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Original Article

Molecular Identification and Genotyping of Theileria Orientalis Type 3 (Buffeli) Isolated from Cattle Using Nested-PCR Assay in Guilan Province, Iran

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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 have been found in cattle, water buffalo, sheep, and goats. *Theileria orientalis* is a non-pathogenic blood protozoan parasite that was detected and identified during a regular investigation of piroplasmida infection in indigenous cattle in the spring of 2019 in Northern Provinces of Iran. In total, 92 blood samples were collected from different areas of Guilan and Mazandaran Provinces, Iran during the spring. The Giemsa stained blood smears did not show any parasitic infection; however, *T. orientalis* was identified by 18S rRNA gene polymerase chain reaction (PCR) and DNA sequencing. The specific sequenced DNA for *T. orientalis* was registered in GenBank under the accession number MN453385. The partial 18S rRNA gene sequence of the obtained DNA showed 100% nucleotide identity with reference sequences for the *T. orientalis* that 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 carriers of Theileria spp. infection. Here, we identified and genotyped *T. orientalis* for the first time as *T. orientalis* type 3 (buffeli) in Iran using molecular phylogenetic analysis and registered the 18S rRNA gene sequence of the *T. orientalis* GC98-01 isolate in GenBank. Moreover, rare T. annulata infection was detected in cattle using semi-nested PCR in Mazandaran (Miankaleh peninsula). The *T. orientalis* can be differentiated from other Theileria and Babesia haemoprotozoan parasites by specific molecular assays.

Keywords: 18S rRNA gene, Cattle, Iran, PCR, *Theileria orientalis*

Identification Moléculaire et Génotypage de *Theileria Orientalis* de Type 3 (Buffeli) Isolé de Bovins à L'aide d'un Test de PCR Emboitée Dans la Province de Guilan, Iran

Résumé: Les parasites protozoaires du genre Theileria sont des parasites transmis par les tiques qui ont été trouvés dans de nombreuses espèces de mammifères. Plus d'une douzaine d'espèces de Theileria ont été trouvées chez les bovins, les buffles d'eau, les moutons et les chèvres. *Theileria Orientalis* est un parasite protozoaire sanguin non pathogène qui a été détecté et identifié lors d'une enquête régulière sur l'infection à piroplasmida chez les bovins indigènes au printemps 2019 dans les provinces du nord de l'Iran. Au total, 92 échantillons de sang ont été prélevés dans différentes régions des provinces de Guilān et Māzandarān, en Iran, au cours du printemps. Les frottis sanguins colorés au Giemsa n'ont montré aucune infection parasitaire; cependant, *T. orientalis* a été identifié par réaction en chaîne par polymérase (PCR) et séquençage de l'ADN du gène ARNr 18S. L'ADN séquencé spécifique pour *T. orientalis* a été enregistré dans la GenBank sous le numéro d'accès

MN453385. La séquence partielle du gène de l'ARNr 18S de l'ADN obtenu a montré une identité nucléotidique de 100% avec des séquences de référence pour le *T. orientalis* qui ont été enregistrées en Europe, en Afrique et en Asie. De plus, des études de phylogénétique moléculaire ont montré que l'isolat de *T. orientalis* Iran GC98-01 appartient au type 3 non pathogène de *T. orientalis* (buffeli). Dans cette étude, les bovins indigènes, le zébu (ou Bos indicus), ont été détectés comme porteurs asymptomatiques de *Theileria spp.* infection. Ici, nous avons identifié et génotypé *T. orientalis* pour la première fois comme *T. orientalis* de type 3 (buffeli) en Iran en utilisant une analyse phylogénétique moléculaire et enregistré la séquence du gène de l'ARNr 18S de l'isolat de *T. orientalis* GC98-01 dans la GenBank. De plus, une infection rare à *T. annulata* a été détectée chez les bovins par PCR semi-emboitée à Māzandarān (péninsule de Miankaleh). Le *T. orientalis* peut être différencié des autres parasites *Theileria* et *Babesia haemoprotozoan* par des dosages moléculaires spécifiques.
Mots-clés: Gène de l'ARNr 18S, Bovins, Iran, PCR, *Theileria orientalis*

1. Introduction

The genus *Theileria* species are intracellular parasites that cause tick-borne diseases and have 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 that make host cell transformation (*T. annulata*, *T. parva*, and *T. lestoquardi*) and 2) those that do not induce host cell transformation (*T. mutans* and *T. orientalis* complex) (Sivakumar et al., 2014).

