PCR-based assay for the mitochondrial cox1 specific amplification of *Eucoleus böhmi*

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Abstract

Eucoleus böhmi (syn. *Capillaria boehmi*) is a trichuroid nematode affecting the epithelium of the nasal turbinates, frontal and paranasal sinuses of wild and domestic canids. Knowledge of the geographic distribution of nasal eucoleosis is fragmentary, despite the infection has been described from Europe and North America. Moreover, gaps exist in information available on the importance of the disease in canine clinical practice. The lack of knowledge on *E. böhmi* is likely due to limitations inherent to diagnostic methodologies. The aim of the present work was to assess a PCR-based assay instrumental to the amplification of a species-specific region of the mitochondrial DNA (mtDNA) gene encoding for the subunit 1 (cox1) gene of *E. böhmi*.

Adult worms of *E. böhmi* from red foxes and dogs from Norway, Serbia and Italy and individual fecal samples from naturally infected dogs from Italy were included in the study. Stool samples from dogs scored negative for *E. böhmi*, but positive for other common parasites in both single and mixed infections, and adult stages of common dog parasites, were used to assess the specificity of this genetic assay. Using the panel of faecal samples, the assay showed a sensitivity of 85.14% and a specificity of 100%.

Keywords: *Eucoleus böhmi*, dog, diagnosis, PCR, cox1

1. Introduction

Nasal eucoleosis is a parasitic disease caused by the trichuroid nematode *Eucoleus böhmi* (syn. *Capillaria boehmi*), which infects the upper respiratory airways of wild and domestic canids. Adult worms live embedded in the epithelium of the nasal turbinates, frontal and paranasal sinuses of the vertebrate hosts. Infected animals (e.g. dogs, foxes, wolves) shed the typical trichuroid-like eggs with the nasal discharges and/or the faeces. Despite a relevant Despite a relevant pathogenic potential, *E. böhmi* is a neglected and underestimated cause of upper respiratory tract disease **(Veronesi et al., 2013, 2014)**.

Life cycle and routes of transmission of nasal eucoleosis are almost unknown **(Conboy, 2009)**. It is argued that the biological cycle of this nematode is similar to that of the more known, closely related, species *Eucoleus aerophilus* (syn. *Capillaria aerophila*), causing lung capillariosis in wild and domestic carnivores. In this case the animal becomes infected either by the ingestion of larvated infective eggs or by the ingestion of invertebrate facultative intermediate/paratenic hosts, e.g. earthworms, whose actual biological role is still to be elucidated **(Campbell and Little 1991; Conboy, 2009; Anderson, 2000; Traversa et al., 2011)**. Knowledge of the geographic distribution of *E. böhmi* is scarcely documented, though infected dogs have been described from Europe and North America **(Schoning et al., 1993; Gajewska et al., 2004; De Liberato et al., 2009; Piperisova et al., 2010; Baan et al., 2011; Di Cesare et al., 2012a,b; Magi et al., 2012; Conboy et al., 2013; Veronesi et al, 2013,2014)**.

Other gaps exist in information available on the factual importance of nasal eucoleosis in canine clinical practice. Symptomatic dogs suffer of damages in the epithelium of the nasal turbinates and sinuses, which induce a rhinitis with varying clinical signs, i.e. sneezing, reverse sneezing, catarrhal blood-stained or muco-purulent nasal discharge, especially when bacterial infections intervene, and impairment of scenting ability (i.e. hypo- or anosmia) **(Evinger et al., 1985; Campbell and Little, 1991; Piperisova et al., 2010; Baan et al., 2011; Veronesi et al., 2013)**. Moreover, the nematode has been recently incriminated as a cause of intracranial disease and meningoencephalitis due to aberrant migration **(Clark et al., 2013)**.

The lack of knowledge on nasal eucoleosis is mainly due to limitations inherent to diagnostic methods. The diagnosis of this disease relies either on the detection of the eggs in fecal samples or in nasal flushings, or *via* the direct rhinoscopic visualization of threaded nematodes **(Veronesi et al., 2014).** Indeed, *E. böhmi* eggs strongly resemble those of other trichuroids which infect companion animals. These overlapping features render the diagnosis complicated especially in the case of mixed infections (e.g. with *E. aerophilus*) and in absence of expertise **(Traversa et al., 2010; Di Cesare et al., 2013b; Veronesi et al., 2014)**.

