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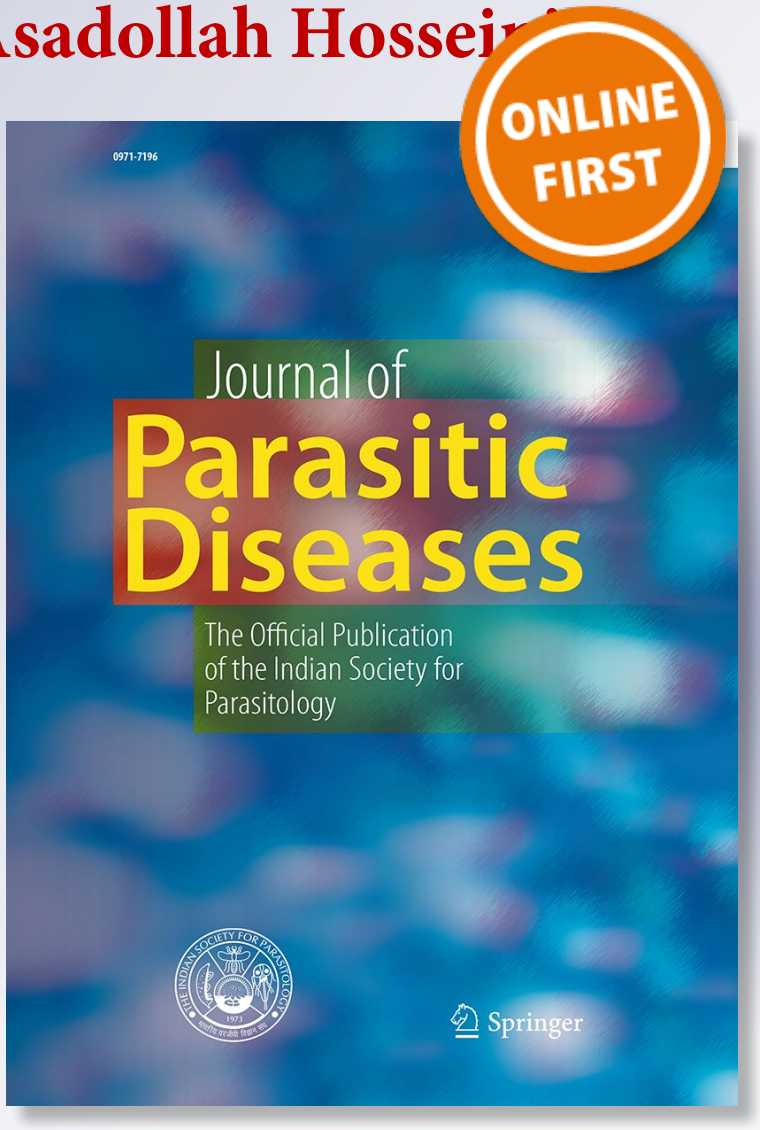

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ORIGINAL ARTICLE

The first molecular detection of a *Theileria*-like species (Apicomplexa: Piroplasmida) in *Meriones persicus* from western Iran

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Abstract The archived blood of rodents species such as *Meriones*, *Mus*, and *Microtus* species was investigated in order to detect any piroplasms species. In this study 18S rRNA target gene of piroplasm parasite was amplified by PCR in a *Meriones persicus*; so, the Locus 1 and Locus 2 of 18S rRNA were sequenced, successfully. A *Theileria*-like taxa was suspected in accordance with the BLAST analysis of 18S rRNA L1 and L2 with 96% and 91% sequence homology, respectively. The present study was the first report of a *Theileria*-like species in *M. persicus* from Iran.

Keywords Haemoparasite · Piroplasm · PCR · Sanger sequencing · Phylogenetic tree · Iran

