Phenotypic characterization of Tunisian sheep breeds for resistance to abomasal parasites

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Abstract

Feces, blood and abomasa were collected from 312 naturally infected and non-infected sheep by abomasal nematodes and slaughtered in eight commercial slaughterhouses across North Tunisia. Haematological biochemichal parameters were assessed. From the same samples of bllod, DNA was extracted and catch-all primers (RLB-F and RLB-R) were used to detect both *Theileria* spp. and *Babesia* spp. piroplasms. For all animals, faecal egg counts (FEC) were qualitatively assessed. Male and Female worms were collected from all abomasa and counted separately under a stereomicroscope. The overall infestation prevalence by coproscopy was 30.82%. Older animal, belonging to the Barbarine breed and from Beja and Tabarka had higher positive coproscopy (p < 0.05). After abomasa recovery, the infestation prevalence was estimated to 76.72%. The overall intensity and abundance were estimated to 198.6 and 152.4, respectively. The kappa concordance coefficient between coproscopy and worm recovery was 0.21. The overall infection prevalence by *Babesia* spp. and *Theileria* spp. was 4.21%. Multiple correlation between haematological and quantitative coproscopic parameters showed statistically significant negative correlations between: red blood cells and total worm count, red blood cells and *H. contortus* count, haemoglobin and total worm counts, haemoglobin and *H. contortus* count and finally between packed cell volume and *H. contortus* counts.

The linear regression model, with dependent variable total worm count, showed a statistically significant relation with haematological and quantitative coproscopic parameters.

The dispersion of observations plots for PCA showed 2 clusters of individuals. Animals having positive *Babesia*/*Theileria* PCR, presence of abomasal worms, high values of albumin and normal haematological parameters (RBC, Hb and PCV). The second cluster represents all the other observations. Genome analysis could be used to provide a better resolution of the sheep genomic profiles.

Keywords: Sheep, abomasal nematodes, Phenotypes, Tunisia

1. Introduction

Over the last decades, sheep population in Tunisia has been increasing reaching over 6.5 million heads in 2016 (Ministry of agriculture, 2016) with the Barbarine sheep as the dominant breed. In Tunisia, sheep population face many health challenges including parasitic infections such as toxoplasmosis (Gharbi et al., 2013), fasciolosis (Akkari et al., 2011), lungworms and gastrointestinal helminths (Akkari et al., 2012). Gastrointestinal nematode (GIN) infections affect the welfare and productivity of small ruminants and are responsible for huge economic losses (Waller, 1997a). These losses are consequent to decreases in weight, reduction in milk yield as well as wool and mortalities can occur (Soulsby, 1983). GIN infections are increased in regions where extensive grazing is practiced (Waller, 1997b). *Haemonchus* spp. is one of the major and the most prevalent abomasal nematodes of small ruminants (O'Connor et al., 2006) and its prevalence increases in countries of temperate regions (Van Dijk et al., 2008). Akkari et al. (2012) showed that, in North Tunisia, 45.5% of the parasite population were found in the abomasum, with the prevalence of *Haemonchus contortus* exceeding 35%. This parasite represents a major problem in most flocks as it has developed resistance against most available anthelmintic drugs.

In sheep and goats, a genetic variation of resistance to GINs has been explored by many authors, it has been demonstrated in several situations that genetic selection could contributes to a better control of sheep haemonchosis. Bishop (2011) describes resistance to infection as the host's ability to interact with parasite and control its lifecycle. The study of nematode resistance is first based on phenotypic measurements. The main indicator used for resistance to GINs is FEC. Nematode resistance assessed by using FEC has a low to high heritability in small ruminants, ranging from 0.01 to 0.65 (Zvinorova et al., 2016).

In addition to FEC, several traits could be considered when studying resistance to GIN. In fact, occurrence of anaemia can be an indicator for resistance in animals infected by *H. contortus*.

Anaemia can be objective by measuring packed cell volume (PCV), erythrocytes and haemoglobinaemia (Baker et al., 2003; Mandonnet et al., 2006; Riley and Van Wyk, 2009).

