Ecole Nationale de Médecine Vétérinaire - Tunisia

CRP livestock genetics flagship ICARDA report

Phenotyping indigenous Tunisian sheep breeds for gastro-intestinal parasite resistance with a special reference to *Haemonchus contortus*

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Background and Rationale

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. This fat tail breed has an adaptive capacity that allows animals to cope under harsh environmental and sub-optimal husbandry conditions (Ben Salem et al. 2011).

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.

The existence of genetic variation amongst sheep and goats in resistance to GINs has been studied by many authors and it has been demonstrated in several situations that genetic improvement could offer a solution to control haemonchosis. Bishop (2011) describes

resistance to infection as the host's ability to interact with parasite and control its lifecycle. In the case of nematode infections, probabilities of ingested larvae, parasite development within the host, parasite mortality, parasite fecundity and faecal egg count (FEC) are included in resistance.

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 indicator traits could be considered in resistance to nematodes. In fact, measurement of anemia can be used as indicator for resistance in animals infected with *H. contortus*. Anemia can be measured using packed cell volume (PCV) or Famacha score which are heritable in sheep (Baker et al., 2003; Mandonnet et al., 2006; Riley and Van Wyk, 2009).

Quantitative trait loci (QTL) mapping can also be used to understand the resistance to parasites. This technique allows identification of candidate genomic regions. Microsatellite markers (Marshall et al., 2009), microarray and genome-wide association studies (Brown et al., 2013) were used in small ruminant breeds to identify genes implicated in the control of resistance. Some of the genes commonly implicated in immune response such as interferon gamma (Dervishi et al., 2011) and major histocompatibility complex loci (Hassan et al., 2011) were also shown to be involved in the genetic resistance to GIN. In addition, several QTL on different regions and chromosomes (OARs) have been reported by many authors (OAR1, 3, 6, 14, 20) (Beh et al., 2002; Dominik, 2005; Crawford et al., 2006; Davies et al., 2006; Matika et al., 2011; Salle et al., 2012). In few studies, some potential candidate genes were identified on OAR8 (Crawford et al., 2006), OAR13 (Beraldi et al., 2007), and OAR22 (Silva et al., 2012).

In Africa, the resistance of the Red Massai breed has been demonstrated (Baker and Gray, 2004). In Tunisia, where GIN represent a huge problem, the genetic resistance to *haemonchus contortus* has never been studied. The aim of this work is to study the genetic resistance of the Barbarine sheep to *Hemonchus contortus* infestation. Phenotypic measurements (age, breed, origin, anthelmintic use, diarrhea, anemia, management type, hematological parameters, biochemical parameters, FEC and abundance of infestation) will be performed and will be compiled in a single phenotype database. Genome analysis will be carried out using the 600K SNP Chip which will provide a better resolution of the sheep genomic profiles.

Materials and Methods

Study area

This study will be carried out in four districts (Tunis, Bizerte, Beja and Jendouba) in the North of Tunisia (Figure 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 have shown that smallholder sheep farmers do not deworm their animals using anthelmintic-based products and because exposure is very high and sheep still manage to survive and produce in these environments. Therefore, the likelihood of finding individuals with some kind of resistance is relatively high.

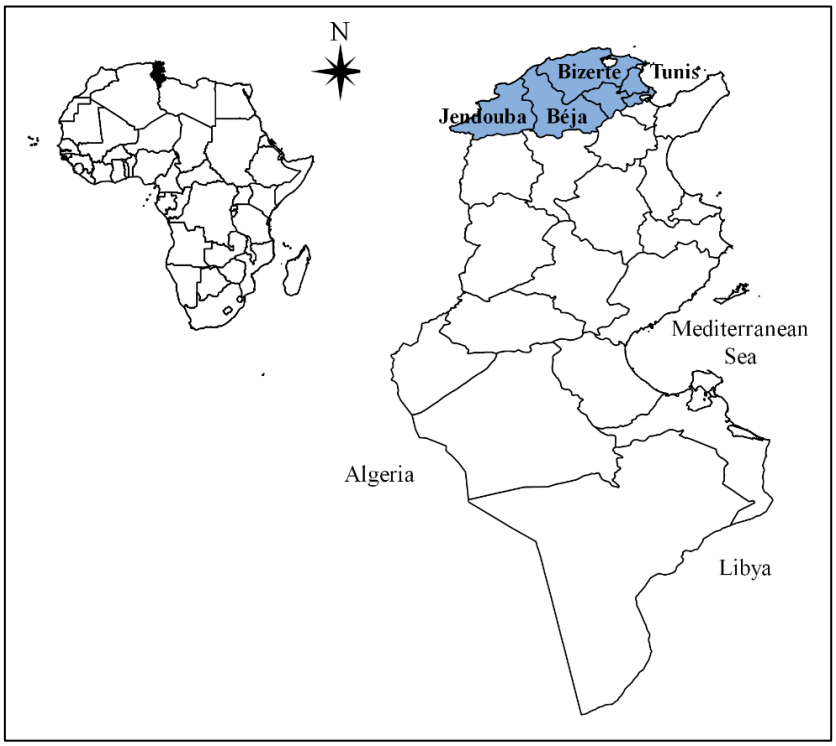

Figure 1. Study area

Sample collection and preparation

During August, September and October 2017, abomasa will be collected from 300 to 400 sheep, aged more than 6 months, from slaughterhouses located in the North of the country (Figure 1).