Introduction

Piroplasms are intra-erythrocytic blood parasites belonging to the subphylum Apicomplexa, class Piroplasmasida. This class contains a single order Piroplasmorida Wenyon, 1926, and encompasses three families: Babesiidae and Theileriidae in warm-blooded vertebrates, and Dactylosomatidae in cold-blooded vertebrates (Levine 1971). *Babesia*, *Theileria* and *Cytauxzoon* are generally considered as piroplasms (Yabsley and Shock 2013). Major economic losses imposed by serious veterinary diseases induced by these organisms can occur worldwide. Piroplasms multiply in the red blood cells and are transmitted by ticks (Levine 1988). More than 100 species of *Babesia* piroplasm have been identified, by which rodent species are infected more than any other species of mammalian hosts (Homer et al. 2000). Order Rodentia is also important as the host of apicomplexa such as; *Hepatozoons* (Harris et al. 2018), *Babesia* (Karbowiak et al. 2010; Manwell and Kuntz 1964), piroplasms (Van Peenen and Duncan 1968; Van Peenen et al. 1977), *Theileria* (Fay and Rausch 1969; Kjemtrup et al. 2001) and recently *Anthemosoma* (Chavatte et al. 2018). Little information is available on the wild animals' piroplasms as well as on their biology and enzootiology. As well as, little is known about the evolutionary history, life cycle including vectors, prevalence, relationships of rodents' piroplasmida and how do they infect rodents are still unclear (Chavatte et al. 2018). However, general view accepted that ixodid ticks may be as the sole biological vector of these parasites. Most piroplasms of domestic animals have more than one ixodid vector (Gunders and Hadani 1974). Recent data on the wild mammals' haemoparasites indicate that they can be considered as a potential risk for the transmission of piroplasms to domestic animals (Barandika et al. 2016). In a large scale investigation conducted by Pasteur Institute of Iran (PII) and Lorestan University of Medical Sciences (LUMS) on rodents in Lorestan province, western Iran, some blood samples of captured rodents were examined using molecular methods. We failed to prepare blood smear

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since the blood samples were clotted; hence, the present study was aimed to detect piroplasms by molecular but not microscopical examination.

Materials and methods

Sample preparation, DNA extraction, and PCR

This study was conducted on blood clot samples (serum-free). Blood samples were freshly collected from rodents in Lorestan province, western Iran (Fig. 1) during 2017–2018. Genomic DNA were extracted from blood samples using CTAB method in accordance with Doyle and Doyle (1987) study accompanied by some modification. Briefly, 1000 µl CTAB extraction buffer was added to 300 µl blood clot into a 2 ml tube. Then, the sample was incubated at 65 °C for 60 min. It was centrifuged at 14,000 rpm for 5 min and supernatant was transferred to a new clean tube and pellet was discarded. An equal volume of Chloroform–isoamilalcohol solution was added to supernatant. The tube was centrifuged at 12,000 rpm for 8 min and supernatant was transferred to a new tube. An equal volume of chilled isopropanol solution was added. Sample was kept overnight in − 20 °C. Then tube was centrifuged at 4 °C and 14,000 rpm for 20 min. The supernatant was discarded and 300 µl of chilled 70% ethanol was added. So, the tube was centrifuged at 4 °C and 14,000 rpm for 5 min. Supernatant was discarded and pellet was let to be dried at room temperature. Pellet was dissolved in 50 µl ddH_2O and DNA solution was stored at − 20 °C. Primer pairs BabfI: 3′-GAC TAG RGA TTG GAG GTC G -5′, BabrI: 3′- GAA TAA TTY ACC GGA TCA CTC G -5′, BabfII: 3′- GCT ACC ACA TCY AAG GAA GGC -5′ and BabrII: 3′- CGT CTT CGA TCC CCT AAC TTT CG -5′ were designed to amplify two different fragments related to various loci in length 561-bp (L1) and 653-bp (L2) on the 18S rRNA gene of any piroplasm (*Babesia*, *Theileria*, *Cytauxzoon,* and *Hepatozoon*). Primer pairs 'BabfI/BabrI' and 'BabfII/BabrII' could successfully initiate the DNA synthesis on positions (bp) 959-77/1588-1609 (Locus 1; L1) and 395-7 to 375-7/899-921 (Locus 2; L2) of GenBank *Babesia* taxa (Accessions: AY046575, DQ329138, AY046577), respectively. To perform the PCR, each 25 µl final volume reaction was performed using 12.5 µl RedMaster PCR 2X (Sinaclon®, Iran), 1 µl from each primer (10 pM), 4 µl of gDNA template (100 ng/µl), and 6.5 µl of D.D. water. The PCR reaction was carried out in a thermocycler (Corbett®, Australia) based on a touchdown temperature profile in 3 min at 94 °C, 11X [45 s. at 94 °C, 50 s. at 60 °C, 60 s. at 72 °C], followed by 24X [45 s. at 94 °C, 50 s. at 50 °C, 60 s. at 72 °C], 3 min at 72 °C. The PCR products were visualized with 1% agarose gel electrophoresis, and the desired bands were purified using the GF-1 Gel DNA Recovery Kit (Vivantis®, Malaysia). Finally, the purified PCR products were submitted to a third-party service provider for sequencing (Faza-Biotech® Inc., Iran).

