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Short Communication

Detection and Discrimination of *Theileria annulata* and *Theileria lestoquardi* by using a single PCR

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ABSTRACT

The aim of this study was to detect and differentiate *Theileria annulata* and *T. lestoquardi* (*hirci*) by PCR. Members of the genus *Theileria* are tick-borne hemoprotozoan parasites; those cause fatal and enervating diseases of cattle and sheep in Iran. In order to develop a specific method for detecting and identification of *Theileria* species, specific primers from the surface protein (SP) sequence were designed that allowed the specific diagnosis of *T. annulata* and *T. lestoquardi* infection simultaneously. *Theileria* surface protein genes have consensus and variable sequences regions that allowed us to design the common primers for both species, which amplified two different PCR products. The results of this study demonstrated that a novel, simple, and high specific PCR for detecting and identifying *T. annulata* and *T. lestoquardi* infection.

Keywords: *Theileria*, *annulata*, *lestoquardi*, PCR, surface protein

INTRODUCTION

Theileria annulata and *T. lestoquardi* (*hirci*) are *tick-born protozoan* parasites that cause malignant theileriosis of cattle and sheep in Iran (Hooshmand-Rad & Hashemi-Fesharki 1971). Theileriosis is broadly distributed in several 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, and vector distribution as well as inspection of Giemsa-stained blood, lymph node and tissue impression smears for the presence of the intraerythrocytic piroplasms and schizont stages of the parasite (OIE 2004). *Theileria* specific antibodies can be detected by using indirect immuno-fluorescent antibody test (IFAT). Since most available tests for the *serological diagnosis* of theileriosis are not sensitive enough to detect all infected cattle, cross–reactions can occur with other species of *Theileria*. In addition, while serodiagnosis does not detect the parasite itself, but the animal may have already cleared the pathogen but remained seropositive. However, the *T. annulata* surface protein (TaSP) was identified by screening a *T. annulata* cDNA expression library with antischizont antiserum (Schnittger *et al* 2002a). TaSP occurs as a single-copy gene and is expressed in the sporozoite and schizont stages of the parasite.

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Homologous of the gene were shown to exist also in other *Theileria* species namely, *T. lestoquardi* and *Theileria sp.* (China) (Schnittger *et al* 2002b), however, stage-specific transcription has not been analyzed. Clearly, there are conserved domains at the N and C termini of the gene, flanking a highly polymorphic central region of the sequence. The essential benefit 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 differentiate *T. lestoquardi* and *T. annulata* infection the causative agents of theileriosis in cattle and sheep, by PCR. The specific primers were derived from the gene encoding the *Theileria* surface protein.

MATERIALS AND METHODS

Parasites. The five *Theileria annulata* and two *T. lestoquardi* isolates were used in this study. The protozoa were originally achieved from different provinces of Iran (Tehran [Vasfenard and Karaj], Fars, Sistan Baluchestan [Zabol] and Qazvin [Boein-Zahra]). *Babesia ovis, Toxoplasma gondii, Neospora caninum, Sarcocystis spp.* and *Leishmania major* DNA were used as closely haemoparasites in this study were.

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

Bioassay Experiment. 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 isolation. Proteinase K and further phenol chloroform purification were performed for DNA extraction (Sambrook 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 phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation, then was dried and resolved in TE buffer. DNA concentration was determined either by agarose gel electrophoresis and spectrophotometry (A_{260}) and measuring the ratio of A_{260}/A_{280}. Quality of the isolated DNA was evaluated by agarose gel electrophoresis.

PCR Primer Design. Specific primers were designed by aligning the two published *Theileria* surface protein gene sequences by *online* tools of *NCBI* (bl2seq) (Figure1). Pairwise alignment exhibited the presence of conserved sequences; those could be used for common primer selection. On the other hand, deletion and/or insertion of nucleotides in some regions made different length sequences in considered sequences, which were limited between two selected oligonucleotide primers. The primers SP1 (5' GCG AAT GTG GTC CAT TTC TTC C) and SP2 (5' GAA GAA TGA TCC ACA ACA TTG CG) were used to amplify bases between 90-651 of the *T.annulata* (AJ316248), and bases 271-710 of the *T. lestoquardi* (AY274335) SP gene sequences and the seven available Iranian *Theileria* stocks.

Polymerase Chain Reaction. 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* DNA 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 35 cycles. Each cycle consisted of a denaturing step of 30 seconds at 94°C, an annealing step of 1 min at 57°C, and an extension step of 1 min at 72°C, followed by final extension step of 5 min at 72 °C.

PCR product detection. Amplified PCR products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized by UV transillumination.

