buffer (8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris–Cl, 20 mM imidazole and pH 8.0) incubated at room temperature (RT) for 90 min, sonicated and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant containing solubilized proteins were subjected to purification by Ni-NTA superflow resin (Qiagen, Germany). Briefly, 1 ml of 50% Ni-NTA slurry was added to 4 ml of lysate and mixed gently by shaking for 30 min at RT. The mixtures were loaded into plastic column and the flow rate was adjusted to 0.5 ml/min. The column was washed with wash buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–Cl, pH 6.3, containing 15 mM imidazole). Bound non-specific bacterial proteins were eluted using elution buffer I, pH 5.9 (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–Cl). The expressed protein was eluted by buffer II, pH 4.5 (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris–Cl), the purified denatured protein was refolded by dialysis against gradually decreasing concentration of urea and concentrated using cut-off device (Pall filter, UK) and stored at -20°C in the presence of cocktail protease inhibitor.

SDS-PAGE and Western blot analysis: The purity of the rTPM was confirmed through SDS-PAGE under the reducing condition using standard protocol. Following electrophoresis, gels were stained with Coomassie brilliant blue R-250 or processed for Western blot analysis. For Western blot analysis, previously raised anti- *H. anatolicum* adult rabbit hyperimmune sera were used.

Following transfer to the nitrocellulose membrane (NCM), one portion of the membrane containing transferred protein was probed sequentially with hyperimmune rabbit sera (dilution 1:1000), peroxidase-conjugated goat anti-rabbit IgG (dilution 1:5000) and chromogenic substance, DAB (diaminobenzidine). While other portion was probed with anti-His tag mAb (Novagen) at the dilution of 1:2500. The reaction was stopped by washing the membrane strips in distilled water.

Animals and experimental design: The study was conducted on adult male Wistar rats (200–250 g) procured from the laboratory animals section of the institute. Rats were kept in polypropylene cages and maintained under standard management conditions. Before the commencement of the experiment, they were kept in laboratory conditions for 7 days for acclimatization. Animals were handled as per the guidelines of Institute Animal Ethics committee.

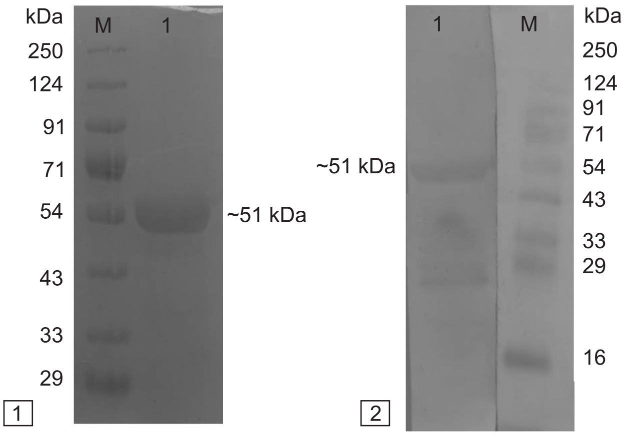
Rats were divided randomly into 4 groups consisting of 5 animals in each group. Group 1, 2 and 3 received 200 µl, 400 µl and 600 µl formulation containing 50 µg, 100 µg and 150 µg of rTropo (recombinant tropomyosin), respectively and MONTANIDE™ ISA 50 V2 as adjuvant. The mixing of adjuvant with rTropo (1:1 ratio), was done using homogenizer. The group IV animals were kept as control and received MONTANIDE™ ISA 50 V2 (Sepic India) adjuvant alone. Each group was immunized three times at two-week intervals.

Rats were bled weekly before and after immunization. Blood was collected from retro-orbital puncture under ketamine (80 mg/kg bw) and diazepam (2.2 mg/kg bw) anesthesia and taken into tubes (BD vacutainer®, 3.5 cm^3) coated with clot activator. The samples were centrifuged at 3,500 rpm for 15 min and serum was stored at -20°C till analysis.

Evaluation of immune response: For the assessment of immunogenicity of rTPM, sera were tested for antibodies against rTPM protein by ELISA and data were analyzed by comparison of mean optical density (OD) values at highest serum dilution amongst the three immunized groups. Initially, variables like, concentration of antigen and secondary antibodies were optimized employing the checkerboard method. The optimized conditions included antigen concentration for coating (400 ng/ml) dilution of secondary antibodies 1:10,000 for whole IgG and primary antibodies was diluted serially from 1:200 to 1: 25,600 to determine antibody titer. The ELISA plates (F96 Maxisorp, Nunc, Roskide, Denmark) were coated with 100 µl of rTPM dissolved in a carbonate buffer (pH 9.6) and stored at 4°C overnight. After washing with PBS with 0.05% Tween 20 (PBS-T), each well was blocked with 200 µl of 5% skimmed milk in PBS-T at 37°C for 2 h. Pre-immunization rat serum was used as negative control. The plates were incubated with 100 µl/well of rat sera in the blocking solution, diluted serially starting at 1:200, at 37°C for 2 h. ELISA plates were washed several times with PBS-T before applying 100 µl of HRP-conjugated rabbit anti-rat IgG (Sigma) in the serum diluting solution (1:10,000 dilution) in each well and then incubated at 37°C for 2 h. After another series of washing, the peroxidase mediated colour was developed for 15 min at RT using ophenylenediamine dihydrochloride (Pierce, USA) in citrate buffer, pH 5.0. The optical density (OD) was read at 492 nm in an ELISA reader (Tecan Sunrise, Austria). Antibody titers between three groups and control rat sera were compared by ANOVA ($P \leq 0.05$).

