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RESEARCH

Molecular cloning and expression of the *ferritin* gene from midgut and salivary glands of brown dog ticks (*Rhipicephalus sanguineus*) in Thailand

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Abstract

Anti-tick vaccine has become an alternative for the control of ticks. However, a monovalent anti-tick vaccine is of particular interest for its efficacy in different geographical areas. An RNAi experiment showed an inhibition effect on the *ferritin* gene expressed in *Ixodes* spp. as a potential candidate antigen for anti-tick vaccines. The aim of the current study was to investigate and characterize a novel antigen, *ferritin* (*FER*) from the midgut and salivary glands of the brown dog tick, *Rhipicephalus sanguineus*, in Thailand. In this study, the complete *ferritin* gene was first amplified and expressed as a recombinant ferritin (rFerritin) protein using the *Pichia pastoris* expression system. The *Pichia* recombinant, KM71H Mut^{S} was expressed in BMMY medium and induced using methanol. Secreted expression proteins in the supernatant were characterized using SDS-PAGE and Western blot analysis. The polyclonal antibodies to ferritin used for Western blot analysis were previously prepared from sera of rabbits exposed to *R. sanguineus*. The Western blot results analysis showed a band of approximately 23 kDa and confirmed that ferritin was able to induce specific immunity to *R. sanguineus* infestation. This study successfully amplified the *ferritin* gene and expressed the ferritin protein from tick midgut and salivary glands. According to the Western blot results, this study also demonstrated that ferritin had antigenicity. Based on these results, the potential use of recombinant ferritin as one of the cocktail antigens to develop a novel multivalent vaccine against *R. sanguineus* will be further tested in animal models.

Keyword: *ferritin*, *Rhipicephalus sanguineus*, *Pichia pastoris*, midgut, salivary glands

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Introduction

Rhipicephalus sanguineus, a brown dog tick, is the most widely distributed tick species and the potential vector for animal and human diseases that causes economic and public health losses. The conventional controls of *R. sanguineus* highlight the potential risks associated with the improper use of acaricides, including environmental pollution, toxicity to humans and other non-target organisms [1]. Tick control is also essential in controlling tick-borne diseases. Ideally, any vaccine should reduce tick infestations and pathogen transmission [2]. Different methods of tick control, such as the use of chemical acaricides and vaccines, have posed several challenges [3]. Vaccination against tick infestations has become an option for the control of ticks and such a vaccine has been developed by the identification of protective antigens using molecular biology techniques [4-7]. For more than 20 years, the only commercially available anti-tick vaccine has been based on the midgut protein, Bm86, from *Rhipicephalus* (*Boophilus*) *microplus* as the antigen [8]. However, it is effective only against the cattle tick in specific geographical areas [6].

Ferritin (*FER*) is a general iron-binding protein consisting of 24 subunits folded in a helical bundle and has a function in iron homeostasis in most organisms [9]. Two types of *ferritin* - an intracellular (*FER1*) and a secretory type (*FER2*) - have been characterized in *Ixodes ricinus* [10,11] and *Haemaphysalis longicornis* [12]. The excess iron from digested blood meals could induce iron toxicity in those ticks. *Ferritin* has a role in the regulation of iron balance and therefore, could be a potential antigen for tick control [13,14]. However, there is little information on *ferritin* in *R. sanguineus*. This study aimed to clone and characterize the cDNA encoding ferritin and to compare the deduced amino acid sequences of ferritins from *R. sanguineus* isolates in Thailand and other previous isolates. The recombinant ferritin produced in *Pichia pastoris* was also tested with polyclonal antibodies of rabbits induced by natural infestation of *R. sanguineus*.

Materials and methods

1. Brown dog ticks

Ticks were obtained from a colony of *R. sanguineus* maintained at the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand. Only female ticks were used in the experiment.

