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Short Communication

A comparative phylogenetic analysis of *Theileria spp.* by using two "18S ribosomal RNA" and "*Theileria annulata* merozoite surface antigen" gene sequences

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ABSTRACT

More than 185 species, strains and unclassified *Theileria* parasites are categorized in the Entrez Taxonomy. The accurate diagnosis and proper identification of the causative agents are important for understanding the epidemiology, prevention and appropriate treatment. This study aims to discuss the importance of two genes of *Theileria annulata* 18S ribosomal RNA (18S rRNA) and *Theileria annulata* merozoite surface antigen (Tams1) for phylogenetic analysis of molecular data. Eight *Theileria annulata* isolates including S15 Iran vaccine strain, six *T. annulata* field isolates and one Iraq *T. annulata* field isolate were studied in this study. The 18S rRNA and Tams1 gene sequences were investigated by phylogenetic analysis after DNA extraction, PCR, and DNA sequencing. Sequence fragments of about 1410 to 1412 bp of the complete 18S rRNA gene and 778 nucleotides of Tams1 gene sequences were used in the *analysis* of nucleotide diversity. Sequence alignment and phylogenetic analysis were performed by constructing the phylogenetic tree and identity matrix based on two above genes. The results of the study revealed, the 18S rRNA is informative in estimation of relationships among *Theileria* species and strains, but the Tams1 seems to be appropriate for analysis of the molecular antigenicity in vaccine development.

Keywords: *Theileria annulata*, Tams1, 18S rRNA, Taxonomy, Phylogenetic analysis

INTRODUCTION

The genus *Theileria* comprises of more than 185 different species, strains and unclassified *Theileria* parasites, are introduced in the Entrez Taxonomy, and associated with a wide variety of animal diseases in domestic and wild animals (www.ncbi.nlm.nih.gov/Taxonomy/). *Theileria annulata* and *T. parva* cause severe disease in cattle, *T. annulata* is the most economically important in tropical theileriosis including Iran (OIE 2008). The parasite causes high morbidity and mortality in exotic cattle (OIE 2008). The recovered animals are theoretically protected against homologous parasite strains (McKeever 2009). Post vaccination surveillance and molecular characterization of isolates circulating in the field are the key measures in controlling the bovine theileriosis (Ali *et al* 2006, Geysen *et al* 1999). Thus, before introduction of immunization it is necessary to use of

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efficacious techniques, such as species specific PCR, to detect and identify *Theileria sp* from the field. Ribosomal RNA is the most abundant constituent of nucleic acids in any non-viral organism with the eukaryotic RNA transcription unit. The rRNA gene has been sequenced from a variety of different organisms, resulting in a large database for sequence comparisons (Chae *et al* 1998, Gubbels *et al* 2000). Moreover, the 18S rRNA gene is valuable for phylogenetic analysis due to its high levels of conservation (Sparagano *et al* 2006). However, the molecule also possesses phylogenetically informative variable regions that are useful for determining relationships among species (Hillis & Dixon 1991). On the other hand, *Theileria annulata* major merozoite-piroplasm surface antigen (Tams1) gene sequence has been used as a specific target for setting up a high sensitive PCR and also for immunological investigations towards of vaccine studies, however there are some molecular epidemiological studies using Tams1 analysis (Habibi *et al* 2007, Kirvar *et al* 2000, Gubbels *et al* 2000, Esmaelizad *et al* 2011).

In this work, it was aimed to discuss about using the two genes of *Theileria annulata*; 18S rRNA and *Theileria annulata* merozoite surface antigen (Tams1) in order to evaluate the prognostic predictive value of phylogenetic analysis of *Theileria spp.* as an analytical tool.

MATERIALS AND METHODS

Strain and field isolates of *Theileria* parasites. One vaccine cell line of *Theileria annulata* (S15 Iran vaccine strain), and six field isolates of *T. annulata* were used in this study. In addition, one field isolated *T. annulata* was obtained from naturally infected cattle in Duhok province, Kurdistan of Iraq.

DNA extraction. DNA extraction was performed using "Proteinase K" method followed by phenol chloroform purification (Sambrook *et al*. 1989). DNA concentration was determined either by agarose gel electrophoresis and spectrophotometry.

