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Research article

GASTROINTESTINAL NEMATODE INFECTIONS IN ANTELOPES FROM MOROCCO: A COPROLOGICAL SURVEY

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This study examined the gastrointestinal parasitological status of three endangered Sub-Saharan antelope species (*Addax nasomaculatus*, *Oryx dammah*, *Gazella dorcas*) hosted at Souss-Massa National Park in Morocco. A total of 254 faecal samples (80 samples from the addax population, 81 from the oryx population and 93 from the dorcas population) were analysed to determine the prevalence and the intensity of the parasites in host faeces (expressed as the mean EPG: *egg per gram*), using microscopic methods (Flotation and McMaster) and the molecular identification of parasites using PCR and sequencing of the second internal transcribed spacer region of the rDNA (ITS-2).

The prevalence results in the addax, oryx and dorcas gazelle were 43.7%, 2.4%, and 61.3%, respectively, for *Nematodirus* spp.; 21.2%, 12.3%, and 16.13%, respectively, for *Trichuris* spp.; and 36.2%, 39.5%, and 53.7%, respectively, for other, undistinguished strongylids.

The means of EPG values for parasites in addax, oryx and dorcas gazelle were 8.9, 2.4, and 61.3, respectively, for *Nematodirus* spp.; 4.3, 2.4, and 4.8, respectively, for *Trichuris* spp.; and 18.1, 16.6, and 50.1, respectively, for other undistinguished strongylids. Sequencing of the ITS-2 rDNA region of the isolated parasites allowed the identification of *Camelostrongylus mentulatus* and *Nematodirus spathiger* in these three antelope species. We can conclude that the studied antelopes are infected at tolerating levels with the first record of *Camelostrongylus mentulatus* and *Nematodirus spathiger* in those antelopes in Morocco.

Keywords: African threatened antelopes; *Camelostrongylus mentulatus*; gastrointestinal nematodes; ITS-2 rDNA; Morocco; *Nematodirus spathiger*.

INTRODUCTION

Morocco is one of the Mediterranean countries with the highest diversity of wild mammals [1]. The current study examined three threatened species of Sahelo-Saharan

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antelopes: *Addax nasomacculatus* (De Blainville, 1816), *Oryx dammah* (Cretzschmar, 1826) and *Gazella dorcas* (Linnaeus, 1788). Before their disappearance from nature, these species inhabited the desert areas of southern Morocco [2]. The three antelopes appear on the red list of threatened species of the International Union for Conservation of Nature (IUCN). According to the list, addax is categorized as "critically endangered", oryx as "extinct in the wild" and dorcas gazelle as "vulnerable" [3-5]. The antelopes were reintroduced successfully two decades ago to Souss-Massa National Park (Agadir, Morocco); addax and oryx were brought from European zoos and dorcas gazelle from the remaining native population [6]. For the purpose of conserving its fauna, Morocco was among the African countries that ratified the agreement for the conservation of sub-Saharan antelopes in the Agadir declaration [7]. In this context, several parks and reserves have been set up to accommodate various endangered species throughout the country. However, animals kept in such conditions of semi-captivity require the control of health management, especially in relation to infectious and parasitic diseases [8,9].

As grazing ruminants, antelopes are exposed to different internal parasitic infections namely coccidian [10], pulmonary nematodes [11], and particularly gastrointestinal nematodes [12,13]. In nature, parasites and hosts maintain a balanced relationship. However, this equilibrium may deteriorate when animals are subjected to conditions such as translocation, sequestration, overcrowding or climate change [14]. Intense parasitism can have pronounced impacts on wildlife host populations; it may affect their fecundity, their survival, their growth or even their behavior [15-17].

This survey aimed to explore the gastrointestinal parasitic status of the studied animals to further, develop a dashboard that will serve the park's health management programs. Many tools may be used to assess the parasite burden in wildlife, by necropsy or by examining some biochemical and serological parameters [18]. However, since the studied antelopes are threatened species, it was more appropriate to use noninvasive methods based on coprological techniques. For that, we first measured the prevalence and the intensity of parasites in the feces of the three species of antelopes. Second, we proceeded to the molecular identification of isolated nematodes.

