



Research Article

Health survey on the wolf population in Tuscany, Italy

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Abstract

The objective of our study was to survey the occurrence of transmissible agents in wolf (*Canis lupus*) population living in the northern Apennines. A total of 703 wolf fecal samples were collected in the Appennino Tosco-Emiliano National Park (ATENP) and the Foreste Casentinesi National Park (FCNP) in Tuscany, Italy. Parasitic forms (eggs or oocysts) were detected in 74.3% of fecal samples, mainly infested by Trichuroidea (60.4%) and Coccidia (27.3%); heavy Trichuroidea and Coccidia infestation were found in 8.5% and 17.4% of samples (the intensity of infestation measured as EPG >1000, OPG >10000). Taking into consideration the main canine viruses, we evaluated the presence of Parvovirus in feces: 54 specimens from the study area in the ATENP and 71 from the study area in the FCNP were negative by PCR for the detection of Parvovirus. Tissue samples from nine wolves found dead were negative for Canine Distemper Virus (CDV), Canine Coronavirus (CCoV), Canine Adenovirus-1 (CAV-1) and Canine Adenovirus-2 (CAV-2). Tissue samples of two dead wolves in the FCNP were positive for Canine Parvovirus (CPV) and the virus was characterized as the antigenic variant 2a. Wild boar is the main component of the wolf's diet in the study areas and 57 out of 135 serum samples (42.2%), collected from wild boars in the surroundings of the FCNP, yielded positive results for the detection of antibodies against Pseudorabies Virus (PrV). Wolves, especially in mountain areas, share the same habitat with dogs: this suggests how useful dog vaccination is for wolf protection.

Introduction

In the last decades, wolves (*Canis lupus*) have expanded their geographic range in Italy on account of their legal protection and the increase in the number of ungulates their diet depends upon. Their number is currently estimated to be between 1260 and 1800 (Galaverni et al., 2015). Furthermore, in mountain areas, where such traditional practices as animal husbandry has been abandoned, a natural reforestation has favored wolf populations (Ciancio et al., 2006). Health monitoring is considered a priority in the Italian wolf conservation plan (Genovesi, 2002), since small pack size and high mortality rates may significantly affect the composition and the stability of wolf populations (Mech and Goyal, 1993).

Among transmissible agents, canine parvovirus (CPV; Protoparvovirus, Parvoviridae), and canine distemper virus (CDV; Morbillivirus, Paramyxoviridae) are the most frequently assessed in health surveys on wolf populations in Europe and North America (Millán et al., 2016; Allison et al., 2013; Almberg et al., 2009; Sobrino et al., 2008; Fico et al., 1996). A wide range of directly and indirectly transmitted gastrointestinal parasites were found in wolves and their role in conservation remains to be clarified (Craig and Craig, 2005; Guberti et al., 1993).

In this study, such non-invasive procedures as the analysis of fecal samples and carcasses were used to survey the presence of transmissible agents in wolf populations in two National Parks located in the Northern Apennines, in Tuscany, Italy. A search for antibodies against the Pseudorabies Virus (PRV) was carried out among wild boars (*Sus*

scrofa), which are epidemiological reservoir of the virus and the main prey of the wolves in the study areas at this location (Lari et al., 2006; Capua et al., 1997; Hahn et al., 1997). Microbial agents can be transmitted between wolves and domestic dogs (*Canis lupus familiaris*). Therefore, information from dog owners was collected to estimate vaccination coverage in dogs sharing wolves' habitat.

Materials and methods

Study areas

The study was carried out in two National Parks in the Northern Apennines, in Tuscany, Italy: the Foreste Casentinesi National Park (FCNP) and the nearby Alpe di Catenaiola area (43°47'26.64" N, 11°43'24.06" E) at an altitude ranging between 300–1700 m above the sea level (a.s.l.); the Orecchiella, Lamarossa, Pania di Corfino Natural Reserves (44°12'15.78" N, 10°21'30.83" E) at an altitude ranging between 1000–2054 m a.s.l. in the Appennino Tosco-Emiliano National Park (ATENP) and the nearby Orrido di Botri Natural Reserve (44°4'56.77" N, 10°36'37.76" E) at an altitude between 900–1300 m a.s.l.

Wolf populations were monitored for several years in both study areas (Mattioli et al., 2011; Reggioni unpublished data 2006; Apollonio et al., 2004) by using indirect procedures (transects, snow tracking and wolf howling). Wolf population was estimated at 2–3 per 100 km² in the ATENP and 8 wolves per 100 km² in the FCNP. Genetic examination in the FCNP identified 35 different genotypes (Caniglia et al., 2014).