2. Midgut and salivary gland dissections

Dissection of ticks was done under a light microscope as described by [15]. Briefly, under a dissection light microscope, partially fed ticks were submerged in phosphate buffered saline (PBS; pH 7.4), held down with a pair of soft tissue forceps. The dorsal cuticle was excised and the salivary glands separated from the rest of the organs with the help of 18-gauge needles. Following dissection, the tissues were transferred into RNA stabilizer reagent and kept frozen at -80 ˚C until use.

3. RNA extraction and RT-PCR

Total RNA was extracted from 0.1 g of *R. sanguineus* midgut and salivary glands by the acid phenol extraction method [16]. Extracted RNAs were amplified by reverse transcription-polymerase chain reaction (RT-PCR). The cDNA strand synthesis reaction was performed using a SuperScript III™ First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The *ferritin*-specific primers were newly designed and synthesized according to *ferritin* cDNA sequences published on GenBank (accession AY277907). The whole *Ferritin* gene was amplified using a forward primer of 5'-GAA-TTC-AAG-AAG-ATG-GCC-GCT-ACT-CA-3' and a reverse primer of 5'-TCT-AGA-TAG-TCC-GAC-AGG-GTC-TCC-TT-3'. The PCR was carried out using Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and hot start PCR with 95 ˚C for 5 min, 35 cycles of 95 ˚C for 1 min, 60 ˚C for 1 min, 74 ˚C for 2 min, and 74 ˚C for 10 min. PCR products were electrophoresed in 1.2% agarose gels and visualized under UV illumination and the target amplicon size was confirmed.

4. Sequencing and nucleotide analysis

The plasmid was extracted using the Qiaprep spin miniprep kit (Qiagen) and was confirmed by restriction endonuclease. Its sequencing was undertaken by FirstBase (Malaysia). Inserted DNA sequences were compared with known *ferritin* sequences on the GenBank database. A phylogenetic tree of the *ferritin* gene was constructed using the distance method in Mega version 5.1 [17].

5. Cloning and expression in *Pichia pastoris*

The *ferritin* coding sequences were amplified using PCR and purified using an UltraClean™ 15 DNA Purification Kit (MO BIO Laboratories, Inc., USA). The purified products were then digested with *EcoRI* and *XbaI* restriction enzymes, subcloned into a *P. pastoris* expression vector pPICZα-A and expressed according to the manufacturer's instructions (Invitrogen® , USA). The KM71H strains (Mut^S, methanol utilization slow) of *P. pastoris* were generated. These strains were used to prepare *Pichia* competent cells. The recombinant plasmids were used to transform *P. pastoris* strain KM71H competent cells using the electroporation protocol (Invitrogen®, USA). The *P. pastoris* positive clones were selected on YPD plates containing 100 µg/ml Zeocin, and checked using the PCR technique. Small-scale expression was performed by induction involving adding 100% methanol to a final concentration of 3% methanol every 24 hours to maintain induction. For secreted expression, the supernatant was transferred to a separate tube and the supernatant was stored at -80 ˚C until used for analysis by SDS-PAGE and Western blotting.

6. Polyclonal antibodies preparation in rabbits

Four rabbits (aged 4 months and weighing 2-2.5 kg) were chosen for preparing antibodies against dog ticks. The rabbits were challenged by tick infestation for 7 days and the infestation was repeated 21 days later. All infestations in each rabbit consisted of 50 females and 50 males which were unfed adult *R. sanguineus*. The serum of these rabbits was used as primary antibodies in both Dot blot and Western blot analysis against ferritin antigen.

7. Immunogenicity

All reagents were purchased from Sigma (St. Louis, MO, USA). For dot blots, secreted protein was dried onto polyvinylidine difluoride (PVDF) membranes and incubated for 1-2 hours with either rabbit hyperimmune serum to *R. sanguineus* (1:50) or mouse anti-polyhistidine immunoglobulin G (IgG) monoclonal antibody (MoAb) (1:3,000). Membranes were then incubated with either goat anti-rabbit (1:3,000) or goat anti-mouse (1:500) secondary antibodies conjugated to horseradish peroxidase (1:500) for 1 h, washed and developed with diaminobenzidine and 1% H_2O_2 for 5-10 min. For Western blotting, secreted protein was analyzed with denaturing 12% SDS-PAGE and stained with Coomassie blue or transferred onto PVDF at semi-dry blotting (Bio-Rad, USA) and developed as described above.