In Africa, the resistance of the Red Massai breed to GIN has been demonstrated (Baker et al., 1999). In fcat, resistance to naturally acquired gastro-intestinal nematode infections was studied in Red Massai, Dorper ans Cross bredd. The result showed that the Red Massai ewes were more resistant to GIN infections than other breeds showing significantly lower FEC and significantly higher PCV at most of the sampling times over the reproductive cycle. Resistnace was also manifested by lower number of ewes having to be treated with an anthelmintic and a lower mortality rate (Baker et al., 1999). In Tunisia, where GINs represent a persistent animal health problem, the genetic resistance of the three Tunisian sheep breeds to GIN has never been studied. The aim of this work is to study the genetic resistance of the Barbarine sheep to GIN infestation. Phenotypic measurements were collected and compiled in a single phenotype database. Parasitological indicators were calculated and some risk factors were analysed. Correlations between haematological parameters and coproscopic results were also analysed.

2. Materials and methods

2.1. Study area and animals

The samples were collected from 312 naturally infected and non-infected sheep by abomasal nematodes and slaughtered in eight commercial slaughterhouses across North Tunisia: Tunis (district of Tunis), Ariana (district of Ariana), Bizerte (district of Bizerte), Mateur (district of Bizerte), Sejnane (district of Bizerte), Béja (district of Béja), Jendouba (district of Jendouba), Tabarka (district of Jendouba) (Figure 1, Table 1). The choice of the geographic area was based on a previous study (Akkari et al. 2012) targeting regions where gastrointestinal parasites, and particularly nematode infestation, represent one of the main constraints in small ruminants'

production. Previous studies showed that infestation risk is very high in this ares (Akkari et al., 2012). Therefore, the likelihood of finding resistant sheep is relatively high.

The samples were collected between September and December 2017, information about the sex, the breed and the age of the animals were collected before animal slaughtering.
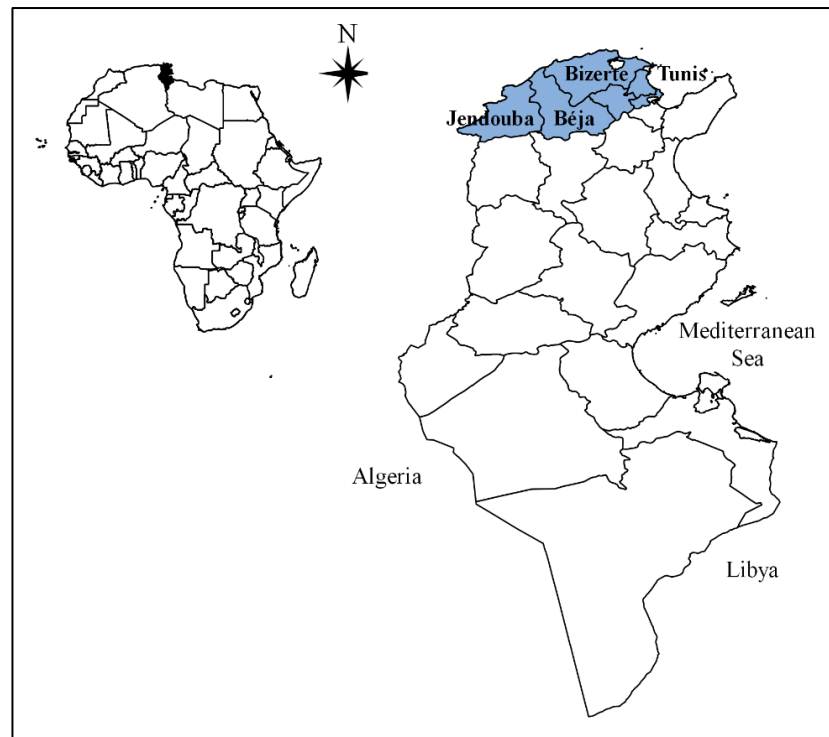

Figure 1. Districts where the sheep samples were collected in North Tunisia

Table 1. Characteristics of the studied area (climatedata.org ; date and time.info ; Ministry of Agriculture, 2016)