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

Theileria spp. were first reported in Iran by Delpy in 1939 who described two pathogenic and benign *Theileria* species. In 1939, Delpy reported two different *Theileria* species in cattle in Iran, including *T. annulata* which was considered a pathogenic species for European breeds and the benign *T. mutans*. Three decades later, in 1963, Rafyi and Maghami confirmed the previous findings and added that *T. mutans* was less frequent than *T. annulata* and was found only in certain parts of the country.

Hooshmand-Rad (1974) believed that the benign *Theileria* was more likely to be *T. sergenti*, due to its distribution that almost coincided with the *Haemaphysalis* tick vector. However, recent studies have documented *T. orientalis* infection in northern regions of Iran (Uilenberg and Hashemi-Fesharki, 1984; Ghaemi et al., 2012; Narimani et al., 2017). There is no published report for the pathogenicity of *T. orientalis* in Iran.

Although the *T. orientalis* complex was recognized to cause less severe and benign infection (oriental theileriosis) in cattle, 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 regions and perform further molecular genotyping based on standard methods. In this study, we have detected and identified the *Theileria* spp. in indigenous cattle from Northern Guilan and Mazandaran Provinces, Iran by nested and semi-nested polymerase chain reaction (PCR) assay and DNA sequencing.

2. Material and Methods

2.1. Blood and Tick Samples

Peripheral ethylenediaminetetraacetic acid-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, 92 blood samples were collected from carrier cattle in Guilan (#53) and Mazandaran (#39) Provinces. The majority of the cattle were indigenous

to the selected regions in Guilan and Mazandaran provinces, while a few of them were crossbred. Moreover, 138 adult ticks were collected from Guilan (#118) and Mazandaran (#20) provinces and preserved in 70% ethanol for morphological identification.

2.2. Microscopic Inspection

The blood films were air-dried, fixed in absolute methanol, and stained with Giemsa stain. Afterward, they were examined at ×1000 magnification using a brightfield microscope (Nikon YS2-T, Japan). In total, 100 fields were observed and the number of red blood cells infected with *Babesia*/*Theileria* was recorded.

2.3. Morphological Tick Identification

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

2.4. Sampling Locations

Four regions were considered for sampling in Guilan (Saravan, Rezvan-Shahr, and Roudsar) and Mazandaran (Miankaleh peninsula) provinces (Figure 1). The altitude of the selected areas from the sea level were -24, +92, +700, and +15 meters for Miankaleh, Saravan, Roudsar, and Rezvanshahr, respectively. 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.

2.5. 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, addition of proteinase K and SDS solution to the pellet, and incubation (one hour at 56ºC) until most of the cellular protein was degraded. Hard ticks were minced using liquid nitrogen, and the smashed ticks were subjected to lysis buffer and proteinase K treatment. The sample digest was deproteinized by phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation, dried, and resolved in deionized-distilled water. The extracted DNA concentration was measured either by agarose gel electrophoresis and spectrophotometry (A_{260}) as well as the ratio of A_{260}/A_{280}. Additionally, the quality of the isolated DNA was estimated by agarose gel electrophoresis.

2.6. Nested Polymerase Chain Reaction

The PCR assay for *T. orientalis* 18S rRNA gene (Accession # U97051) was performed using two sets of primers. First, the *Theileria* 18S Ext1: GGC GGC GTT TAT TAG ACC and *Theileria* 18S Ext2: CCT TGT TAC GAC TTC TCC primer pair were used to amplify the external fragment (1538 bp). Afterward, 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 (358 bp).

The PCR was performed in a final reaction volume of 20 µl containing 1X PCR premixed YektaTajhiz™, 6 ul ddH_2O, 10 pmol of each primer, and 2 µl of DNA template. The reactions were performed in an automatic DNA thermal cycler (Techne, Germany) with the first denaturation at 94 ºC for 3 min followed by 35 cycles. Each cycle consisted of a denaturing step for 10 sec at 94 ºC, an annealing step for 20 sec at 56 ºC, and an extension step for 40 sec at 72 ºC followed by the final extension step for 5 min at 72 ºC.

