A study of hematological changes in sheep naturally infected with *Anaplasma* spp. and *Theileria ovis*: Molecular diagnosis

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Key words:
Anaplasma, hematology, sheep, *Theileria*

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Received: 17 November 2014
Accepted: 18 January 2015

Abstract:

BACKGROUND: Ovine anaplasmosis and theileriosis are important tick-borne diseases of sheep and goats which are distributed in the tropical and subtropical areas of the world. **OBJECTIVES:** This study was performed to assess hematological status in sheep naturally infected with *Anaplasma* and *Theileria* spp. to clarify the pathogenic aspects of various species involved in ovine anaplasmosis and theileriosis in Ahvaz region. **METHODS:** 109 sheep were sampled, and blood parasite infections were diagnosed by microscopic examination and PCR. The blood samples were also subjected to hematologic assessment. **RESULTS:** PCR analysis revealed *A. ovis* infection in 86.2% of sheep, while mixed infections with *A. marginale* were also detected in 53.2% of them. However, *Anaplasma* inclusion bodies were only observed in 32.1% of the tested animals. *T. ovis* were found in 88% of the inspected sheep by PCR, and 67.8% of them were detected microscopically, as well. Hematologic assessment showed that mean RBC, PCV, Hb, and MCHC were significantly lower, whereas MCV and RDW were higher in the animals with mixed infections of *Anaplasma* with parasitemia and *Theileria*, compared to the uninfected sheep and groups with single infection or without parasitemia. **CONCLUSIONS:** In brief, it seems that *Anaplasma* can be activated and induce its pathogenesis in the presence of other infective agents in the carrier or asympthomatic animals. It can also be concluded that mixed infections of *Anaplasma* with parasitemia and *Theileria* may induce a regenerative anemia which is most likely attributable to a combined effect of the two.

Introduction

Anaplasma and *Theileria* are tick-borne pathogens that infect wild and domestic animals in the tropical and subtropical areas of the world (Aktas et al., 2005; Rymaszewska and Grend, 2008).

Ovine anaplasmosis is a disease mainly caused by intracellular rickettsia *A. ovis* (Rymaszewska and Grend, 2008). Although *A. ovis* may infect domestic sheep and goats without clinical signs (Splitter et al. 1956), animals experimentally infected, display depression, debility, weight lose, fever, and pro-

gressive anemia in acute phase of the disease which can cause considerable losses in farming stock (Melendez, 2000; Rymaszewska and Grend, 2008; Yasini et al., 2012).

Theileriosis, an important hemoprotozoal disease of sheep and goats (Altay et al., 2007), is caused by several species of *Theileria*, of which, *T. lestoquardi* (syn. *T. hirci*) is considered highly pathogenic. The other species such as *T. ovis* cause subclinical infection in small ruminants (Aktas et al., 2005).

Ahvaz, the capital of Khuzestan province, is a tropical area in southwest Iran which is of great importance in livestock industry. As the hot and humid weather is a predisposing factor, parasitic infections and tick-borne diseases are highly prevalent in this region (Zaeemi et al., 2011; Jalali et al., 2013).

This study was carried out to evaluate the hematological changes in sheep naturally infected with *Anaplasma* and *Theileria* spp. in order to clarify the pathogenic aspects of various species involved in ovine anaplasmosis with or without theileriosis in Ahvaz region which was performed for the first time.

Materials and Methods

Collection of blood samples: This study was carried out in Ahvaz and surrounding area, in the southwest of Iran, which is a tropical endemic area of ovine tick-borne diseases.109 sheep (55 male and 54 female, 3 months to 9 years of age) were sampled during the tick activity season, July to September 2011. The temperature and humidity of Ahvaz in the mentioned period were between 26.3 to 47.3°C, and 10to 48%, respectively. Sampling was performed in suspicious farms with the history of the outbreak of tick-borne diseases and in animals with tick infestation.

Blood samples were collected from jugular vein into sterile tubes with anticoagulant (EDTA) for hematologic and molecular assessment.

Blood parasite infected sheep were diagnosed by microscopic examination of thin blood smears and PCR analysis.