MATERIALS AND METHODS

Area of study

Created in 1991, the National Souss-Massa Park (SMNP) covers an area of 33 800 ha, along the Atlantic coast, 70 km long and 7 km wide. It is located between the cities of Agadir, to the North, and Tiznit, to the South (Figure 1). It is considered a wetland, irrigated with two seasonal rivers, Souss and Massa, whose deltas are included in the park area. It has sandy soil and a savannah-like ecosystem, containing diversified endemic Mediterranean and Saharan flora (e.g., *Argania spinosa* and *Atriplex halimus*…). In addition to the three antelopes, the park supports a diverse fauna with a large variety of endemic reptiles, birds, small mammals and other endangered species, such as the bald ibis (*Geronticus eremita*) and the red-necked ostrich (*Struthio camelus camelus*).

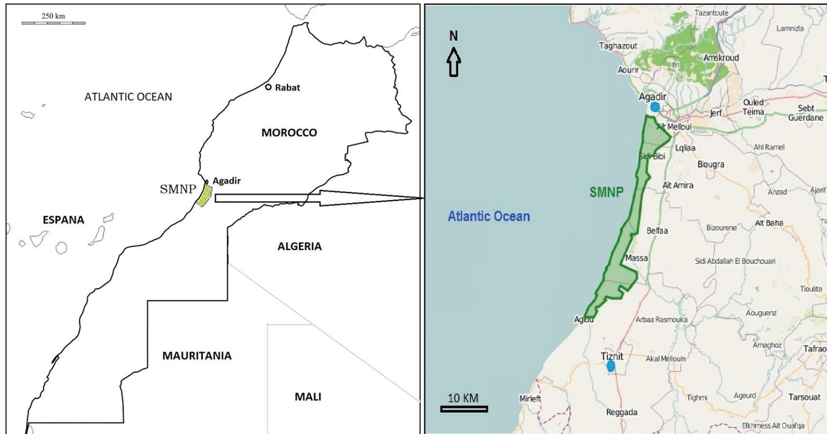

Figure 1. Map showing the localization of the park within Morocco (left) and its geographic extent (right)

Animals and sampling procedure

To avoid conflict and hybridization-related problems between oryx and addax, the animals are hosted in two separate geographical spots within the SMNP. The addax is housed in the Rokein reserve with a part of dorcas gazelles, while the oryx is housed in the Arrouais reserve with the other part of dorcas gazelles. The current population sizes of antelopes are estimated at 440 individuals for addax, 250 individuals for oryx, and 850 individuals for dorcas gazelle.

According to their availability, morning fresh individual fecal pellets of herds were randomly collected from the ground (regardless of the age and sex of the animals). The fecal pellets of each species are easily distinguishable by their size; pellets of gazelles are smaller than those of oryx and addax. A total of 254 samples were collected from January to July 2015 (80 samples from the addax population, 81 from the oryx population and 93 from dorcas gazelle population). All samples were labeled, transported immediately to the laboratory, and kept in the refrigerator at 4°C until used (no more than 2 weeks).

Recovery and eggs counting

Nematode eggs were detected by simple test tube flotation and counted by the McMaster method to measure the fecal eggs per gram (EPG), as in domestic ruminants [19]. Sheather's sugar solution (454 g of granulated sugar, 355 ml of tap water and 6 ml of formaldehyde), with a specific gravity of 1.27 was used in this study [20]. Briefly, the fecal suspension was prepared by adding 4 g of feces to 56 ml of flotation solution and sieved with a tea strainer, and then microscope slide examinations were done at 100-400 x magnifications for both the tube flotation and McMaster method.

Larvae isolation for DNA extraction

For L3 stage larvae culture, crushed and moistened fecal pellets were deposited into glass Petri dishes and incubated at 27°C for 7 days for the majority of Trichostrongylidae nematodes, and after 14 days for *Nematodirus* spp. following a laboratory guide for domestic ruminants [21], then the L3 larvae were collected by the Baermann funnel method [22]. A volume of 20 μl of L3 larval suspension was examined on a glass slide under an inverted microscope at 250 x magnification. Next, one unique larva was isolated by aspiration with a P10 micropipette, washed twice in water and then conserved in 10 μl of water in a 0.5 ml microtube at -20°C.

DNA Extraction Procedure

DNA extraction was done following the tissue protocol of the QIAGEN QIAamp DNA Mini Kit (QIAGEN Lake Constance GmbH, Germany) with a slight modification in the tissue digestion step. A 0.5 ml microtube containing a larva or an egg was thawed, and then 180 μl of buffer ATL and 20 μl of Proteinase K were added. The microtube was vortexed and centrifuged for a few seconds, and then the contents were transferred to a 2 ml microtube containing 0.5 mm Zirconia/Silica beads (BioSpec®, BioSpec Products, Inc., USA) and incubated for 1 h at 56°C and 900 rpm in a microtube thermal shaker (CAT®-H26). Then we collected 200 μl volume of the digestion product and followed the next steps of the QIAGEN protocol.

