Lost and found: helminths infecting invasive raccoons introduced to Italy

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Abstract

North American raccoons (*Procyon lotor*) have been introduced to several European countries, where they may represent a sanitary threat as hosts of several pathogens such as the zoonotic ascarid *Baylisascaris procyonis*. We carried out parasitological analysis on raccoons introduced to Italy to verify whether the species had carried along *B. procyonis* or any other gastro-intestinal helminths that may threaten humans, livestock or native wildlife. We examined 64 raccoons culled in northern Italy during control activities and 3 roadkills opportunistically sampled from a separate population located in central Italy. Helminths were collected from the gastro-intestinal tract through standard parasitological techniques and identified based on a combination of morphology and molecular methods. Overall, examined raccoons showed a poor parasitic fauna, with almost 30% of individuals free of any helminth infection. The most prevalent species were the nematodes *Strongyloides procyonis* (26.9%), *Aonchotheca putorii* (25.4%) and *Porrocaecum* sp. (19.4%). *Plagiorchis* sp. trematodes were also common (13.4%), whereas cestodes were scarcely represented. With the exception of *S. procyonis* introduced from North America, all the other identified taxa have either a European or a Palearctic distribution. Despite not finding any *B. procyonis* in the examined raccoons, passive surveillance for this parasite should be implemented, especially in Tuscany, since the limited host sample examined in the present survey does not allow to exclude its presence.

Keywords

alien species; macroparasites; biological invasions; gastro-intestinal helminths; *Baylisascaris procyonis*; *Procyon lotor*

The northern raccoon (*Procyon lotor*) is a carnivore native from North America that has been introduced in several European countries, the Caucasian region, Japan and some Caribbean islands, either deliberately as a game species or through releases from pet or fur trade [1]. Impacts by invasive raccoons may include predation on native fauna and competition with other small- and medium-sized carnivores, such as the endangered European mink (*Mustela lutreola*) [1]. From a sanitary point of view, raccoons represent a risk towards humans, domestic animals and wildlife as they are known to harbour a great variety of pathogens and are an opportunistic species that can adapt to a wide range of habitats, including urban and peri-urban environments [2]. In its native range the species is considered a reservoir for both rabies and canine distemper virus and is the definitive host of the zoonotic ascarid *Baylisascaris procyonis* [2].

Adult *B. procyonis* inhabit the small intestine of raccoons and the infection is usually subclinical: in North American populations, this nematode may naturally infect up to 90% of raccoons [3]. However, birds and mammals other than raccoons, humans included, may act as paratenic or dead-end hosts and become infected with *B. procyonis* in the larval stage. In these hosts somatic migration of larvae can sometimes cause extensive tissue damage and granulomatosis, potentially resulting in visceral, ocular or neural Larva Migrans Syndrome (LMS) [3]. Compared to other ascarids, *B. procyonis* larval migration appears particularly aggressive and leads more frequently to severe or fatal neural damage [3]. Additionally, the opportunistic behaviour of raccoons coupled with the high reproductive output of *B. procyonis* and the high resistance of its eggs to environmental degradation, result in a relevant risk of exposure for humans and domestic animals [3]. Other than in raccoons' native range, adult *B. procyonis* have been reported so far in feral raccoon populations in Germany and Denmark [2,4] and eggs have been detected in raccoons' latrines in Poland [5].

In Italy, raccoons have currently established two separate populations of different origin: a first one that has been expanding since 2004 along the southern part of the Adda river (North Western Italy, [6]) and a second one of more recent origin (2013) in Tuscany (central Italy, [7]). In the European Union, the species is listed among the invasive species of European concern (EU Regulation 1143/2014), for which management by member countries is mandatory. In 2016, Adda Nord Regional Park and Lombardy region implemented a control program aimed at eradicating the main raccoon population located in Northern Italy [6]. We surveyed the gastro-intestinal parasite community of culled Italian raccoons, to verify if they introduced *Baylisascaris procyonis* or if they harbour any other helminth that may represent a threat to humans, domestic animals or wildlife.

