Molecular Identification and Genotyping of *Theileria orientalis* type 3 (buffeli) from cattle in Guilan Province of Iran, using Nested-PCR assay

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**Abstract**

Protozoan parasites of the genus Theileria are tick-borne parasites that have been found in many species of mammals. More than a dozen species of Theileria occur in cattle, water buffalo, sheep and goats. *Theileria orientalis* is a non-pathogen blood protozoan parasite, was detected and identified during a regular investigation of piroplasmida infection in indigenous cattle in spring season of 2019 from Northern Provinces of Iran. A total of 92 blood samples were collected in the spring season from different areas of Guilan and Mazandaran Provinces, Iran. The Giemsa stained blood smears did not show any parasite infection but *T. orientalis* was identified by 18S rRNA gene PCR and DNA sequencing. The specific sequenced DNA for *T. orientalis* was registered in GenBank under accession number of MN453385. The partial 18S rRNA gene sequence of obtained DNA has showed 100% nucleotide identity with reference sequences for the *T. orientalis* have been registered from Europe, Africa and Asia. Additionally, molecular phylogenetic studies have shown that *T. orientalis* Iran GC98-01 isolate belongs to nonpathogenic *T. orientalis* type 3 (buffeli). In this study, the indigenous Bos indicus cattle were detected as asymptomatic carrier state for Theileria spp infection. Here we identified and genotyped *T. orientalis* for the first time as *T. orientalis* type 3 (buffeli) from Iran using molecular phylogenetic analysis and the 18S rRNA gene sequence of the *T. orientalis* GC98-01 isolate was registered in GenBank. Moreover, rare *T. annulata* infection was detected in cattle.
using semi nested-PCR in Mazandaran (Miankaleh peninsula). T. orientalis can be differentiated from other Theileria and Babesia haemoprototozoan parasites by specific molecular assay.

Keywords. Theileria orientalis, 18S rRNA gene, PCR, Cattle, Iran

Introduction
The genus Theileria species are intracellular parasites that causing tick-borne diseases and has a significant impact on livestock production due to economic losses (Ahmed et al., 2008). Theileria spp can be classified into two major groups based on their pathogenicity: (1) those making host cell transformation (T. annulata, T. parva and T. lestoquardi) and (2) those do not (T. mutans, T. orientalis complex) (Sivakumar et al., 2014).

The benign form of bovine theileriosis is categorized differently, T. buffeli in Australia, T. sergenti in Japan and East Asia, and T. orientalis in many other regions (Hammer et al., 2015).

The first investigations for Theileria spp in Iran came back to reports of Delpy, Rafyi, Maghami, and Hooshmand-Rad who described two pathogenic and benign Theileria species. Delpy in 1938 has reported two different Theileria species in cattle from Iran; T. annulata as a pathogenic species for European breeds, and the benign T. mutans (Delpy, 1938). Three decades later Rafyi and Maghami have confirmed the previous findings and added that T. mutans was less frequently found than T. annulata and occurred only in certain parts of the country (Rafyi and Maghami, 1963). Hooshmand-Rad was believed that the benign Theileria was more likely to be T. sergenti, due to its distribution almost coincided with Haemaphysalis tick vector (Hooshmand-Rad, 1974).

However, recent studies have documented T. orientalis infection from northern area of Iran (Uilenberg and Hashemi-Fesharki, 1984; Ghaemi et al., 2012; Narimani et al., 2017). There is no published report for pathogenicity of T. orientalis to data in Iran.

Although T. orientalis complex were recognized to cause a less severe and benign infection (oriental theileriosis) in cattle, but pathogenic genotypes are now identified and reported in many
countries (Watts et al., 2016). The pathogenic genotypes are now identified and described as emerging parasitic agents for cattle in some regions (Perera et al., 2014).

Therefore, it is very important to detect and identify *Theileria* species in flat, Sub Mountain and forest area and further molecular genotyping by standard methods. Here, in this study we have detected and identified the *Theileria* spp in indigenous cattle from Northern Guilan and Mazandaran Provinces of Iran by nested and semi nested PCR assay and DNA sequencing.

**Materials and Methods**

**Blood and tick samples.** Peripheral EDTA-anticoagulated whole blood samples, air-dried blood smears and skin attached ticks were taken from asymptomatic free-living cattle. Between April 2019 and July 2019, a total of 92 blood samples from carrier cattle were collected from Guilan (#53) and Mazandaran (#39) Provinces. The majority of the cattle were indigenous and a few crossbred in all selected area in Guilan and Mazandaran Provinces. Moreover, 138 adult ticks were prepared from Guilan (#118) and Mazandaran (#20) Provinces and preserved in 70% ethanol for morphological identification.

