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Original Article

PCR-RELP for detecting of *Theileria annulata* infection in cattle and *Hyalomma* species in Kermanshah Province, Iran

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ABSTRACT

Bovine theileriosis is important disease in tropical and subtropical areas with great economic losses in livestock husbandry in Iran. The aim of study was to assess the prevalence of *Theileria annulata* infection in cattle and *Hyalomma* species of Kermanshah Province, Iran. A number of 138 blood samples were randomly taken from examined cattle. The genomic DNA was extracted and PCR was performed to specifically amplify a 721-bp-long fragment of the 30 Kilo Dalton major merozoite surface antigen (30 KDa msa) of *T. annulata*. The amplified products were digested with Taq*I*, Ras*l*, and Alu*I* restriction enzymes. Overall prevalence was 9.44% (13/138) with lymphadnopathy (1.17%) and pale mucosal membrane (1.9%) in Holstein cattle aged <1 year and more than 5 years-old, respectively. Five species of genus *Hyalomma* (18.8%, 141/750) including *Hyalomma anatolicum anatolicum* (30.4%), *H. anatolicum excavatum* (31.2%), *H. marginatum* (36.8%), *H. asiaticum asiaticum* (0.7%), and *H. detritum* (0.7%) were identified. The tick indices for each *Hyalomma* species were ranged from 0.01 to 0.36. PCR findings indicated that 3 out of 138 blood samples (2.17%) and 19 out of 141 *Hyalomma* ticks (4.13%) were infected with *T. annulata*. Amplified PCR products from blood samples generated similar RFLP patterns, but different RFLP pattern for *T. annulata* from *H. anatolicum anatolicum* (9.21%) and *H. anatolicum excavatum* (4.2%). The RFLP patterns of the amplified fragment of the 30 KDa msa of *T. annulata* indicated the circulation of four different *T. annulata* isolates of *H. anatolicum anatolicum* and *H. anatolicum excavatum* in the region.

Keywords: PCR-RELP, Cattle, *Theileria annulata*, *Hyalomma*, Iran

INTRODUCTION

Theileriosis is estimated to be responsible for the annual loss of thousands of dollars in Iranian agricultural industry (Aeschliman *et al* 1990). The hemoprotozoan *Theileria annulata* is the etiological agent of theileriosis which infects cattle in tropical and subtropical areas (Norouzi *et al* 2007). In Iran, cattle theileriosis is mainly caused by *T. annulata* (Hashemi-Fesharaki 1988). However, the occurrence of *T. orientalis* has been reported from Iran (Ulinberg & Hashemifesharaki 1984, Azizi *et al* 2008). Two families out of three established ticks' families were identified with 896 species. Out of those 193 ticks are

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in the Argasidae family (soft ticks) and 702 species in the Ixodidae family (hard ticks) (Guglielmone *et al* 2010). Ixodid ticks play an important role in transmission of blood hemoprotozoan and considered as a significant threat to livestock (Aeschliman *et al* 1990, D' Oliveria *et al* 1995). The *Hyalomma* species of this family have been reported from different parts of Iran, most of which were found to be the main vectors for *T. annulata* or *T. orientalis* (Mazlum 1972, Razmi *et al* 2003, Azizi *et al* 2008, Tavassoli *et al* 2011). Of those, *H. anatolicum anatolicum* (Koch 1844), *H. asiaticum asiaticum* (Schulze & Schlottke 1930), *H. anatolicum excavatum* (Koch 1844), *H. dromedarii* (Koch 1844), *H. detritum* (Schulze 1919), and *H. marginatum* (Koch 1844) are the most widely distributed ixodid ticks throughout the country (Yakhchali *et al* 2012). For many decades, microscopic examinations (blood smear and lymph node examination) were the most frequent methods to detect *Theileria* infection in definitive hosts (Uilenberg & Hashemifesharaki 1984). However; these methods had low sensitivity due to difficulties in *Theileria* species discrimination and asymptomatic carriers' detection (Kirvar *et al* 1998). For this reason, molecular tools (PCR, Semi- Nested PCR, Nested PCR, PCR-RFLP) have been recently employed to detect *Theileria* infections in Iranian cattle population (Uilenberg & Hashemi-Fesharaki 1984, Azizi *et al* 2008). According to the geographical distribution of *Hyalomma* species and bovine theileriosis pattern in different parts of Iran, collection of accurate data on ixodid ticks is crucial to estimate the potential infection risk for cattle (Azizi *et al* 2008). Thus, it was aimed to determine prevalence, *Hyalomma* species diversity, and PCR-RFLP pattern of *T. annulata* isolates in Kermanshah province, Iran.