Results

Amplification and sequence analysis

In this study, two full-length cDNAs encoding ferritin were identified and cloned from the EST database of *R. sanguineus*. The full-length *ferritin* gene we identified contains 516 bp (Fig.1) and phylogenetic analysis of the *ferritin* gene demonstrated the genetic similarity between Thai *R. sanguineus* isolate (KP688390) and a reference DNA sequence (AY277907) which is a *R. sanguineus* isolate from the USA (Fig. 2). The nucleotide sequence homologies between the Thai and AY277907 ferritin were 99.6%. The predicted start codon is at 139–141 bases, the stop codon is at 655–657 bases, and an ORF extends from position 139 to 657 that encodes for 172 amino acid residues (Fig.3). The molecular mass of the deduced amino acid sequences was predicted about 23 kDa and compared with a ferritin amino acid sequence available in the GenBank database. The deduced ferritin amino acid sequence in the present study had 100% identity to *R. sanguineus* isolate from the USA (AAQ54715).

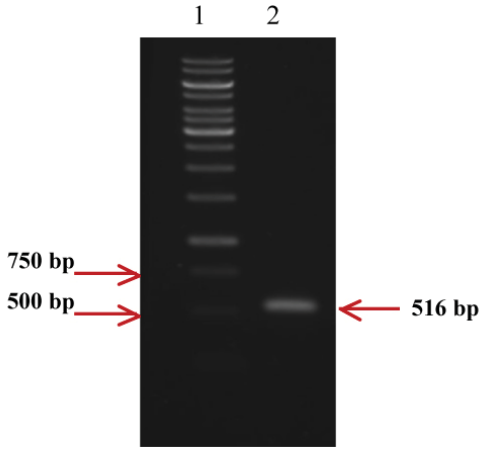

Fig. 1 Amplification of the ferritin transcript by PCR
Lane 1 = 100 bp plus ladder DNA marker; Lane 2 = amplification product from *R. sanguineus* cDNA with Ferritin-specific primers described in the text.

In vitro expression of recombinant ferritin (rFerritin)

Recombinant ferritin (rFerritin) was expressed *in vitro* using the pPICZαA expression vector and the *P. pastoris* strain KM71H expression system. Recombinant protein was expressed as secreted into media culture. The molecular weight of rFerritin was 23 kDa on 12% polyacrylamide gel, which correlated with the expected molecular mass 20.5 kDa considering that the expression vector produced a recombinant protein fused with a 2.5 kDa vector protein (Fig. 3).

Detection of rFerritin expression by Dot blot and Western blot analysis

Detection of rFerritin proteins was determined by Dot blot and Western blot techniques. Supernatant was separated from media culture and routinely electrophoresed on 12% polyacrylamide