From 2017 to 2019, we examined a total of 64 raccoons (27 males and 37 females) culled in Lombardy region. Additionally, we examined three road-killed animals opportunistically sampled from the Tuscany population, where culling activities are not yet in place. All the animals from Lombardy were captured with live-traps (model 205, Tomahawk Live Trap Co., Tomahawk, Wisconsin, U.S.A.) baited with pet food and euthanised on the field by means of CO_2 overdose, following AVMA guidelines [8]. Permits were issued by the Lombardy Region (permit n° 198, 13/01/2017) and ISPRA (Istituto Superiore per la Ricerca Ambientale – National Institute of Environmental Research; permit n° 66230, 15/11/2016). Carcasses were stored at -20°C until examination, during which the gastro-intestinal tract was removed, dissected longitudinally and its content flushed with tap water through two sieves (lumen 0.4 and 0.038 mm, respectively), to filter out coarser material. All the culled animals were in good condition and did not show any clinical signs of disease at post-mortem examination. The whole gastro-intestinal content was examined under a stereomicroscope and all the helminths counted and stored in ethanol 70% for morphological identification based on keys by [9–11]. Inconclusive or uncertain identifications were confirmed through molecular analyses. Based on morphological features, helminths found within a single host were pooled in groups of 1-8 specimens, ethanol was removed and pools were subjected to crude DNA extraction performed in 15 µl of Tris-HCl buffer (10 mM, pH 8) and 2 µl of proteinase K (20 mg/ml), with overnight incubation at 56°C. Samples were put at 95°C for 10 minutes to inactivate proteinase K, then centrifuged at 20000 g for 10 minutes and stored at -20 °C until subsequent analyses. The supernatant (undiluted and 1:10, 1:100 serial dilutions) was subjected to qualitative PCR analyses using primers reported in Supplementary Table 1 (primers final concentration 1 µM). PCR products were loaded on agarose gel, excised and gel-purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and Sanger sequenced. The electropherograms were manually corrected and sequences were subjected to BLAST analysis (https://blast.ncbi.nlm.nih.gov/).

Forty-eight (71.6%) out of the 67 examined raccoons (46/64 from Lombardy and 2/3 from Tuscany) were infected by gastro-intestinal helminths. We found a total of at least 11 different helminth taxa, with richness in infected animals ranging from 1 to 5 taxa (mean value: 1.2 ± 0.1 SE). *B. procyonis* was not detected in any of the examined individuals. Detailed results of parasitological examination are reported in Table 1.

Overall, the most prevalent species found in Italian raccoons was the North American nematode *Strongyloides procyonis* (prevalence 26.9%), which was also the only gastro-intestinal helminth detected in roadkills sampled from the Tuscany population. The amplification and sequencing of partial 18S rRNA, 28S rRNA and Internal Transcribed Spacer 1 (ITS1) showed 100% identity scores with *S. procyonis* sequences present in GenBank (AB205054; [12]). Accession numbers of of newly obtained sequences at GenBank are shown in Supplementary Table 2. *S. procyonis* is a nematode specific to raccoons [13] and, to the best of our knowledge, this represents its first report in Italy. The species has also been reported in invasive raccoons in Japan [12] and Poland [5]. With the exception of *S. procyonis*, which has a clear North American origin, all the other taxa identified in the present survey have been recorded in Europe, having a Palearctic or a wider Holarctic distribution. This is the case for *Aonchotheca putorii*, which was the second most prevalent species in our sample (25.4%). *A. putorii* is a capillariid nematode that frequently infects mustelids and other medium-sized carnivores, raccoons included, in both North America and Eurasia [10,14]. The genetic sequences of our specimens (Suppl. Table 2) showed respectively 100% and 98.12-98.44% identity with 18S rDNA and cytochrome *c* oxidase subunit I (*COXI*) *A. putorii* sequences present in GenBank (e.g. LC052363, KC355430). Larvae belonging to the cosmopolitan genus *Porrocaecum* were also rather common in our sample (19.4%). The obtained 18S rRNA, 5.8S rRNA and cytochrome *c* oxidase subunit 2 (*COXII*) gene fragment sequences were deposited in GenBank (Suppl. Table 2). However, molecular analyses were not able to discriminate the analyzed specimens at the species level. *Porrocaecum* spp. are ascarid nematodes with earthworms and birds of prey as intermediate and definitive hosts, respectively [11]. By feeding on earthworms, small and medium-sized mammals may become infected with the parasite in the larval stage and act as paratenic hosts. Larval stages of *Porrocaecum* spp. have been reported also in raccoons introduced in Japan [15]. Eight raccoons were found to harbour immature *Hystrichis tricolor* individuals, which were identified based on keys by [9]. A fragment of the 18S rRNA of this species was amplified, sequenced and deposited in GenBank (Suppl. Table 2). Similarly to *Porrocaecum* spp., this nematode has earthworms as intermediate hosts and the infection in raccoons appears of accidental nature, since adults of this species normally infect waterfowl and have been very rarely reported in mammals [11].