MATERIALS AND METHODS

Field study area. The study was carried out in Kermanshah province, which was located in an important livestock production region in western Iran. Ecologically, this area is classified as a semi-arid zone. Cattle raising is a very important economically occupations in this province. According to Iranian Veterinary Organization (I.V.O., 2010), an average population of 8.74 million cattle and calves have been distributed in Iran in which Kermanshah province has approximately 3.2% of these cattle population.

Animals, collection of blood and ticks. During the course of the study from March 2011 to February 2012, a total of 138 cattle (54% male and 46% female) were randomly selected and clinically examined. Data pertaining to each examined animal (animal location, management system, time of day, tag number, breed, age and sex) were recorded. The cattle were raised following traditional practices, with animals grazing during the day in less than three seasons of the year (spring, summer, and fall). Cattle breeds were Holstein, cross-breed and indigenous. The blood samples were also taken and stored at -20 °C until DNA extraction. To estimate parasitemia, blood smears were prepared, stained with Giemsa, and examined at 1000×. The place of study were divided into three sub-areas, i.e. west (40 cattle), center (60 cattle), and east (38 cattle) (Table 1). The animals were also divided in four age groups on the basis of eruption of permanent incisor teeth (Smallwood 1992) (Table 1). Ixodid ticks were directly collected from the body surface of examined animals by robbing alcohol pads surrounding the skin to remove embedded living ticks (Yakhchali *et al* 2012). The data pertaining to the predilection sites, stages of hard ticks (larva, nymph, adult), and recent use of acaricides were recorded. The hard ticks were placed into labeled glass vials with 70% ethanol (Merck, Germany) and *Hyalomma* species were identified using identification keys as described by Soulsby (1982) and Walker et al. (2003). Tick salivary glands of *Hyalomma* ticks were also dissected out (Oliver *et al* 2005) and stored in 70% ethanol (Merck, Germany) for DNA extraction.

Molecular procedures: DNA extraction from blood and ticks samples. The genomic DNA extraction of *T. annulata* was performed by using Genomic DNA Purification Kit (Fermentas, Germany). To extract DNA from tick salivary glands, each sample was washed several times in 0.01 M phosphate-buffered saline (PBS, pH 7.2) and digested by using lyses buffer (4 M Sodium chloride, 10 mM tris-HCL, 2 mM EDTA, 400 mg/ml proteinase K) at 56 °C for 1hour.

PCR and RFLP analysis. A pair of primers, (Forward: 5'GTAACCTTTAAAAACGT-3' and Reverse: 5'GTTACGAACATGGGTTT-3') were used to amplify a 721 bp fragment of the large subunit rRNA gene sequences encoding the 30-KDa major merozoite surface antigen of *T. annulata*. The primer's specificity and sensitivity was assessed by D'Oliveria *et al.* (1995). PCR reaction was carried out in 25µl reaction mixture containing 2µl (100 ng) of genomic DNA (diluted 1:30), 1.5U of *Taq* DNA polymerase (Fermentas, Germany), 50mM of each dNTPs (CinnaGen, Iran), 2mM of $MgCl_2$, 2.5µl of PCR reaction buffer (10×) and 0.2µM of each primer with positive and negative controls. The reaction was performed in a Bioer XP thermal cycler. The samples were subjected to an initial denaturation step at 94 ºC for 2 min, followed by 30 cycles of 60 s at 94 ºC, 60s at 48 ºC and 60s at 72 ºC, and a final extension step at 72 ºC for 5 min. A volume of 10µl of each PCR product was analyzed by electrophoresis on 1.5% (w/v) agarose gel for approximately 1.5hrs at 90V. The gels were visualized by staining with ethidium bromide (1 µg/ml). The amplified products were digested with Taq*I*, Ras*l*, and Alu*I* restriction enzymes (http://www.mbio.ncsu.edu) as described by the manufacturer, and analyzed using 2% agarose gel. For restriction digestion, a total volume of 15µl of digestion reaction containing 5µl of PCR product, 1µl of restriction enzyme, 1.5µl of enzyme buffer (Fermentas, Germany) and 8µl ddH_2O was prepared. The reaction tubes were incubated at 37 ºC for 12 hours.

RESULTS

Clinical findings. Microscopic examination of thin blood smears showed parasitemia in infected cattle ranging from 8.6% to 61.21%. Of 138 examined blood samples, 13 (9.44%) were positive for piroplasm with lymphadnopathy (1.17%) and pale mucosal membrane (1.9%) in Holstein cattle aged <1 year and more than 5 years-old (Table 1).