Specificity of the PCR. PCR specificity was shown *in vitro* by using other apicomplexan protozoa DNA for example *Babesia ovis, Toxoplasma gondii, Neospora caninum, Sarcocystis spp*., cattle and sheep genomic DNAs as well as negative control. Moreover, specificity of the PCR was determined by SP amplicons sequencing**.** Sequence analysis of SP gene obtained from seven studied *Theileria* isolates were sequenced and analyzed by "Blast n" program.

RESULTS AND DISCUSSION

The result of SP gene amplification. The specific primer pair was designed for SP gene in order to detection and discriminating two species of *T. annulata* and *T. lestoquardi* simultaneously. The amplicons were recognized by size on agarose gel electrophoresis. The size of PCR product was 561 bp for *T. annulata* SP gene, and 440 bp for *T. lestoquardi* (Figure 2).

The Specificity of the PCR. Analysis of designed SP1 and SP2 primers by "blast n" demonstrated all of 17 deposited *T. lestoquardi* and *T. annulata* SP gene sequences in GenBank have 100 percent homology with designed SP1 and SP2 primers that reveals the presence of consensus sequence regions in SP gene. *In silico* study showed the expected size of amplicons, 440 bp for *T. lestoquardi* and 506 to 607 bp for *T. annulata*. However, the PCR products for seven Iranian available isolates were 440 bp for *T. lestoquardi* and 561 bp for *T. annulata*. SP1 and SP2 primers were used to amplify specifically both *Theileria* species DNA and no amplification was shown by other available apicomplexan protozoa DNA including *Babesia ovis, Toxoplasma gondii, Neospora caninum, Sarcocystis spp*., and healthy cattle and sheep genomic DNA as well as negative control (Figure 3). The results of SP PCR products sequencing and further analysis by using "Blast n" program showed specific amplification of *Theileria* SP gene. The sequenced *T. annulata* and *T. lestoquardi* SP gene sequences vaccine strain and wild isolates were deposited in GenBank as the following: *T. lestoquardi* vaccine strain, Acc# EF092924; *T. lestoquardi* Zabol isolate, Acc# EF092923; *T. annulata* Boein Zahra C2 isolate, Acc# EF092922; *T. annulata* Boein Zahra C1 isolate, Acc# EF092921; *T. annulata* Karaj/78 isolate, Acc# EF092920. *Theileria annulata* and *T. lestoquardi* cause malignant theileriosis in cattle and sheep in Iran, both are transmitted by *Hyalomma* ticks (Hashemi-Fesharki 1988). Recently, it has been shown that *T. lestoquardi* can infect bovine monocytes, therefore it is important to find a simple, effective and specific method for detecting and discriminating these two parasites in ruminant hosts and tick vectors (Leemans *et al* 1999). *T. annulata* and *T. lestoquardi* have the same morphological characters in microscopic inspection. Moreover, both *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). On the other hand, for deciding to control program including treatment and preventive strategy (i.e. the specific vaccination regime) it is essential

