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Research Note

Pearsonema plica and *Eucoleus böhmi* infections and associated lesions in wolves (*Canis lupus*) from Italy

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Summary

Reports of *Pearsonema plica* and *Eucoleus böhmi* infections in wolves (*Canis lupus*) in Europe are limited and data on associated lesions are lacking. In the present study urinary bladders, nasal turbinates and faecal samples from 8 necropsied wolves were examined for *P. plica* and *E. böhmi* infections and associated lesions. *P. plica* was identified in the bladder of four wolves. At histological examination, follicular chronic cystitis and eosinophilic cystitis were found. *E. böhmi* nematodes and eggs were identified from the nasal turbinates and rectal faecal samples of three wolves. Worms and eggs were found embedded in the mucosa among the nasal bone laminae. Two wolves were found coinfected by *P. plica* and *E. böhmi.* This is the first report of *P. plica* and *E. böhmi* infections in wolves from Italy and the first description of pathological lesions associated with *P. plica* infection in wolves.
Keywords: *Pearsonema plica; Eucoleus böhmi;* wolf *(Canis lupus);* cystitis; Italy

Introduction

In Europe, the wolf (*Canis lupus*) is globally classified as "Least Concern" by the International Union for Conservation of Nature, but several regional populations are considered at a higher risk of decline. Despite a recent increase in population range and numbers, the Italian wolf population is classified as "Vulnerable" (Large Carnivore Initiative for Europe, 2007). Indeed, carnivore-livestock conflicts determine a high human pressure on wolf populations and illegal killings, by poaching and poisoning are considered a main threat to wolf conservation (Large Carnivore Initiative for Europe, 2007). Since the wolf is a highly protected species and a host of conservation concern, studies aimed to investigate diseases and helminthoses are of relevant interest. Furthermore, similarly to what observed after the increase of red fox (*Vulpes vulpes*) populations in Europe (Sréter *et al*., 2003; Saeed *et al*., 2006; Magi *et al*., 2014), also the recovery of wolf populations may potentially be responsible for an increased risk of helminthic diseases transmission from wolves to humans and to domestic carnivores (Guberti *et al*., 1993; Eleni *et al*., 2014).

Among helminthic infections of wolves from Italy, several cestode (*Dipylidium caninum*, *Echinococcus granulosus*, *Mesocestoides lineatus*, *Taenia hydatigena*, *Taenia multiceps*, *Taenia pisiformis*, *Taenia ovis*) and nematode (*Ancylostoma caninum*, *Dirofilaria immitis, Toxascaris leonina*, *Toxocara canis*, *Trichinella britovi, Trichuris vulpis* and *Uncinaria stenocephala*) species have been identified (Guberti *et al*., 1993; Pozio *et al.,* 1996; Guberti *et al.,* 2004; Pascucci *et al*., 2007). The nematode species *Thelazia callipaeda* and *Angiostrongylus vasorum* have also been recently reported (Otranto *et al.,* 2009; Eleni *et al.,* 2014).

Among extraintestinal nematodes of carnivores, *Pearsonema plica* and *Eucoleus böhmi* have been reported in wolf populations from Europe (Zarnoswski & Patik, 1960; Bagrade *et al.,* 2009).

P. plica infects the urinary tract of domestic and wild carnivores worldwide (Basso *et al.,* 2013). The adult worms are thread-like, whitish nematodes that live superficially attached to or are buried within the bladder mucosa whereas the ureters and renal pelvis are seldom affected (Callegari *et al.,* 2010). *P. plica* is considered to be of low pathogenic significance and in most cases parasite establishes only asymptomatic infections (Fernández-Aguilar *et al.,* 2010). However, in heavy parasite loads *P. plica* has been found to be responsible for urinary signs and lesions in both carnivore pets (Callegari *et al.,* 2010; Rossi *et al.,* 2011; Basso *et al.,* 2013) and wild canids (Fernández-Aguilar *et al.,* 2010; Bork-Mimm & Rinder,

