Original Article

Establishment and Validation of *Theileria annulata* Sporozoite Ak-93 Infection in Laboratory-Reared *Hyalomma anatolicum* Tick Using In Vivo and In Vitro Assays

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(Received 15 Jun 2019; accepted 27 Sep 2020)

Abstract

Background: Tropical Theileriosis caused by *Theileria annulata* is a tick-borne disease which transmitted by the ixodid tick members of the genus *Hyalomma*. Studies on different aspects of disease require to access infective sporozoite of parasite which produced by tick vector. This study was carried out to establish of *T. annulata* life cycle to achieve *T. annulata* infected ticks.

Methods: Laboratory rabbit and calf were used for rearing of *Hyalomma anatolicum* different instars. Unfed nymphs were fed on *T. annulata* infected calf. Clinical signs, Giemsa stained smears and Polymerase Chain Reaction (PCR) methods were used for detection of infection in blood and tick specimens. Susceptible calf was used for confirmation of sporozoites maturation and infectivity in bioassay test.

Results: *Hyalomma anatolicum* two and three-host strategies of life cycle was lasted 90 and 116 days respectively. The PCR confirmed *T. annulata* infection in blood and tick samples. Maturation of *T. annulata* sporozoites was confirmed in bioassy test. First clinical symptom of disease was seen earlier in the case of transmission of disease through feeding of live ticks in comparison with blood injection method.

Conclusion: Complete life cycle of *T. annulata* was done and confirmed by clinical signs, microscopic examination, molecular methods and bioassay test. According to published reports to date, this is the first report of establishment of *H. anatolicum* tick infection with *T. annulata* using susceptible calf under controlled conditions in Iran.

Keywords: *Theileria annulata*; *Hyalomma anatolicum*; Tick rearing; Polymerase Chain Reaction (PCR); Bioassay

Introduction

Bovine tropical theileriosis caused by *Theileria annulata* is one of the most important tick borne disease in cattle. The disease is transmitted trans-stadially by species of ixodid ticks of the genus *Hyalomma* and it is distributed in tropical and subtropical areas from northern Africa, southern Europe, the Middle East (including Iran) to Far East (1, 2). Complex life cycle of *T. annulata* involve schizogony and merogony in vertebrate host and sporogony in invertebrate hosts. Sporozoites of *T. annulata* enter the blood stream of host by feeding of infected tick on cattle and multiply as schizonts in lymphocytes and macrophages and from these stages merozoites developed in red blood cells that called piroplasm or erythrocytic forms. Some of these differentiate to gametocytes. By uptake of blood with ticks, the gamogony and sporogony stages occurred in tick midgut lumen and salivary glands of tick respectively (3). Lymph nodes enlargment, fever, anemia and icterus are main clinical signs of disease (4). Tropical theileriosis can be controlled by methods such as application of acaricides for

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Published Online: September 30, 2020

tick vector control, administration of live attenuated vaccine or by combination of both acaricides and vaccination (1). In case of infected animals the specific treatment against *T. annulata* is recommended by using parvaquone and buparvaquone (5). *Hyalomma anatolicum* as main vector of *T. annulata* is predominant species of *Hyalomma* ticks in domestic ruminants in Iran (6). Succesfull laboratory rearing of *H. anatolicum* tick using white rabbit has been reported (7). Artificial tick feeding has been used in some studies (8, 9). In this study laboratory animals were used as host for nymph and larva of ticks and preservation of tansstadial transmission of *T. annulata*. Sprozoite stage of *Theileria annulata* that present in salivary glands of tick vector is important in parasite life cycle (10). The maturation is necessary for infectivity of sporozites and occurred in tick salivary glands by both incubation at 36–37 °C or feeding on rabbit for 4–5 days (11, 12). Infective sporozoites can be used in many studies on different features of disease specially in vaccine efficay test (13). This study was carried out to establish of sexual stage of *T. annulata* life cycle using laboratory white rabbit and *T. annulata* infected calf for rearing of different tick instars to achieve *H. anatolicum* ticks infected by sporozoites of *T. annulata* AK-93 local strain. Although the bovine theileriosis vaccine immunity assay has performed by inoculating cryopreserved *T. annulata* infected blood stabilates, but here for the first time we tried to install the process of preparation and confirmation of *T. annulata* infected tick by in vitro molecular methods and in vivo assay by inoculation to susceptible calf.