PCR. Two oligonucleotide primers were designed to setting up the specific PCR based on the major merozoite surface antigen gene of *Theileria annulata* (TAU22887) (CinnaGen, Iran). The external primers were: Tms1 (5' ATG TTG TCC AGG ACC ACC CTC AAG) (nucleotides 55 to 78) and Tms2 (5' TTA AAG GAA GTA AAG GAC TGA TGA GAA GAC G) (nucleotides 870 to 900) (Habibi *et al* 2007).

The specific primers were designed based on *Theileria annulata* 18S ribosomal RNA gene sequence (accession # EU083801). Two primer pairs were designed in order to span the major hypervariable regions along the 18S ribosomal RNA gene sequence (Gene Runner program, Version 3.05). The first pair of primers, F1 (5’ GGC GGC GTT TAT TAG ACC 3’) and R1 (5’ TCA ATT CCT TTA AGT TTC AGC C 3’) were used to amplify bases between 186-1093 and the second primers, F2 (5’CAG ATA CCG TCG TAG TCC 3’) and R2 (5’ CCT TGT TAC GAC TTC TCC 3’) were applied to amplify bases between 945-1714 of *Theileria annulata* 18S ribosomal RNA gene sequence (EU083801) and these two primers sets covered the majority length of the 18S ribosomal RNA gene sequence with 127 bp overlapping. The length of the 18S rRNA gene of all *Theileria spp*. involved in this study was around 1,400 bp.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 (Habibi *et al* 2007).

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 demonstrated 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 positive and negative controls.

Sequence alignment and phylogenetic analysis. The DNA sequences of 18S rRNA gene obtained from two studied *Theileria annulata* samples and 42 sequences of 18S rRNA gene sequences including 36 *Theileria spp.*, 4 *Babesia spp.* and two Coccidian protozoa were accessed from GenBank. The sequences were aligned by Clustal W multiple alignments program. The alignment was manually edited in BioEdit and truncated to the size of the smallest sequence (~1,400bp). Phylogenetic tree was constructed by using DNADist Neighbor-Joining method (version 3.6a2.1), sequence identity matrix of all sequences were computed as well (BioEdit phylogeny package, Version 7.0.1) (Habibi *et al* 2011). Phylogenetic analysis of *Theileria sp* was performed on the hypervariable regions of the 18S rRNA gene positions 240-1650 to accurately predict the evolutionary relationship of *Theileria spp.* Therefore, if there are some previous data that support using the minimum acceptable window to provide meaningful phylogenetic analysis, the partial gene sequence can be used instead of whole or the majority of the gene sequence length (Vauloup-Fellous *et al* 2010). To the best of our knowledge, there are no published reports based on minimum acceptable 18S rRNA gene sequence window which produced *Theileria* phylogenetic analysis. Thus, the present study was carried out to analysis of *Theileria spp* phylogenetic.

RESULTS AND DISCUSSION

Results for Tams1 gene phylogenetic analysis. Totally seventeen Tams1 gene sequences were analyzed for phylogenetic study including four sequences belonging to Iran and 13 previously registered sequences in GenBank. The constructed phylogenetic tree showed the *T. annulata* Tams1 sequences were distributed into two major groups. There was no distinct classified clusters for studied Tams1 sequences derived from three continents (Asia, Africa and Europe), the sequences were scattered in two clusters. Interestingly, two sequences from Iran were classified in the first group near the European and African sequences, and two another Iranian isolates (Boein-Zahra C1/C2) were grouped in the second sub-group, close to the European and African Tams1 sequences (Figure 1).

Results for 18S rRNA gene phylogenetic analysis. Sequence alignment and phylogenetic analysis were performed by constructing the phylogenetic tree based on 18S ribosomal RNA gene for *Theileria annulata* Iran S15 vaccine strain, Iraq isolate, and 36 *Theileria spp.* as well as sequences including *Babesia spp., Toxoplasma gondii* and *Isospora suis* sequences. The results clearly revealed the entire eight *T. annulata* 18S rRNA gene sequences from China, Iran, Iraq, Italy and Spain were classified in one clade (Figure 2).

Theileria spp. is intra-cellular protozoa, transmitted by *ixodid ticks*, which recognize and invade specific host cells (Shaw 2003); therefore, the presence of tick was strongly associated with bovine theileriosis. This association makes a broad geographical distribution of the *Theileria* species and numerous strains with a wide range of virulence around the world (Shaw 2003).