Microscopic examination: Blood smears were prepared and fixed with methanol for 1 min and stained with 5% Giemsa solution for 20 min and then examined for the presence of blood parasites or rickettsiae (*Anaplasma*, *Theileria* or *Babesia* spp.) under immersion oil lens (×100).

Parasitemia ratio was assessed by counting the number of infected red blood cells on examination of at least 200 microscopic fields. The number of infected cells was then expressed as a percentage (Jalali et al., 2014).

PCR analysis: DNA extraction was performed by molecular biological system transfer kit (MBST- Iran), according to the manufacturer's instructions.

A PCR method was carried out to detect *Anaplasma* spp. (*A. ovis* and *A. marginale*) using one pair of primers, based on the MSP4 gene sequence of *Anaplasma* spp. Primers were forward strand primer 5'-TTGTTTACAGG-GGGCCTGTC- 3' and reverse strand primer 5'- GAACAGGAATCTTGCTCCAAG-3'. *A. ovis* and *A. marginale* were differentiated from each other by PCR-RFLP using HpaII enzyme (Ahmadi-Hamedani et al., 2009; Jalali et al., 2014).

Theileria and *Babesia* infections were diagnosed by a PCR technique using forward strand primer FThBab 5′-GCATTCG-TATTTAACTGTCAGAGG-3′ and reverse strand primer RThBab 5′- GATAAGGTTCA-CAAAACTTCCCTAG-3′ which were specific for 18SrRNA gene sequence of *Theileria* and *Babesia* spp.

PCR-RFLP was done to differentiate *Theileria* and *Babesia* and also various *Theileria* species which infect sheep (*T. ovis* and *T. lestoquardi*) using Hind II and VspI enzymes, respectively (Jalali et al., 2014).

Hematological assessment: Hematological parameters including total erythrocyte count

(RBC), hematocrite value (HCT), hemoglobin concentration (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), and total white blood cells (WBC) were determined by the BC-2800Vet hematology analyzer (Mindray, China). Differential leukocyte counts were also estimated manually, and the erythrocytes morphology was examined as described by Meyer and Harvey (2004).

Statistical analysis: Analysis of variance (ANOVA) and Tukey's Post Hoc tests were used to compare and determine statistical differences in laboratory-obtained values between groups. All values were expressed as mean and standard error (SE), and $p<0.05$ was considered as statistically significant.

Results

PCR analysis of 109 blood samples obtained from different parts of Ahvaz and the surrounding area revealed that 86.2% (94/109) of sheep were infected with *Anaplasma* spp., whereas inclusion bodies were only observed in 32.1% (35/109) of the tested animals. All the positive blood samples were identified as *A. ovis* in PCR-RFLP, while mixed infections with *A. marginale* were also detected in 53.2%(50/94) of them.

Theileria infections were found in 88% (96/109) of the inspected sheep by PCR, and 67.8% (74/109) of them were detected microscopically, as well. In enzymatic digestion of PCR products, all *Theileria* positive samples were identified as T.ovis.

Babesia spp. piroplasms were neither detected in the blood smear examination nor in PCR analysis.

The sampled animals were divided into 14 groups based on *Anaplasma* spp. and/or T.ovis infections in PCR and microscopic examination (table 1). However, groups 7, 10. and 12 were excluded in statistical analysis due to inadequate sample size.

All the blood samples were evaluated to determine hematologic parameters and the data were expressed as mean ± SE for each group (Table 2, 4 and 5).

The analysis of hematology parameters showed that groups 11, 13, and 14 had the lowest RBC counts among all, while HCT values and Hgb concentrations in groups 8, 11, 13, and 14 were significantly lower than groups 1,

Table 1. Animal groups based on *Anaplasma* spp. / *Theileria* ovisinfection.