Tick infestation of examined animals. Out of 750 collected ixodid ticks, 141 (18.8%) were belonging to genus *Hyalomma* with five species, i.e. *Hyalomma anatolicum anatolicum* (30.4%), *H. anatolicum excavatum* (31.2%), *H. marginatum* (36.8%), *H. asiaticum asiaticum* (0.7%), and *H. detritum* (0.7%). The highest tick infestation was belonging to *H. marginatum* and inner thigh was the most infested body site with *H. anatolicum anatolicum* (11.35%, 16/141). The tick indices (number of ticks per infested animals) for each *Hyalomma* species were ranged from 0.01 to 0.36. The highest ixodid ticks infestation was recorded in spring (6.53%) than summer (2.91%) (Table 2).

Molecular findings. The PCR findings revealed that 3 out of 138 blood samples (2.17%) and 19 out of 141 *Hyalomma* ticks (4.13%) were infected with *T. annulata* (Figure 2). Of all infected ticks, *H. anatolicum anatolicum* (9.21%) and *H. anatolicum excavatum* (4.2%) had the highest prevalence in central parts of the region. PCR-RFLP pattern of salivary glands revealed the circulation of four different isolates of *T. annulata* (Figures 1 and 2).

DISCUSSION

Bovine *Theileria* infection is an important hemoprotozoan infection causing tropical theileriosis in Iranian cattle (Tavasoli *et al* 2011). In Iran, the first cases of cattle theileriosis were recorded in 1935 and so far there are lots of reports on economical losses, e.g. high mortality (<5-90%) and morbidity (40-80%) (Hashemi-Fesharki 1988, Yakhchali *et al.* 2012). In the present study, it seems that the occurrence of bovine theileriosis has a seasonal pattern which starts in April with a peak from June to July (spring) and gradually goes down during August and September (summer). This may be as a result of temperature decreasing and low ixodid tick vectors population on the pastures during summer in the region (Hashemi-Fesharki 1988, Rahbari *et al* 2007). Furthermore, *H. marginatum* was

Table1. The prevalence of *Theileria annulata* infection in cattle based on sex and breeding in different age groups (n=138).

Season	No. of examined animals	Prevalence (%)(*n/N*)	Age (year) (%)				Sex (%)		Breeding (%)			Clinical findings (%)				
			<1	1-2	3-4	>5	M	F	Ho	Na	Hy	C	J	Pa	Ly	Pe
Spring	35	6.53	1.45	1.45	0.73	2.9	2.54	3.99	5.07	0.73	0.73	1.12	1.17	1.9	1.17	1.17
Summer	34	2.91	0	1.45	1.46	0	1.45	1.46	2.91	0	0	0	0	0.73	0.73	1.45
Fall	35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Winter	34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	138	9.44	1.45	2.9	2.19	2.9	3.99	5.45	7.98	0.73	0.73	1.12	1.17	2.63	1.9	2.62

Notes: C, cough; F, Female; J, Jaundice; Ly, lymphadnopathy; M, male; Pa, pale mucosa; Pe, petechiae, n, animals infected with *Theileria annulata*; *N*, total animals examined.

Table2. The percentage and body site distribution of *Hyalomma* species in infested cattle of Kermanshah province, Iran.

Hyalomma species	Tick indices	No. of unfed ticks	Prevalence (%)	Body site distribution (%)					
				Ey	Er	It	Te	Ta	U
H. anatolicum anatolicum	0.31	43	30.4	0	0	11.35	10.64	0	8.51
H. asiaticum asiaticum	0.01	1	0.7	0	0	0.71	0	0	0
H. anatolicum excavatum	0.31	44	31.2	0	0	7.8	12.06	0	11.35
H. detritum	0.01	1	0.7	0	0	0.71	0	0	0
H. marginatum	0.36	52	36.8	0	0	10.64	16.31	0	9.93
Total		141	18.8	0	0	31.21	39.01	0	29.79

Notes: Er, Ear; Ey, Eye; It, inner thighs; Neg, negative; Po, positive; Te, Testis; Ta, tail; U, udder.