Sample collection

Before slaughter, animals will be identified and blood samples collected in EDTA, heparin and dry tubes via jugular venipuncture. Fecal samples will also be collected from each animal. Information concerning age, sex and breed. The presence of diarrhea, anemia or other symptoms will be noted in the data sheet (Annex 1).

Worm recovery

Abomasa will be ligated at both ends then transported to the Laboratory of Parasitology at the National Veterinary School of Sidi Thabet (Tunisia) in cooler boxes. Larvae (L3) (both *Haemonchus* and other parasite species: *Ostertargia* and *Trichostrongylus*) will be collected from the abomasum. Parasites will be preserved in 70% ethanol until examination.

Parasite identification

The identification of gastrointestinal parasites will be made using a microscope equipped with eyepiece micrometer. Prevalence, intensity and abundance of infestation will be estimated as follows:

Prevalence of infestation = 100 x number of infested sheep/number of examined sheep

Infestation intensity = number of collected larvae/number of infested sheep

Abundance of infestation = number of collected larvae/number of examined sheep

Phenotypic traits

Hematological parameters

The following hematological parameters will be estimated for each animal: White blood cells (10^9 l^{-1}), hematocrit (PCV) (%), red blood cell count ($\times 10^{12}$ m^{-1}), hemoglobin (g d^{-1}), Mean Corpuscular Volume (MCV) (fl), Mean Corpuscular Hemoglobin (MCH) (pg), Mean Corpuscular Hemoglobin Concentration (MCHC) (g dl^{-1}), Red Blood Cell Distribution Width (RDW), Index of Red Blood Cells Distribution (IDR) (%), Platelets (10^9 l^{-1}), Average Platelet Volume (VPM) (fl), Index of Platelets Distribution (IDP) and Plaquettocrite (Pct) (%) will be estimated using an Auto Hematology analyser BC-2800Vet® (ShenzenMindray Bio-Medical Electronics Co., Ltd, Hamburg, Germany).

The presence and type of anemia will be recorded (Blood & Radostitis, 1989).

Biochemical parameters

Plasma will be recovered from the heparin tubes and used to estimate biochemical indicators: albumin (g/L) and total proteins (g/L) with a Random Access Clinical Autolyzer® (Dialab, Vienna, Austria).

Coprology: Fecal Egg Count (FEC)

Fecal samples will be processed by flotation (McMaster Egg Counting Technique). Fecal Egg Count (FEC) will be performed on 5 g of feces using saturated solution and each egg counted representing 50 eggs. In order to identify eggs, coprocultures from each animal will be prepared

and incubated at 22–25 ◦C for 7–10 days to provide third stage larvae (L3) which will be harvested by the Baermann technique. The composition of each coproculture will be examined by microscopically. The identification of *fasciola* and other gastro-intestinal parasites will be performed.

DNA extractions

Animal's DNA extraction

DNA will be extracted from 300 µL of the blood of each sheep using the Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions and stored at -20°C for genome analysis.

Parasite's DNA extraction

DNA will be extracted from the collected *H. contortus* and other worms. After washing in PBS, worms will be ground with a pestle in liquid nitrogen in 1.5 ml microcentrifuge tube. Proteinase K will be added and the mix will be incubated overnight at 56°C. DNA will then be extracted using Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions then stored at -20°C until used.

Tick-borne pathogens

In order to verify the presence of piroplasms (*Babesia* and *Theileria*) and if anemia is only caused by gastrointestinal parasitism, Giemsa-stained blood smears will be examined under a microscope with immersion oil at 1000 magnification. For each slide, 50 microscopic fields will be examined.

In addition, Catch-all primers (RLB-F and RLB-R) which detect *Theileria* spp. *Babesia* spp. and *Anaplasma*/*Ehrlichia* spp. pathogens will be used. Reactions were performed in 25 µl volume containing 1 x PCR buffer, 1.5 mM $MgCl_2$, 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). Forty PCR cycles will be performed with a thermocycler (ESCO Swift MaxPro). Each cycle consist of a denaturing step of 1 min at 94°C, an annealing step of 1 min at 50°C, and an extension step of 1.5 min at 72°C. A final extension step of 10 min at 72°C will complete the program.

