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PCR-based detection of *Theileria* infection and molecular characterization of Tams1 *T. annulata* vaccine strain

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ABSTRACT

In order to develop a method for detecting and identification of *Theileria annulata*, the specific primers from the major merozoite-piroplasm surface antigen sequence of *Theileria* (Tams1) were used to detect the *T. annulata* by nested-PCR technique. The assay provides a valuable tool for the identification of *Theileria annulata* directly from clinical samples and enables determination of the infecting species by a facile technique with high sensitivity and specificity power. The sensitivity of the PCR was determined up to 1.34 pg infected DNA, and specificity of the PCR was confirmed by DNA sequencing. The Tams1 nested-PCR assay will facilitate parasite infection follow up and might improve diagnosis and therapeutic approach of bovine tropical theileriosis. Moreover, multiple alignment and phylogenetic analysis of Tams1 sequences of available strains/isolates showed that there is a particular restriction site in *T. annulata* Iran-vaccine strain could be recognized by *AvaII* enzyme. These findings can be used in further disease control and prevention program as well as epidemiological studies.

Keywords: *Theileria annulata*, PCR, phylogenetic

INTRODUCTION

Theileria annulata is tick-born protozoan apicomplexan parasite of cattle that cause malignant theileriosis of large ruminants in Iran (Hooshmand-Rad & Hashemi-Fesharki 1971). Theileriosis is widely distributed in many tropical regions of the world, extending from southern Europe to southern Asia including Iran. Mortality varies from 90% in introduced exotic breeds to 5% or less in indigenous cattle breeds (Katzer *et al* 1998). Diagnosis of Theileriosis is based on clinical signs, knowledge of disease, and vector distribution as well as examination of Giemsa-stained blood, lymph node and tissue impression smears for the presence of the piroplasm and schizont stages of the parasite (OIE 2004). Serological tests such as indirect immuno-

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fluorescent antibody test (IFAT) can be detected antibodies. Serologic diagnosis may not be sensitive enough to detect all infected cattle, and cross–reactions can occur with other species of *Theileria*. Moreover, since serodiagnosis does not detect the parasite itself, whereas the animal may have already cleared the pathogen but remained seropositive. The central advantage that today's molecular techniques offer is that they allow researchers to study DNA directly. Polymerase chain reaction (PCR), reverse line blot assay and DNA probes are developed to identify *Theileria* species (Bekker *et al* 2002).

This study was performed to detect *T. annulata* infection the causative agent of theileriosis in cattle, by nested-PCR. Primers were derived from the gene encoding the *Theileria* major merozoite-piroplasm surface antigen (Tams1) gene. Multiple alignments of amplified Tams1 sequences were used for discriminating *T. annulata* vaccine strain from other *Theileria* strains (isolates).

MATERIALS AND METHODS

Parasites. The seven stocks of *Theileria* parasites were used in this study. The protozoa were originally obtained from different parts of Iran (Tehran [Vasfenard], Karaj, Fars, Zabol and Qazvin [Boein-Zahra]). Two *Theileria annulata* and *T. lestoquardi* samples were isolated from infected blood; two other were vaccine strains and two *T. annulata* infected cell lines were grown in tissue culture. Other hemoparasites DNA used in this study were *Babesia ovis, Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis spp*. and *Leishmania major.*

Growing protozoa in tissue culture medium. *T. annulata* infected cell line (vaccine strain) and two isolated from Qazvin (Boein-Zahra) were cultivated *in vitro*. Cell suspension was propagated and maintained at 37 ^{o}C in stoker media (Razi Institute, Iran) supplemented with 10% heat-inactivated horse/bovine serum, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Experimental infection. Three infected blood isolates from Karaj and Qazvin (Boein-Zahra) were derived from calves with clinical Theileriosis were subjected to experimentally splenectomized calves to study the biological features of Theileriosis and propagation of parasites *in vivo*. Calves were inoculated via intravenous injection with infected calf blood. Monitoring of experimental animals was carried out by taking of daily rectal temperatures, blood smears, and biopsy smears from swollen lymph nodes. Two-milliliters of blood aliquots were collected in disodium EDTA containers and stored at -70 ^{o}C until required for DNA extraction.