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′) and Tms92R (5′- TTA AGT GGC ATA TAA TGA CTT AAG C -3′) while the internal forward primer was Tms92nF as a 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 ºC.

2.7. Polymerase Chain Reaction 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, extracted from agarose gel, and submitted for bidirectional DNA sequencing using the chain termination method (Takapouzist, Bioneer, South Korea). 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.

2.8. DNA Sequence Analysis

Basic Local Alignment Search Tool (BLAST), which is an online program, was used to find regions of local similarity between sequences. For this purpose, the nucleotide or protein sequences were compared to the sequence databases and the statistical significance of matches was calculated (https://blast. ncbi. nlm. nih. gov/).

2.9. Phylogenetic Analysis and Genotyping

The obtained 18S rRNA gene sequence from the studied *T. orientalis* infected sample was registered in the GenBank database. Afterward, the sequence was analyzed by phylogenetic software for pairwise alignment. The BLAST program calculates a pairwise alignment between a query and the searched database sequences. 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 (1993) in MEGA (Version 5). It should be mentioned that all positions with gaps and missing data were eliminated (Tamura et al., 2011).

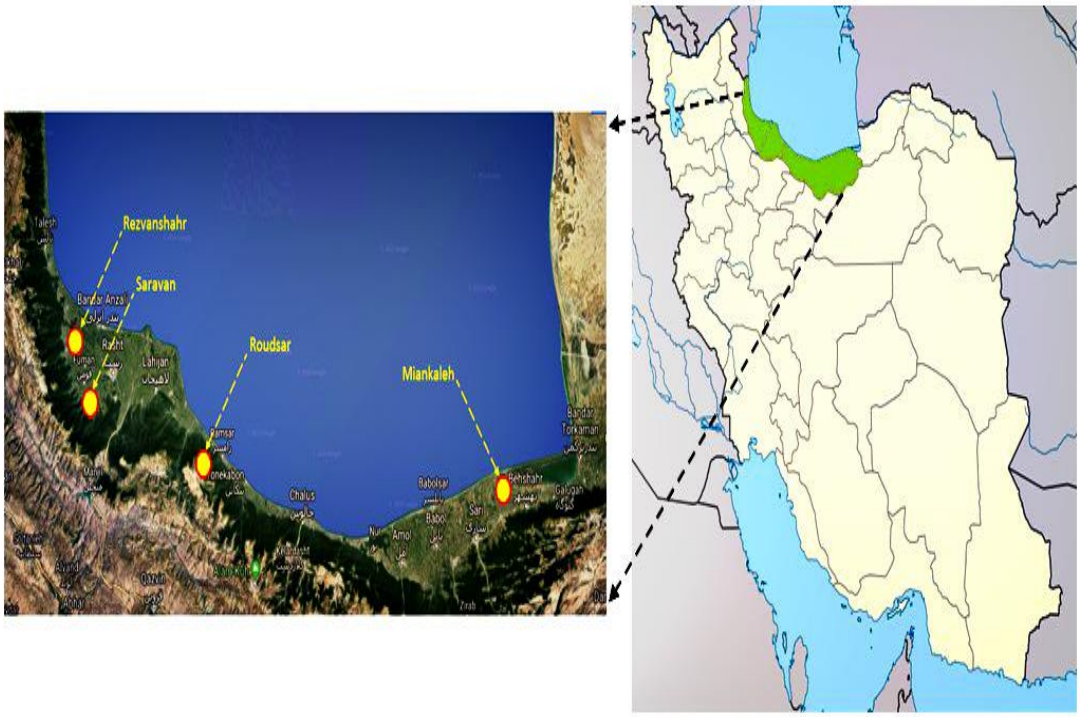

Figure 1. Four selected areas for sampling in Guilan and Mazandaran Provinces, Iran. The forest, plains, and coastal regions have a high livestock population and numerous indigenous cows with free-living life. According to the established *T. orientalis* type3 infection in Guilan Province, the region is considered as an enzootic province for this nonpathogenic haemoprotozoan parasite (maps.google.com and www.wikipedia.org).