PCR amplification conditions

PCR assays targeted the internal transcribed spacer (ITS-2) regions of the ribosomal DNA of nematodes. For that purpose, universal primers were used: the forward primer NC1: 5'ACGTCTGGTTCAGGGTTGTT and the reverse primer NC2: 5'-TTAGTTTCTTTTCCTCC GCT-3', described previously [23].

PCR amplification was performed using the AgPath-ID™ kit (Ambion®-Applied Biosystems) in a total volume of 50 μl per reaction, which contained 10 μl of DNA extract, 2 μl of each 10 μM primer, 2 μl of 25X RT-PCR Enzyme Mix, 25 μl of 2X RT-PCR Buffer and 9 μl of water. PCR was carried out in a Bio-Rad iCycler® Thermal cycler with an initial denaturation at 94°C for 10 min followed by 40 cycles, 45 s each, of denaturation at 94°C, annealing at 55°C and extension at 72°C, with a final extension at 72°C for 5 min. PCR amplicons were visualized by electrophoresis in a 2% agarose gel; positive samples were identified by the presence of a band of ~300 bp.

DNA sequencing and phylogenetic analysis

Positive PCR products were submitted to a sequencing service provider (BIO BASIC®, Markham ON, Canada) for purification and Sanger dideoxy sequencing using the same primers for PCR. Electropherograms were examined by Sequence Scanner Software v1.0 (Applied Biosystems). The GenBank database was searched for sequences that

matched the sequencing results using the BLAST algorithm hosted by the national center for biotechnology information (NCBI) network server [24]. Sequences alignments were performed using *BioEdit* v7.2.6 software, and phylogenic analysis by MEGA v7.0 software [25]. Phylogenic trees were constructed by the neighbor-joining method after 1000 bootstraps [26]. Seventeen sequences were deposited in the GenBank database: [MH047854-MH047855], [MH047838-MH047839], [MH047843-MH047844], [MH047850], [MH047830-MH047831], [MH047835], [MH047846-MH047849], and [MH047840-MH047842].

Statistical analysis of data

Coprological data are presented as percentages for prevalence and means ± standard deviation for parasite intensity. The differences in prevalence were analyzed using the χ^2 test (95% confidence interval), and parasite intensity values among the three populations of antelopes were compared with one-way analysis of variance (ANOVA) and the Newman-Keuls multiple comparison test, with P-values of <0.05 indicating significance (GraphPad PRISM® v5.00 software, USA).

RESULTS

Prevalence and intensity

The coprological examination revealed various forms of gastrointestinal nematode eggs of different nematode genera: *Nematodirus* spp., *Trichuris* spp. and other undistinguishable strongylids. The prevalence and the intensity values varied according to the parasite and its host (Table 1).

Table 1. Prevalence and intensity values (EPG) for parasites recovered from fecal samples of antelopes.

	Strongylids		***Nematodirus* spp.**		***Trichuris* spp.**	
	Prevalence %	**Intensity mean ±SD**	**Prevalence %**	**Intensity mean ±SD**	**Prevalence %**	**Intensity mean ±SD**
Addax nasomaculatus	36.2 (29/80)	18.1±34.9	43.7 (35/80)	8.9±25.1	21.2 (17/80)	4.3±14.2
Oryx dammah	39.5 (32/81)	16.6±32.6	2.4 (2/81)	1.8±16.7	12.3 (10/81)	2.4±10.9
Gazella dorcas	53.7 (50/93)	50.1±143.8	61.3 (57/93)	12.3±33.5	16.13 (15/93)	4.8±16.5

For prevalence rates, we noticed that there were no significant differences across the three antelope populations in *Trichuris* spp. or strongylid nematodes. In contrast, the *Nematodirus* spp. prevalence rate in oryx was significantly lower than in addax and dorcas gazelle.

Data analysis of intensity means revealed no significant differences in *Trichuris* spp. among the three antelopes, but the *Nematodirus* spp. intensity mean was significantly

lower in oryx than in gazelles. We noticed also that the strongylids intensity mean was significantly higher in dorcas gazelle than in addax and oryx.

Molecular identification and phylogenetic analyses

The sequences alignment results allowed the identification of many isolates: *Camelostrongylus mentulatus* and *Nematodirus spathiger* in the three antelope species and other *Trichostrongylus* spp. in dorcas gazelle and oryx.

