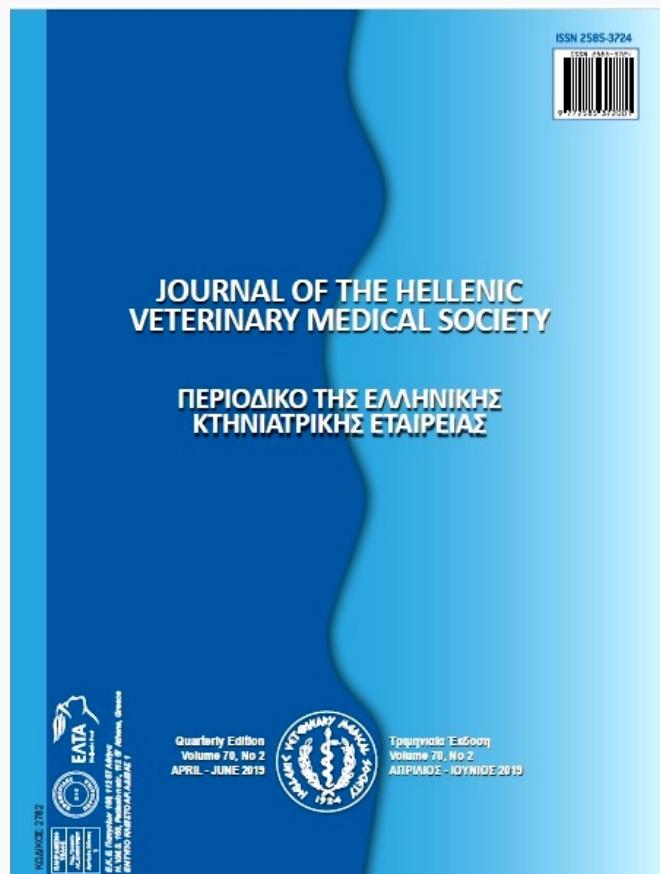


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## ***Giardia* sp. and *Cryptosporidium* sp. in Iberian Wolf**

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**Abstract:** A subsample consisting of fifty fecal samples from wild Iberian Wolf (*Canis lupus signatus*), from the northwest of Spain were collected in the field. The samples were analyzed for cysts of *Giardia* sp. and oocysts of *Cryptosporidium* sp. using a direct immunofluorescence antibody test (IFA). *Giardia* sp. and *Cryptosporidium* sp. were found in 20.0 % of the samples examined. Simple infections were more frequent (90.0 %) with seven (14.0 %) positive for *Giardia* sp. and two (4.0 %) positive for *Cryptosporidium* sp. To the authors' knowledge, this is the first report of occurrence of *Cryptosporidium* sp. in Iberian Wolf.

**Keywords:** *Cryptosporidium*; *Giardia*; Spain; Parasites; Wolf

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## INTRODUCTION

The Iberian Wolf (*Canis lupus signatus*), one of the major wildlife predator that circulates between Portugal and Spain in the Iberian Peninsula, carries a cultural and historical stigma that contributed to its historical decline. However the populations are currently increasing across Europe (Chapron et al., 2014). In human-dominated landscapes, the occurrence of wolves is the result of a complex interaction among several environmental and human factors (Llaneza et al., 2012). It is recognized that wolves can be a reservoir of many zoonotic agents (Torres et al., 2000) but on the other hand, humans and/ or livestock can be a risk for wolves like other wildlife species (Castro-Hermida et al., 2011).

*Giardia* sp. and *Cryptosporidium* sp. are considered ubiquitous protozoan parasites and can be a cause of gastrointestinal diseases in many mammals (Reboredo-Fernández et al., 2014). Both protozoan are included in the WHO Neglected Diseases Initiative in 2004 (Savioli et al., 2006) with special concern for the species of *Giardia duodenalis* and *Cryptosporidium parvum*, that can be a cause of human mortality (Plutzer and Karamis, 2009). Cysts of *Giardia* sp. and oocysts of *Cryptosporidium* sp. (infective stages) can feature a high fecal excretion in the environment and can remain on the ground surface or soil even after the feces have decomposed (Kloch et al., 2005; Ryan and Cacciò, 2013).

## MATERIALS AND METHODS

The fecal samples were collected in the Northwest of Spain. Given the nature of the sampling procedure no information was available regarding the sex or age of the animals. Differentiation of the wolf's feces from, namely dog feces, was achieved by indirect signs such as the composition/ morphology of feces (shape, size, and contents like hair and pieces of bones with a characteristic odor) and typical localization (Llaneza et al., 2012). Samples were collected during the summer (May, June, July and August) and autumn (September and October) seasons of two consecutive years (2013 and 2014). The collected fecal samples were frozen at -20 °C, and maintained in this condition until analysis.

From total fecal samples collected, fifty samples were randomly selected to determine the presence of *Cryptosporidium* oocysts and *Giardia* cysts. For that purpose, a commercial direct immunofluorescence assay (Cellabs® Pty Ltd, Brookvale, Australia) was used.

Feces (50 µL or 5 mm diameter) were diluted (1:10) with phosphate-buffered saline (PBS). PBS was prepared by diluting 0.2 g potassium chloride, 0.2 g potassium dihydrogenphosphate, 1.2 g anhydrous disodium hydrogen phosphate and 8 g sodium chloride in 1 L of distilled water, with pH adjusted to 7.4 through the use of 1 M HCl and 0.1 M NaOH. In a microscope slide, 20 µL of the fecal specimen was placed and allowed to completely air dry. The slides were fixed for five minutes in acetone and allowed to air dry. The probed-antibody (25 µL) was added to the fixed specimen and positive control, covering all area. The slides were incubated at 37 °C in a humid chamber for 30 minutes, and then rinsed gently in a bath of PBS for one minute. The slide was drained and the excess moisture around the well was removed with tissue. A drop of mounting fluid (RMG) was added to the slide well, and a cover-slip was placed on top of the drop and the air bubbles were removed. The entire specimen was immediately scanned using a fluorescence microscope.