**Microscopic inspection.** The blood films were air dried, fixed in absolute methanol, and stained with Giemsa stain, then were examined at ×1000 magnification using a brightfield microscope (Nikon). A total of 100 fields were observed, and the number of red blood cells (RBC) infected with *Babesia/Theileria* recorded.

**Morphological tick identification.** The collected ticks were examined for morphology of dorsal surface, the basis capitulum, mouth parts, eyes, anal and adanal plates, genital orifice, spiracle and festoons (Animal Health Diagnostic Center).

**Sampling places.** Four regions were considered for sampling in Guilan (Saravan, Rezvan-shahr and Roudsar) and Mazandaran (Miankaleh peninsula) Northern Provinces of Iran (Figure 1). The altitude of the selected areas from the sea level were -24 meters for Miankaleh, +92 meters for Saravan, +700 meters for Roudsar and +15 meters for Rezvanshahr. The geographic coordinates
were 53.64 and 36.87 for Miankaleh, 49.61 and 37.14 for Saravan, 50.25 and 37.02 for Roudsar and 49.14 and 37.55 for Rezvanshahr.

**DNA isolation.** Genomic DNA was extracted using Proteinase K and further phenol chloroform purification (Sambrook et al., 1989). In brief, the cells were destructed by lysis buffer, followed by centrifugation, adding proteinase K and SDS solution to the pellet, and then was incubated (one hour at 56°C) until most of the cellular protein was degraded. Hard ticks were minced using liquid nitrogen, and then the smashed ticks were subjected to lysis buffer and proteinase K treatment. The sample digest was deproteinized by phenol/chloroform/isomyl alcohol extraction, recovered by ethanol precipitation, then was dried and resolved in deionized-distilled water. The extracted DNA concentration was measured either by agarose gel electrophoresis and spectrophotometry (A260) as well as the ratio of A260/A280. Additionally, quality of the isolated DNA was estimated by agarose gel electrophoresis.

**Nested Polymerase Chain Reaction (nPCR).** PCR assay for *T. orientalis* 18S rRNA gene (Accession # U97051) was performed using two sets of primers; first assay used the *Theileria* 18S Ext1: GGC GGC GTT TAT TAG ACC and *Theileria* 18S Ext2: CCT TGT TAC GAC TTC TCC primer pair to amplify external fragment the 1538 bp. Then, the second primer pair, *Theileria* 18S Int1: GGT AAT TCC AGC TCC AAT AGC G and *Theileria* 18S Int2: GAA GCG TCC TTG GCA AAT GC were applied to amplify the internal fragment the 358 bp. PCR was performed in a final reaction volume of 20 µl containing 1X PCR premixed YektaTajhiz™, 6 ul ddH₂O, 10 pmol of each primers, and 2 µl of DNA template. The reactions were performed in an automatic DNA thermal cycler (Techne, Germany) with the first denaturation at 94°C for 3 min and were followed by 35 cycles, each cycle consisted of a denaturing step of 10 seconds at 94°C, an annealing step of 20 seconds at 56°C, and an extension step of 40 seconds at 72°C, followed by final extension step of 5 min at 72°C. The specific primers for *T. annulata* and PCR conditions were used as previously described (Habibi, 2016). Briefly, in a semi-nested PCR, three oligonucleotide primers for detection of *T. annulata* Tams-1 gene sequence (GenBank accession no. TAU22888) were designed to amplify 597 and 470 bp DNA fragments in first and semi-nested PCR respectively. The external primers were; Tms92F (5’-GAG ACA AGG AAT ATT CTG AGT CC-3’), Tms92R (5’- TTA AGT GGC ATA TAA TGA CTT AAG C -3’) and the internal forward primer Tms92nF as semi-nested PCR primer (5'
CGG CAC TGG AAA GAA GTA CAC C 3'). The cycling conditions were as mentioned for *T. orientalis* except the annealing temperature at 54 °C.

**PCR product detection and sequencing.** Amplified PCR products were separated by electrophoresis on 1.5% agarose gel, stained with RedSafe™ (Nucleic Acid Staining Solution), and visualized by UV transillumination. The semi nested-PCR product in size of 358 bp was cleaned up, extracted from agarose gel and was submitted for bidirectional DNA sequencing using chain termination method (Takapouzist, Bioneer, South Korea). Since the *T. orientalis* infestation rate was very low as carrier state, therefore we had to amplify the target DNA by semi nested PCR to have enough material for DNA sequencing.

**DNA sequence analysis.** The online program of blastn “Basic Local Alignment Search Tool (BLAST)” was used to find regions of local similarity between sequences by comparing the nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches (https://blast.ncbi.nlm.nih.gov/).