All six *C. mentulatus* sequences isolated from the three antelope species showed a percentage of identity higher or equal to 98% compared to the GenBank reference sequence [KY930444], with a fixed three-nucleotide deletion (152_154delGTA) and a nucleotide substitution (246T>A); in addition, the three sequences isolated from *G. dorcas* [MH047837, MH047838, MH047839] showed a fixed nucleotide substitution (213A>G). The phylogenetic tree and alignment results for these sequences are shown in Figures 2 and 3.

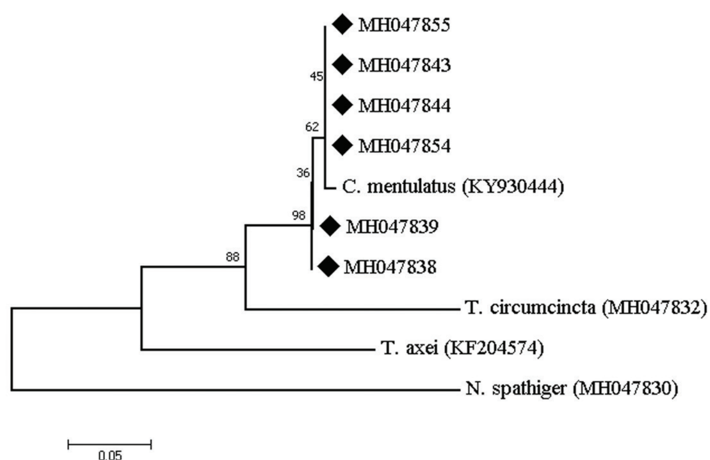

Figure 2. The phylogenetic relationships of *Camelostrongylus mentulatus* based on ITS-2 sequences of the rDNA, using the Neighbor-Joining method with the Tamura 3-parameter method. The percentages of replicate trees in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 10 nucleotide sequences (6 from antelopes, and 4 from Genbank). Evolutionary analyses were conducted in MEGA7.

For *N. spathiger*, the four sequences isolated from antelopes showed a percentage of identity higher than 99% when compared to the reference sequence isolated in *G. dorcas* from Tunisia [KY930420]. Similarities between isolates and the reference sequence are represented as a phylogenetic tree in Figure 4.

Figure 3. Alignment, done in BioEdit v7.2.6, showing substitutions and deletions, between Moroccan antelope sequences of *Camelostrongylus mentulatus* (MH047838, MH047839, MH047843, MH047844, MH047854, MH047855) and the Tunisian Addax sequence (KY930444). Dots, residues that match the consensus exactly.

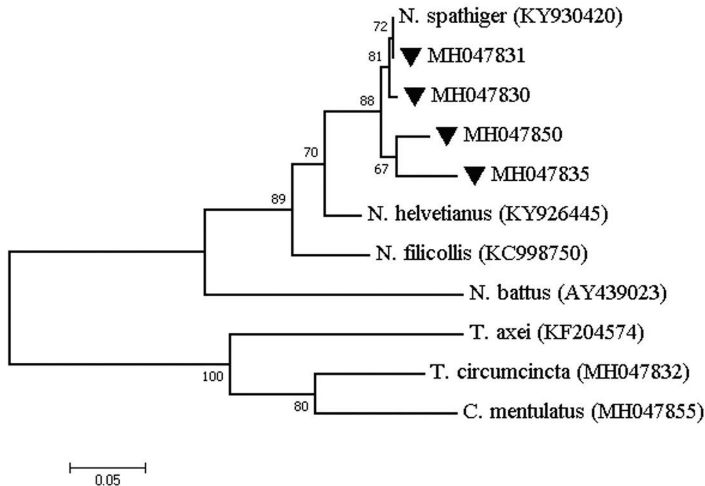

Figure 4. The phylogenetic relationships of *Nematodirus spathiger* based on ITS-2 sequences of the rDNA, using the Neighbor-Joining method with the Tamura 3-parameter method. The percentages of replicate trees in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 11 nucleotide sequences (4 from antelopes, and 7 from Genbank). Evolutionary analyses were conducted in MEGA7.

The phylogenetic analysis of the *Trichostrongylus* spp. sequences showed that they cluster well in the same clade with other species belonging to *Trichostrongylus* genus. Figure 5 provides information on genetic topology across these isolates.