Materials and Methods

Parasite

A highly virulent *T. annulata* infected blood AK-93 strain has been previously cryopreserved in cell bank unit of the Department of Parasite Vaccines Research and Production, Razi Vaccine and Serum Research Institute, (RVSRI). The virulent *T. annulata* Ak-93 strain has been isolated from an infected calf with severe theileriosis from Takestan County in Qazvin Province of Iran in 2014. The infectivity and virulence of the Ak-93 strain was earlier confirmed by in vivo assay through subcutaneously injection to a healthy intact calf (14).

Tick rearing

The adult engorged female *Hy. anatolicum* tick obtained from Department of Parasitology, Razi Vaccine and Serum Research Institute, incubated for oviposition. All incubation conditions in tick rearing experiments were carried out in constant temperature 28 ºC and relative humidity 85% unless specifically noted. To avoid fungal contamination the tick was rinsed by distilled water, dried in room temperature and kept in glass tube before incubation. The glass tube outlet closed by cotton for good ventilation.

Egg hatching

After complete egg laying, the dead tick was removed from tube and eggs were incubated in the same condition as mentioned in the case of oviposition.

Larvae feeding

Three month old white laboratory rabbit was provided from Department of Laboratory Animal Production of Razi Vaccine and Serum Research Institute 1.5 to 2kg of weight was used for feeding of tick larvae. The ear's hair of rabbit and also calves completely shaved and cleaned before releasing of larvae on animal's ears. Aproximately 1000 larvae released on rabbit ears using cotton earbags.

Moulting of larvae

The free detached engorged larvae have been collected from rabbit's earbag and kept in glass tube and incubated for moulting.

One of six-month-old calves was injected with Ak-93 virulent strain of *T. annulata* for infection induction. Another calf was remained safe for future challenge of the infective tick.

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Experimental infection of calf

Experimental infection with *Theileria annulata* in calf was done by subcutaneous injection of 20ml of virulent *T. annulata* infected blood (AK-93 strain) around prescapular area. The clinical features of bovine theileriosis in inoculated calf such as fever and lymph node enlargement were monitored daily. Giemsa stained smears prepared from aspirate of swelling lymph node were used for early detection of disease.

Feeding of nymphs

Unfed nymphs were divided in two groups, one for feeding on intact calf and another for feeding on *T. annulata* experimentally infected calf using earbags as described before in the case of larvae feeding.

Moulting of nymphs

Both groups of engorged nymphs collected from intact and *Theileria* infected calves were incubated under conditions similar to moulting of larvae.

Feeding of adults and maturation of sporozoites

Adult male and female unfed *Hyalomma anatolicum* ticks that obtained from moulted nymphs were used for rearing of next generation of tick by feeding on intact calf, as well as for *T. annulata* sporozoites maturation in tick's salivary glands by incubation at 37 ºC, relative humidity 85% for 3 days in the case of ticks that their nymphal stage had been fed on *Theileria* infected calf.

Bioassay test

Unfed adult *T. annulata* infected ticks were fed on intact six-month-old calf for confirmation of sporozoite maturation as bioassay test and the remainder ticks were used for stabilate preparation that could be applied in other future studies.

Animal working

All the procedures were accomplished in accordance with Animal Care and Ethics Committee (ACEC) of Razi vaccine and serum research institute instructions. There was no deviation of planned protocols and working methods and no animal wasting in research was occurred. In the last part of the tests, all rabbits and calves were dismissed according to the standard protocols.

Molecular test

Primer design

Two sets of primers were used: (i) TAMS (*Theileria annulata* merozoite surface antigen) for confirmation of *Theileria annulata* infection in calf blood and nymph specimens and (ii) HAEC (*Hyalomma anatolicum excavatum* calreticulin gene) for PCR and Reverse Transcription Polymerase Chain Reaction (RT-PCR) internal control of *Hyalomma* ticks. The forward and reverse HAEC primers designed from two exons of mentioned gene (in order to produce two different PCR product lengths) on genomic DNA and cDNA in PCR/RT-PCR reactions (Table 1).