This molecular technique will detect the presence of tick-borne pathogens in sampled animals.

Work progress

Sample collection

Blood, Feces and abomasa were collected from 304 animals belonging to mainly four breeds: Barbarine breed, the main sheep breed in the country which is at-tailed breed characterized by metabolic and digestive adaptations to the contrasting environmental conditions in Tunisia (Ben Salem *et al*. 2011), Queue fine de l'Ouest breed, Cross bred individuals between the two previous breeds and finally Noire de Thibar, mostly present in the North of the country. Age, sex and breed were recorded for each animal.

Worm recovery

All abomasa were opened along the greater curvature and the contents were washed into a bucket and then they were carefully examined for the presence of *Haemonchus* and other gastro intestinal worms. The counting of the parasites of each abomasum was realized, males were separated from females and will be used for morphological identification. All worms from each animal were preserved in 70% ethanol in order to be used for DNA extraction.

The number of parasites in each abomasum of each animal was recorded in the database.

Parasite identification

Haemonchus parasites are already identified and the number of this species in each abomasum was recorded. For other gastrointestinal nematodes males are used for identification and the work is in progress.

Hematological parameters

All hematological parameters (haematocrit (PCV), red blood cell count, haemoglobin, Mean Corpuscular Volume, Mean Corpuscular Hemoglobin, Mean Corpuscular Hemoglobin Concentration, Red Blood Cell Distribution Width, Index of Red Blood Cells Distribution, Platelets, Average Platelet Volume, Index of Platelets Distribution and Plaquettocrite (Pct) were estimated for each animal using an Auto Haematology analyser BC-2800Vet® (ShenzenMindray Bio-Medical Electronics Co., Ltd, Hamburg, Germany) and were recorded in the database.

Biochemical parameters

Albumin (g/L) and total proteins (g/L) were estimated with a Random Access Clinical Autolyzer® (Dialab, Vienna, Austria) and recorded in the database.

Coprology: Fecal Egg Count (FEC)

The coproscopic survey was realized for each feces sample and qualitative coprology allowed identification of gastro intestinal eggs and other eggs which are Trichures, Cocidies, Nematodirus, Moneizia and pulmonary larvae. Quantitative corology examination allowed the counting of these eggs and this information is uploaded in the database.

Histological study

After dissection of the abomasa, 240 samples were collected from normal and affected tissues. Tissue samples were fixed in 10% formalin and processed for a routine histological examination. Samples will be stained with hematoxylin Eosine and examined under microscope for the presence of specific lesions. A correlation will be established between the infestation pattern and the microscopic lesions.

DNA extractions

Animal's DNA extraction

DNA from the blood of each animal was extracted using the Wizard® Genomic DNA purification kit (Promega, Madison, USA) according to the manufacturer's instructions and the concentration was measured using a Spectrophotometer. DNA concentration was recorded in the database. DNA was stored at -20°C for genome analysis.

Parasite's DNA extraction

Parasite's DNA extraction will be done at the end of the step of the morphological identification.

Tick-borne pathogens

Catch-all PCR using RLB-F and RLB-R primers which detect *Theileria* spp. *Babesia* spp are yet to be carried out.

Conclusion

The database on phenotyping indigenous sheep breeds for gastro-intestinal parasites concerned 304 sheep from 4 different breeds and several locations, all in the North of Tunisia. Compilation of the database has now well progressed and should be finalized soon. In summary, phenotyping includes information related to each individual animal which are age, breed, origin, anthelmintic use, slaughter reason, feeding scheme, diarrhea, anemia, management type

(grazing, grazing and supplementation, intensive), hematological parameters, biochemical parameters, fecal egg count, abundance of infestation and existence of parasite-induced lesions in the abomasa. DNA, for genome analysis using the 600K SNP Chip, was extracted from the blood of each sheep and first data on genotyping should be made available in 2018.

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Annexes:

1. Data collection form in the slaughterhouse

2. Approximate work plan

Num	Age	Breed	Origin	Anthelmintic use, if yes when	Slaughter reason	Diarrhea	Anemia	Other	Grazing	Grazing +supp	Intensive	Observations
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												

Annex 1. Information sheet

Date:……***Slaughterhouse:*** ……

Annex 2. Work plan

Activities	June	July	August	September	October	November	December
-Preparation	x						
- Reagent purchase	x						
- Contact slaughterhouses and regional veterinarians	x						
- Sampling		x	x	x	x		
- Complete blood count		x	x	x	x		
- Worm recovery		x	x	x	x		
- Parasites identification		x	x	x	x		
- FEC		x	x	x	x		
- DNA extraction (blood + parasite)					x	x	x
- Catch-all PCR					x	x	x
- Biochemical parameters					x	x	x
- Giemsa-stained blood smears					x	x	x
Report writing							*