Locality	District	Localization	Mean annual rainfall (mm)	Mean annual temperature (°C)	Mean temperature during winter (°C)	Mean temperature during summer (°C)	GPS coordinates	Estimated sheep population
Ariana	Ariana	North-east	449	18.0	11.3	24.9	36°51′N 10°11′E	32,400
Beja	Beja	North-west	662	17.7	9.9	26.1	36°44′N 09°11′E	357,560
Bizerte	Bizerte	North	527	18	11.6	24.5	37°16′N 9°52′E	199,670
Jendouba	Jendouba	North-west	504	18	10.4	26.6	36°29′N 08°47′E	232,550
Mateur	Bizerte	North	539	18	11.2	25.1	37°02′N 9°39′E	199,670
Sejnane	Bizerte	North	527	18	11.2	25.1	37°3'N 9°14'E	199,670
Tabarka	Jendouba	North-west	865	18.2	11.8	24.8	36°57′N 8°45′E	232,550
Tunis	Tunis	North-east	448	18.1	11.4	25.2	36°48′N 10°10′E	18,880

2.2. Sample collection and preparation

2.2.1. Haematological analyses

Blood samples were collected from each sheep by puncture of the jugular vein using sterile EDTA and heparinized tubes. EDTA tubes were used for haematological parameters and red blood cell count (RBC) ($\times 10^6$/mL), haematocrit (PCV) (%) and haemoglobin (Hb) (g/dL) were estimated using an Auto Haematology analyser BC-2800Vet® (Shenzen Mindray Bio-Medical Electronics Co., Ltd, Hamburg, Germany). Plasma samples were collected from heparinized tubes and were used to estimate biochemical indicators: albumin (g/L) and total proteins (g/L) with a Random Access Clinical Autolyzer® (Dialab, Vienna, Austria). Threshold values used for haematological and biochemical parameters are shown in Table 2 (Blood & Radostits 1989).

Table 2. Hematological and biochemical threshold values (Blood & Radostitis 1989)

Parameter	Threshold value
Haematological parameters	
Red blood cells (10^{12}/L)	5.0 - 14.0
Haemoglobin (g/dL)	9.0 - 15.5
Haematocrit (%)	26.0 - 45.0
Biochemical parameters	
Albumin (g/L)	24 – 30
Total proteins (g/L)	60 – 79

2.2.2. DNA extraction and PCR amplification of *Theileria/Babesia* 18S rRNA gene

DNA was extracted from 300 µl of EDTA blood using Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions and stored at -20°C until used. Catch-all primers (RLB-F and RLB-R) which detect both *Theileria* spp. and *Babesia* spp. piroplasms were used (Gubbels et al., 1999) (Table 2). These primers amplify the hypervariable V4 region of the 18S ribosomal ribonucleic acid (rRNA) gene of *Theileria* spp. and *Babesia* spp.. Reactions were performed in 25 µl volume containing 19 µl PCR buffer, 1.5 mM MgCl2, 200 µM of each deoxyribonucleotide triphosphate, 0.125 µg of Taq hot start Ab,

0.1 U of Uracil DNA glycosylase, 25 pmol of each primer and 1.25 U of Super Taq DNA polymerase (Vivantis, Chino, CA, USA) and 3 µl of sampled DNA (Gubbels et al., 1999). PCR products were examined by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light.

2.2.3. Faecal Egg Counts

For all animals, faecal egg counts (FEC) were qualitatively assessed using 5 g of stool collected from each sheep by flotation technique to verify whether the presence of gastrointestinal parasite eggs. For positive samples, the quantification was done with the McMaster technique (Raynaud, 1970).

2.2.4. Abomasa collection and worm recovery

As soon as the animal is slaughtered and directly after removal of the intestinal tract, each abomasum was legated at both ends then cut and transported to the Laboratory of Parasitology at the National Veterinary School of Sidi Thabet (Tunisia).

In the laboratory, each abomasum was opened along its greater curvature and the contents were washed into a bucket. Male and Female worms were collected and counted separately under a stereomicroscope.

2.3. Statistical analyses

The infestation prevalence, intensity and abundance of infestation were respectively estimated as follows: (i) 100 x number of infested sheep/number of examined sheep (ii) number of collected larvae/number of infested sheep (iii) number of collected larvae/number of examined sheep (Margolis et al., 1982).