DNA extraction

Phenol-chloroform method were used for genomic DNA extraction from blood and ticks samples (15). Each tick was grounded by mortar and pestle in 2ml of PBS (Phosphate-Buffered Saline) and supernatant was used for DNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from the tick stabilate using the One Step RNA reagent (YTzol Pure RNA, Yekta Tajhiz Azma, Iran) in accordance with the manufacturer's instruction. DNase treatment was done to assure the RNA response in RT-PCR assay (DNase I, Jena Bioscience, Germany). The cDNA synthesis and further PCR were performed using the M-MuLV reverse transcriptase and PCR mix (Yekta Tajhiz Azma, Iran) according to the manufacturer's instructions. Briefly, 2µg of total DNase treated RNA and 160pm of oligo $(dT)_{15}$ primer were incubated at 70 ºC for 5 minutes

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and then 4ul RT buffer 5X, 20U RNase Inhibitor, 200 Units of M-MuLV reverse transcriptase, 500µM each of dNTP were added in a total volume of 20µl. The reaction was incubated at 42 ºC for 1 hour, followed by 10min at 70 ºC to inactivate the enzyme.

PCR and RT-PCR

DNA and cDNA obtained from nymphal stages of *H. anatolicum* ticks before and after feeding on experimentally infected calf were used in PCR and RT-PCR reactions. Each reaction preformed in total volume of 20µl containing 10µl of master mix (Yekta Tajhiz Azma, Iran), 7µl of DEPC water, 1µl of each forward and reverse of mentioned primers and 1µl of DNA and synthesized cDNA in PCR and RT-PCR reactions, respectively. Both PCR andRT-PCR cycling started an initial denaturation at 94 ºC for 2min followed by 38 cycles of denaturation at 94 ºC for 30sec, annealing at 60 ºC for 1min and extension at 72 ºC for 1min. final extension preformed at 72 ºC for 7 minutes. Expected lenght of PCR and RT-PCR products were detected by running on 1.5% agarose gel for 60 minutes.

Results

Tick rearing

The adult *H. anatolicum* preoviposition and complete ovipostion periods lasted 7 and 14 days respectively. The maximum egg laying occurred in days 4–7 of oviposition period. The egg hatching was initiated after incubation for 7 days and lasted for 12 days. The resulted larvae were kept for 8 days for pre-feeding period. During larvae feeding on rabbit's ear, two and three-host behaviors were seen. Due to needs for unfed nymphs according to transstadial transmission nature of *Theileria* parasites, to get unfed infected adult ticks for bioassay test, some engorged larvae detatched manually from rabbit's ear in day 6 after releasing and remainder larvae moulted to nymphs on rabbit and engorged nymphs dropped and collected in day 13 of feeding period. After incubation, the resulted unfed nymphs and adults were fed on experimentally infected and intact calves respectively for 8 days. The obtained engorged nymphs and adults were incubated for moulting and oviposition. Complete two and three-host life cycle of *H. anatolicum* tick lasted 90 and 116 days. Some stages of tick rearing are shown and results of tick rearing in two and three-host manner were summarized in Table 2.

Experimental infection of calf

Absence of previous exposure to *T. annulata* in calf was confirmed with PCR using species specific TAMS primers (Table 1). Prescapular lymph node enlargement were observed in 15th day after injection of infected blood. The schizont stage of *T. annulata* was detected using Giemsa stained biopsy smear (Fig. 2 left). Parasitemia (Fig. 2 right) and fever above 40 °C (up to 42) were observed at day 20 post infection.

Polymerase Chain Reaction and Reverse Transcription Polymerase Chain Reaction

The expected PCR/RT-PCR products resulted from TAMS (597bp) and HAEC (480/330bp) primers on DNA/cDNA of nymphs samples before and after infection with *T. annulata* were seen in agarose gel (Fig. 3).

Bioassay test

Theileria annulata infected nymphs moulted to unfed adults after incubation. Additionally, to maturation of sporozoites of *T. annulata* in tick salivary glands, the adult ticks incubated at 37 ºC and RH 85% and then three males and four females ticks were used for confirmation of sporozoite maturation by in vivo bioassay test using intact calf (Fig. 4) and remaindor tick will used in stabilate preparation according OIE's instruction for vaccine efficacy test. The absence of *Theileria* infection in experimental calves was confirmed by specific PCR assay. Clinical signs such as lymph node enlargement and fever occurred

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Published Online: September 30, 2020

around in days 10 and 11 after releasing of ticks, however, the *T. annulata* infection was verified by PCR and Giemsa blood and lymph node smear staining. The clinical signs of Theileriosis in experimental calves using two employed methods were summarized in Table 3.