DNA extraction. DNA extraction was performed using "Proteinase K" method followed by phenol chloroform purification (Sambrook *et al* 1989). Briefly, after removing the RBCs with lysis buffer, followed by centrifugation, the cell pellet was placed in a solution of proteinase K and SDS then incubated until most of the cellular protein was degraded. The digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extractions, recovered by ethanol precipitation, then was dried and resuspended in TE buffer. DNA concentration was determined either by agarose gel electrophoresis and spectrophotometry.

PCR. Two oligonucleotide primers were designed based on Tams1 encoding gene of *Theileria annulata* in order to detect the protozoa (CinnaGen, Iran). Two primer pairs were designed to setting up the nested-PCR based on the major merozoite surface antigen gene of *Theileria annulata* (Tams-1). The external primers were: Tms1 (5' ATG TTG TCC AGG ACC ACC CTC AAG) and Tms4 (5' GAT AAG TTG TTA CGA ACA TGG), and the internal primers were: Tms3 (5' CGA GAC CTA CTA CGA TGA AG) and Tms2 (5' TTA AAG GAA GTA AAG GAC TGA TGA GAA GAC G). PCR was performed in a final reaction volume of 20 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM (each) deoxynucleoside triphosphate, 0.5 U of *Taq*

polymerase (CinnaGen, Iran), 10 pmol of each primers, and 2 microliter of template. The reactions were performed in an automatic DNA thermal cycler (techne, Germany) for 30 cycles. Each cycle consisted of a denaturing step of 30 seconds at 94 ^{o}C, an annealing step of 1 min at 58^{o}C (Tams1 primers), and an extension step of 1 min at 72 ^{o}C, followed by final extension step of 5 min at 72 ^{o}C. PCR product sequences submitted to be aligned for multiple sequence alignment and phylogenetic study of *Theileria* strains/isolates.

Detection of PCR products. Amplified PCR products were separated by electrophoresis on a 1.5 % agarose gel, stained with ethidium bromide and visualized by UV transillumination.

Specificity of the PCR. PCR specificity was shown by using other apicomplexan protoza DNA such as *Babesia ovis*, *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis spp*., and healthy cattle, and sheep genomic DNA as well as negative control. To compare the analytical sensitivity of the nested-PCR, serial dilutions of known amounts of schizont-infected cell DNA were performed. Then, PCR was carried out by external and the PCR products were subjected to a second round of PCR (nested-PCR).

Sequence alignment and phylogenetic analysis. The amplicons of Tams1 gene obtained from seven studied *Theileria* strains (isolates) were sequenced and aligned by Clustal W multiple alignments program. The multiple alignments of six Iranian Tams1 sequences and thirteen Tams1 sequences from other countries (Spain, Portugal, Italy, Turkey, Sudan, Mauritania, Tunisia, India, and Bahrain) were used for phylogenetic analysis using DNADist Neighbor-Joining method (Thompson 1994). Phylogenetic tree was constructed using DNADist (version 3.6a2.1) program to compute distance matrix across the interval from 36 to 813 of the Tams1 gene by the Neighbor-Joining /UPGMA, and a dendrogram was plotted (all programs are from the BioEdit phylogeny package,Version 7.0.1).

RESULTS

Result of DNA extraction. DNAs were extracted and purified from three *Theileria* infected blood samples and four *Theileria* infected cell lines by proteinase K and phenol/chloroform/isoamylalcohol successfully. Quality of extracted DNA was evaluated by agarose gel electrophoresis and quantified using UV spectrophotometry (A260) and measuring the ratio of A260/A280.

The result of amplification for *Theileria* Tams1 gene. Specific PCR using Tams1 gene for available *Theileria* isolates (strains) yielded amplicons with the same size for both *Theileria* species (~846 bp PCR product length) by primers Tms1 and Tms2 (Figure 1), but PCR was specific for *T. annulata* by using primers Tms3 and Tms4 (490 bp PCR product length (Figure 2).