Phylogenetic analysis

Sequences were manually checked using FinchTV® software (www.geospiza.com) to modify any probable source

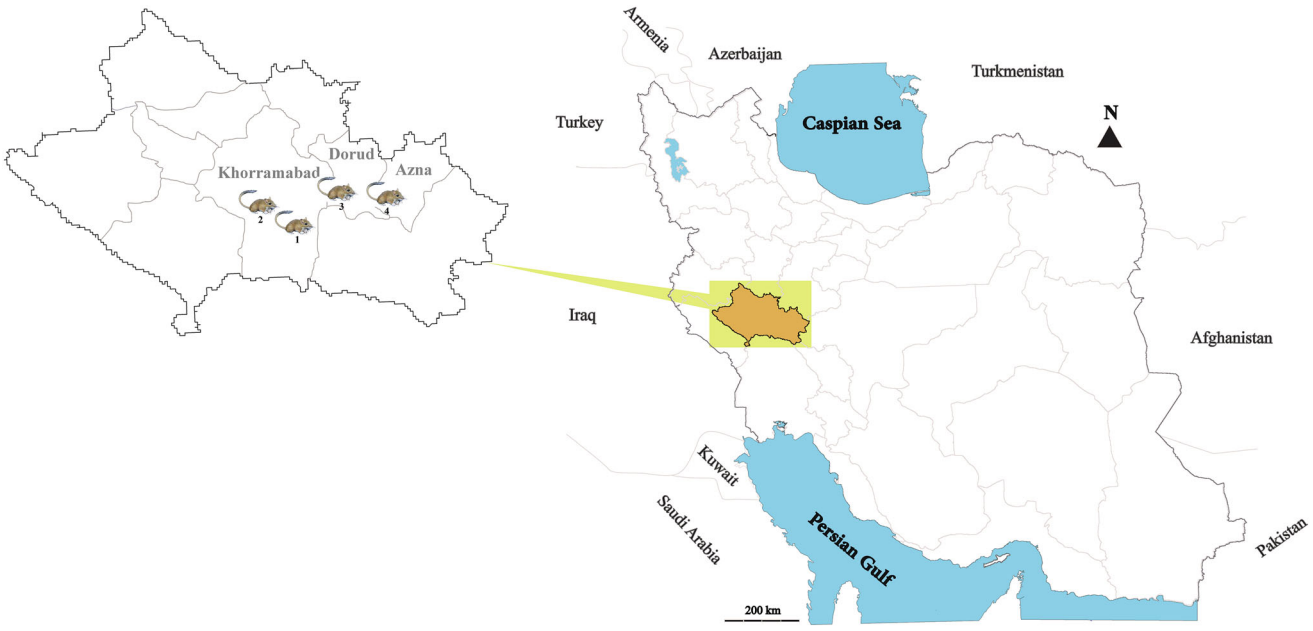

Fig. 1 Map of rodent collection sites in Lorestan province, Iran; Numbers stand for the sites summarized in Table 1

Table 1 Rodent collection data from Lorestan province, Iran; Parenthesized site numbers are mapped in Fig. 1