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Field data collection

Wolf fecal samples

Wolf fecal samples (n=703) were collected during wolf population monitoring activities, on transects selected by convenience sampling criteria, in either one or two monthly sessions in 2006 and 2007 (CFS — State Forestry Police). Fecal samples were attributed to wolves on the basis of physical criteria (Caniglia et al., 2014; Darimont et al., 2008) in the ATENP (n=439) and by means of genetic analysis in the FCNP (n=264). Feces were stored at -80°C for 10 days to neutralize *Echinococcus* spp. eggs and then at -20°C prior to laboratory analysis (Hildreth et al., 2004). Data obtained from the analysis of fecal samples from the ATENP and the Orrido di Botri Natural Reserve were combined as ATEPN data, since the two areas are relatively close (≈ 20 km distance).

Wolf tissue samples

Nine wolves (one from the ATENP, eight from the FCNP), which were found dead in the study areas between 2005 and 2007, underwent necropsy. Causes of death included car accidents and poaching. Tissue samples were taken from kidneys, bladder, liver, lungs, mesenteric lymph nodes, spleen, small intestine, and brain. Samples were frozen and submitted to the Department of Veterinary Medicine, Aldo Moro University of Bari, for laboratory analysis. Owing to the poor conservation status of the carcasses, no sample for histopathology was taken.

Wild boar blood samples

Blood samples were collected from the cardiac clot of 135 wild boars during the 2005 hunting season in the surroundings of the FCNP. Blood serum was stored at -20°C prior to laboratory analysis.

Information on domestic dogs

One of the authors interviewed 81 dog owners living in remote areas where dogs share part of wolves' habitat. Data were collected on the vaccination status of dogs against the major viral diseases (parvovirus, paramyxovirus; vaccination against rabies is generally not carried out, since Italy is rabies-free).

Laboratory analyses

Genetic analysis of wolf fecal samples

To identify individual wolves and characterize their circulating genotypes, DNA was extracted from 264 fecal samples collected in the FCNP and submitted for genetic analysis by using a panel of 12 canine microsatellite PCR primers (Galaverni et al., 2012). All of the 35 genotypes identified were matched with the database which is comprehensive of all Italian wolves (Caniglia et al., 2014). No hybrid was found.

Parasitological analysis

Fecal samples (2 g) were examined for parasite eggs or oocysts by flotation in a saturated solution of zinc sulphate (1200 density). Identification was based on morphology and size (Sloss and Kemp, 1978). Protozoa and Nematoda eggs/oocysts were classified by genus, whereas Ascaridae by species (Stronen et al., 2011; Kloch et al., 2005; Gompper et al., 2003). Fecal eggs/oocyst count was carried out by using McMaster's technique (Whitlock, 1948) and expressed as eggs/oocysts per gram (EPG/OPG).

Virological analysis

Wolf fecal samples (including 54 specimens from the ATENP and 71 from the FCNP) were analyzed in pools of five to detect CPV by using laboratory techniques, as described in Decaro et al. (2006, 2005a). DNA was extracted from tissue samples by employing the DNeasy Tissue Kit (QIAGEN S.p.A. Milan, Italy), whereas RNA purification was obtained by using the QIAamp® Viral RNA Mini Kit (QIAGEN S.p.A.).

Wolf tissues samples were analyzed by using molecular methods to detect the viral agents CPV, CDV, CCoV, and CAdV: a real-time PCR (Decaro et al., 2005b, 2006); TaqMan-based real-time RT-PCR (Elia et al., 2006); duplex real-time PCR assay (Dowgier et al., 2016).

Virological analysis

Wild boar serum samples were tested for antibodies against PRV by employing IgB antibody ELISA test (IDEXX antiPRV gB). The ELISA colorimetric reaction was measured by using a spectrophotometer (BIO-RAD 680 Microplate Reader).

Statistical analysis

For each parasite group, the prevalence of positive fecal samples (in which at least one parasitic form was detected) was obtained with the FREQ procedure in the SAS System. The median and third quartiles (Q1, Q3) of the number of EPG/OPG were calculated (PROC MEANS, SAS, 2011). Furthermore, the prevalence of samples with high burden (EPG>1000; OPG>10000) were calculated to assess the frequency of wolf scats which were highly infested. We chose the cut-off of 1000 EPG (Urquarth et al., 1998) and 10000 OPG for *Coccidia* infection, to define the parasitic infection as serious. Confidence intervals of prevalence were not calculated, since multiple samples might have belonged to the same individual. Thus, no statistical analysis was carried out to test differences in prevalence and in the number of EPG/OPG. Therefore, data analysis was limited to descriptive statistics. Prevalence of each viral agent in wolf tissue samples and antibodies against PRV in wild boars' serum sample was also obtained.

