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Research paper

Protozoan and helminth parasite fauna of free-living Croatian wild wolves (*Canis lupus*) analyzed by scat collection

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ABSTRACT

The European wolf (*Canis lupus*) is a large carnivore species present in limited areas of Europe with several small populations still being considered as endangered. Wolves can be infected by a wide range of protozoan and metazoan parasites with some of them affecting free-living wolf health condition. On this account, an epidemiological survey was conducted to analyze the actual parasite fauna in Croatian wild wolves. In total, 400 individual faecal samples were collected during field studies on wolf ecology in the years 2002–2011. Parasite stages were identified by the sodium acetate acetic acid formalin (SAF)-technique, carbolfuchsin-stained faecal smears and *Giardia*/*Cryptosporidium* coproantigen-ELISAs. A subset of taeniid eggs-positive wolf samples was additionally analyzed by PCR and subsequent sequencing to identify eggs on *Echinococcus granulosus*/*E. multilocularis* species level. In total 18 taxa of parasites were here detected. *Sarcocystis* spp. (19.1%) occurred most frequently in faecal samples, being followed by *Capillaria* spp. (16%), ancylostomatids (13.1%), *Crenosoma vulpis* (4.6%), *Angiostrongylus vasorum* (3.1%), *Toxocara canis* (2.8%), *Hammondia*/*Neospora* spp. (2.6 %), *Cystoisospora ohioensis* (2.1%), *Giardia* spp. (2.1%), *Cystoisospora canis* (1.8%), *Cryptosporidium* spp. (1.8%), *Trichuris vulpis* (1.5%), *Taenia* spp. (1.5%), *Diphyllobothrium latum* (1.5%), *Strongyloides* spp. (0.5%), *Opisthorchis felineus* (0.5%), *Toxascaris leonina* (0.3%), *Mesocestoides litteratus* (0.3%) and *Alaria alata* (0.3%). Some of the here identified parasites represent relevant pathogens for wolves, circulating between these carnivorous definitive hosts and a variety of mammalian intermediate hosts, e. g. *Taenia* spp. and *Sarcocystis* spp., while others are considered exclusively pathogenic for canids (e.g. *A. vasorum, C. vulpis, T. vulpis, Cystoisospora* spp.). This study provides first records on the occurrence of the two relevant anthropozoonotic parasites, *Giardia* spp. and *Cryptosporidium* spp., in wild wolves from Croatia.

1. Introduction

The European wolf (*Canis lupus*) used to be present in most areas of Europe but during the 20th century it disappeared from many European countries (Segovia et al., 2001; Stronen et al., 2011; Szafranska et al., 2010). Nowadays, the wolf is only present in limited areas of Europe and respective populations are still considered as seriously endangered (Guerra et al., 2013; Segovia et al., 2001). Wolves in Croatia belong to the large stable Dinaric-Balkan population of calculated 3900 individuals (Kaczensky et al., 2012). In 1995, when a residual population of only 30 animals was left, wolves became a protected species in Croatia. Nowadays its population has slightly recovered from recent hunting and deforestation, and has now re-settled in the mountainous regions of Lika, Gorski Kotar and Dalmatia (Frkovic and Huber, 1992; Kusak et al., 2005). Currently, the population ranges between 200 and 220 animals (Štrbenac et al., 2010).

As large carnivores, wolves are natural hosts for a wide range of gastrointestinal parasites, some of them with zoonotic potential such as *Echinococcus* spp. (Guerra et al., 2013; Ito et al., 2013; Schurer et al., 2014) and *Toxocara canis* (Segovia et al., 2001; Szafranska et al., 2010). So far, studies on wolf parasite infections were performed in Nearctic (Choquette et al., 1973; Custer and Pence, 1981; Holmes and Podesta, 1968; Messier et al., 1989; Samuel et al., 1978) and Palearctic regions (Craig and Craig, 2005; Guberti et al., 1993; Ito et al., 2013; Papadopoulos et al., 1997; Schurer et al., 2014; Shimalov and Shimalov, 2000) and para-