Voucher No.	Species	Locality (Location No.)	GPS coordinates (NE)
MHHM0521	*Meriones persicus*	Khorramabad, Vark waterfall road (2)	33° 19′ 16.73″ 48° 25′ 41.14″
MHHM0525	*Meriones persicus*	Khorramabad-Chambagh village (1)	33° 20′ 57.98″ 48° 32′ 40.13″
MHHM0534	*Meriones persicus*	Khorramabad–Dorud road, Miangaran village (3)	33° 29′ 15.30″ 48° 35′ 39.90″
MHHM0550	*Meriones persicus*	Khorramabad–Dorud road, Miangaran village (3)	33° 29′ 15.30″ 48° 35′ 39.90″
MHHM0563	*Meriones persicus*	Khorramabad–Dorud road (3)	33° 31′ 8.80″ 48° 45′ 21.20″
MHHM0544	*Meriones tristrami*	Dorud-Azna road (4)	33° 28′ 42.20″ 49° 10′ 37.90″
MHHM0545	*Meriones tristrami*	Dorud-Azna road (4)	33° 28′ 42.20″ 49° 10′ 37.90″
MHHM0547	*Meriones tristrami*	Dorud-Azna road (4)	33° 28′ 42.20″ 49° 10′ 37.90″
MHHM0557	*Meriones tristrami*	Dorud-Azna road (4)	33° 28′ 42.20″ 49° 10′ 37.90″
MHHM0575	*Microtus* sp. *(Sumeriomys)*	Dorud-Azna road (4)	33° 28′ 42.20″ 49° 10′ 37.90″
MHHM0530	*Mus macedonicus*	Khorramabad-Chambagh village (1)	33° 20′ 57.98″ 48° 32′ 40.13″
MHHM0536	*Mus macedonicus*	Khorramabad–Dorud road, Miangaran village (3)	33° 29′ 15.30″ 48° 35′ 39.90″
MHHM0548	*Mus macedonicus*	Khorramabad–Dorud road, Miangaran village (3)	33° 29′ 15.30″ 48° 35′ 39.90″
MHHM0549	*Mus macedonicus*	Khorramabad–Dorud road, Miangaran village (3)	33° 29′ 15.30″ 48° 35′ 39.90″
MHHM0552	*Mus macedonicus*	Khorramabad–Dorud road (3)	33° 29′ 2.60″ 48° 39′ 41.50″

of error or ambiguity. Homologies with the sequence data available in GenBank were checked using the BLAST analysis. So, each different fragments of 18S rRNA was BLASTed individually. Finally, sequences were submitted to the GenBank, and accession numbers were assigned. The sequences were aligned using SeaView4 software (Gouy et al. 2010), and the genetic distances among the sequences were calculated by a Maximum Composite Likelihood (MCL) model in the MEGA7 software (Kumar et al. 2016). To construct phylogenetic trees, the aligned sequences of each gene were analyzed based on the Bayesian Inference (BI) method using BEAST® software (version 2.5.1) (Bouckaert et al. 2014). An appropriate substitution model was selected using online FINDMODEL program (http://hiv.lanl.gov/content/sequence/findmodel/findmodel.html) (Posada and Crandall 1998). The Jukes-Cantor (JC69) was found as the appropriate model with a –lnL score of 1804.91 (L1) and 1420.25 (L2). The BI method employs the Markov chain Monte Carlo (MCMC) algorithms and extracts the most credible tree given the posterior probabilities of alternative tree topologies. For this purpose, 23 (L1) and 27 (L2) taxa (including sequences found in the present study as well as the comparable GenBank data sequences as in- and out-group members) were used. The clades in the phylogenetic tree were arranged and labelled based on two criteria, including posterior probability $> 99\%$ support value and the reasonable genetic distance differences within and between the clade members. A *Plasmodium* taxon was examined as out-group in 18S rRNA phylogenetic trees.