The results of phylogenetic analysis based on 18S rRNA gene sequence clearly shows close relationship of different species, *T. annulata*, *T. lestoquardi*, *T. parva* and *T. taurotragi* in constructed tree the entire are within cluster "A" (Figure 2). The interesting finding was that all pathogenic *Theileria* species including *T. annulata*, *T. parva* and *T. lestoquardi* were classified in one cluster. The comparison of the Tams1 gene sequences derived from distant and far geographical

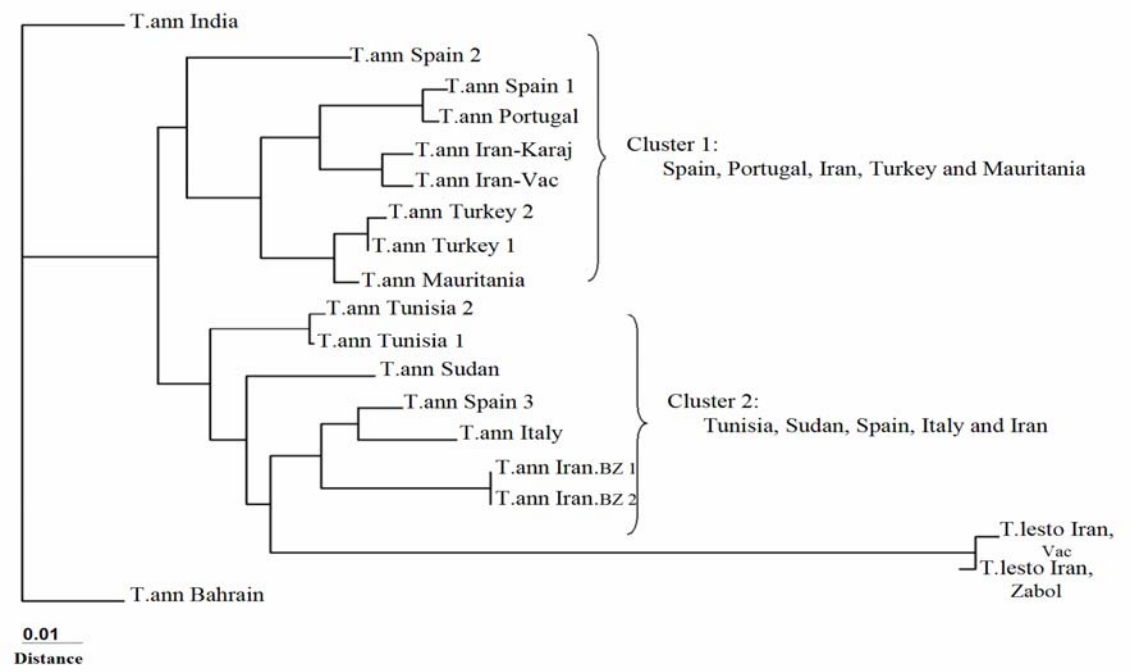

Figure 1. The phylogenetic tree of selected seventeen *Theileria annulata* strains and isolates from three continents of Asia, Africa and Europe based upon Tams1 gene sequences. An unrooted tree was constructed using DNADist program to compute distance matrix across the interval 778 nucleotides of Tams1 gene by the BioEdit Sequence Alignment Editor.