The infection prevalence percentages were compared with the chi square of Mantel–Haenszel using Epi Info 6 (Dean et al., 2011). The level of agreement between coproscopy and worm recovery results was assessed with the Kappa test (Toma et al., 2007). The interpretation of the Kappa values was as follows: 0.00–0.20 = weak, 0.21–0.40 = fair, 0.41–0.60 = moderate, 0.61–0.80 = good and 0.81–1.00 = very good agreement (Landis and Koch, 1977). Correlations and linear regression were made using SPSS for Windows. Principal component analyses (PCA) was also performed using SPSS and considering 6 variables: Babesia /Theileria infection, presence or absence of abomasal worms, Albumin, RBC, Hb and PCV. Outliers were not took into consideration. A cut-off value of 0.05 was considered for all the statistical tests (Schwartz, 1993).

3. Results

3.1. Parasitological indicators

For the 292 faeces collected samples only 90 samples were positive for abomasal nematodes by coproscopy giving an overall infestation prevalence of 30.82%. Animal of 12 months or more had higher positive coproscopy than animal aged of less than 12 months (46.67 and 25.22, respectively) (p=0.001). Animals belonging to the Barbarine breed had higher infestation prevalence by coproscopy (55.56%) (p=0.047). Finally, infestation prevalence of animals from Beja and Tabarka was higher than the other localities (56.00 and 58.33%, respectively) (p<0.001). There was no significant difference of infestation prevalence according to sex (Table 3).

Among the 312 sampled sheep, only 305 abomasa were collected and among them 234 were infested by at least one abomasal helminth, corresponding to an overall infestation prevalence of 76.72%. There was no significant difference between the infestation prevalences according to both sheep breeds and genders (p>0.05). The overall intensity and abundance were estimated

to 198.6 and 152.4, respectively. The highest worm infestation intensity was recorded in a sheep from Bizerte district (n=2991).

The kappa concordance coefficient between coproscopy and worm recovery was 0.21 which is a fair agreement between the two techniques according to the interpretation of Cohen's Kappa values.

Among the 312 sampled animals, only 309 blood samples were collected and DNA extracted. The overall infection prevalence by *Babesia* spp. and *Theileria* spp. was 4.21% (13/309). There was not significant difference in this prevalence according to sex, breed, age and locality (p>0.05) (Table 3).

3.2. Relation between haematological parameters and coproscopic results

Multiple correlation between haematological and quantitative coproscopic parameters showed statistically significant positive correlations between haematological parameters and negative correlations between: red blood cells and total worm count, red blood cells and *H. contortus* count, haemoglobin and total worm counts, haemoglobin and *H. contortus* count and finally between packed cell volume and *H. contortus* counts (Table 4).

The linear regression model, with dependent variable total worm count, showed a statistically significant relation with haematological and quantitative coproscopic parameters (p=0.001) (Table 5).

Two components were retained for PCA analyses and explained 61.7% of the variance, with component 1 showing the direction of the most variation (41.65%) and component 2 showing the direction of the second most variation (20.10%). The component plot showed that haematological parameters (RBC, Hb and PCV) were positively correlated with component 1 and *Babesia*/*Theileria*, negative or positive abomasa for worms and albumin were positively correlated with component 2 (Figure 2).

Each subject of the data was plotted on the bases of its first and second components values. The dispersion of observations plots for PCA showed 2 clusters of individuals. The first one (presented in red colour) represents animals having positive *Babesia*/*Theileria* PCR, presence of abomasal worms, high values of albumin and normal haematological parameters (RBC, Hb and PCV). The second cluster (presented in green colour) represents all the other observations.

3.3. Biochemical and haematological results according to PCA results

According to dispersion of observations obtained by PCA, animals were divided into two groups (i) animals infected by *Babesia*/*Theileria* and positive abomasa for worms (cluster 1) (ii) other animals (cluster 2) (Figure 3). Haematological and biochemical results in the two groups and other phenotypic traits (age, sex, breed) according to the two groups were summarised in Table 6. For the first cluster where animal were infected by *Babesia*/*Theileria* and abomasal worms, all haematological and biochemical parameters were normal.