Table 1. Specifications of primers used in this study

Primer	Sequence 5'-3'	Expected size of Product		GenBank reference accession no.
		PCR	RT-PCR	
$TAMS_F$ $TAMS_R$	GAG ACA AGG AAT ATT CTG AGT CC TTA AGT GGC ATA TAA TGA CTT AAG C	597bp	597bp	Z48739.1
$HAEC_F$ $HAEC_R$	CAT TTT GCT TCC CCT GGT CGG CTG CAG TCG AAC AGC TTG AGG	488bp	330bp	AY395259.1

Table 2. Duration of rearing of tick's different developmental stages

Developmental stage	Duration (day)	
	Two-host manner	Three-host manner
Adult female preoviposition	7	7
Oviposition	14	14
Pre-hatching	7	7
Hatching	12	12
Larvae pre-feeding	8	8
Larvae feeding		6
Larvae pre-moulting		5
Larvae moulting	13	7
Nymph pre-feeding		10
Nymph feeding		8
Nymph pre-moulting	7	9
Nymph moulting	7	9
Adult pre-feeding	7	7
Adult feeding	8	7
Total life cycle	90	116

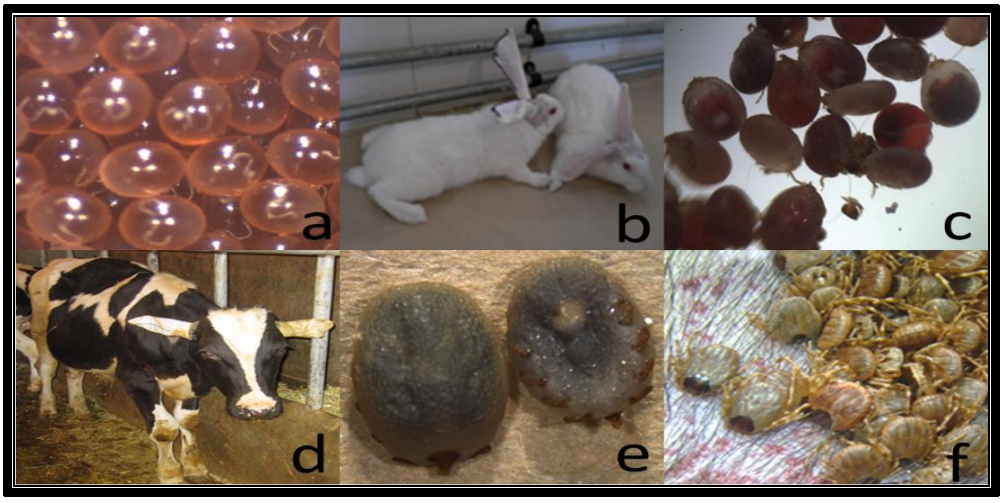

Fig. 1. Different stages of *Hyalomma anatolicum* tick rearing: a (eggs contain larvae), b (feeding of larvae on rabbit), c (engorged larvae), d (nymph and adult feeding on calf), e (engorged nymphs before molting) and f (adult tick feeding on calf's ear)

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Table 3. Comparative clinical signs of theileriosis using two infecting methods

Method of infection	Day of observation Onset of clinical signs		
	Lymph node enlargement	Fever	Prasitemia
Subcutaneously injection of blood	15	19	20
Feeding of live infected ticks	10	11	13