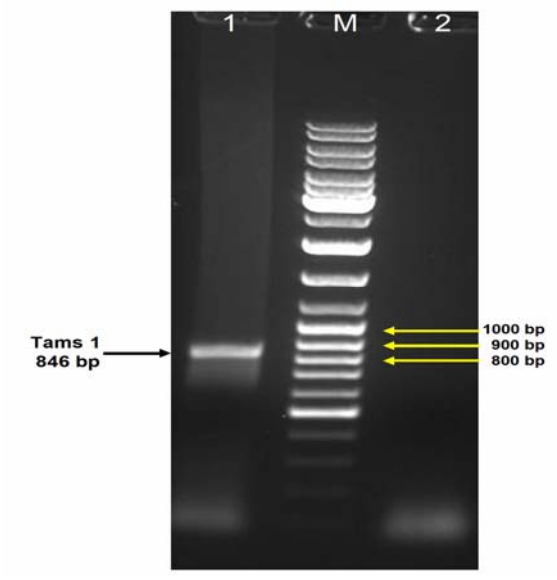

Figure 1. Gel agarose (1.5 %) electrophoresis of amplified Tams1 using Tms1 and Tms2 primers. 1, the amplified *Theileria annulata* Tams1/Ms1-2 in *T. lestoquardi*. *Lane* M, 100 bp DNA ladder as size marker, and *Lane* 2, negative control (no DNA)**.**

The specificity of the PCR. No amplification was shown by other apicomplexan protozoa such as *Babesia ovis, Toxoplasma gondii, Neospora caninum, Sarcocystis spp*., and healthy cattle and sheep genomic DNA as well as negative control using Tms3 and Tms4 primers (Figure 2).

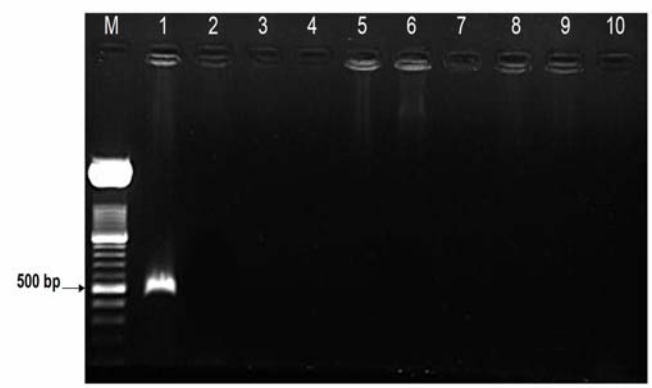

Figure 2. The specificity of the PCR for *Theileria annulata* detection using protozoan DNAs, calf DNA and negative control (no DNA). *Lanes* 1 is *T.annulata* amplified Tams1 gene. *Lane* 2, *T. lestoquardi* DNA; *Lane* 3, *B. ovis* DNA; Lane 4, *T. gondii* DNA; ane 5, *N. caninum* DNA; *Lane* 6, *L.major* DNA; *Lane* 7, *Trypanosoma spp.* DNA; Lane 8, *Sarcocystis spp.* DNA; *Lane* 9, Calf thymus DNA; and *Lane* 10, negative control (no DNA).

The sensitivity of the PCR. The sensitivity of was performed using nested-PCR for Tams-1 gene PCR of *Theileria annulata* (using primers Tms1, 4 as external and primers Tms2,3 as internal primers). Diluted DNA solution was used for determining the limit of detection, and nested-PCR was carried out first by external primers. The result of the first PCR demonstrated the sensitivity of the PCR was 134 pg for infected DNA, but in the second round of the PCR (nested-PCR) the sensitivity was up to 1.34 pg (Figure 3).

Sequence alignment and phylogenetic analysis. The six Iranian Tams1 sequences and thirteen published from three continents were compared in this study. The length of aligned sequences was 778 nucleotides. Multiple alignments of the nineteen Tams1 sequences were performed for discriminating the vaccine strain from other Iranian isolates by phylogenetic analysis. This comparison showed there is a unique restriction site in Tams-1 gene of *T. annulata* Iran vaccine strain recognized by *AvaII*, but not existed in other examined strains (isolates) (Figures 4, 5 and 6).