Trematodes were found in 13.4% of examined raccoons but, based on molecular analysis, were identified as *Plagiorchis* sp., a ubiquitous genus in European freshwater ecosystems [16]. Unfortunately, it was not possible to further define our specimens, due to contrasting sequencing results of 28S rDNA, ITS2, and *COXI* (Suppl. Table 2). Finally, Cestodes were found only in five hosts. Morphological identification and molecular analyses placed the cestode specimens obtained from three of these raccoons in the genus *Dilepis*. However, the obtained gene sequences (Suppl. Table 2) did not match with *Dilepis undula* (the only representative species whose sequences are available in GenBank), showing the following identities scores with it: 99.64% for the 12S rDNA, 100% for the 18S rDNA gene, 92.49-95.43% for the *COXI* gene (reference sequences used for comparison: L49457, AF286981 and EU665471, respectively). This genus includes mostly species parasitising birds and small rodents [17] and, to the best of our knowledge, has never been previously reported in raccoons. Unfortunately, the specimens found in the two remaining raccoons were not identified due to morphological and DNA degradation. Overall, our findings show that raccoons introduced to Italy have lost many gastro-intestinal parasites commonly found in their native range (e.g. [18,19]). As a result, despite having acquired a few European taxa, they have an impoverished community compared to North American populations [cf. 18, 19], both in terms of species richness and parasite abundance. Indeed, almost 30% of examined individuals resulted entirely free from gastro-intestinal parasites. The exact origin of the two Italian raccoon populations is unclear, but this parasite loss is likely the result of a combination of founder effect and anthelmintic treatments, as the two populations were both probably founded by a few pets escaped or released from captivity. Introduction of parasites by invasive host species depends indeed on several factors, but it is first and foremost a stochastic process [20]. For instance, as mentioned above, raccoons established in central Europe carried along *B. procyonis* from their native range, but the infection appears to be absent from wild populations in other introduction ranges, such as Japan [15, 21]. Whatever the cause, this reduced parasite load could represent an advantage for invading hosts (enemy-release hypothesis, [20]). The introduction of North American *S. procyonis* to Italy does not appear particularly alarming from a sanitary point of view, as in humans this parasite is known to cause a mild dermatitis at the most [13]. However, its presence deserves further attention because its potential impact on naïve native hosts is unknown. In particular, the risk for spillover would be higher towards other small- and medium-sized carnivores that share their habitat with the invader, such as stone and pine martens, weasels, badgers, foxes or even domestic dogs. Indeed, the conspicuous presence of *A. putorii* in our raccoon population suggests that interspecific transmission with native mustelids and other wild carnivores is already occurring. Finally, our survey suggests that *B. procyonis* is absent from Italian raccoons, but this result should be taken with caution. First of all, we cannot draw any conclusion about the Tuscany population, because of the very limited number of individuals examined through opportunistic sampling. Hence, further parasitological analyses need to be carried out to specifically ascertain the absence of the ascarid from this nucleus, which has a distinct origin from the North Italian one. Secondly, assuming intermediate to low prevalences of *B. procyonis,* the Lombardy sample size appears adequate for a parasitological survey. However, modelling data on *B. procyonis* dynamics suggest that, during the initial stages of host invasion, the parasite might persist at very low prevalences that may hinder its detection [22]. As a consequence, ongoing passive surveillance is recommended to rapidly detect any emergence of *B. procyonis* in Northern Italy [23].

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Table 1 Gastro-intestinal helminths detected in raccoons (*Procyon lotor*, N=67) introduced to Italy: number of infected hosts (n), prevalence of infection (% infected hosts/examined hosts) and mean intensity of infection (no. parasites/infected hosts); SE=Standard Error.

Taxon	n	Prevalence ± SE	Mean intensity ± SE
Nematoda			
Strongyloides procyonis	18	26.9 ± 5.4	11.7 ± 5.5
Aonchotheca putorii	17	25.4 ± 5.3	7.2 ± 1.9
Porrocaecum sp. (larvae)	13	19.4 ± 4.8	14.7 ± 5.3
Hystrichis tricolor (immature)	8	11.9 ± 4.0	8.5 ± 6.8
Trichuridae spp. [a]	2	3.0 ± 2.1	*1; 2* [b]
Trichostrongylidae spp. [a]	2	3.0 ± 2.1	*2; 3* [b]
Ancylostomatidae sp.	1	1.5	*1* [b]
Oxyuridae sp.	1	1.5	*1* [b]
Trematoda			
Plagiorchis sp.	9	13.4 ± 4.2	7.0 ± 3.9
Cestoda			
Dilepis sp.	3	4.5 ± 2.5	*4; 1; 11* [b]
Unidentified	2	3.0 ± 2.1	*1; 26* [b]

[a] uncertain number of species, single or two; [b] intensity of infection in each infected host