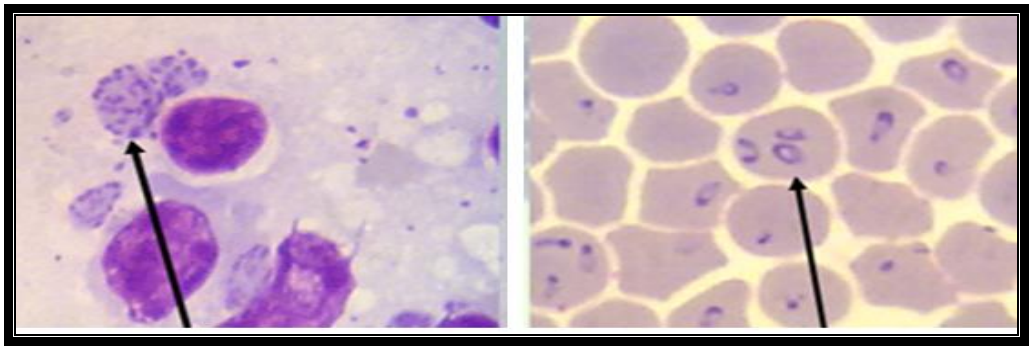

Fig. 2. *Theileria annulata* in Giemsa stained lymph node (Left) and blood smear (Right). Macroschizont in infected leukocytes and merozoites in RBCs are shown in lymph node biopsy and blood smears respectively

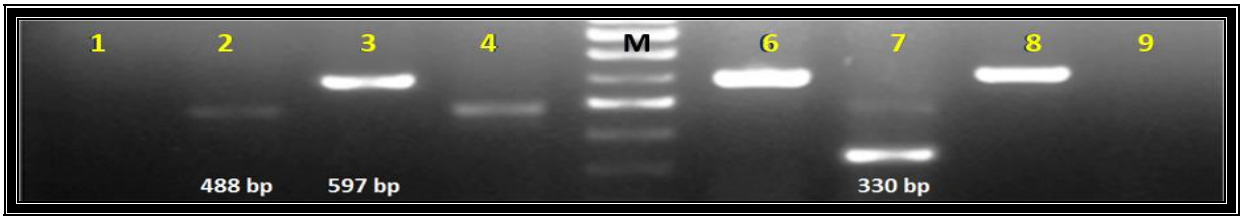

Fig. 3. Agarose gel electrophores of TAMS (1, 3, 6, 8) and HAEC (2, 4, 7) products in PCR (1–4) and RT-PCR (6-9) reactions on *Hyalomma anatolicum* nymphs before (1, 2) and after (3, 4, 6, 7), *Theileria annulata* vaccine strain as positive control (8), no cDNA as negative control (9) and 100 bp DNA ladder (M)

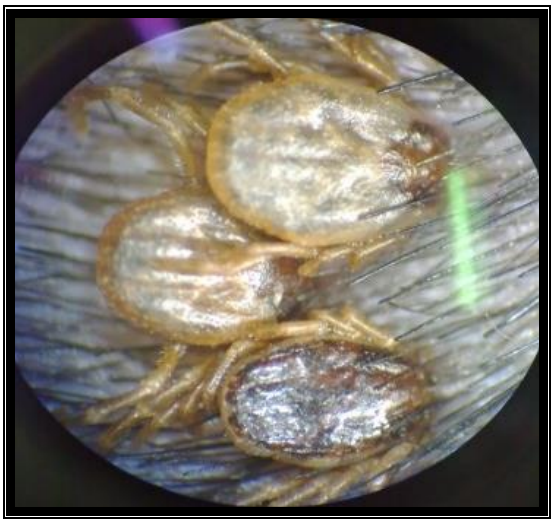

Fig. 4. Adult unfed infected *Hyalomma anatolicum* male and female ticks in bioassay test

Discussion

Here in this study, *T. annulata* sporozoite Ak-93 Infection was established and validated in laboratory-reared *Hy. anatolicum* tick vector by in vivo and in vitro techniques. Since, there is no appropriate laboratory animal model for bovine theileriiosis, the susceptible Taurine calves breeds are used for in vitro establishment of parasite's life cycle. Many efforts were performed to feed tick larvae on calf's ear in this study, but were not successful. However, the laboratory rabbits were used for feeding of larval stage and calves for nymph and adult stages of *H. anatolicum* tick. The results of laboratory tick rearing studies re-

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vealed that *H. anatolicum* tick has two-host behavior (7). A similar behavior was observed here in this study. Depending on the geographical distributions of *Hyalomma* in Iran, the minimum duration of complete life cycle of *H. anatolicum* ticks have been reported from 76–100 days (16). In the present study, according to the stable conditions for temperature and relative humidity and regardless of times elapsed for prefeeding periods, the complete life cycle for *H. anatolicum* continued 75 and 91 days in two and three-host behaviors respectively. In addition, as we have to provide unfed nymphs to further feeding on experimentally *T. annulata* infected calves, the engorged larvae were detached and collected manually from rabbit's ear and this physical intervention resulted in prolongation of complete life cylce in three-host manner.