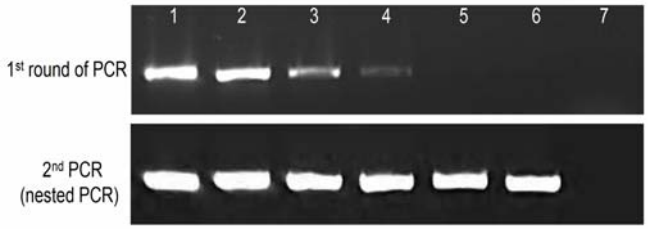

Figure 3. Determination of sensitivity of the designed nested-PCR for detecting *Theileria annulata* Tams1 gene. Extracted DNA was quantified and serial dilutions were prepared and the first and second rounds of PCR were carried out. *Lanes* 1 to 6 exhibits the 10^{-1} to 10^{-6} dilutions of *T. annulata* infected DNA; and *Lane* 7 is negative control. The results showed the sensitivity for the 1st round of PCR was 134 picogram (pg) *T. annulata* infected DNA, and 1.34 pg infected DNA for nested-PCR.

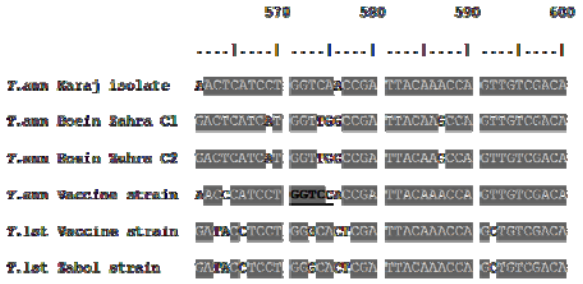

Figure 4. Alignment of sequenced *Theileria annulata* and *T. lestoquardi* Iranian strains and isolates using "Multiple Alignment Method" by "Clustal W" program. The nucleotide number 575 of Tams1 sequence exhibited a specific difference in comparison with other Iranian Tams1 sequence. The nucleotides between 571 to 575 are in restriction enzyme cut site of "Ava II".

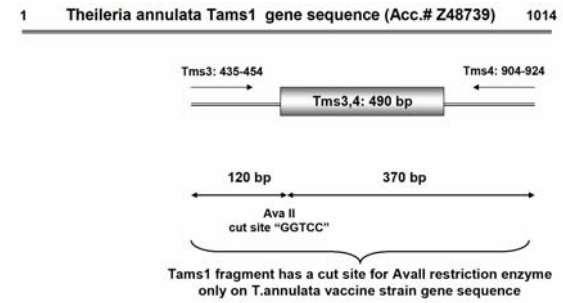

Figure 5. Map of *Theileria annulata* Tams1 sequence and the presence of one cut site for Ava II restriction enzyme in *T. annulata* vaccine strain.

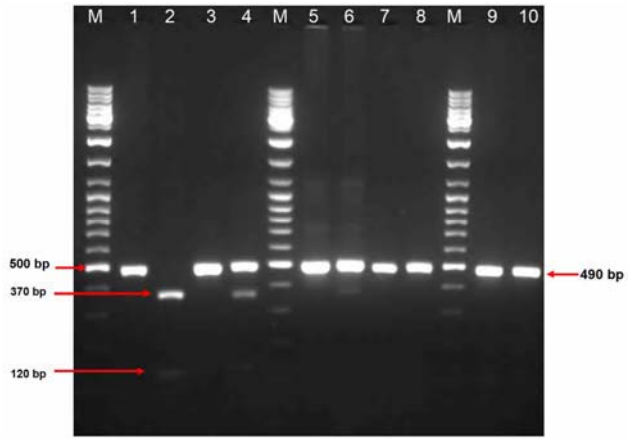

Figure 6. Gel agarose electrophoresis of restriction enzyme digestion of Tams1 *T.annulata* Vaccine strain sequence and other Iranian *T.*ann isolates. *Lanes* 1 and 2 show the result of restriction analysis of Tams1 sequence based on "Ava II" before and after digestion respectively. Results for other examined isolates: *Lanes* 3 and 4 for Karaj isolate, *Lanes* 5 and 6 for Boein-Zahra C1, *Lanes* 7 and 8 for Boein-Zahra C2 and *Lanes* 9 and 10 for Zabol isolate.