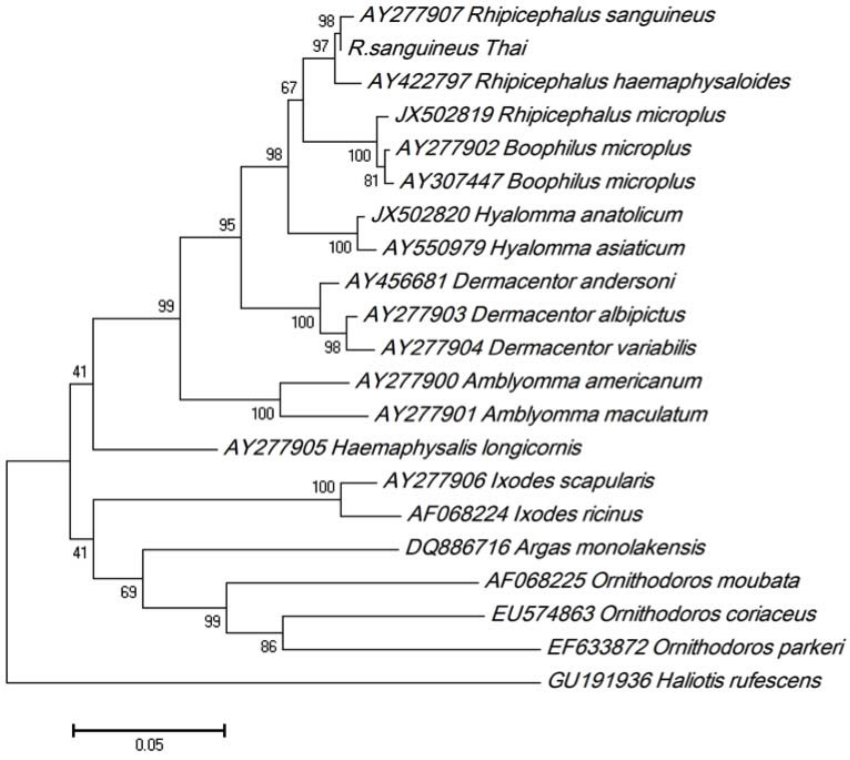

Fig. 2 Neighbor-Joining phylogenetic tree of the *ferritin* gene of *R. sanguineus* isolates in Thailand and validated *ferritin* genes of other tick species
Percentage bootstrap support (>40%) from 1,000 pseudoreplicates is indicated at the left of the supported node.

gel. Following electrophoresis, the transferred proteins were electro-blotted onto a PVDF membrane. The membrane was incubated in mouse-anti-histidine IgG monoclonal antibody and goat-anti-rabbit serum polyclonal antibody. The result showed the specific band at the same size presented in the SDS-PAGE (Figs. 4 and 5).

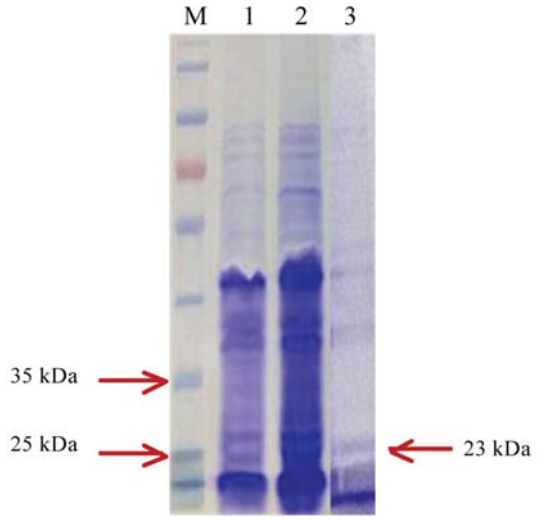

Fig. 3 SDS-PAGE analysis of rFerritin protein from transformed *P. pastoris* induced with 3% methanol final concentration
SDS-PAGE result demonstrates the size of rFerritin protein (7 days) stained with Coomassie brilliant blue. Lane M: molecular protein marker, Lane 1: Wild type protein, Lane 2: 23 kDa crude rFerritin protein and Lane 3: 23 kDa supernatant rFerritin protein (arrow).

Discussion

Genetic analysis of the *ferritin* gene was performed in both the DNA and predicted amino acid sequences. In this study, the phylogenetic analysis of the *ferritin* gene retrieved from various tick isolates revealed that these genes are conserved within tick genera and species (Fig. 2). Based on the Neighbor-Joining tree constructed in this study, Thai *ferritin* genes formed a distinct relationship in the genus pattern. However, the low ranged diversities of this gene were evident among ticks so that this gene can be used as a candidate antigen for anti-tick vaccine against *R. sanguineus*. The identity of this gene between Thai *R. sanguineus* isolates and USA (AY277907) [18] was shown in this study and there was no geographical relationship of this gene in different tick isolates.