2011; Alić *et al.,* 2015). In wolves, *P. plica* infection has been previously reported in Belarus (Shimalov & Shimalov, 2000), Spain (Segovia *et al.,* 2001) and Latvia (Bagrade *et al.,* 2009). However, the associated pathological lesions were never evaluated before.
E. böhmi is a neglected nematode infecting domestic and wild canids (Perrucci *et al.,* 2014). Adults of *E. böhmi* live threaded through the mucosa of nasal turbinates, frontal and paranasal sinuses (Piperisova *et al.,* 2010). *E. böhmi* eggs may be found in nasal discharge and in faeces of infected animals, and are passed in a partial stage of embryonation with the developing larva often appearing square or rectangular (Supperer, 1953; Campbell & Little, 1991). In dogs, *E. böhmi* has been recently reported as a cause of respiratory signs with varying degrees of severity, associated with nasal discharge and chronic rhinitis (Baan *et al.,* 2010; Piperisova *et al.,* 2010; Veronesi *et al.,* 2013). Convulsive seizures and meningoencephalitis following intracranial migration of *E. böhmi* eggs have been also observed in an infected dog (Clark *et al.,* 2013). In the wolf, *E. böhmi* has been previously reported only in a single study in Poland (Zarnoswski & Patik, 1960).
Considering the lack of data in Italian wolf populations, the main aim of the present study was to evaluate *P. plica* and *E. böhmi* infections and associated lesions in wolves from Italy.

Material and Methods

Between March 2013 and October 2014, 8 (W1-W8) traumatically or unlawfully killed wolves from Central Italy were submitted by the local authorities for *post mortem* examination to ascertain the cause of death. Animals were frozen (-20°C) and allowed to thaw overnight before necroscopic examination. For each wolf the sex was recorded and animals were assigned to an age class (adult or juvenile) according to tooth eruption and tooth wear (Ballard *et al.,* 1995). From all animals the bladder was removed, opened, examined for gross lesions and washed with 70 % ethanol. The bladders and lavage fluids were first observed under a stereomicroscope for presence and collection of nematodes. Then adult nematodes were counted, while the lavage fluids were centrifuged at 2000g for 2 minutes and the sediment microscopically examined for capillariid eggs. Under light microscope the isolated adults and eggs were identified at the species level on the basis of their morphometrical features (Levine, 1968). From 6 wolves (W3 – W8), the opened urinary bladder was fixed in 10 % neutral buffered formalin, embedded in paraffin wax, sectioned at 4 µm, stained with haematoxylin-eosin and examined for histopathological lesions. From all wolves rectal faecal samples were also collected and examined for the presence and number of *E. böhmi* eggs. Flotation test and a modified McMaster test with sensitivity of 20 eggs per gram of faeces (EPG) with low density solution (SG 1.2) according to Perrucci *et al.* (2014) were used. Based on morphology of egg content and wall surface the *E. böhmi* eggs were distinguished from eggs of the other trichuroid nematodes (Di Cesare *et al.*, 2011). From four wolves (W4, W5, W6, W8), the skull was sawn and nasal cavities and sinuses were examined under a stereomicroscope to assess the presence of adult nematodes. Isolated parasites were collected and adult stages were counted and microscopically identified at the species level on the basis of their morphometrical features (Supperer, 1953). Samples of nasal turbinates were formalin-fixed, decalcified with specific solution (K-EDTA Decalcifier, Kaltek, Italy) and processed for histopathological examination as previously described.