Table 3. Association between *Babesia* spp./*Theileria* spp. infection and faeces analyses for GIN in sheep and different parameters

Parameter		*Babesia* spp./*Theileria* spp. PCR		Faeces analyses for GIN	
		Positive/examined (%)	P value	Positive/examined (%)	P value
Sex	Male	2/90 (2.22)	0.26	21/85 (24.71)	0.14
	Female	11/219 (5.02)		69/207 (33.33)	
Breed	Barbarine	1/37 (2.70)	0.9	20/36 (55.56)	0.047
	Cross breed	6/142 (4.23)		34/137 (24.82)	
	Noir de Thibar	3/59 (5.08)		20/58 (34.48)	
	Queue Fine de l'Ouest	3/71 (4.23)		16/61 (26.23)	
Locality	Ariana	0/33 (0)	0.64	6/29 (20.69)	<0.001
	Beja	3/54 (5.56)		28/50 (56.00)	
	Bizerte	3/29 (10.34)		10/29 (34.48)	
	Jendouba	1/49 (2.04)		9/46 (19.57)	
	Mateur	2/50 (4.00)		6/49 (12.24)	
	Sejnane	2/40 (5.00)		10/39 (25.64)	
	Tabarka	1/25 (4.00)		14/24 (58.33)	
	Tunis	1/29 (3.45)		7/26 (26.92)	
Age group	[0,12[	10/242 (4.13)	0.9	58/227 (25.55)	0.001
	[12-120]	3/67 (4.48)		32/65 (49.23)	
Overall		13/309 (4.21)		90/292 (30.82)	

Table 4. Multiple correlations between haematological and quantitative coproscopic parameters

	FEC	RBC	Hb	PCV	TWC	HCC
RBC	-0.015					
p	0.797					
Hb	-0.010	0.662				
p	0.859	0.000**				
PCV	-0.014	0.851	0.670			
p	0.815	0.000**	0.000**			
TWC	0.148*	-0.175	-0.118	-0.103		
p	0.012	0.002**	0.041*	0.073		
HCC	0.019	-0.183	-0.134	-0.165	0.458	
p	0.749	0.001**	0.020*	0.004**	0.000**	
Breed	0.025	0.029	0.018	-0.080	-0.055	0.071
p	0.667	0.612	0.748	0.160	0.342	0.215

FEC: Faecal EGG Count
RBC: Red Blood Cells
Hb: Haemoglobinaemia
PCV: Packed Cell Volume
TWC: Total Warm Count
HCC: *Haemonchus contortus* Count
p: significance
*: Significant $0.05 < p < 0.001$
**: Highly significant $p < 0.001$

Table 5. Linear regression between hematologic and quantitative coproscopic parameters (dependent variable: TWC)

Model	Sum of squares	df	Mean square	F	p
Regression	2407433.390	4	601858.347	4.725	0.001*
Residual	$3.567E^7$	280	127376.784		
Total	$3.807E^7$	284			

Model	Unstandardized coefficients		Standardized coefficients		
	B	Std. Error	Beta	t	p
(Constant)	422.878	138.017		3.064	0.002*
FEC	0.024	0.009	0.146	2.528	0.012*
RBC	-55.815	20.394	-0.315	-2.737	0.007*
Hb	-5.786	13.567	-0.034	-0.426	0.670
PCV	9.431	5.999	0.182	1.572	0.117