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sitoses are increasingly recognized for their profound influences on individual or population health (Daszak et al., 2000; Hudson et al., 2006). Furthermore, parasite-host interactions are shaped by a multitude of factors, including host availability, parasite community structure (Jolles et al., 2006; Telfer et al., 2010) and environmental conditions (Biek and Real, 2010). Moreover, parasitoses are increasingly associated with human-modified ecological transition zones (Despommier, 2007) and are of growing concern for isolated wilderness reserves (Stronen et al., 2011). Parasitic diseases can therefore severely reduce isolated populations even within protected geographical areas (see Despommier et al., 2006; Foreyt and Jessup, 1982; Leon-Vizcaino et al., 1999; Stronen et al., 2011). As an example, mange due to *Sarcoptes* infestation is discussed to have substantial impacts on wolf populations in Spain (Oleaga et al., 2011) as was also reported for red foxes (*Vulpes vulpes*) (Al-Sabi et al., 2014; Nimmervoll et al., 2013).

Up to date, some investigations on wild wolf gastrointestinal parasites have been performed in other European countries, such as Belarus (Shimalov and Shimalov, 2000), Spain (Segovia et al., 2001), Portugal (Guerra et al., 2013), Greece (Papadopoulos et al., 1997), Italy (Guberti et al., 1993), Poland (Kloch et al., 2005; Szafranska et al., 2010), Latvia (Bagrade et al., 2009) and Finland (Stronen et al., 2013). Most investigations analyzed hunted or dead animals (Eleni et al., 2014; Otranto et al., 2007, 2009; Segovia et al., 2001) or animals kept in captivity (Andre et al., 2010; Erdelyi et al., 2014; Szafranska et al., 2010). So far, a survey on trichinellosis (Beck et al., 2009) and on visceral leishmaniosis (Beck et al., 2008) have been performed in Croatian wolves. The present study aimed to identify gastrointestinal parasitoses of free-ranging and alive wolves by analyzing a high number of individual scat samples focusing on both helminths and protozoans. Furthermore, we considered the role of these wild canids in the maintenance of synanthropic parasite cycles as discussed elsewhere (Guerra et al., 2013; Ito et al., 2013; Schurer et al., 2014; Segovia et al., 2001).

2. Material and methods

2.1. Faecal samples and study area

In total 400 *C. lupus* scat samples were collected in 2002–2011 during field studies on wolf ecology and behavior in the mountainous areas of Risnjak, Sniezik, Krasno, Paklenica, Suho, Snjeznik and Nic (Gorski Kotar region, Croatia). All scat samples were immediately fixed in 90% ethanol until further investigation. Fixed faecal samples were thereafter shipped to the Institute of Parasitology, Justus Liebig University Giessen, Germany, for further coproscopical analysis. Coproscopical analyses included the standard sodium acetate acetic acid formalin (SAF) technique applying ethyl acetate (Yang and Scholten, 1977; Young et al., 1979) for the detection of parasite eggs, larvae, cysts, sporocysts and oocysts. In addition, carbol-fuchsin- stained faecal smears according to Heine (1982) were carried out for the detection of *Cryptosporidium* spp. oocysts. Moreover, commercial coproantigen-ELISAs for the detection of *Giardia* and *Cryptosporidium* infections (ProSpecT®, Oxoid) were performed. The total subset of samples being microscopically positive for taeniid eggs ($n = 7$) was further analyzed by PCR and sequencing in order to identify taeniid eggs to *Echinococcus granulosus/E. multilocularis* species level.