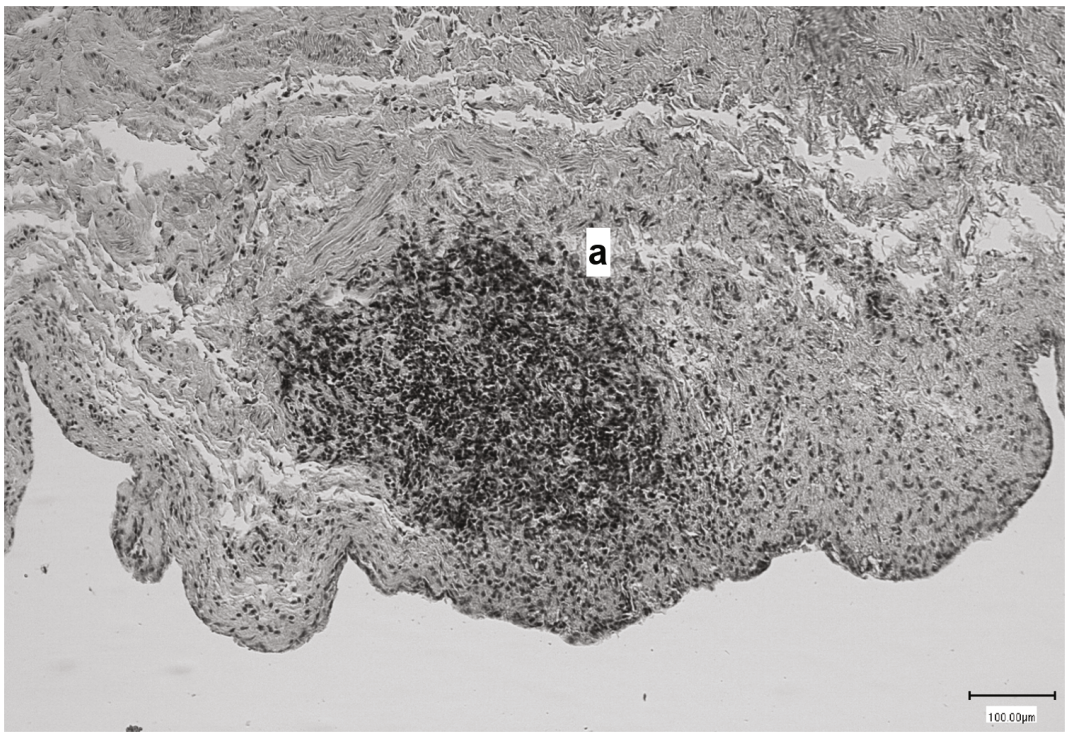

Fig. 1. Urinary bladder (wolf W4). Hyperplastic lymphocytic nodule (a) in the submucosa found associated to *Pearsonema plica* infection. (Hematoxylin-Eosin), *bar* 100 µm

Results

Animals were 4 adult males (W1, W2, W7, W8), 3 adult females (W3, W4, W6), and a juvenile male (W5). At microscopic examination, the urinary bladder mucosa and the bladder lavage fluids of wolves W1, W4, W7 and W8 revealed the presence of adult worms and of oval, colourless capillariid eggs showing a thick wall and bipolar plugs. More precisely, 15, 12, 2 and 4 adult stages were isolated from wolves W1, W4, W7 and W8, respectively. While eggs measured 58.5 – 62.4 X 26 – 31.2 µm and showed a thick wall and bipolar plugs, adult female parasites (30 – 60 mm X about 60 µm) showed a cylindrical appendage near the end of the oesophagus. Adult male parasites (15 – 30 mm X about 48 µm) showed a long spicule with an unarmed and punctate spicule sheath. On the basis of their morphological features isolated worms and eggs were identified with *P. plica*. Gross lesions characterized by 1 – 2 mm scattered foci of mucosal bluish pigmentation were observed only in the bladder mucosa of one infected wolf (W4).. At histological examination, follicular chronic cystitis with scattered hyperplastic lymphocytic nodules in the sub-mucosa (Fig. 1) was found in two wolves (W4 and W7). Nematodes cross-sections and eggs were also observed within the lumen or embedded in the mucosa epithelium of ureter in wolf W4. A mild diffuse eosinophilic cystitis was found in one wolf (W8). At histopathology, no lesions were observed in the remaining three wolves found negative at parasitological examination.

Capillariid nematodes threaded through the mucosa of the nasal turbinates were isolated after microscopical examination from W4, W5 and W8. In particular, 15, 8 and 3 adult nematodes were isolated from W4, W5 and W8, respectively. Adult females (37 – 40 mm X about 170 µm) showed a finely striated cuticle. The vulva did not protrude beyond the surface of the body and was located directly behind the passage of the oesophagus in the intestine and the pitted eggs measured 61 µm X 30.42 µm in average. Adult males (about 21mm X 110 µm) showed a delicate weakly chitinised spiculum. The everted portion of the sheath of the spiculum was about 500 µm long and completely covered with long spines. At coprological examination capillariid eggs, measuring 56 – 60.2 µm X 29 – 31 µm, with polar plugs and containing a developing larva were found in faecal samples from W4 (900 EPG), W5 (540 EPG) and W8 (280 EPG). Eggs had a thimble-like surface showing a large number of small depressions. Morphological and metric features of adults and eggs were in accordance with the original description of *E. böhmi* by Supperer (1953). Gross lesions of nasal cavities were not assessable in W4 due to putrefactive changes and presence of abundant necrophagous entomofauna. Only small amounts of catarrhal exudate were observed in W5 and W8. At histological examination cross-sections of adult worms and eggs were seen among the nasal bone laminae, embedded in the turbinates mucosa (Fig. 2). Wolves W4 and W8 were found coinfected by both *P. plica* and *E. böhmi*.