The tick colonies in our laboratory have been maintained by feeding on rabbits. The tick infestations have been repeated for several cycles in the same hosts. We observed that the last few generations of ticks had slow growth, a decreased survival rate and decreased ability to attach to the host compared to previous generations of ticks (data not shown). Accordingly, we decided to use

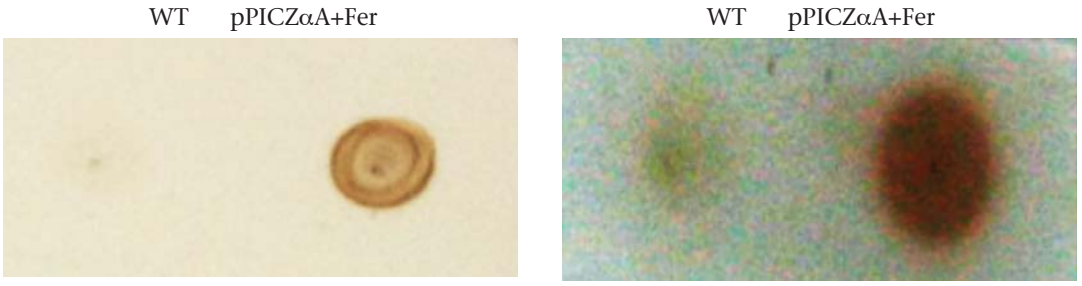

Fig. 4 Recombinant plasmid Ferritin (rFerritin) expression in *Pichia pastoris*
Dot blot analysis of recombinant proteins secreted into supernatant at day 7 of wild type (WT) and transformed *P. pastoris* (pPICZαA+Fer) using monoclonal antibody against the histidine tag (A) and pooled immune serum from rabbit infested with *R. sanguineus* (B).

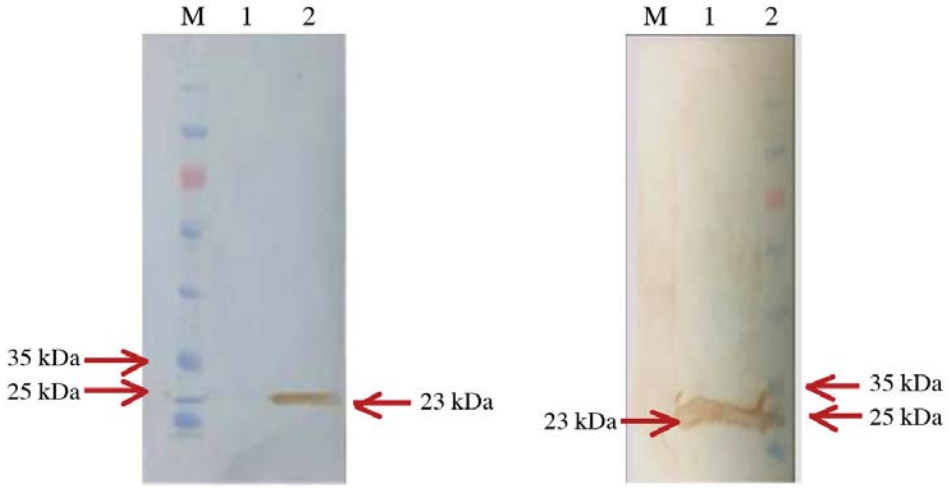

Fig. 5 Determination of immunogenicity of rFerritin by Western blot
Lane M: molecular marker; Lane 1: Wild type protein; Lane 2: rFerritin protein (arrows) using monoclonal antibody against the histidine tag (A) and pooled immune serum from rabbit infested with *R. sanguineus* (B).

tick-infested rabbits' sera for primary antibodies in Dot blot and Western blot analysis against rFerritin and the results were clearly demonstrated in this study.