FEC: Faecal EGG Count
RBC: Red Blood Cells
Hb: Haemoglobinaemia
PCV: Packed Cell Volume
df: Degrees of Freedom
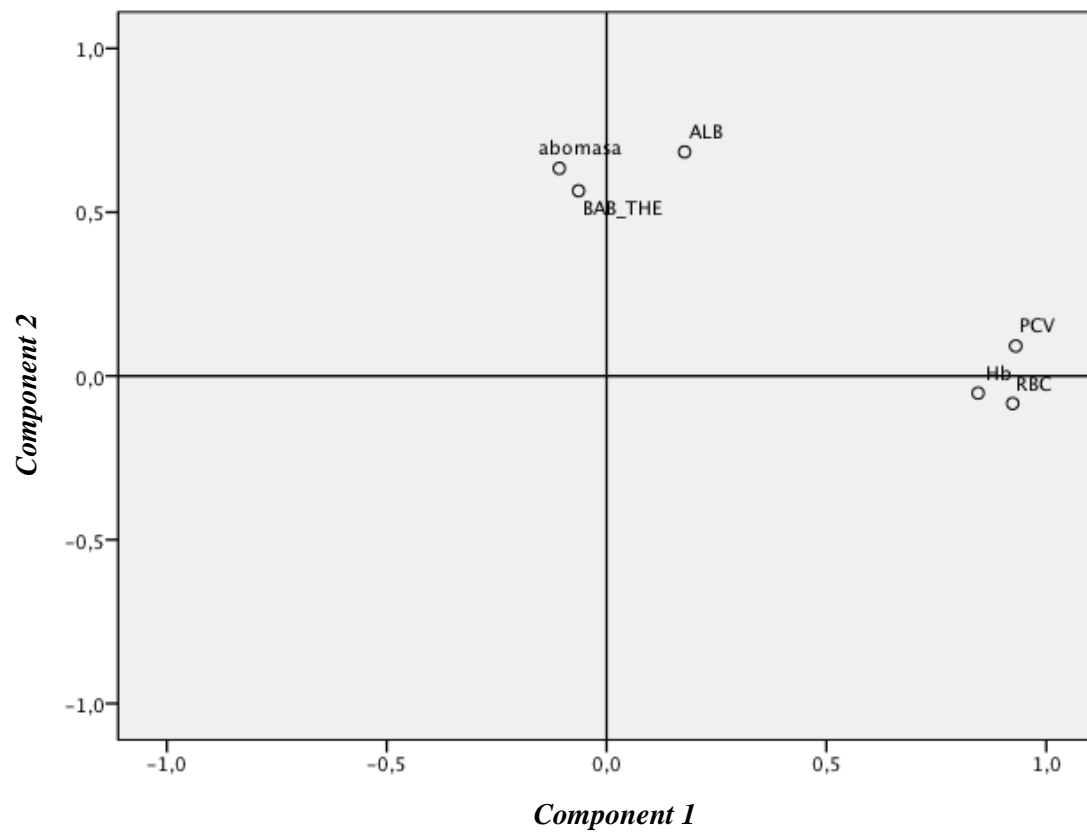**p**: Significance

Figure 2. Component plot: haematological parameters (RBC, HB and PCV) positively correlated with component 1 and the result of PCR for *Babesia*/*Theileria*, negative or positive abomasa for worms and albumin positively correlated with component 2

Figure 3. Dispersion of observations plots for PCA: there are 2 clusters of individuals
Cluster one (red circle): animals infected by both *Babesia*/*Theileria* and abomasal worms with high
values of albumin and normal values of haematological parameters (RBC, Hb and PCV)
Cluster two (green circle): all other animals.

Table 6. Haematological and biochemical results according to PCA results

		Cluster 1	Cluster 2
PCR for *Babesia* and *Theileria*	Positive	7/7 (100%)	6/299 (2.01%)
	Negative	0/7 (0%)	293/299 (97.99%)
Abomasa for worms	Positive	7/7 (100%)	225/297 (75.76%)
	Negative	0/7 (0%)	72/297 (24.24%)
Haematological parameters under threshold values	Red Blood Cells (RBC)	0/7 (0%)	1/299 (0.33%)
	Haemoglobin (HB)	0/7 (0%)	38/296 (12.84%)
	Packed Cell Volume (PCV)	0/7 (0%)	3/299 (1.00%)
Biochemical parameters under threshold values	Albumin	0/7 (0%)	148/292 (50.68%)
	Total Proteins	0/7 (0%)	47/292 (16.10%)
Sex	Male	1/7 (14.28%)	89/302 (29.47%)
	Female	6/7 (85.71%)	213/302 (70.53%)
Breed	Barbarine	0/7 (0%)	36/302 (11.92%)
	Cross breed	4/7 (57.14%)	137/302 (45.36%)
	Noir de Thibar	1/7 (14.28%)	58/302 (19.21%)
	Queue Fine de l'Ouest	2/7 (28.57%)	71/302 (23.51%)
Locality	Ariana	0/7 (0%)	34/302 (11.26%)
	Beja	2/7 (28.57%)	51/302 (16.89%)
	Bizerte	2/7 (28.57%)	28/302 (9.27%)
	Jendouba	1/7 (14.28%)	48/302 (15.89%)
	Mateur	0/7 (0%)	50/302 (16.56%)
	Sajnène	1/7 (14.28%)	38/302 (12.58%)
	Tabarka	1/7 (14.28%)	24/302 (7.95%)
	Tunis	0/7 (0%)	29/302 (9.60%)
Age group	[0,12[	4/7 (57.14%)	238/302 (78.81%)
	[12-120]	3/7 (42.85%)	64/302 (21.19%)

4. Discussion

In this study, FEC showed that 30.82% of animals presented positive faeces for gastrointestinal nematodes. This result was lower than the prevalence found by Akkari et al. (2012) (92 % in ewes). Compared to other countries such as Spain or Morocco, this study showed lower infestation with GIN (87.9 % and 60 %, respectively) (Martinez-Gonziilez et al., 1998; Pallargues, 2007).