Results

In both study areas, eggs of Tricuridae and Strongylidae were the most prevalent Nematode parasitic forms. Although the prevalence of Ascarididae was relatively low, presence of Ascarididae eggs was highest in positive fecal samples (Tab. 1). Infections with EPG >1000 were detected in 8.5% of specimens for Trichuridae. The intensity of infection was also detected (UPG>1000, OPG>10000): in particular, 8.5% of samples showed high levels of Trichuridae, 1.4% of Strongylidae, 22.2% of Ascarididae eggs, and oocysts were found in 17.4% of fecal samples.

Virological analysis

Twenty-five pools, each composed of five fecal samples (for a total of 125 samples) showed negative PCR for Parvovirus. Tissue samples from the nine wolves which were found dead were negative for CDV, CCoV, CAdV-1, and CAdV-2. Samples from intestine, spleen and mesenteric lymph nodes of two wolves (of an approximate age of 6 and 18 months) found dead in the FCNP were positive for parvovirus (CPV). Viral DNA titers were generally low, ranging from 7.19×10^3 (intestine of one individual) to 2.71×10^4 (intestine of the other wolf) per mg of feces. Pooled spleen and lymph nodes of the same individuals were positive, though they displayed lower CPV DNA loads (2.84×10^3 and 6.12×10^3 per mg of feces, respectively). Both strains were characterized as type 2a, but viral DNA loads were too low to allow any sequencing of informative regions for subsequent evolutionary studies.

ELISA for the detection of antibodies against PRV yielded positive results on 57 out of 135 serum samples (42.2%), which were collected from wild boars in the surroundings of the FCNP.

Table 1 – Prevalence of fecal samples with at least one parasitic form, and median (Q1, Q3) numbers of parasite eggs or oocysts per g in positive wolf fecal samples in the study areas in the Northern Apennines, Italy.

Parasite group	Study area (n=703)					
	FCNP (n=264)			ATENP (n=439)		
	%	Median	Q1, Q3	%	Median	Q1, Q3
Ascaridia	2.3	180	30, 6570	0.68	120	90, 1350
Tricuridae	67.8	150	45, 480	56	90	45, 255
Strongil	12.5	30	15, 60	6.8	30	30, 120
Cestoda	7.2	105	30, 540	9.6	60	30, 180
Coccidia	33.3	2000	1000, 7500	23.7	2600	1000, 6900