Most concealed antigens are commonly found on the tick's gut wall and hidden from host immune responses [19]. In contrast to the concealed antigens, the exposed antigens are mostly found in the salivary gland and are normally excreted in saliva during tick feeding on the host [19]. In this study, the RT-PCR amplifications targeting the *ferritin* gene were performed separately between the salivary gland and midgut of *R. sanguineus*. The detection of the mRNA of the *ferritin* gene in both the salivary gland and midgut confirmed that this gene regularly transcribes in both organs. Dot blot and Western blot analysis in this study confirmed that natural tick infested in rabbits induced antibody that was recognized as ferritin. These results clearly showed that ferritin antigens generally expose to the host immune system. Ferritin has been classified as a concealed antigen in previous studies [19,20]. According to our findings in this study, we propose that ferritin could be identified as having the combined properties of both exposed and concealed antigens and that this third group of candidate antigen is preferred as a broad-spectrum anti-tick vaccine. In addition, the phylogenetic analysis constructed in this study revealed the highly conserved property of this gene among tick isolates.

The genetically conserved property of this gene also suggested the possibility to use rFerritin produced in this study for anti-tick vaccine in various species of the genus *Rhipicephalus* including *R. sanguineus*, *R. haemaphyloides* and *R. microplus*. Although the ferritin antigens are grouped into *FER1* (intracellular) and *FER2* (secretory), cross immunity responses between the two types have been demonstrated [20].

Gene regulation of the tick *ferritin* was associated with rickettsial infections of *Dermacenter variabilis* [20]. Ferritin of *D. variabilis* acted as a stress response protein to *Rickettsia montanensis* infections in those ticks. The iron concentration is able to mediate the function of the *ferritin* family genes of both tick vectors and some

pathogenic bacteria [21,22]. The tick ferritin antigens interacting with the host's specific immunoglobulins could establish excess iron-mediated cell damage in both tick vectors and tick-borne pathogens [21,22].

To our knowledge, this is the first report of the cloning, sequencing and expression of ferritin from *R. sanguineus* indigenous to Thailand. In this paper, we have described for the first time the complete sequences of ferritin from Thai *R. sanguineus*. Sequence similarity suggests that the Thai strain ferritin is a priority target as a candidate vaccine antigen in Thailand.

Conclusion

cDNA clones of ferritin from TSG collected from a dog tick colony at the Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand were isolated by RT-PCR. The *ferritin* cDNA was 518 bp in length, which encoded a ferritin protein of 172 amino acid residues whose deduced amino acid had 100% identity to *R. sanguineus* (accession number AY277907). Based on this result, the Thai *ferritin* gene was homologous to *R. sanguineus* (accession number AY277907). This study indicated a potential possible use of recombinant ferritin as a vaccine against *R. sanguineus*. In this study, the recombinant protein could be detected by Dot blot and Western blot. Based on these results, this study has established a potential use of recombinant ferritin as one of the candidate antigens for a cocktail anti-tick vaccine. However, it requires further vaccine trials in natural host animals to prove its efficacy against *R. sanguineus* tick infestations.