The aligned Tams1 sequences were subjected to determine the genetic relationships between 19 Tams1 gene (homologous Ms1-2 gene in *T. lestoquardi*) sequences by construction of a neighbor-joining tree. The resulting phylogenetic tree is shown in figure 7.

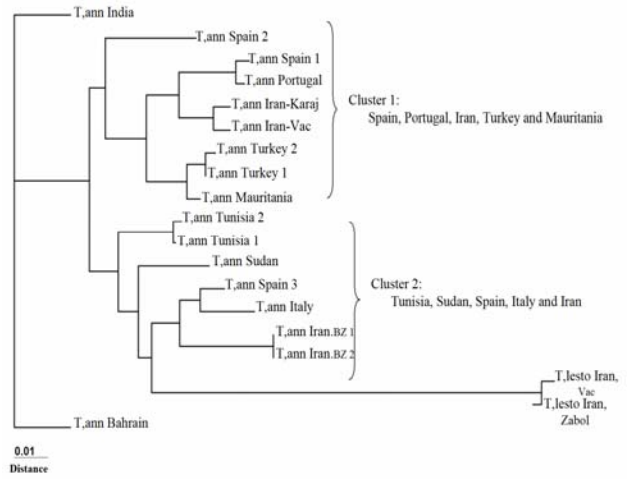

Figure 7. Phylogenetic tree of 19 *Theileria* sequences, listed in table 1. Unrooted tree were constructed using DNADist program to compute distance matrix across the interval 778 nucleotides of Tams1 gene by the Neighbor phylogenetic tree.

A pair wise comparison of the Tams1 sequences obtained from the recognized Iranian *Theileria* species revealed DNA similarity values ranging from 83.9 to 99.8 % (Table 1).

Table1. Sequence identity matrix of Iranian *Theileria* isolated and sequenced Tams1 (Ms1-2) gene.

T. annulata Karaj	ID					
T. annulata Boein-Zahra C1	0.895	ID				
T. annulata Boein-Zahra C2	0.896	0.998	ID			
T. annulata Vaccine	0.985	0.901	0.903	ID		
T. lestoquardi Vaccine	0.845	0.865	0.866	0.843	ID	
T. lestoquardi Zabol	0.841	0.860	0.862	0.839	0.987	ID
Sequence	T.annulata Karaj	T. annulata Boein-Zahra C1	T. annulata Boein-Zahra C2	T. annulata Vaccine	T. lestoquardi Vaccine	T. lestoquardi Zabol

Table 2. GenBank accession numbers, host and origin of the Tams1 gene sequences used in the phylogenetic tree analysis.

Species	Origin	Accession number	Host
T. annulata	Bahrain	AF214802	Cattle
T. annulata	Caceres (Spain)	AF214807	Cattle
T. annulata	Caceres (Spain)	AF214812	Cattle
T. annulata	Cadiz (Spain)	AF214816	Cattle
T. annulata	Portugal	AF214829	Cattle
T. annulata	Sudan	AF214834	Cattle
T. annulata	Turkey	AF214839	Cattle
T. annulata	Hissar (India)	AF214844	Cattle
T. annulata	Mauritania	AF214858	Cattle
T. annulata	Italy	AF214863	Cattle
T. annulata	Tunisia	AF214907	Cattle
T. annulata	Ankara (Turkey)	AF214918	Cattle
T. annulata	Tunisia	AF214920	Cattle
T. annulata	Karaj (Iran)	EF092915	Cattle
T. annulata	Boein-Zahra (Iran)	EF092918	Cattle
T. annulata	Boein-Zahra (Iran)	EF092919	Cattle
T. annulata	Tehran (Iran)	EF092914	Cattle
T.lestoquardi	Fars (Iran)	EF092917	Sheep
T.lestoquardi	Zabol (Iran)	EF092916	Sheep