3. Results

3.1. Results of Microscopic Inspection of Giemsa-stained Blood Smears

No parasites and infection were seen in red blood cells by light microscopic examination.

3.2. Results of Morphological Identification of Ticks

As mentioned earlier, 138 ticks were collected and studied for identification and tick-borne parasites (118 ticks from Guilan and 20 from Mazandaran). All ticks were examined and 30 of them were identified as *Haemaphysalis* spp. according to the key criteria. 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 in a way that the capitulum anterior to the basis capitulum appears to be triangular. There are no eyes in either gender while festoons are present.

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

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

3.3. Results of DNA Isolation and Nested Polymerase Chain Reaction

Genomic DNA was extracted from 92 blood samples and 138 ticks. The first and second rounds of PCR were performed for all DNA samples, and most of the DNAs (blood and tick) were determined as positive for piroplasmida infection. A noticeable number of samples were positive for *T. orientalis* as detected by gel agarose electrophoresis (Figure 2). In total, 8 out of the 53 blood samples collected from cattle in Guilan Province were found to be infected with *T. orientalis* using semi-nested PCR. Moreover, one of the positive samples was amplified and subjected to PCR product bidirectional DNA sequencing. The employed semi-nested PCR showed that seven out of the 39 blood samples taken in Mazandaran Province were infected with *T. annulata* (Figure 3).

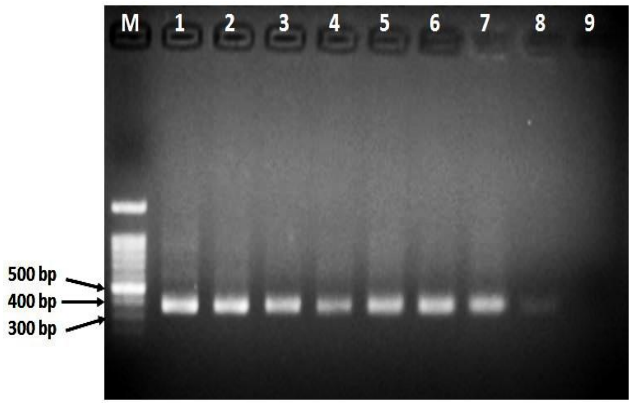

Figure 2. Gel agarose electrophoresis for *Theileria orientalis* specific PCR on cattle blood DNA. Lanes 1-8 are *T. orientalis*-specific nested PCR for (positive samples, the expected size is 358 bp). Lane M is 100 bp DNA size marker and lane 9 is the negative control.

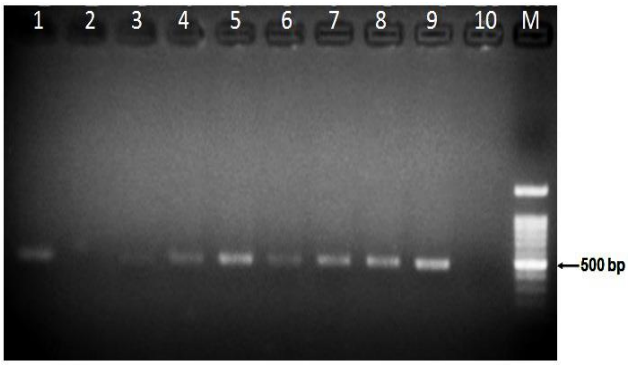

Figure 3. Gel agarose electrophoresis for *Theileria annulata*-specific PCR on cattle blood DNA. Lanes 1-9 are *T. annulata*-specific semi-nested PCR for (all are positive except lane 2, the expected size is 470 bp). Lane M is 100 bp DNA size marker and lane 10 is the negative control.

3.4. DNA Sequence Analysis

According to the results of the comparison of the sequenced PCR product using Blastn, CG98-01 Iran isolate belongs to the *T. orientalis*/ *sergenti*/ *buffeli* group. It has been registered in GenBank under accession number MN453385.

3.5. 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 the construction of a phylogenetic tree. The local isolate was placed close to the *T. orientalis* type 3 sequences in a distinct clade and separate from *T. orientalis* type1 and type 2 (Figure 4).