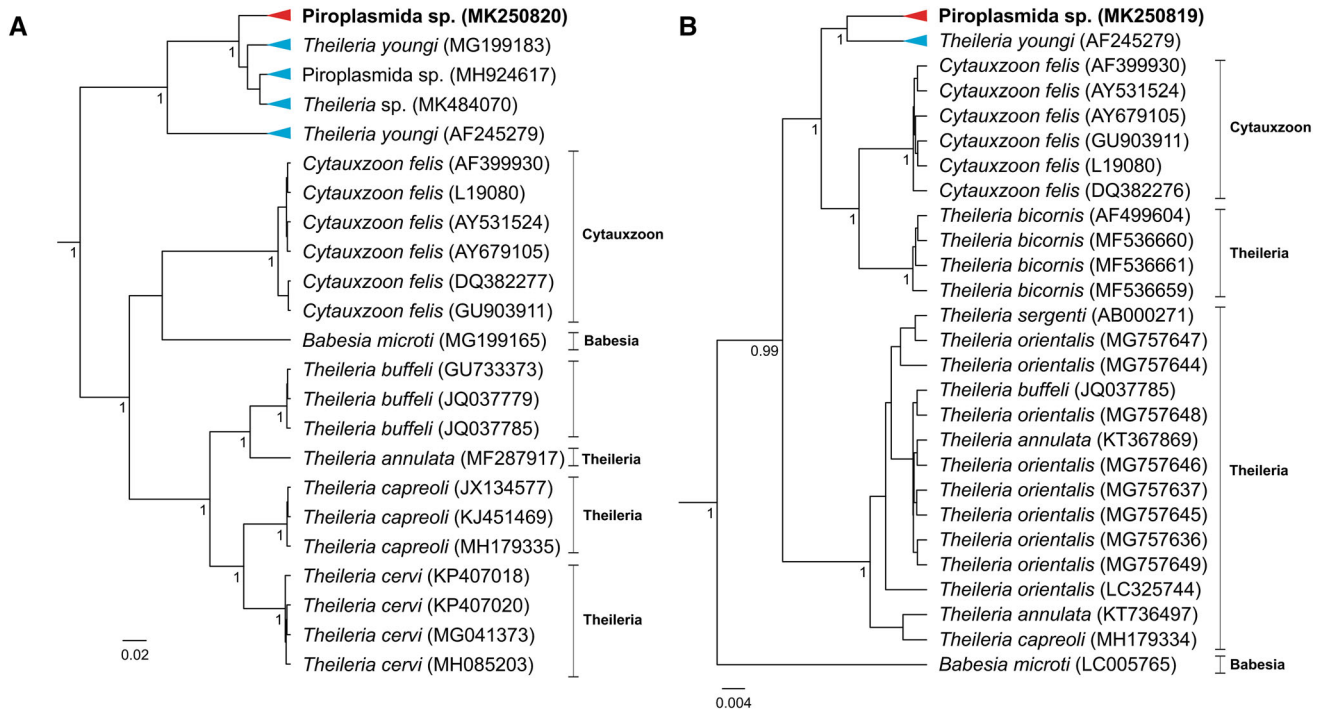

Fig. 2 Phylogenetic relationships among piroplasm taxa derived from Bayesian inference (BI) method and generated based on the analysis of L1 (A) and L2 (B) 18S rRNA; Numbers below each node represent posterior probability value in BI analysis (10 million reiterations). Taxon labels specify the species' names followed by (in turn) GenBank accession numbers in parentheses; Taxa sequenced in the present study are in bold. The phylogeny is rooted with a clade of *Theileria* (A) and *Babesia microti* (B). Branch lengths are proportional to the evolutionary changes

Results

Rodent collection and PCR detection

The blood samples were collected from four rodent species namely *Meriones persicus*, *M. tristrami*, *Mus macedonicus,* and *Microtus* sp. (*Sumeriomys*) to detect any piroplasm species. The 18S rRNA target gene of piroplasm parasite was amplified by PCR in a *M. persicus* specimen collected from Khorramabad–Dorud road (Site 3, 33° 31′ 8.80″ N 48° 45′ 21.20″ E) (Fig. 1 and Table 1). Then the L1 and L2 of 18S rRNA were successfully sequenced. PCR reactions were negative for other species/specimens collected from other sites in Lorestan province. Two sequences were assigned in GenBank under the accession numbers MK250819-20.

Phylogenetic tree analysis

Two individual phylogenetic trees were constructed according to the L1 and L2 of partial 18S rRNA sequence data (Fig. 2). L1 18S rRNA phylogenetic tree shows three clades (namely *Theileria*, *Babesia*, and *Cytauxzoon)* with five distinct and monophyletic branches: a piroplasmida sp. sequence from the present study (MK250820), *Theileria youngi* (MG199183, AF245279), a *Theileria* sp. (MK484070), and a piroplasmida sp. (MH924617). According to L2 18S rRNA phylogenetic tree, two clades (i.e., a *Cytauxzoon* and a *Babesia)*, as well as two divergent branches (i.e., a piroplasmida sp. sequence from this study (MK250819) and a *T. youngi* (AF245279)), were detected. According to the BLAST analysis and homology with *Theileria* clades of 18S rRNA phylogenetic tree, it was assume that the piroplasmida sp. sequence from this study might be a *Theileria* sp. (Table 2).