Molecular detection of *T. annulata* infection using specific primers designed for various genes such as Tams (17), internal transcribed spacers (ITS1-ITS2) (18) and sporozoite surface antigen (SPAG) (19). In the present study, the species specific Tams primers were used for detection of *T. annulata* infection in calves and tick specimens. Additionally, RT-PCR reactions using Tams and HAEC genes were carried out in order to confirm the presence of active parasite infection (sporozoites formation) in prepared samples. It should be noted that the calreticulin is a multifunctional gene that expressed in all eukaryotic cells and has a 158bp intron spanned between two exons in *H. anatolicum* (20). This specific character is useful for RT-PCR assay. Moreover, Giemsa-stained blood and lymph node needle biopsy smears were also examined for early detection of *T. annulata* infection in calves.

In acute tropical theileriosis, death may be occurred between 5 and 7 days after onset of clinical signs in calves (21). Thus, early detection of infection is important for well-timed releasing of nymphs on infected calf according to the required time for nymphs feeding.

Sporozoite maturation could be achieved in tick's salivary glands by two methods; feeding of infected ticks on vertebrate hosts for several days or incubation of infected ticks at 36–37 ^{o}C in laboratory condition. However, it has been reported that the sporozoite maturation could be better formed by feeding on live animals than in vitro incubation (12). Since, the infected ticks supposed to be used in bioassay test, thus the ticks were incubated for 3 days to induce sporozoites maturation. This period has been reported for six days in *H. a. excavatum* as a very close species to *H. anatolicum* (11). Comparison of clinical symptoms in two methods of disease transmission including injection of *T. annulata* infected blood and through live infected ticks indicates the severity of disease was more by *T. annulata* sporozoites transfer via live infected ticks.

Tropical theileriosis in Iran was described for the first time in 1935. Control of disease has been performed using live attenuated Theileriosis vaccine manufactured by Razi Vaccine and Serum research Institute initiated since 1973 and continues to date (1). According to OIE's recommendation, vaccine efficacy test has to be done by challenge test using live infected ticks or *T. annulata* sporozoite tick stabilate. Despite the absence of international agreed standards for the challenge dose size, 5 to 10 females and the same number of infected unfed male *Hyalomma* ticks have to be used for infection of cattle (13). Here in this study, we used three male and four female unfed infected adult *Hyalomma* ticks for sporozoite maturation by subjecting to an intact naïve calf in bioassay test. Therefore, the presence of clinical signs of theileriosis after feeding of ticks has verified the sporozoite maturation and successful complete formation of *T. annulata* life cycle in calf and tick under in vitro controlled condition. Similar results have been reported in transmission of *T. annulata* by *H. dromedary* (22) and *Theileria hirci* by *H. anatolicum* previously (23). In recent years, due to ethical, economical and practical problems associated with tick rearing using ani-

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Published Online: September 30, 2020

mals led researchers to use artificial membranes for tick feeding as a new approach (24). *Hyalomma anatolicum* tick adult stage was successfully fed artificially through silicone membrane, although this method did not worked for nymphs, however, replacement of silicone membrane by mouse skin sheet showed satisfactory results (9). Although artificial tick feeding was used for adults and a little for nymph stages, but rabbit utilization for rearing of larval stage is unavoidable. According to OIE recommendation (13) the efficacy test of theileriosis vaccine must be conducted using live or stabilte of infected tick thus the obtained *Theileria annulata* infected tick from this study will used in vaccine efficacy test in future studies. Based on the available published reports to date, this is the first report of establishment of *H. anatolicum* tick infection with *T. annulata* under controlled condition in Iran.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article and all experiments on laboratory animals were performed according to regulations set by Ethical Committee of Razi Institute.

Acknowledgements

The authors thank Dr Ghader Khezrian for providing blood sample. This study was supported by the Parasitic Vaccine Research and Production Department, Razi Vaccine and Serum Research institute. This study was supported by the Razi Vaccine and Serum Research Institute, Iran with the research project code of 2-18-18-94123. The authors declare that they have no conflict of interest.

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