The sequenced *T. annulata* and *T. lestoquardi* Tams1 (Ms1-2) gene sequences for two *Theileria* vaccine strains and five isolates were deposited in GenBank as the following: *T. lestoquardi* vaccine strain Ms1-2 gene, Acc# EF092917, *T. lestoquardi*

Zabol isolate Ms1-2 gene, Acc# EF092916; *T. annulata* Boein Zahra C2, Tams1 gene Acc# EF092919; *T. annulata* Boein Zahra C1, Tams1 gene Acc# EF092918; *T. annulata* Karaj/78, Tams1 gene Acc# EF092915; *T. annulata* vaccine strain, Tams1 gene Acc# EF092914 (Table 2).

DISCUSSION

This study describes the development of a simple, rapid, sensitive and specific method for detection and identification of *Theileria annulata*. *Theileria* species are transmitted by *Hyalomma* ticks. Protozoa can not be distinguished in the salivary glands of infected ticks by traditional staining methods such as methyl green-pyronin because of morphological similarity (Kirvar *et al* 1998). The therapeutic and preventive strategy needs to epidemiological findings about prevalence and distribution of protozoan parasite in ticks and/or carrier livestock. Therefore, having a specific, sensitive and simple method is very important for detecting and discriminating *T. annulata* from other *Theileria* species.

The results of this study clearly demonstrated the high sensitivity and specificity of the designed PCR for detecting *Theileria annulata* species based on Tams1 gene for high sensitive detection of the parasite. Moreover, multiple alignments of amplified sequences showed a specific nucleotide substitution in *T. annulata* vaccine strain Tams1 gene sequence. This finding was proved by restriction analysis. Digestion with appropriate restriction enzyme (*AvaII*) cleaved the *T. annulata* vaccine strain Tams1 gene sequence, but did not for other available strains (isolates).

Kirvar *et al* (2000) used the 30 kDa merozoite surface protein gene as a detective target sequence by designed specific primer to amplify only *T. annulata* or *T. lestoquardi*. Gubbels *et al* (1999) showed the probe hybridization for detecting *Theileria annulata* had a cross reaction with *T. lestoquardi* DNA, and Schnittger *et al* 2004 demonstrated the prepared specific *T. lestoquardi* probe cross reacted with *T. annulata* DNA. Both results might be due to differences in *Theileria* genome. Here, we showed the specific nested-PCR for detecting of *T.annulata* specifically.

Iranian Tams1 gene sequences and 13 registered Tams1 sequences were analyzed for phylogenetic study using Clustal W for multiple alignment and phylogenetic tree was constructed using Neighbor-Joining phylogenetic tree method by DNADIST version 3.5c (Figure 7). As shown in the phylogenetic tree, *T. annulata* Tams-1 sequences were clustered into two major sub-groups, apart from Bahrain and Indian isolates. There is no distinct classified clusters for studied Tams1 sequences derived from three continents, the sequences were scattered in two main sub-groups and two sequences were apart of these two clusters. Interestingly, an isolate and one strain from Iran were classified in the first sub-group near the European and African sequences, and two another isolates (Boein-Zahra C1/C2) were grouped in the second sub-group, close to the European and African Tams1 sequences.

In conclusion, we developed a molecular diagnostic technique for detecting *Theileria annulata* with high sensitivity and specificity. The sensitivity of the PCR (limit of detection) was determined by preparing DNA in serial dilution and observing the PCR results. The specificity of the PCR was validated by sequencing of the amplified fragments and using available apicomplexan protozoa DNAs as well as healthy ruminants DNA. The discrimination of vaccine strain from other examined *Theileria* strains (isolates) was shown by restriction analysis. This is the first report, shows the difference between *Theileria* vaccine strains from other available strains (isolates) in Iran. The phylogenetic analysis demonstrated the scattered pattern of *T. annulata* strains (isolates) near the other European and African strains (isolates).

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