## RESULTS

*Cryptosporidium* oocysts (2-6 µm in size) appeared with a round or oval shape with bright green fluorescence. A fold or suture could be seen on the surface. *Giardia* cysts appeared elliptical in shape, with bright green fluorescence. The test was considered positive if one or more oocysts and cysts were present. Of the fifty samples analyzed, ten (20.0 %) were positive for both *Giardia* sp. and *Cryptosporidium* sp. *Giardia* cysts and *Cryptosporidium* oocysts were detected in simple infections with more frequency (90.0 %), with seven (14.0 %) samples positive for *Giardia* sp., and two (4.0 %) samples positive for *Cryptosporidium* sp. and one sample (2.0 %) positive for both agents.

## DISCUSSION

Our results show that *Giardia* sp. were more frequently found than *Cryptosporidium* sp. but on the other hand most of the studies on wolves do not have a large number of samples, especially in Europe where this specie (*Canis lupus*) is endangered and the samples are difficult to collect. We found a lower prevalence compared to other studies (Kloch et al., 2005; Paziewska et al., 2007; Stronen et al., 2011) which may be due to the fact that our samples were frozen and the sensitivity may be lower. However, this study reveals that Iberian wolf is susceptible to the presence of these agents and at the same time can play a role on dissemination, as it was reported for other species from different environments in Spain.

In northwest of Spain, these protozoa were previously reported in some wild species. In otters (*Lutra lutra*), a prevalence of 6.8 % (30/437) for *Giardia* sp. and 3.9 % (17/437) for *Cryptosporidium* sp. was reported by Méndez-Hermida et al. (2007). Reboredó-Fernández et al. (2015) analyzed 70 fecal samples of aquatic species, found that 2.8 % were positive for *Giardia* sp. and 5.7 % positive for *Cryptosporidium* sp. In addition, *Giardia* sp. (6.0 %) and *Cryptosporidium* sp. (9.0 %) were detected in common dolphins (*Delphinus delphis*). In eight of the analyzed samples (n=133) *G. duodenalis* was identified whereas *C. parvum* was identified in three samples (Reboredó-Hermida et al., 2014). Castro-Hermida et al. (2011) compared different environments (635 samples collected from a coastal area and 851 samples from an inland area) with samples of wastewater (untreated and treated). The results showed a difference between coastal (positive for *Giardia* sp. 15.9 % and positive for *Cryptosporidium* sp. 9.2 %) and inland area (positive for *Giardia* sp. 26.7 % and positive for *Cryptosporidium* sp. 13.7 %). In wild birds 2.1 % (9/433) of the fecal samples were found positive for *Giardia* sp. and 8.3 % (36/433) positive for *Cryptosporidium* sp. *Giardia* sp. was identified in two species of raptors and *Cryptosporidium* sp. was identified in 7.1 % of the samples (Reboredó-Fernández et al., 2015). In roe deer (*Capreolus capreolus*), Castro-Hermida et al. (2011) also reported *Giardia duodenalis* (5.3 %) and *Cryptosporidium parvum* (1.3 %). The same study detected *Giardia* sp. (1.3 %) and *Cryptosporidium* sp. (7.6 %) in 381 fecal samples of wild boars. Recently, a study in Spain, in wild carnivores reported one positive sample for *Giardia duodenalis*, out of six analysed fecal samples of wolves (Mateo et al., 2017).

Outside Spain, both protozoan parasites have already been reported in wolves. In Poland, Kloch et al. (2005) found a prevalence of 45.5 % for *Giardia* sp. and 54.9% for *C. parvum* (n=57). Also in Poland, Paziewska et al. (2007) reported *Cryptosporidium* sp. with a prevalence of 35.5 % (n=14). In Canada, the protozoan parasites were also detected by Stronen et al. (2011) with a prevalence of 29.5 % for *Giardia* sp. and 1.2 % for *Cryptosporidium* sp., but with a larger number of samples (n=601).

To the authors' knowledge, no studies reported the presence of *Cryptosporidium* oocysts in the wolf packs of Iberian Peninsula. *Giardia* sp. in canids can cause chronic diarrhea, weight loss, lethargy and growth retardation. *Cryptosporidium* sp. is normally

asymptomatic in canids, but in immunosuppressed animals can be a cause of chronic diarrhea (Taylor et al., 2007) From the obtained results, it is reasonable to suggest that accidental infection of humans, livestock or companion animals can ensue, especially *Giardia duodenalis*, which has been reported in many mammals, including humans (Ryan et al., 2013). On the other hand, *Cryptosporidium* sp. has a negative impact in immunocompromised population, humans being infected by many species of *Cryptosporidium* (Plutzer and Karanis, 2009). The social behavior of wolf packs can further contribute to the dissemination due to their ability to migrate for long distances and the increasing proximity with humans and livestock. Wolf marking behavior by means of visual and scent marks such as feces and urine (Mech and Boitani, 2003; Zub et al., 2003; Llaneza et al., 2014) in unpaved roads and trails, and that could further contribute to the dissemination, as cysts and oocysts can remain in the soil for long periods of time, even under adverse conditions, such as rain or snow.

## CONCLUSIONS

More studies using molecular assays are needed to characterize the parasite fauna of remote and endangered wildlife especially to assess to infectious status in living animals. Furthermore, molecular assays can clarify the trophic relationships of wild carnivores and their prey species especially in human-dominated landscapes and with these studies we can establish the source of the infection of the wolves.

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## CONFLICT OF INTEREST

The authors report no conflicts of interest

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