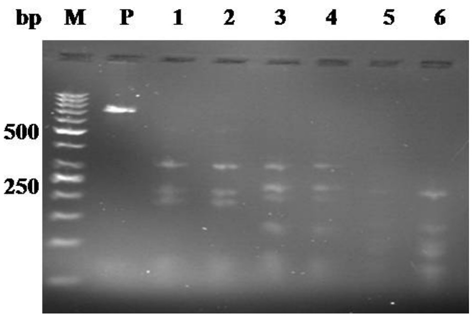

Figure 1. RFLP pattern of PCR products of *Theileria annulata* from infected cattle after digestion with restriction enzymes (lanes 1-2: *Alu*I; lanes 3-4: *Rsa*I, lanes 5-6: *Taq*I), 721-bp-long PCR product of *T. annulata* (lane P), 50 bp DNA size marker (lane M).

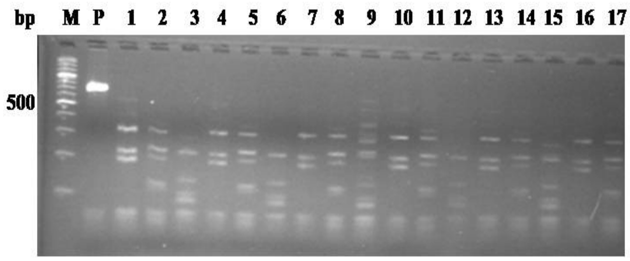

Figure 2. RFLP pattern of PCR products of *Theileria annulata* (lanes: 1-8) from infected *Hyalomma anatolicum anatolicum* and *H. anatolicum excavatum* (9-17) after digestion with restriction enzymes (*Taq*I: lanes 1, 4, 7, 10, 13, 15; *Rsa*I: 2, 5, 8, 11, 14, 17; *Alu*I: lanes 3, 6, 9, 12, 16:), 721-bp-long PCR product of *T. annulata* (lane P), 100 bp DNA size marker (lane M).

found to be prevalent specie in Kermanshah province. According to Azizi and Yakhchali (2006) *Hyalomma* species play an important role as vectors of tropical theileriosis in Iran. So far, 14 species of family Ixodidae have been reported from different parts of Iran (Mazlum 1972, Rahbari 1995, Yakhchali & Hajihasanzadehzarza 2004, Yakhchali *et al* 2011, 2012). The predominant tick infestation in Iranian cattle was *H. anatolicum anatolicum* from East, Center, North West, South, and West (Mazlum 1972, Razmi *et al* 2003, Rahbari *et al* 2007, Noaman *et al* 2007, Tavasoli *et al* 2011, Yakhchali *et al* 2011, 2012). *H. marginatum* were also reported from different parts of the country, while *H. detritum* exists in Caspian Sea coast of northern Iran (Mazlum 1972, Razmi *et al* 2003, Rahbari *et al* 2007). In the semi-arid areas of Mediterranean region, *H. marginatum* and *H. anatolicum excavatum* were reported as important vectors of *T. annulata* (Viseras *et al* 1999). During the past decades, microscopic examination was the frequent methods to detect *T. annulata*. However, the sensitivity and/or specificity of these methods were low because of difficulties in detection and differentiation of *Theileria* infection (Nikpay *et al* 2007). The MGP was previously useful tool to detect tick infection with sporozoite-like of *T. annulata* (Walker *et al* 1979). However, molecular tools were reported more sensitive and specific to detect *T. annulata* infection in hard ticks (Oliveira *et al* 2005, Azizi *et al* 2008, Hoghoghi-Rad *et al* 2011). In this work, molecular findings indicated that *H. anatolicum anatolicum* was prevalent tick vector for *T. annulata*. According to Aktas et al. (2004) and Tavassoli et al. (2011), infection rate of *T. annulata* was higher in *H. anatolicum anatolicum*. Razmi et al. (2003) reported that *H. anatolicum excavatum* (51%) was predominant specie using G staining. In earlier study, Jacquiet et al. (1997) reported that *H. dromedarii* and *H. marginatum* infection rate with *T. annulata* was respectively 73.08% and 15% using MGP. The RFLP-based assay has been previously employed by Spitalska et al. (2004) as a method in which organisms may be differentiated. The similarity of the PCR-RFLP patterns generated can be used to differentiate species and genotypes by analysis of patterns derived from cleavage of their DNA. Tavasoli et al. (2011) noted that one genotype of *T. annulata* existed in North West and West of Iran. While in this study, the RFLP patterns elucidated the circulation of four different genotypes of *T. annulata* in West part of Iran. From the results of this work, it was concluded that *Hyalomma* species may play an important role in transmission of different isolates of *T. annulata* in west part of Iran. Further studies are recommended to determine of which genotypes of *T. annulata* may cause of tropical theileriosis in cattle of the region.

Ethics

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

Conflict of Interest

Hereby, I declare "no conflict of interest exists" regarding submitted article.

Acknowledgments

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