Fig. 2. Nasal turbinates (wolf W4). Cross-sections of *Eucoleus böhmi* (**a**) among the nasal bone laminae. (Hematoxylin-Eosin), *bar* 10 µm

At faecal analysis wolves W4 and W8 were found also positive for *Sarcocystis* sp. sporocysts and *Eucoleus aerophilus* and *Ancylostoma*/*Uncinaria* eggs. W4 was found also infected with *Aonchoteca putorii, w*hile W5 was found coinfected only by *Sarcocystis* sp. Microscopic examination of faecal samples from all the remaining wolves was negative for *E. böhmi*, but *Sarcocystis* sp. sporocysts were identified in faecal samples from W6 and W7. From the latter wolf, *Ancylostoma*/*Uncinaria* and *Taenia* sp. eggs were also isolated. No positive results were obtained at coprological examination of faecal samples from W1, W2 and W3 (summarised in Table 1).

Table 1. Parasites isolated from 8 deceased wolves (W1-W8) from Italy examined for *Pearsonema plica* and *Eucoleus böhmi* occurrence and intensity (number of isolated *P. plica* and *E. böhmi* adult stages and *E. böhmi* eggs/gram of faeces) and associated lesions. A= adult; blf= bladder lavage fluid; blfm= microscopical examination of bladder lavage fluid; EPG = eggs per gram of faeces; F= female; f= faeces; ft= faecal flotation test; J = juvenile; M= male; mm= McMaster test; nc= nasal cavities; ncg= gross lesions of nasal cavities; nch= histopathology of nasal cavities; ub= urinary bladder; ubg= gross lesions of urinary bladder; ubh= histopathology of urinary bladder; ubm= microscopical examination of urinary bladder.

Wolf	Gender	Age	Collected samples	Parasite	Parasite Intensity (N. of adult stages of *P. plica* and *E. böhmi* and of *E. böhmi* EPG)	Anatomo-Histopathological Examination
W1	M	A	blf, f	***P. plica*** (blfm)	15	_
W2	M	A	blf, f	_		_
W3	F	A	blf, f, ub	_		_
W4	F	A	blf, f, nc, ub	***P. plica*** (blfm, ubm) ***E. böhmi*** (ft, mm) *Eucoleus aerophilus* (ft) *Sarcocystis* sp. (ft) *Aonchoteca putorii* (ft) *Ancylostoma/Uncinaria* (ft)	12 15 (900 EPG)	**ubg:** 1-2 mm scattered foci of mucosal pigmentation; **ubh**: follicular chronic cystitis and nematode cross-sections embedded in bladder and ureter mucosa **ncg** and **nch**: not assessable due to putrefactive changes and presence of abundant necrophagous entomofauna
W5	M	J	blf, f, nc, ub	***E. böhmi*** (ft, mm, nc) *Sarcocystis* sp. (ft)	8 (540 EPG)	**ncg:** catarrhal exudate **nch**: worms and eggs embedded in the turbinate mucosa
W6	F	A	blf, f, nc, ub	*Sarcocystis* sp. (ft)		
W7	M	A	blf, f, ub	***P. plica*** (blfm, ubm) *Sarcocystis* sp. (ft) *Ancylostoma/Uncinaria* (ft) *Taenia* sp. (ft)	2	**ubh:** follicular chronic cystitis
W8	M	A	blf, f, nc, ub	***P. plica*** (blfm, ubm) ***E. böhmi*** (ft, mm, nc) *Eucoleus aerophilus* (ft) *Sarcocystis* sp. (ft) *Ancylostoma/Uncinaria* (ft)	4 3 (280 EPG)	**ubh:** mild eosinophilic cystitis **ncg:** catarrhal exudate **nch**: worms and eggs embedded in the nasal turbinate mucosa