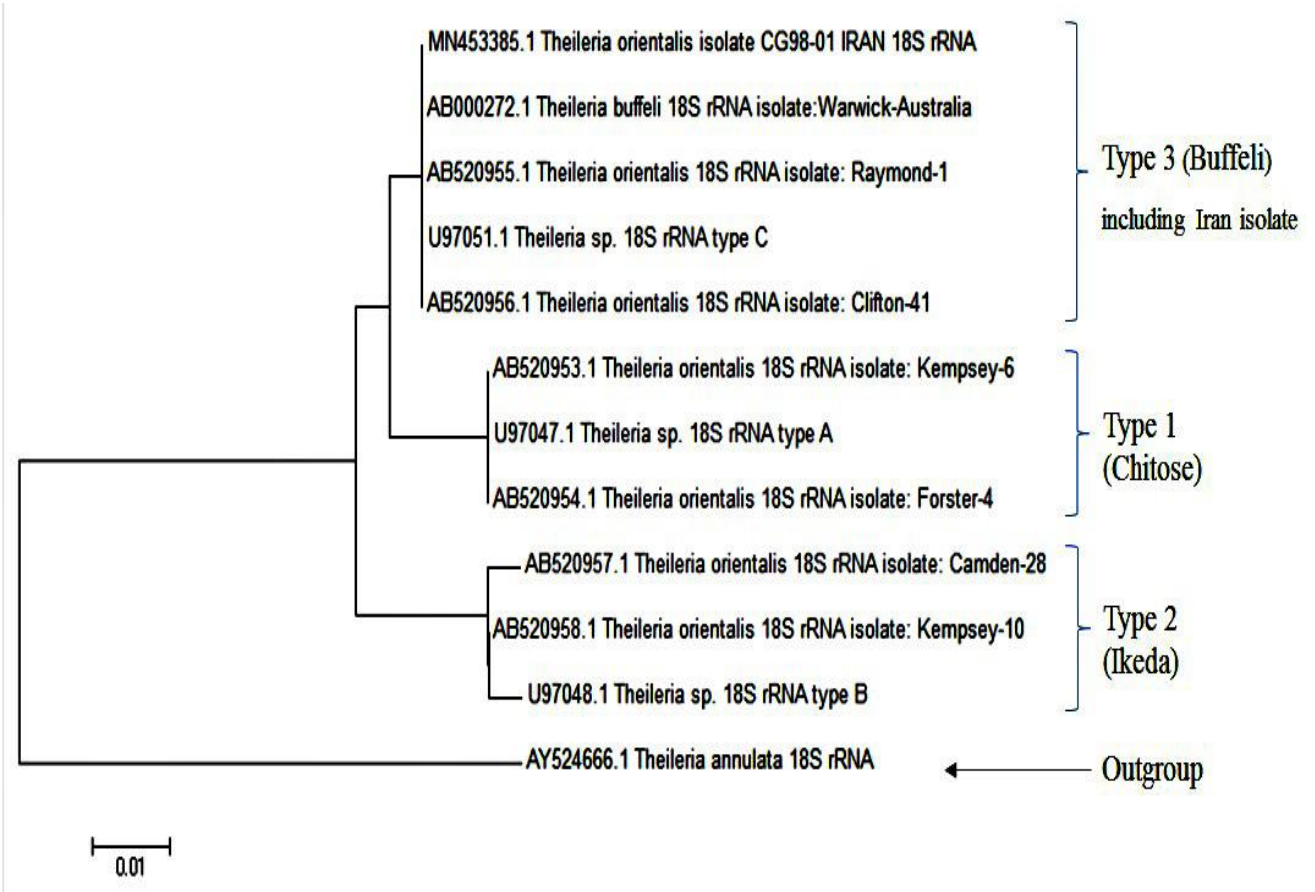

Figure 4. Phylogenetic relationships among *Theileria orientalis* types and new isolated types in Northern Province of Iran based on 18S rRNA gene sequences. This tree shows Buffeli, Chitose, and Ikeda types in three different clades. One corresponding sequence from *T. annulata* (AY524666) served as the 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 and 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 MEGA (version 5).