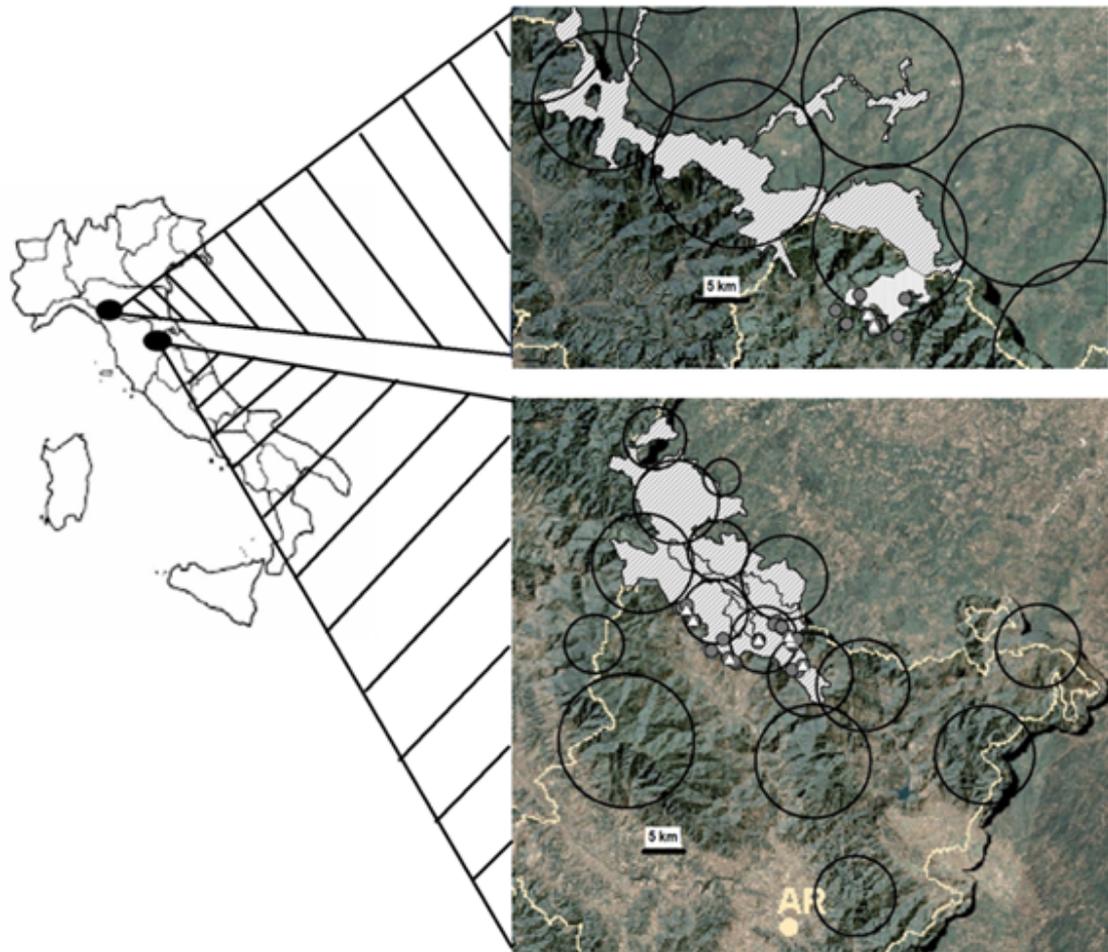


Figure 1 – Dogs and wolves share the same territory: the circles represent the home range of different wolf packs obtained from snow tracking, wolf howling and genetic analysis data (LIFE07NAT/IT/000502 “Improving the conditions for large carnivore conservation — a transfer of best practices” LIFE EX-TRA). The white and grey dots represent the location/position of dogs. The grey dots represent unvaccinated dogs.

Dog owner survey

Based upon the analysis of questionnaires administered to dog owners, in the ATENP, 39% of dogs were correctly vaccinated (three vaccinations administered at four, eight, and sixteen weeks of age), 37% received only the first two vaccinations, and 24% were unvaccinated. In the PNFC, 45% of dogs were correctly vaccinated, 35% were vaccinated only twice, whereas 20% were unvaccinated.

Discussion

Parasitological examination of wolf feces in the two study areas showed that Trichuridae eggs were the most common Nematode parasitic forms, followed by Strongylidae eggs. Such finding might be accounted for by the resistance of eggs of these parasites in the environment, favoring the transmission of infection within wolf populations (Capelli et al., 2003). Conversely, eggs of Ascaridae were relatively rare in the samples examined; in fact, despite being common in carnivores and previously reported in wolves (Bryan et al., 2012; Byman et al., 1977; Holmes et al., 1968), Ascaridae are not considered dominant in this species (Segovia et al., 2003, 2001; Guberti et al., 1993). Moreover, in our study, the frequency of Ascaridae might have been underestimated owing to a relatively low likelihood of collecting feces from juvenile wolves, which are more liable to be infected. In fact, scats found on transects tend to be part of scent-marking by adult wolves (Mech and Boitani, 2003; Vilà et al., 1994). With respect to the detection of Cestoda (Gori et al., 2015; Guerra et al., 2013; Guberti et al., 1993), the relatively low prevalence obtained in our study might be due to the poor sensitivity of copromicroscopical analysis by flotation in the diagnosis of these parasites (Poglayen et al., 2017; Villeneuve et al., 2015). We observed relatively high burdens of *Coccidia* oocysts; however, none

of the parasitic forms identified belonged to *I. canis*, which was previously shown as a pathogen associated with the death of wolf puppies in North America (Mech and Kurtz, 1999).

The relatively low proportions of wolf feces characterized by great parasitic counts (EPG>1000 and OPG>10000) suggest an aggregate distribution of parasites among hosts (Tompkins et al., 2002; Wilson et al., 2002; Anderson and May, 1978). Under these circumstances, the few hosts harboring the majority of parasites might be weakened and particularly vulnerable to secondary pathogens (Stronen et al., 2011; Scott, 1988).

The high prevalence of seropositivity to PRV in wild boars, which is consistent with the findings in other areas of Italy (Lari et al., 2006), suggests that wolves are exposed to the agent of Aujeszky's disease, given that wild boar is the main component of a wolf's diet (Davis et al., 2012; Mattioli et al., 2011, 1995). Although PRV was identified in the brain of a wolf which showed nervous signs after being fed with wild boar offal in a wildlife park in Belgium (Verpoest et al., 2014), consequences of exposure to PRV for free living wolf populations remain to be clarified and are likely scarce.