Group	*Anaplasma*			*Theileria*		
	PCR		Parasitemia	PCR	Parasitemia	
	A.ovis	A.marginale				Number
1						4
2				+		4
3				+	+	7
4	+					4
5	+			+		8
6	+			+	+	16
7	+	+				2
8	+	+		+		6
9	+	+		+	+	23
10	+		+			2
11	+		+	+	+	14
12	+	+	+			1
13	+	+	+	+		4
14	+	+	+	+	+	14

Table 2. Hematological indices (mean ± SE) in different sheep groups.(*) Different letters in rows indicate significant difference between groups.

Group	RBC (×106 /µL)	PCV (%)	Hb (g/dL)	MCHC (g/dL)	MCV (fL)	RDW (%)
1	11.55 ± 0.47 [a*]	31.53 ± 0.74 [a]	8.93 ± 0.29 [a]	27.93 ± 0.29 [ab]	26.70 ± 0.51 [ab]	19.46 ± 1.14 [ab]
2	11.58 ± 0.85 [a]	28.76 ± 1.77 [ab]	8.43 ± 0.63 [a]	29.23 ± 0.56 [a]	24.96 ± 1.26 [a]	17.16 ± 0.23 [a]
3	10.14 ± 0.91 [ab]	28.88 ± 2.20 [ab]	8.10 ± 0.60 [a]	28.10 ± 0.54 [ab]	28.87 ± 1.16 [ab]	16.57 ± 0.65 [a]
4	9.91 ± 0.28 [ab]	29.35 ± 0.68 [ab]	9.15 ± 0.21 [a]	31.15 ± 0.70 [a]	29.77 ± 1.44 [ab]	16.40 ± 0.39 [a]
5	12.08 ± 0.63 [a]	30.05 ± 1.29 [a]	8.78 ± 0.37 [a]	28.18 ± 0.23 [ab]	25.02 ± 0.68 [a]	18.85 ± 0.80 [ab]
6	10.88 ± 0.39 [a]	30.85 ± 1.21 [a]	9.01 ± 0.34 [a]	29.22 ± 0.31 [a]	28.41 ± 0.58 [ab]	16.98 ± 0.19 [a]
8	8.77 ± 0.86 [ab]	25.02 ± 2.48 [b]	6.84 ± 0.71 [b]	27.26 ± 0.70 [b]	28.58 ± 0.40 [ab]	17.66 ± 1.02 [a]
9	9.28 ± 0.38 [ab]	27.34 ± 1.01 [ab]	7.71 ± 0.32 [ab]	25.11 ± 0.33 [b]	29.70 ± 0.69 [ab]	17.66 ± 0.44 [a]
11	7.81 ± 0.72 [b]	23.40 ± 1.73 [b]	6.42 ± 0.47 [b]	27.58 ± 0.52 [b]	30.88 ± 0.99 [b]	18.15 ± 0.52 [ab]
13	7.76 ± 1.57 [b]	24.45 ± 2.37 [b]	6.57 ± 0.75 [b]	26.70 ± 0.59 [b]	33.27 ± 2.96 [b]	21.50 ± 2.11 [b]
14	8.03 ± 0.52 [b]	25.05 ± 1.08 [b]	6.80 ± 0.31 [b]	27.05 ± 0.36 [b]	32.17 ± 1.49 [b]	19.57 ± 1.07 [b]

Table 3. Number and percentage of anemic animals (PCV < 27%) in each group.

Group	Number of animals with PCV< 27%	Percentage
1	1/4	25
2	1/4	25
3	3/7	42.85
4	0/4	0
5	1/8	12.5
6	2/16	12.5
8	2/6	33.33
9	12/23	52.17
11	10/14	71.42
13	3/4	75
14	8/14	57.14

5, 6 and 1, 2, 3, 4, 5, 6, respectively. Concomitant decreases in MCHC values in groups 8, 9, 11, 13, and 14 were also observed which were significantly different from the mean values in groups 2, 4, and 6.

There were significant rises in mean MCV of groups 11, 13, and 14 and mean RDW of groups 13 and 14 compared to other groups (Table 2).

Basophilic stippling, polychromasia, and reticulocytosis were observed in microscopic examination of anemic sheep blood samples.