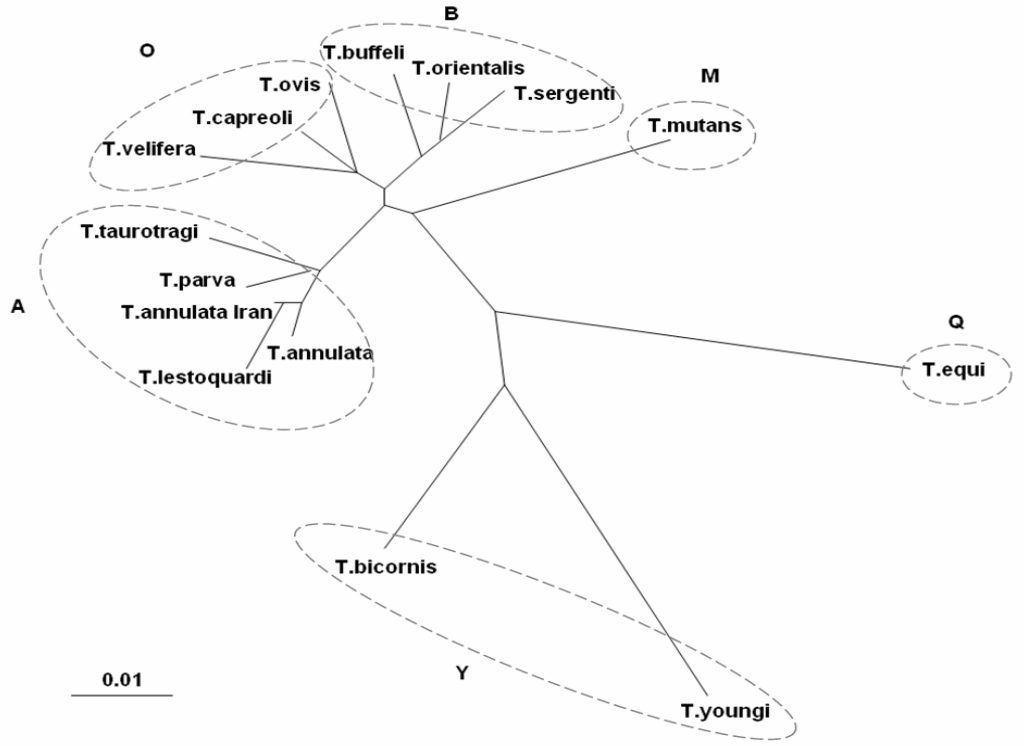

Figure 2. The unrooted phylogenetic tree of *Theileria* 18S rRNA gene sequences. The analyzed sequences were clustered in six major groups, based on Theileria 18S rRNA gene sequences. Scale bar represents nucleotide substitutions per position.

geographical regions determined that there was no particular sequence type belongs to a definitive region. Identical Tams1 sequences were found in widely separate regions such as Spain and India (Gubbels *et al* 2000). Similar findings were obtained by us, for instance Tams1 gene sequences from two continents; Spain-1 (AF214807) and Iran-Karaj (EF092915) isolates or Turkey (AF214918) and Mauritania (AF214858) isolates showed the close classification, but the other studied sequences from a certain region, i.e. Iran-Boein Zahra (EF092918 and EF092919) and Iran-Karaj sequences were placed in two distinct clusters. Although, the 18S rRNA has a crucial role in the conclusion of relationships among *Theileria* species, but the question is here that, why some researchers have been used the immunodominant Tams1 gene sequence instead of 18S rRNA gene sequence for phylogenetic analysis?

Dickson and Shiels have characterized the 30-kDa merozoite surface antigen of *Theileria annulata* (Ankara). The merozoites and piroplasms antigens were strongly identified by serum from an immune cow. Moreover, the molecule was found to be antigenically divergent (Dickson & Shiels 1993).

Another group of researchers have analyzed a population sample of nucleotide sequences of Tams1 (Gubbels *et al* 2000). They have studied a total of 129 Tams1 sequences from parasites isolated in Bahrain, India, Italy, Mauritania, Portugal, Spain, Sudan, Tunisia and Turkey. To investigate the generation of Tams1 molecular diversity they have sequenced a number of variants from a range of isolates obtained across a wide geographical distribution of the parasite and compared the data with a registered Tams1 gene sequences in GenBank (Gubbels *et al* 2000). Interestingly, significant sequence diversity was found both within and between isolates and many of the sequences was unique. Briefly, no geographical specificity of Tams1 gene sequence types was observed and almost identical sequences occurred in different geographical areas and a panmictic (it is the ability of individuals in a population to move freely within their habitat, and thus breed with other members of the population that defines panmixia) population structure is suggested by studied results (Gubbels *et al* 2000). Gubbels has hypothesized the reasons for diversity at the amino acid level. The results have verified that selection of amino acid replacements has occurred within variable domains of the Tams1 molecule and provide evidence that these domains are exchanged during sexual recombination, generating a mosaic pattern of molecular diversity (Gubbels *et al* 2000).

It is generally believed that the resulting diversity is positively selected since it allows evasion of the host immune response. Therefore, that generation of Tams1 diversity permits the parasite to escape from of host immunity. This knowledge may provide valuable information on the antigenic structure and might be helpful in vaccine design.

In conclusion, the small subunit rRNA (18S) has played a dominant role in the informative estimation of relationships among *Theileria* species from molecular data, but the Tams1 as a known immunodominant surface antigen has been used for investigation of the molecular diversity, and antigenicity in vaccine purposes.

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