The detection of CPV in tissues from two wolves confirmed the circulation of such agent within the wolf population of the Northern Apennines, where it was previously found in fecal samples by Martinello et al. (1997). The presence of CPV had never been investigated before in the ATENP, where all fecal and tissue samples yielded negative results. The viruses detected in the present study belong to the variant CPV-2a. It is unlikely that CPV contributed to the death of the two positive wolves, since viral DNA loads were very low and the wolves were found dead with lesions attributable to car accidents. The most recent report on CPV in wolves in Italy dates back to 2001 and describes the detection of four CPV-2b strains in 1995 (Battilani et al.,

2001). More recently, a CPV-2a strain was reported in a wolf in Bulgaria (Filipov et al., 2016). Based upon genetic characterization of the viruses we identified, both individuals harbored one of the antigenic variants circulating within the dog population. A CPV-2b strain was recently detected in a wolf found dead in the Campania region, Italy, which was co-infected by pantropic CCov and CA2V-2 (Alfano et al., 2019).

All of the fecal samples examined were negative for parvovirus. The virus was seldom found in feces, in spite of the frequent detection of antibodies against CPV and the endemic CPV presence in a number of wolf populations in North America and Europe (Alfano et al., 2019; Millán et al., 2016; Molnar et al., 2014; Almberg et al., 2009; Sobrino et al., 2008). This is due to the viral excretion, which may occur only during the first phase of the infection. Furthermore, viral excretion in a population with endemic parvovirus is likely reduced by immunity, including the protection of puppies by passive immunization (Mech et al., 2012). Serological analysis on live trapped individuals may reveal valuable information regarding the health status of wolves (Zarnke and Ballard, 1987; Goyal et al., 1985; Choquette and Kuyt, 1974), though it sparks a debate on the considerable financial outlays required as well as on ethical considerations (Darimont et al., 2008).

Dogs vaccinated against the old strain CPV-2 may a new infection by new viral reservoirs, which may emerge following be affected by a mutation, as shown by the viral lineages identified in the last years (CPV-2a, b, c). This might explain the occurrence of outbreaks of infection in breeding kennels, where dogs were regularly vaccinated with the old-type vaccines, based on the classical lineage CPV-2, which is now extinct (Decaro and Buonavoglia, 2012; Müller et al., 2011; Decaro et al., 2009, 2008). We cannot exclude the establishment of a sylvatic cycle of parvovirus transmission (Santos et al., 2009; Sobrino et al., 2008).

Other canine viruses were not detected in the samples analyzed, although it was reported that CDV, CA2Vs (Dowgier et al., 2018) and CCov are able to infect and cause diseases in wild carnivores (Alfano et al., 2019; Zarnke et al., 2001; Laursen et al., 1998; Stephenson et al., 1982; Choquette and Kuyt, 1974). Distemper is due to a highly pathogenic virus (Almberg et al., 2009), which needs a large host population in order to be maintained. Dog populations were considered the cause of distemper outbreak on several occasions (Di Sabatino et al., 2014; Decaro et al., 2004; Cleaveland et al., 2000); in a number of cases, the existence of a multi-host population was presumed, with dogs thus playing a secondary role. The vaccination against distemper grants a good immunity level for many years (Martella et al., 2008). Since the herd immunity against CDV was sufficient to control the disease despite infectious pressure in dogs as long as herd immunity was slightly over 70% of the vaccinated dogs (Rikula et al., 2007), we can suppose that the dogs vaccinated until now were sufficient to build a barrier against the disease and that the population of non-vaccinated dogs was not sufficient to constitute CDV reservoir. Further research should include fox populations in health surveys in the study areas, considering that, since 2006, red foxes in Northern Italy experienced an epidemic of canine distemper (Loots et al., 2017; Martella et al., 2010), and given the recent introduction and spreading of novel, or re-emerging, CDV strains in Europe, carried by dogs imported by Eastern Europe (Mira et al., 2018).

The proximity of such wolf's preys as deer and wild boars to human settings might favor the transmission of microbial agents between wolves and domestic dogs. Therefore, dog populations might be considered an important component of wolf ecosystem and can be used as sentinels for wolf health (Di Sabatino et al., 2015). The Authorities of National Parks, in collaboration with public veterinaries, should pay particular attention to local dog populations, reduce the number of roaming dogs, promote appropriate dog vaccination and education programs and protect the wolf population from CDV and new strains of CPV outbreak. 🐾

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