Animals of 12 months or more had higher number of faeces presenting eggs of GIN than younger animals. This finding was similar to that obtained by Akkari et al. (2012) who found an average FEC higher in ewes (620) than lambs (311). This could be explained by a decrease of adults' immunity (Mage, 1998). This decrease in immunity is due to the unset of the reproductive function in adults, aggravated by malnutrition since animals pasture through over the year and are rarely supplemented. Animals belonging to the Barbarine breed presented higher number of faeces positive for GIN eggs (55.56%) (p=0.047). This could be related to breed differences, to the exposure of the animals or simply to animals management. Animals from Beja and Tabarka were more infested with GIN than other localities. These two localities presented higher mean annual rainfall and this climate is favourable for sheep infestation by gastrointestinal nematodes. In fact, the presence of rain in autumn increases the parasitic burdens.

The prevalence of infestation evaluated by worm count in abomasa was 77%. This result was higher than the prevalence found by Akkari et al. (2012). In fact, these authors found that 45.5 % of the parasitic population of lambs were present in the abomasum. As this survey took place between September and December, the higher prevalence in comparison with the study of Akkari et al. (2012) could be explained by the fact that the total worm count decreases during the dry seasons and increased during the wet seasons. In the study of Akkari et al. (2012) total worm count peaked during winter (November-December).

The kappa concordance coefficient between coproscopy and worm recovery was 0.21 which is a fair agreement. This could be explained by the prepatent period necessary for egg shedding. In addition, the number of female present in the abomasa and their prolificity influences the result of coproscopy.

The overall prevalence of *Babesia* and *Theileria* was 4.21%. This prevalence was compared to that obtained by Rjeibi et al. (2016) by Giemsa-stained blood smears. In fact, the overall infection prevalence by *Babesia* spp. and *Theileria* spp. in sheep was 2.9% (8/270) and 4.8% (13/270) respectively (Rjeibi et al., 2014). The molecular results showed higher prevalence 16.3% and 7.8% for Theileria ovis and Babesia ovis, respectively) (Rjeibi et al., 2016). In another study conducted by Rjeibi et al. (2014), The overall infection prevalence of piroplasms in Giemsa-stained blood smears was 3.2% which is similar to the prevalence obtained in this study.

In this trial, negative correlations between: red blood cells and total worm count, red blood cells and *H. contortus* count, haemoglobin and total worm count, haemoglobin and *H. contortus* count and finally between packed cell volume and *H. contortus* count were highlighted.

This finding is evident since infestation by *H. contortus* causes significant changes in haematological parameters and causes mainly anaemia (Al-Quaisy et al., 1987).

In others studies, the experimental infestation of Sahabadi sheep by *H. contortus* induced a significant decrease in haemoglobinaemia.

In Merino sheep and with an infestation of 11 000 L3 of *H. contortus*, the mean values of PCV and haemoglobinaemia decreased progressively. When the infestation persisted, PCV and haemoglobin values continued to decline (Allonby and Urquhart, 1975).

PCA showed two clusters of animals. On the first hand, animals who presented positive PCR for *Babesia* and *Theileria* and positive abomasa for worms and did not present anaemia. This group of individuals present may be a genetics of resistance to parasite infection. On the other

hand, the second group contains several cases which could be interesting to analyse for resistance to abomasal parasites. In fact, animals which are hyper infested (high number of adults in the abomasum) shedding few eggs when analysed by coproscopy are very interesting since the prolificity of the parasites could be repressed by the immune system of the host which is a pattern of resistance. Animals presenting a low prevalence of infestation with no impact on the haematological parameters are also resisting since their immune system did not allow the multiplication of parasites. These groups of resistant animals could be compared to the class of infested animals with an impact on haematological parameters and without being positive for *theileria* and *babesia*. Genome analysis could be used to provide a better resolution of the sheep genomic profiles and to explain the presence of this resistance in some animals.

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