Additionally, as it can be seen in Table 3, the percentage of anemic animals (with PCV < 27%) were higher in groups 11, 13, and 14, with group 13 having the highest percentage (75%) among the three (Table 3).

There were no significant differences in WBC counts, lymphocytes, neutrophils, eosinophils, and monocytes (Table 4).

Discussion

Ovine tick-borne diseases are widespread in Iran and cause high economic losses especially in tropical areas. Theileriosis, due to *T. lestoquardi*, causes avirulent disease in sheep; however, other species such as *T. ovis* are believed to be non-pathogenic (Aktas et al. 2005; Hashemi Fesharaki, 1997).

Anaplasmosis is usually a subclinical or mild condition in sheep; nonetheless, moderate to severe clinical disease may occur with fever and a variable degree of anemia and icterus (Stoltsz, 2004).

It was found in this study that *T. ovis* was the most prevalent *Theileria* spp. in sheep in Ahvaz region. Ovine anaplasmosis caused by *A. ovis* and *A. marginale* was also present and highly prevalent in this area.

Absence of clinical signs in most sheep may be associated with the carrier state of these animals. This may also explain the extremely high prevalence of ovine anaplasmosis and theileriosis in our study.

Although ovine anaplasmosis is rarely associated with marked clinical signs, apparent disease with fever and anemia can be observed

Table 4. Leukocytes total and differential counts (mean ± SE) in different sheep groups.

Group	WBC ($\times10^3/\mu L$)	Lymphocyte (µL)	Neutrophil Seg. (µL)	Neutrophil Band (µL)	Eosinophil (µL)	Monocyte (µL)
1	10.80 ± 0.17	4514.0 ± 1355.4	5826.0 ± 1461.9	125.6 ± 177.0	246.0 ± 194.5	37.0 ± 37.0
2	11.76 ± 4.90	6711.6± 2657.3	4724.7 ± 2464.7	124.6 ± 42.1	117.6 ± 49.0	88.0 ± 52.0
3	11.12 ± 1.44	4581.4± 707.0	5884.1 ± 1123.2	94.14 ± 71.7	431.5 ± 125.6	101.6± 41.3
4	10.45 ± 0.60	4517.7± 806.5	5539.0 ± 808.2	48.0 ± 48.0	295.5 ± 125.7	49.7 ± 28.7
5	12.48± 0.48	5119.5± 596.0	6935.7±531.1	69.7±53.6	261.7±65.4	99.0± 60.1
6	12.10 ± 0.58	5351.5±422.5	6214.0 ± 579.8	33.37± 15.5	439.0±88.9	61.7± 25.3
8	14.52 ± 1.47	5166.0 ± 783.0	8468.8 ± 868.9	168.0 ± 65.3	643.0 ± 303.5	74.2 ± 45.4
9	12.66 ± 0.93	5713.0 ± 488.0	6454.6 ± 731.7	71.7 ± 28.3	383.8 ± 87.2	32.4 ± 17.8
11	11.32± 0.94	5433.1±625.2	5473.0± 781.7	46.7± 24.8	306.1±95.4	69.4± 28.0
13	10.37 ± 0.65	5308.5± 790.9	4717.5 ± 352.9	72.7 ± 47.5	228.5 ± 80.0	47.7 ± 27.6
14	11.57± 1.56	4303.0 ±413.9	6770.21± 146.2	99.4±63.5	380.9± 138.7	25.0±9.4

in certain situations especially when concomitant infections occur (Stoltsz, 2004; Stuen and Longbottom, 2011).

Hematologic analysis of sheep blood samples showed that the most significant decreases in RBC counts, HCT values, Hgb concentrations, and MCHC values were in groups 11, 13, and 14 with concurrent *Theileria* and *Anaplasma* infection accompanied by parasitemia. MCV and RDW values were also significantly higher in the mentioned groups compared to uninfected ones. These indices revealed the presence of macrocytic hypochromic anemia in the corresponding animals. Reticulocytosis, basophilic stippling, and polychromasia in anemic animals indicated the regenerative response (Meyer and Harvey, 2004).