**Phylogenetic analysis and Genotyping.** The obtained 18S rRNA gene sequence from the studied *T. orientalis* infected sample was registered in GenBank database. Then the sequence was analyzed by phylogenetic software for pairwise alignment. The BLAST program calculates a pairwise alignment between a query and the database sequences searched. The number of previously registered *T. orientalis* sequences in GenBank as different known types (Buffeli, Chitose and Ikeda) were used for molecular genotyping. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). (Molecular Evolutionary Genetic Analysis, Ver. 5 [MEGA5]). All positions containing gaps and missing data were eliminated (Tamura et al., 2011).

**Results**

**The results of Microscopic inspection of Giemsa stained Blood smears.** No parasites and infection were seen in red blood cells by light microscopic examination.

**The results of morphological identification of ticks.** As mentioned earlier, the number of 138 ticks were collected and studied for identification and tick-borne parasites (118 ticks from Guilan and 20 from Mazandaran Provinces). All ticks were examined and according to the key criteria the numbers of 30 of them were identified as *Haemaphysalis spp*. The species of *Haemaphysalis*
Genus are small inornate ticks with short mouthparts. The basis capitulum is rectangular and the base of the second palpal segment is expanded, projecting laterally beyond the basis capitulum. The second and third palpal segments taper anteriorly so that the capitulum anterior to the basis capitulum appears to be triangular. There are no eyes in either sex. Festoons are present.

Most of the isolated ticks in Miankale region were identified as *Hyalomma detritum* and a few were diagnosed *Rhipicephalus bursa* and *Boophylus sp.*, but in Guilan three genera were identified as *Boophylus sp, Haemaphysalis sp*, and *Ixodes sp*.

*Haemaphysalis inermis* was diagnosed from indigenous cattle in Guilan Province. *H. inermis* also known as the Winter tick, that can infest Cattle, horse, sheep, deer, dog, fox, humans. *H. inermis* is generally found in forest areas. The adult tick shows long and narrow palps, cornua absent, trochanter I has a small posterior facing spur.

**Results of DNA isolation and nested-PCR.** Genomic DNA was extracted from 92 blood samples and 138 ticks. First and second round of PCR were performed for all DNA samples, most of DNAs (Blood and tick) were determined as positive for piroplasmida infection. A noticeable number of samples were positive for *T. orientalis* as shown in gel agarose electrophoresis (Figure 2). Of the 53 blood samples were collected from cattle in Guilan Province, eight samples were identified as *T. orientalis* infected using semi-nested PCR. One of the positive samples was amplified and subjected for PCR product bidirectional DNA sequencing. Of the 39 blood samples taken in Mazandaran Province, the employed semi-nested PCR showed seven samples were infected to *T. annulata* (Figure 3).

**DNA sequence analysis.** The results of comparison of the sequenced PCR product using Blastn, has revealed that the CG98-01 Iran isolate, belongs to the *T. orientalis/sergentil buffeli* group, and then has been registered in GenBank under accession number of MN453385.

**Phylogenetic analysis and Genotyping.** The obtained 18S rRNA gene sequence from the *T.orientalis* Iran isolate (MN453385) was compared to various previously registered 18S rRNA gene sequences in GenBank, for construction of a phylogenetic tree. The local isolate was placed close to the *T. orientalis* type3 sequences in a distinct clade separate from *T. orientalis* type1 and type2 (Figure 4).
Discussion

In this study, *T. orientalis* was detected by nested PCR from indigenous cattle in Guilan Province of Iran, and the molecular phylogenetic analysis by 18S rRNA gene sequencing has revealed the parasite is classified among the non-pathogenic *T. orientalis* type3 (buffeli). Additionally, *T. annulata* rare infection was recognized in cattle living in Mazandaran Province.

The majority of the cattle population in Iran is indigenous zebu breed, though a huge part are imported *Bos taurus* breed and their crosses (Hashemi-Fesharki, 1988). In Iran, cattle are infested by several species of hard ticks (Acari: Ixodidae) including *Hyalomma* spp, *Haemaphysalis* spp, *Ixodes* spp, *Rhipicephalus* spp and *Boophylus* spp. (Rahbari et al., 2007, Nabian et al., 2009).

Tick-borne parasites have long been a subject of interest in the past. Delpy (1938) has reported two species of *Theileria* in cattle in Iran, *T. annulata* that is pathogenic to animals belonging to European breeds, and the non-pathogenic species; *T. mutans*. Later, Rafyi and Maghami (1963) confirmed these findings and added that *T. mutans* was less frequently found than *T. annulata* and occurred only in certain parts of the country, according to Hooshmand-Rad (1974) only in the Caspian Sea area. Recently, it has been discussed that the name of *T. sergenti* is invalid for a *Theileria* species of cattle and that the correct name for the benign species associated with *Haemaphysalis* ticks in Eurasia and Australia should be *T. orientalis* (Uilenberg and Hashemi-Fesharki, 1984).