Discussion

The Italian wolf population is estimated to be around 500 – 800 individuals distributed along the Apennines (Large Carnivore Initiative for Europe, 2007) and it is protected by EU and national laws. The International Union for Conservation of Nature classifies the Italian population as "Vulnerable", stressing out that it may easily reverse from its current favourable status due to high human pressure (Large Carnivore Initiative for Europe, 2007). Limited data are available on *P. plica* and *E. böhmi* infections of wolves in Europe and they are completely lacking for the Italian wolf population. In the present study eight wolves were examined for *P. plica* and *E. böhmi* infections and associated lesions.

Four out of the eight examined wolves were infected with *P. plica* and in three of them (W4, W7, W8) histopathology revealed follicular or eosinophilic cystitis, despite gross lesions being observed only in one of these wolves. Lesions of the urinary bladder associated with *P. plica* infection previously observed in dogs (Senior *et al.,* 1980) and in an arctic fox (*Alopex lagopus*) (Fernández-Aguilar *et al.,* 2010) were characterized by predominant eosinophilic inflammation along with infiltration of lymphocytes or occasional plasma cells. Chronic follicular cystitis has been recently described in infected red foxes, but it has been considered a less common finding compared to the eosinophilic cystitis (Alić *et al.,* 2015). Data from this study are the first report of lesions associated with *P. plica* infection in the wolf.

A correlation between adult parasite load and the presence of gross lesions in the urinary bladder mucosa was observed in red foxes by Bork-Mimm and Rinder (2011). In the present study,

gross lesions were only observed in the bladder mucosa of one wolf (W4). Despite the difficulties in the determination of the exact number of worms embedded in the bladder and ureter mucosa, the number of *P. plica* adults isolated from this subject was higher (12) than that found in the other infected wolves. Furthermore, in this same animal cross-sections of nematodes were histologically observed not only embedded in the bladder mucosa, but also in the ureter mucosa.

In this study, three out of the eight wolves were found positive for *E. böhmi* adults and eggs. *E. böhmi* has been recently reported as a cause of respiratory signs in dogs where nasal chronic inflammation was observed (Baan *et al.,* 2010; Piperisova *et al.,* 2010; Veronesi *et al.,* 2013). In the present study, in one (W4) of the infected wolves histopathological lesions were not assessable whilst in the remaining two positive wolves (W5 and W8) the inflammatory lesions were not observed. We hypothesise that in these latter wolves infection by *E. böhmi* could be recently established and therefore chronic inflammatory lesions reported were not present yet.

In the red fox, prevalence and pathogenic potential of *P. plica* and *E. böhmi* are quite high and this canid species is considered a reservoir of *P. plica* and *E. böhmi* infections for companion and hunting dogs in Europe (Davidson *et al.,* 2006; Bork-Mimm & Rinder, 2011; Magi *et al.,* 2014). Prevalence of *P. plica* infection in red foxes in Italy have been reported to be 56.8 % (Magi *et al.,* 2014), but it is difficult to obtain similar data concerning prevalence of infection in the wolf population, since *Canis lupus* is a protected species for which there are no culling plans as for red foxes. Nevertheless, a high prevalence of *P. plica* (41.4 %) was observed in wolves from Latvia (Bagrade *et al.,* 2009). Worldwide, only one previous record on *E. böhmi* infection in wolves from Poland is available (Zarnoswski & Patik, 1960), but it is not known if wolves are ancestral hosts of this parasite or whether infection may be acquired from red foxes. Routine necroscopic procedures do not usually include opening of nasal cavities, mucosal scrapings and microscopic examination of isolated organs. This may contribute to the underestimation of *P. plica* and *E. böhmi* infections in wolves.

Although the evaluation of *Eucoleus aerophilus* and *Aonchoteca putorii* infections was not included in the aims of the present study, data here reported provide the first record of these helminthic species in wolves from Italy.

In conclusion, previous data and findings from the present study stimulate a reflection on pathology and actual frequency of *P. plica* and *E. böhmi* infections among European wolves and on the potential role of this carnivores being a reservoir of these infections along with red foxes.

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