RESULTS AND DISCUSSION

In early 1990, BM86 based vaccines created significant breakthrough in arthropod vaccine research but reported to have limited cross-protective efficacy. To overcome this problem, research is going on in identification of conserved genes across the tick species/haematophagus arthropods to develop cross protective vaccines. Tropomyosin is one of the highly conserved molecule involved in both polymerization and stabilization of F-actin filament and regulates actin binding proteins including myosin in muscle and non-muscle cells (Schmidt *et al.* 2015).



Figs 1–2. **1.** SDS PAGE analysis of purified recombinant tropomyosin (TPM) protein of *Hyalomma anatolicum*. M-3 color pre stained protein ladder [Genetix]. **2.** Western blot analysis of recombinant tropomyosin (TPM) protein probed with rabbit anti-*H. anatolicum* antibodies. M-3 color pre stained protein ladder [Genetix].

To exploit the potentiality of the conserved molecule as cross-protective tick vaccine candidate, the tropomyosin gene was amplified using degenerate primers derived from TPM consensus sequences yielded a specific product using optimized PCR conditions with cDNA from the *H. anatolicum* as template. The complete cds of HaTPM (*H. anatolicum* tropomyosin) was amplified as 901 bp product and cloned gene was sequence submitted to GenBank (accession no. KU297197.1). Further, rHaTPM protein was expressed in expression vector as a ~51 kDa including a 6× Histag and was detected by SDS-PAGE (Fig. 1). Western blot analysis of the protein showed a specific and strong reaction at ~51 kDa with hyperimmune sera raised against antigen prepared from partially fed adults of *H.anatolicum* (Fig. 2) and anti-His tag mAb.

In order to identify a suitable tick vaccine candidate, it is necessary to evaluate the level of immune response generated following inoculation in the experimental model (Mallon 2016). The role of humoral antibody response in conferring immunity against ticks has been established in many immunization study using tick antigens and vaccine efficacy was directly related to strong and sustained antibody response (Bian *et al.* 2011, Carreon *et al.* 2012, de la Fuente *et al*. 2011). With the introduction of very strict guidelines regarding the use of animals for experimentation, it has become mandatory to establish the potentiality of candidate molecule in *in-vitro* model, followed by small animal experimentation. Accordingly, RNAi protocol for *H. anatolocum* has been established in the laboratory and was used for functional characterization of the gene (Ghosh *et al*. 2016). The encouraging RNAi data stimulated the group to characterize the further immunization potential of the target. As a part of regulatory authority guidelines, three immunization dosages were selected and were tested in rat model. Fold rise in total IgG levels at maximum dilution (1:25,600) of sera was determined and was statistically analyzed. Statistically insignificant variations were recorded in initial serum dilutions (1:200 to 1:12,800) of all the three immunized groups. The mean antibody response was dramatically increased in all the treatment groups. After 15 days of 2nd booster dose, the mean response in group 2 was significantly higher in comparison to 1 and 3 animal groups. Antibody titer was increased significantly after 2nd booster dose in all the three groups. The antibody responses in all the three immunized groups were showing statistically significant variation compared to control group (Table 1).

Table 1. Analysis of antibody response before and after 2nd booster dose of rHaTPM

Weeks of serum collection	Gr I (TD 150 µg) Mean ± SE	Gr II (TD 300 µg) Mean ± SE	Gr III (TD 450 µg) Mean ± SE	Control group Mean ± SE
4th; before 2nd booster	0.317 ± 0.006	0.814 ± 0.042***	0.706 ± 0.02	0.16 ± 0.016
5th; 1st week of 2nd booster	0.614 ± 0.036	0.92 ± 0.073***	0.84 ± 0.003	0.162 ± 0.012
6th; 2nd week of 2nd booster	0.52 ± 0.052	0.89 ± 0.026***	0.58 ± 0.04	0.166 ± 0.014

***Significant at P<0.001 as compared to A; TD, total dose.

Earlier functional characterization study of TPM of *H. longicornis* and vaccination of rabbit with rTPM reported a significant reduction of female engorgement weight and oviposition and concluded that the antigen, rTPM may play a role in the physiological activities of ticks and thus can be considered as a potential vaccine candidate (Tian *et al.* 2015).

The results obtained from our study are in conformity with Kaewhom *et al*. (2009), who assessed the immunogenicity of rSERPIN from *R*. (*B*). *microplus* in rabbit model and concluded that 300 µg TD of rSERPIN was capable of eliciting good antibody response. Likewise, Merino *et al*. (2011) recorded high anti-rSUB antibody titer following first and subsequent immunization and linked with 42% protection against challenged infestations. Memom *et al*. (2015) used salivary gland extract as vaccine prepared from *H. anatolicum* tick in rabbit model and concluded that vaccine containing 7.5 mg or more antigens induced detectable level of anti-tick salivary gland vaccine antibodies. Similarly, Aguirre *et al*. (2016) evaluated immune response in cattle against *R. microplus* by using a synthetic ATAQ peptide, which is present in the gut and malpighian tubes of ticks and showed consistent immune response in cattle detected by ELISA. The antigen with good performance in vaccine trails is not enough to meet the commercial success. Conducting experiments on animal model before immunization trial on natural host is necessary to study the safety and level of protective immune response. In the present study, the high titre obtained in the initial trial, if replicated in the large animal model may provide protective response against experimental challenge infestations. The antibody titre based selection of dose and adjuvant has provided a strong foundation for getting final clearance from regulatory authority regarding the use of large animals in immunization and challenge study.

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