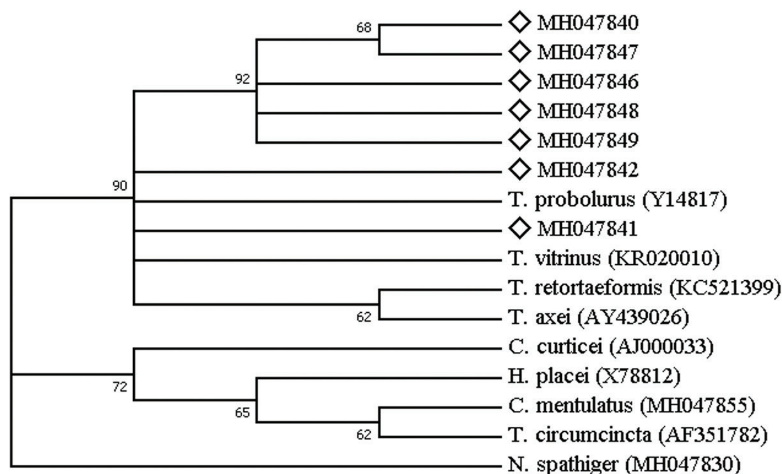

Figure 5. The phylogenetic relationships of *Trichostrongylus* spp. based on ITS-2 sequences of the rDNA, showing topology among species, using the Neighbor-Joining method with the Tamura 3-parameter method. The percentages of replicate trees in the bootstrap test (1000 replicates) are shown next to the branches. The analysis involved 16 nucleotide sequences (7 from antelopes, and 9 from Genbank). Evolutionary analyses were conducted in MEGA7.

DISCUSSION

The presented results clearly and accurately demonstrate through molecular tools, that all three antelope species are infected by *C. mentulatus* and *N. spathiger*, in addition to other *Trichostrongylus* spp. isolates detected in oryx and gazelles.

Geographically very widespread, *C. mentulatus* inhabits the abomasum and small intestine of ruminants. It has been reported in several species: in small domestic ruminants [27], in camelids [28] and in various wild ruminants [29]. In antelopes, this parasite causes abomasitis lesions and presents ostertagiosis-like symptoms: diarrhea, emaciation or even collapse in juveniles [30]. This study is in agreement with previous studies that mentioned *C. mentulatus* as a prevalent nematode species in African gazelles [13], in addax [31] and in oryx [32].

Unlike *C. mentulatus*, *N. spathiger*, which inhabits the small intestine of camelids and ruminants with a worldwide geographic distribution [33-35], does not usually cause clinical disease in adult ruminant hosts, but may cause dehydration syndrome in calves and lambs, attributable to larval migration-induced damage to the intestinal mucosa [36]. We can thus confirm by this study that *N. spathiger* is a nematode that is very common in Sub-Saharan antelopes, as previously reported in many studies [12,27].

Trichostrongylus species are ubiquitous among herbivores worldwide [12,30,37,38]. Generally, the adult parasites are encountered in the abomasum or in the small intestine of their hosts, in which they may cause weight loss, diarrhea and reduced growth rates [36]. In addition, some species of *Trichostrongylus* are related to many reported food-borne zoonosis cases [39,40].

Trichuris species, revealed by microscopy in the current study, inhabit the large intestine of their hosts and are also common parasites of many terrestrial mammals worldwide; the three species that may infect ruminants are *T. ovis, T. discolor* and *T. globulosa*, with light or asymptomatic infection [36].

Despite the scarcity of parasitological epidemiology studies in Sub-Saharan antelopes, the intensity and prevalence of parasites revealed in our study are globally comparable to those of previous studies of antelopes held under similar conditions. In fact, a study conducted in three African gazelle species (*G. dorcas*, *G. cuvieri* and *G. damah mhorr*) from Spain revealed close EPG values, for *Trichuris* spp. (>100), for *Nematodirus* spp. (<50) and for other Trichostrongylids (>50), especially in dorcas gazelles [37]. In other studies in scimitar-horned oryx, for *Nematodirus* spp. (=150) was also so closer to ours in which concern EPG value [41] or prevalence [30].