Although *T. orientalis* has long been considered as a benign haemoparasite, but pathogenic genotypes are now reported in many countries including Australia, New Zealand, Japan, China, Korea and the United states (Oakes et al., 2019).

The diagnosis of oriental theileriosis is based on the observation of clinical signs, the detection of piroplasms of *T. orientalis* in blood films, and/or the use of serological test or molecular techniques (Kakuda et al., 1998). Clinical signs of *T. orientalis* infection are mostly associated with the consequence of anaemia. These signs are lethargy, weakness, anorexia, pale mucous membranes, lymph node swelling, tachypnoea, tachycardia, dyspnoea, jaundice, late-term abortion, dystocia, pyrexia, and mortality (Aparna et al., 2011). However, no symptoms were observed in any of the cases studied in this investigation.
To date, there are 11 genotypes of *T. orientalis* complex have been identified (Chitose (type 1), Ikeda (type 2), Buffeli (type 3), types 4 to 8, and N-1 to N-3) using a number of molecular markers, including major piroplasm surface protein (MPSP), 23-kDa piroplasm membrane protein (p23), small-subunit (SSU) rRNA gene (18S rRNA), and the first and second internal transcribed spacers of nuclear ribosomal DNA (ITS-1 and ITS-2, respectively) (Perera et al., 2015).

The Ikeda genotype is more associated with clinical disease than Chitose, and Buffeli in Australia, and this genotype also occurs internationally where clinical cases of *T. orientalis* have been reported (Aparna et al., 2011).

It is clear that genotype 3 (buffeli) and its phylogenetic relative, type 5 have not been associated with clinical disease and are considered benign (Eamens et al., 2013).

In Asia, Australia, and New Zealand, the primary tick vector for the *T. orientalis* Ikeda genotype is *Haemaphysalis longicornis*, which is also known as the Asian longhorned or bush tick (Oakes, 2019). Here, the *Haemaphysalis inermis* tick was isolated and identified from the free living cattle in Guilan Province. However, the role of this tick in the life cycle of *T. orientalis* in Iran remains to be studied in future.

*T. orientalis* infections may occur due to a combination of various genotypes, which can cause evasion of the host immune system similar to the apicomplexan parasites. The infected cattle seem to preserve the parasite for a long period of time, perhaps for lifetime (Jenkins and Bogema, 2016). The majority of the herds in enzootic area are expected to be infected and some of them are developed a degree of resistance to disease. In these circumstances, *T. orientalis* infection produces a carrier state in resistant cattle (Jenkins and Bogema, 2016). This is exactly what happened here in infected indigenous cattle in Guilan Province of Iran.

Healthy and naïve cattle those recently entered to *T. orientalis* infected area, or having predisposing factors including stress, immunodeficiency disorders or pregnancy cause animals to be susceptible for developing clinical disease (Eamens et al., 2013). These factors must be reduced in order to prevent the disease to happen. Additionally molecular typing of the parasite should be defined regularly for monitoring of the circulating genotype(s) in the region.

In conclusion, the indigenous *Bos indicus* cattle were detected as asymptomatic carrier state for *Theileria* spp infection. We have identified and genotyped *T. orientalis* for the first time as *T.*
orientalis type3 (buffeli) using molecular phylogenetic analysis and the 18S rRNA gene sequence of the *T. orientalis* GC98-01 Iran isolate was registered in GenBank (MN453385). Moreover, rare *T. annulata* infection was detected in cattle using semi nested-PCR in Mazandaran Province (Miankaleh peninsula) of Iran. Molecular methods for diagnosis of species and genotypes of parasites are recommended to prevent geographical expansion in the country.

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**References**


Figure 1. The four selected areas for sampling in Guilan and Mazandaran Provinces, Iran. The forest, plain and coastal regions has large livestock population and numerous indigenous cows with free living life. According to established *T. orientalis* type 3 infection in Guilan Province, the region is considered as an enzootic province for this nonpathogenic haemoproteoan parasite (maps.google.com and www.wikipedia.org).

Figure 2. Gel agarose electrophoresis for *Theileria orientalis* specific PCR on cattle blood DNA. Lanes 1 to 8 are specific nested PCR for *T. orientalis* (positive samples, the expected size is 358 bp), M is 100 bp DNA size marker and lane 9 is the negative control.
Figure 3. Gel agarose electrophoresis for *Theileria annulata* specific PCR on cattle blood DNA. Lanes 1 to 9 are specific semi-nested PCR for *T. annulata* (all are positive except lane 2, the expected size is 470 bp), M is 100 bp DNA size marker and lane 10 is the negative control.
Figure 4. Phylogenetic relationships among *Theileria orientalis* types and new isolates in Northern Province of Iran based on 18S rRNA gene sequences. This tree shows buffeli, chitose, and ikeda types in 3 different clades. One corresponding sequence each from *T. annulata* (AY524666) served as outgroup. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 240 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.