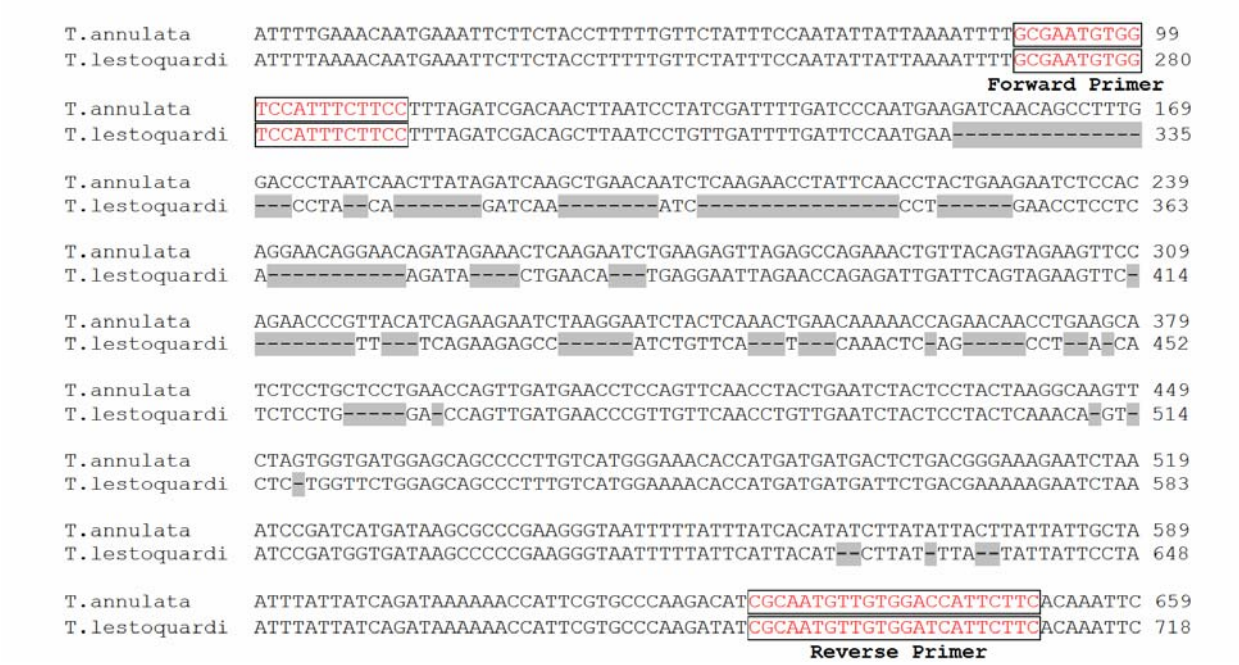

Figure 1. Pairwise alignment of *T.annulata* and *T.lestoquardi* Surface Protein gene sequences. Nucleotides deletions in *T. lestoquardi* SP gene sequence (highlighted regions) or base insertion in *T.annulata* SP gene sequence created different PCR products length (561 and 440 bp for *T.annulata* and *T.lestoquardi* respectively) limited between two designed oligonucleotide primers. The common forward and reverse primers for two *Theileria* species were shown in boxes. (Reference sequences accession numbers are; *T.annulata* acc# AJ316248 and *T. lestoquardi* acc# AY274335).

is essential knowing the epidemiological data based on prevalence and distribution of protozoan parasite in ticks or carrier livestock. Therefore, having a specific, sensitive and uncomplicated method is very important for simultaneous detecting and distinguishing these two *Theileria* species. Here we propose a convenient and single PCR protocol using specific primers from the *Theileria* Surface Protein gene sequence for detecting and discriminating two similar *Theileria* species concurrently with no more enzymatic reaction or probe hybridization. Spitalska *et al* in 2004 demonstrated the restriction fragment length polymorphism (RFLP) method to discriminate two *Theileria* species by enzymatic digestion of amplified PCR products of the 18S rRNA gene. (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 *T. 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.

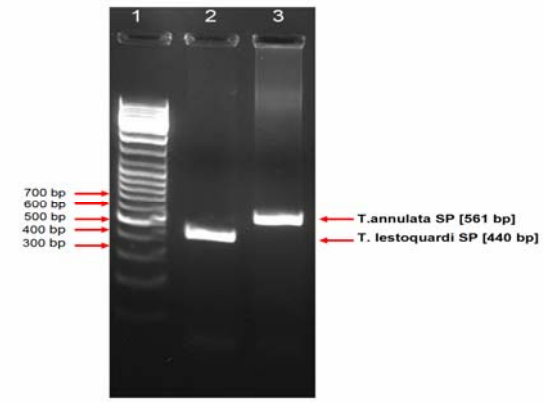

Figure 2. Gel agarose (2 %) electrophoresis of amplified SP (Surface Protein) gene of *Theileria annulata* and *T. lestoquardi* using SP1 and SP2 primers. Lane 1, 100 bp DNA ladder as size marker; lane 2, the amplified *Theileria lestoquardi* SP gene; and lane 3, the amplified *Theileria annulata* SP gene.

Here, we showed conserved regions in SP gene that used for primer designing for two *Theileria* species, and many nucleotide substitutions in SP gene sequence that caused the two different PCR fragment lengths. In conclusion, we have developed an efficient molecular diagnostic technique for detecting *T. annulata* and *T. lestoquardi* with high specificity. The specificity of the PCR was validated by sequencing of the amplified fragments and using available apicomplexan protozoa DNAs as well as healthy host DNA.

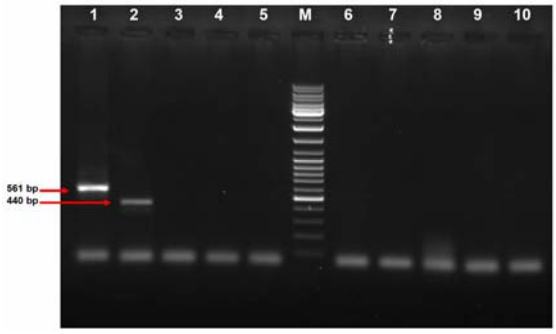

Figure 3. The specificity of the PCR for *Theileria annulata* and *T. lestoquardi* detection using protozoan DNAs, calf DNA and negative control (no DNA). Lanes 1 and 2 are *T.annulata* and *T. lestoquardi* amplified SP gene. Lane 3, *B. ovis* DNA; lane 4, *T. gondii* DNA; lane 5, *N. caninum* DNA; lane 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).

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