The relatively lower infection levels recorded in antelopes from SMNP, by analogy to domestic ruminants, may be explained by various factors. The most plausible explanation is related to spatial dimensions of host-parasite interactions, stating that host population density is positively correlated with their parasite burden [41,42]. The antelope population density within the park seems still to be low, which keeps the density of free-living forms of parasites (L3: third-stage infective larvae of nematodes) low in the pasture and avoids a continuous process of reinfection. These free-living forms of gastrointestinal nematodes require adequate conditions of moisture and temperature for their development from egg hatching to third larva molting [43,44]. The semiarid climate of the geographical area of the SMNP thus favors the host too, by reducing the infectivity potential of the free-living forms of parasites. Nevertheless, some of them can survive under extreme conditions, especially the larvae of *N. spathiger*, whose development takes place within eggs, where they are protected by the shell during the cold season until hatching in summer [45]. Other intrinsic factors, related to parasite developmental behavior, may explain the lower infection levels; *C. mentulatus* is characterized by its L3-L4 hypobiotic larvae, which, like those of *Ostertagia* spp., can delay their development and survive for several months in the mucosa of the abomasum before becoming adults [46]. The social behavior of the host can also explain such variation in the parasitic burden. The three studied African antelopes are recognized to be gregarious and to have a determined social organization, which makes parasite prevalence correlates positively with the size of the group in those African wild bovides, and territorial gazelle males are more likely to have higher parasites intensities than the rest of the herd [47]. This social behavior may explain the different levels of infections among the three populations of antelopes noted in our results. We

have to take into consideration, also, that inbreeding in wildlife, especially in captivity and semi-captivity is a factor that makes animals more susceptible to parasitism [48].

In addition, food composition may directly or indirectly interact with gastrointestinal parasites. Some plants containing tannins, which are part of the ruminant diet, may help animals to improve their gastrointestinal immunological response or even have a direct negative effect by counteracting the growth of gastrointestinal nematodes [49]. At the SMNP, the flora is very diverse, and we find some tannin-rich species that are occasionally grazed by animals, such as *Argania spinosa, Acacia gummifera, Retama monosperma*, *Ononis natrix*, *Atriplex halimus,* etc., and thus could help antelopes to maintain low parasitic infection levels.

Based on the results of the current study and according to the host-parasite database [50], we recorded, for the first time, the prevalence of *C. mentulatus* and *N. spathiger* in the three antelope species in Morocco. These three antelopes can now be considered reservoirs for these parasites that should be taken into consideration for any further projected animal translocation or reintroduction operations.

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Authors' contributions

AS and WO collected the fecal samples. AS carried out the laboratory analyses (Coprology and molecular biology). RM, FH, and WO participated in the design of the study, then prepared and critically revised the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ISPITIVANJE INFEKCIJE GASTROINTESTINALNIM NEMATODAMA KOD ANTILOPA U MAROKU

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U studiji je sprovedeno ispitivanje parazitološkog statusa intestinalnog trakta kod tri ugrožene vrste subsaharske antilope (*Addax nasomaculatus, Oryx dammah, Gazella dorcas*) koje su se nalazile u Souss-Massa nacionalnom parku u Maroku. Da bi se ispitala prevalencija i intenzitet infestacije parazitima kod životinja, ukupno je ispitano 254 uzoraka fecesa (80 uzoraka iz *addax* populacije, 81 uzoraka iz *orix* populacije i 93 uzoraka iz *dorcas* populacije) pri čemu su rezultati prikazivani kao srednja vrednost EPG (jajašca parazita po gramu fecesa). Korišćene su mikroskopske metode (Flotacija i McMaster) kao i molekularna identifikacija parazita upotrebom PCR testa i sekvencioniranjem rDNK (ITS-2).

Rezultati prevalence *Nematodirus* spp kod *addax*, *oryx* i *dorcas* gazela bili su: 43,7%, 2,4% odnosno 61,3%; za *Trichuris* spp vrste 21,2%, 12,3% i 16,3%, a za strongilidne vrste koje nisu mogle da se determinišu: 36,2%, 39,5% i 53,7%.

Srednje EPG vrednosti za parazite kod *adax*, *oryx* i *dorcas* gazele bili su za *Nematodirus* spp vrste 8,9, 2,4 i 61,3; za Trichuris 4,3, 2,4 i 4,8 a za na strongilidne vrste koje nisu mogle da se determinišu, 18,1, 16,6 odnosno 50,1. Rezultati sekvencioniranja ITS-2 regiona rDNK izolovanih parazita omogućio je identifikaciju *Camelostrongylus mentulatus* i *Nematodirus spathinger* u navedene tri vrste antilopa. Može da se zaključi da su infestacije parazitima kod ove tri vrste antilopa na podnošljivom nivou pri čemu je po prvi put dijagnostikovana infestacija antilopa u Maroku sa *Camelostrongylus mentulatus* i *Nematodirus spathinger*.