Acknowledgments

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References

1. Graf JF, Gogolewski R, Leach BN, Sabatini GA, Molento MB, Bordin EL, *et al*. Tick control: an industry point of view. Parasitology. 2004;129:S427-42.
2. Merino O, Alberdi P, de la Lastra JMP, de la Fuente J. Tick vaccines and the control of tick-borne pathogens. Front Cell Infect Microbiol. 2013;3:30.
3. Willadsen P. Tick control: thoughts on a research agenda. Vet. Parasitol**.** 2006;138: 161-8.
4. Tellam RL, Smith O, Kemp OH, Willadsen P. Vaccination against ticks. In: Yong WK (ed). Animal Parasite Control Utilizing Biotechnology. London: CRC Press: 1992.
5. Riding GA, Jarmey J, McKenna RV, Pearson R, Cobon GS, Willadsen P. A protective "concealed" antigen from *Boophilus microplus*. Purification, localization, and possible function. J. Immunol**.** 1994;153:5158-66.
6. De la Fuente J, Kocan KM. Strategies for development of vaccines for control of ixodid tick species. Parasite Immunol. 2006;28: 275-83.
7. De la Fuente J, Estrada-Peña A, Venzal JM, Kocan KM, Sonenshine DE. Overview: ticks as vectors of pathogens that cause disease in humans and animals. Front Biosci. 2008;13:6938-46.
8. Willadsen P, Bird P, Cobon GS, Hungerford J. Commercialisation of a recombinant vaccine against *Boophilus microplus*. Parasiology. 1995;110:43–50.
9. Arosio P, Ingrassia R, Cavadini P. Ferritins: a family of molecules for iron storage, antioxidation and more. Biochim Biophys Acta. 2009;1790:589-99.

10. Hajdusek O, Sojka D, Kopacek P, Buresova V, Franta Z, Sauman I, *et al*. Knockdown of proteins involved in iron metabolism limits tick reproduction and development. *Proc Natl Acad Sci*. 2009;106:1033-8.
11. Hajdusek O, Almazan C, Loosava G, Villar M, Cannales M, Grubhoffer L, *et al*. Characterization of ferritin 2 for the control of tick infestations. Vaccine. 2010;28:2993-8.
12. Galay RL, Miyoshi T, Umemiya-Shirafuji R, Maeda H, Kusakisako K, Tsuji N, *et al*. Evaluation and comparison of the potential of two ferritins as anti-tick vaccines against *Haemaphysalis longicornis*. Parasites & Vectors. 2014;7:482-92.
13. Sonenshine DE. Biology of tick. Vol. 1. New York: Oxford University Press; 1991. p 159-88.
14. Petr K, Jana Ž, Toyoshi Y, Christoph W, Natasha R, John HL. Molecular cloning, expression and isolation of ferritins from two tick species - *Ornithodoros moubata* and *Ixodes ricinus*. Insect Biochem Mol Biol. 2003;33:103–13.
15. Jittapalapong S, Jansawan W, Gingkaew A, Barriga OO, Stich RW. Protection of dairy cows immunized with tick tissues against natural *Boophilus microplus* infestations in Thailand. Ann N Y Acad Sci. 2004;1026:289-97.
16. Chomczynsky P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-choroform extraction. Anal Biochem. 1987;168:156-9.
17. Koichiro T, Daniel P, Nicholas P, Glen S, Masatoshi N, Sudhir K. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731-9.
18. Xu G, Fang QQ, Keirans JE, Durden LA. *Ferritin* gene coding sequences are conserved among eight hard tick species Ixodida: Ixodidae. Ann Entomol Soc America. 2004;97:567-73.
19. Nattall PA, Trimnell AR, Kazimirava M, Labuda M. Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases. Parasite Immunol. 2006;28:155-63.
20. Galay RL, Miyata T, Umemiya-Shirafuji R, Maeda H, Kusakisako K, Tsuji N, *et al*. Evaluation and comparison of the potential of two ferritins as anti-tick vaccines against *Haemaphysalis longicornis*. Parasites & Vectors. 2014;7:482-91.
21. Mulenga A, Macaluso KR, Simser JA, Azad AF. Dynamics of *Rickettsia*-tick interactions: identification and characterization of differentially expressed mRNAs in uninfected and infected *Dermacentor variabilis*. Insect Mol Biol. 2003;12:185-93.
22. Waidner B, Greiner S, Odenbreit S, Kavermann H, Velayudhan J, Stähler F, *et al*. Essential role of Ferritin Pfr in *Helicobacter pylori* iron metabolism and gastric colonization. Infect Immun. 2002;70:3923-9.