Discussion

The DNA of piroplasm parasite was detected using PCR and Sanger sequencing method in *Meriones persicus* collected from western Iran. A *Theileria*-like taxa was suspected according to the BLAST analysis of 18S rRNA L1 (AF245279) and L2 (MG199183) with 96% and 91% sequence homology, respectively (Table 2). This is the first report on the existence of a *Theileria*-like species in *M. persicus*. Rodents are recognized as the host of more than 60 zoonotic diseases so that they are considered as a serious threat to human health (Morand et al. 2015). Various piroplasm species have been detected in rodent species

Table 2 Summary description of BLAST results between the sequences of this study with GenBank piroplasms sequence data originated from various rodent hosts

Host[a]	18S rRNA Locus I (MK250819)		18S rRNA Locus II (MK250820)	
	QC	Id.	QC	Id.
Meriones	*B. divergens* (MG944238)		*B. divergens* (MG944238)	
	99	89	54	92
Apodemus	*B. microti* (AY144700)		*B. microti* (MG199165)	
Mesocricetus	99	91	100	81
Microtus				
Peromyscus				
Rattus				
Mus	*B. rodhaini* (AB049999)		*B. rodhaini* (DQ641423)	
	99	91	100	80
Rattus	*T. youngi* (AF245279)		*T. youngi* (MG199183)	
	99	96	100	91
			T. youngi (AF245279)	
			100	85

Accession numbers in parentheses, *B.*: *Babesia*, *T.*: *Theileria*, QC: Query cover (%), Id.: Identity (%)

[a]Host of piroplasm species

such as *Babesia microti* from *Apodemus* (Beck et al. 2011), *Microtus*, *Myodes* (Obiegala et al. 2015), *Peromyscus* (Hofmeister et al. 1998), *Rattus* (Chao et al. 2017), *T. youngi* (*Rattus*) (GB accession MG199183), piroplasmida sp. (*Xerus*) (Harris et al. 2018) and *Theileria* sp. (unknown rodent species) (GB accession MK484070), and *Hepatozoon* sp. (*Calomys*) (GB accession KP757838). The piroplasms of wild mammals such *Neotoma*, *Peromyscus*, *Microtus*, *Spermophilus*, and *Sylvilagus* were studied by Van Peenen and Duncan (1968) in California, and they concluded that all of the isolated piroplasms were *B. microti*. In fact, this southern Californian piroplasm may be similar to *T. youngi*. Kjemtrup et al. (2001) described *T. youngi* from an infected cricetid rodent, *Neotoma fuscipes*. They assigned this organism to *Theileria* based on molecular phylogenetic analysis of the 18S rRNA gene and its morphological features. In both L1 and L2 phylogenetic trees, a *Theileria*-like taxa found in this study diverged from *Theileria* species isolated from large mammals closely related to *Cytauxzoon* clade. The phylogenetic analysis suggests that *T. youngi* (*Theileria*) may be closely related to (*Cytauxzoon*) *C. felis* with regard to the sequence analysis of the 18S rRNA (Kjemtrup et al. 2001; Conrad et al. 2006) so that the two genera were later synonymized with *Theileria* (Levine 1971). Based on the multi-gene analyses, the order Piroplasmida includes three genera: *Babesia*, *Theileria*, and *Cytauxzoon* (Jalovecka et al. 2018). Divergent branches of phylogenetic trees indicate various genetic distances ranging from 4% to 17% among taxa, which represent different *Theileria* species. Generally, *Theileria* behaves as paraphyletic clades in our phylogenetic analysis. Allsopp et al. (1994) amplified small subunit ribosomal RNA (srRNA) genes of three *Theileria* species from large ungulates (namely one *Cytauxzoon* and four *Babesia* species). They indicate the *Theileria* form a monophyletic taxon which was extracted from a paraphyletic group containing *B. equi*, *C. felis* and *B. rodhaini* species. Future researches are recommended to microscopically examine the blood samples of rodents collected from the same area in Lorestan province for a specific detection of the piroplasma species.

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Author contributions AH-C designed the study. AH-C, SZH, FK, AKR, EM, and MHK collaborated to the sample collection, laboratory assays and the manuscript writing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval We hereby declare all ethical standards have been respected in preparation of the submitted article. This research is not involving human participants. We follow the guideline for the care and use of laboratory animals National Research Council (2010). Ethical approval of Lorestan University of Medical Sciences for the research project is: LUMS.REC.1395.104.

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