4. Discussion

In this study, *T. orientalis* was detected by nested PCR in indigenous cattle in Guilan Province. According to the molecular phylogenetic analysis by 18S rRNA gene sequencing, the parasite is classified among the non-pathogenic *T. orientalis* type 3 (buffeli). Additionally, a rare infection by *T. annulata* was recognized in cattle living in Mazandaran Province.

The majority of the cattle population in Iran is indigenous zebu breed, while a significant number of them are imported *Bos taurus* breed and their crosses (Hashemi-Fesharki, 1988). In Iran, the 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).

Tick-borne parasites have been a subject of interest for a long time. In 1939, Delpy reported two species of *Theileria* in cattle in Iran, including *T. annulata* that is pathogenic to animals belonging to European breeds, and *T. mutans* which are the non-pathogenic spp. Later, Rafyi and Maghami (1963) confirmed these findings and added that *T. mutans* was less prevalent than *T. annulata* and found only in certain parts of the country. According to Hooshmand-Rad (1974), *T. mutans* were only found in the Caspian Sea region. Recently, it has been discussed that the name of *T. sergenti* is not valid for the *Theileria* spp. found in cattle and that *T. orientalis* is the correct name for the benign species associated with *Haemaphysalis* ticks in Eurasia and Australia (Uilenberg and Hashemi-Fesharki, 1984).

Although *T. orientalis* has long been considered as a benign haemoparasite, pathogenic genotypes are now reported in many countries, including Australia, New Zealand, Japan, China, Korea, and the United States (Oakes et al., 2019). Oriental theileriosis is diagnosed based on the observation of clinical signs, detection of piroplasms of *T. orientalis* in blood films, and/or the use of serological tests or molecular techniques (Kakuda et al., 1998). Clinical signs of *T. orientalis* infection are mostly associated with 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 examined in this study.

To date, 11 genotypes of *T. orientalis* complex have been identified [Chitose (type 1), Ikeda (type 2), Buffeli (type 3), types 4-8, and N-1 to N-3] using a number of molecular markers, including major piroplasm surface protein, 23-kDa piroplasm membrane protein, small-subunit, 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).

In Australia, the Ikeda genotype is more associated with clinical disease than Chitose and Buffeli. Moreover, this genotype has been found 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 long-horned or bush tick (Oakes et al., 2019). In this study, the *Haemaphysalis inermis* tick was isolated from free living cattle in Guilan Province. However, the role of this tick in the life cycle of *T. orientalis* in Iran needs to be studied in the future.

The *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, even for a lifetime (Jenkins and Bogema, 2016). The majority of the herds in enzootic areas are expected to be infected and some of them have developed a degree of disease resistance. In these circumstances, *T. orientalis*

infection produces a carrier state in the resistant cattle (Jenkins and Bogema, 2016). This is exactly what happened here in the infected indigenous cattle in Guilan.

Healthy and naive cattle who recently enter a region infected with *T. orientalis* or have predisposing factors, including stress, immunodeficiency disorders, or pregnancy, are susceptible to the development of clinical diseases (Eamens et al., 2013). To prevent the disease, such factors must be reduced. Additionally, molecular typing of the parasite should be defined regularly to monitor the circulating genotype(s) in the region.

In conclusion, the indigenous *Bos indicus* cattle were detected as asymptomatic carriers of *Theileria* spp. infection. We identified and genotyped *T. orientalis* for the first time as *T. orientalis* type3 (buffeli) using molecular phylogenetic analysis and registered the18S rRNA gene sequence of the *T. orientalis* GC98-01 Iran isolate in GenBank (MN453385). Moreover, rare *T. annulata* infection was detected in cattle using semi-nested PCR in Mazandaran Province. Molecular methods for the diagnosis of species and genotypes of parasites are recommended to prevent their geographical expansion in the country.

Authors' Contribution

Study concept and design: Gh. H. and A. Sh.
Acquisition of data: Gh. H. and A. Sh. and Q. Kh.
Analysis and interpretation of data: Gh. H. and A. Sh. and A. A.
Drafting of the manuscript: Gh. H.
Critical revision of the manuscript for important intellectual content: Gh. H.
Statistical analysis: Gh. H. and A. A.
Administrative, technical, and material support: Gh. H. and A. Sh., A. A., S. B. and Q. Kh.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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