Yasini et al. (2012) investigated the hematologic and clinical effects of experimental ovine anaplasmosis caused by *A. ovis*. Their findings revealed marked normocytic normochromic anemia at the beginning of the infection which became macrocytic normochromic by the development of the disease. They also reported reticulocytosis and basophilic stippling in the animals that were infected experimentally. There were negative correlations between parasitemia and RBC, PCV, and Hgb values.

An experimental infection in pregnant goats with *A. ovis* was created by Barry and Van Niekerk (1990). While the infected goats showed few noticeable signs of anaplasmosis, a high body temperature, severe anaemia, and abortion were observed in half of the examined animals.

Extra-vascular hemolytic anemia is a key feature of anaplasmosis (Latimer et al., 2003). Although the exact mechanism is not clear, the anemia can be attributed to erythrophagocytosis by reticulo-endothelial cells, immune-mediated destruction of non-parasitized erythrocytes besides parasitized erythrocytes, oxidative damage and poor antioxidant status (De et al., 2012; Nazifi et al., 2008).

Recently, Nazifi et al. (2008) demonstrated parasitemia caused by *A. marginale* increases the mean corpuscular fragility of red cells and LDH activity. They recorded negative correlations among parasitaemia and RBC count, Hb, and PCV.

Additionally, it has been shown that erythrocytes infection with *A. marginale* can change phosphofructokinase enzyme activity and reduce ATP component which in turn may lead to defects in RBC membrane and metabolism (Rodestits et al., 2007; Stoltsz, 2004).

In a research conducted by Ahmadi-Hamedani et al. (2011) hematological analysis of *A. ovis*-infected group of goats indicated significant decreases in RBC counts, HCT values, and hemoglobin concentrations compared to the uninfected group. These data were similar

to the results obtained in this study.

On the other hand, *Theileria* infections possibly paly a role in hematologic signs observed in the current study.

It has been demonstrated that the decrease in RBC, PCV, and hemoglobin level observed in *Theileria* infected animals might be due to erythrophagocytosis in lymph nodes, spleen, and other organs of monocyte-macrophage system. Immune mediated hemolysis and oxidative damage to erythrocytes are also the possible mechanisms contributing to anemia induced by this infection (Nazifi et al., 2011; Latimer et al., 2003; Stockham and Scott, 2002).

T. lestoquardi is the most virulent species among all *Theileria* species infective in small ruminants but other species, such as *T. ovis* and *T. separata* are considered less pathogenic (Hooshmand-Rad and Hawa, 1973; Uilenberg, 1997).

However, in an experimental infection with *T. ovis*, the infected sheep developed a febrile response and reductions in hemoglobin level and RBC count were observed (Li et al., 2010).

The changes in leukocyte counts, lymphocytes, neutrophils, eosinophils, and monocytes were not statistically significant in this study. It may be attributable to the subclinical nature of the diseases induced by *Anaplasma* spp. and T.ovis in sheep. These results were consistent with other researchers' findings.

Ahmadi-Hamedani et al. (2011) reported leukopenia, lymphopenia, neutrophilia, eosinopenia, and monocytopenia in the *A. ovis* infected goats; nevertheless, none of these changes were significant compared to uninfected goats.

Yasini et al. (2012) also did not detect any significant changes in the mean of WBC count during the course of the disease in sheep experimentally infected with *A. ovis*.

In brief, it seems that *Anaplasma* can be activated and induce its pathogenesis in the presence of other infective agents in the carrier or asympthomatic animals. It can also be concluded that mixed infection of *Anaplasma* with parasitemia and T.ovis may induce anemia which is greater than animals with single infection of *Theileria* or *Anaplasma*. Therefore, this is most likely caused by a combined effect of the two.

Acknowledgments

This study was supported by the Faculty of Veterinary Medicine, University of Tehran, Iran. The authors would like to acknowledge all veterinarians and technicians in Veterinary Organization of Ahvaz and Faculty of veterinary Medicine, Shahid Chamran University of Ahvaz who helped in sample collection. We also thank the staff in Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, for excellent technical assistance. The authors declare that there is no conflict of interest.

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