2.2. DNA extraction from faecal samples

DNA was extracted from faecal samples using the QIAamp DNA Stool Mini kit (Qiagen, Germany) following glass bead homogenization as described by Nunes et al. (2006). 1 g of ethanol-preserved faecal samples was weighted into a 15 ml plastic tube, equilibrated in phosphate buffered saline at 4 °C overnight, centrifuged and resuspended in ASL extraction buffer using a glass rod. Then ~30 glass beads of 4 mm diameter (Carl Roth, Germany) were added. The sample was homogenized by horizontal vortexing (Vortex Genie 2 equiped with a 13000-V1-15 adapter, MO BIO Labs) and incubated at 70 °C for 15 min. 2 ml of this homogenate were transferred to a reaction tube, incubated at 95 °C for 10 min and after full speed centrifugation (8000 x *g*, 10 min), and 1.2 ml of the supernatant was further processed according to the manufacturers protocol by adding the inhibitor tablet and extracting the DNA.

2.3. Polymerase chain reaction and sequencing

For the molecular characterization of taeniid egg-positive samples, nested PCRs being specific for the mitochondrial 12S rDNA of *E. multilocularis* and *E. granulosus* sensu stricto were performed according to Dinkel et al. (1998, 2011) and Stefanić et al. (2004) with minor modifications. For the first, cyclophyllid-specific PCR a revised P60.short.for (according to von Nickisch-Rosenegk et al., 1999; Dinkel et al., 1998) forward primer (EmgrrnSs: 5′- TGACAGGGATTAGATACCC-3′) was used in combination with the reverse primer P375.short.rev (5′-TGACGGGCGGTGTGTACC-3′; Dinkel et al., 2011). For the specific nested PCRs we used the following primer combinations: *E. multilocularis* – Em-nest for (5′-GTGAGTGATTCTTGTTAGGGGAAGA-3′, Stieger et al., 2002; Dyachenko et al., 2008) and Pnest.rev. (5′-ACAATACCATATTACAACAATATTCCTATC-3′, Dinkel et al., 1998); *E. granulosus* – Eg1f (5′- CATTAATGTATTTTGTAAAGTTG-3′) and Eg1r (5′- CACATCATCTTACAATAACACC-3′, both Stefanić et al., 2004). The PCR reaction was performed in a 25 μl volume containing 2.5 μl copro-DNA, 5 μl 5x Hot FIREPol Blend master mix, 0.5 μl 7.5 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 0.25 μl 10 mM BSA and 0.5 μl of each 10 μM primer. Amplification conditions for the first PCR were 15′ 95 °C initial denaturation, followed by 35 cycles of 15″ 95 °C, 30″ 58 °C and 30″ 72 °C and a final extension of 5′ 72 °C. For the nested PCRs 1 μl of the first PCR was used at 62 °C annealing for *E. multilocularis* and 58 °C for *E. granulosus*. For a further characterization of *Echinococcus*-negative samples, a semi-nested cyclophyllid PCR with the forward primer Cest5 (5′-GCGGTGTGTACMTGAGCTAAAC-3′, Trachsel et al., 2007) and P375.short.rev was run with annealing at 58 °C. PCR amplicons were gel-purified and sent to a commercial service (LGC Genomics, Berlin, Germany) for direct sequencing. Sequences were analyzed by BLAST search of the GenBank database.

3. Results

3.1. Wolves

A total of 400 individual wolf faecal samples were collected during the years 2002–2011. The spatial analysis of the tracking data and the sample distribution revealed the presence of approximaltely 48 packs within the area of investigation. The wolf pack ranges were estimated by telemetric analyses within the Gorski Kotar and Dalmatia area (Kusak et al., 2005). Pack territories consisted mainly of mountainous mixed and coniferous forests, valleys, meadows and villages. The total wolf population was estimated 220 in the Gorski Kotar and Dalmatia regions (Štrbenac et al., 2010). In the Gorski Kotar area, roe deer (*Capreolus capreolus*), deer (*Cervus elaphus*), wild boars (*Sus scrofa*), hares (*Lepus europaeus*), and rabbits (*Oryctolagus cuniculus*) served as prey animals. In Dalmatia, most of the wolf diet referred to domestic animals (Huber, personal communication).