Furthermore, the rhinoscopic examination requires general anesthesia, is expensive and time-consuming, and may be limited by the inaccessible location of the parasite in the caudal part of the nasal cavity, along with the presence of abundant mucus hampering a correct airways visualization **(Veronesi et al., 2014)**.

The characterization of the mitochondrial DNA (mtDNA) gene encoding for the cytochrome oxidase (*cox*1) 1 gene of *C. aerophila* recently provided powerful markers for the molecular diagnosis of lung capillariosis **(Di Cesare et al., 2013a).** Given the merit in answering several questions on basic and applied knowledge of nasal eucoleosis, the present work aimed at characterizing the *cox*1of *E. böhmi* and at assessing a mtDNA-based assay for new molecular studies on nasal eucoleosis.

2. Materials and methods

2.1 Samples

Single adult stages of *E. böhmi* were collected from red foxes in Norway (n. 2), Serbia (n. 6), Italy (n. 5) and from dogs in Italy (n. 5).

Individual fecal samples were collected from 14 naturally infected dogs diagnosed with nasal eucoleosis at the microscopic examinations of eggs in faeces or nasal flushings. In most cases, the diagnosis was confirmed at the rhinoscopic visualization of adult stages or at the detection of eggs in nasal flushings. In this latter case, eggs were identified by their morphological and morphometric features **(Di Cesare et al., 2012b)**. Out of these 14 dogs, 4 were positive for other trichurids including *E. aerophilus* (n. 3) and *Trichuris vulpis* (n.1) (Table 1). Stool samples from 20 dogs copromicroscopically negative for *E. böhmi*, but positive for other common endoparasites in both single and mixed infections (Table 2),), and DNA extracted from adult and/or immature stages of common dog parasites (i.e., *Ancylostoma caninum, Toxocara canis, T. vulpis, Dirofilaria immitis, Dirofilaria repens, Angiostrongylus vasorum, Taenia* spp. and *Dipylidium caninum*) were also used to assess the specificity of the genetic assay (section 2.2.3).

2.2 Molecular protocols

2.2.1 Characterization of a region internal to the cox1 gene of Eucoleus böhmi

Genomic DNA was individually extracted from each adult stage of *E. böhmi*. All DNA samples were subjected to a PCR specific for a 344-bp-long region internal to the *cox*1 gene using the degenerated set of primers Cox1NEMF and Cox1NEMR previously designed on the basis of Capillarinae Subfamily **(Di Cesare et al., 2012b)**. PCRs were carried out as previously described for *C. aerophila* **(Di Cesare et al., 2012b)** and amplicons were purified using a QIAquick® Gel Extraction Kit (Qiagen, GmbH, Hilden, Germany) and then sequenced directly using BigDye Terminator v.3.1 chemistry (Applied Biosystems, USA). Sequences were determined in both strands, aligned using BioEdit software 7.0 **(Hall, 1999)** and then compared with each other and with those of the Capillariinae *cox*1 available in GenBank using the Nucleotide–Nucleotide ‘‘Basic Local Alignment Search Tool’’ (BLAST; http://www.ncbi.nlm.nih.gov/BLAST).

2.2.2 Assessment of a semi-nested PCR for the specific identification of Eucoleus böhmi

The primer EboIntR (forward 5'- TTTTAGGTTTTCTTCTTTTATTT -3') was designed internal to the analyzed *cox*1region of *E. böhmi* following the criteria of Sharrocks (1994).

The ability of a semi-nested PCR protocol for the specific identification of a 324-bp-long fragment internal to the *cox* gene1 of *E. böhmi* was evaluated using the DNA extracts from adult nematodes (section 2.2.1). The primer set Cox1NEMF and Cox1NEMR was used in the first step, while the reverse primer EboIntR was used together with primer Cox1NEMF in the second step.

All PCR mixtures were prepared in 50 μl reaction containing 100 pmol of each primer in both steps, 4 μl of DNA extract in the first step and 4 μl of template (1:20) in the second step, 25 μl of AmpliTaq Gold (Applied Biosystem), adding distilled water provided by the same manufacturer. PCRs were performed in a thermal cycler (2700, Applied Biosystems, Foster City, CA, USA) using the following cycling protocol: 10 min at 95 °C, 40 cycles at 94 °C for 1 min, 48 °C (first step) or 50 °C (second step) for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. To check the presence of any contaminations, a negative control sample containing all the reaction reagents with sterile distilled water to substitute the template, was added to each PCR run. Amplicons were sequenced and sequences analyzed as described in section 2.2.1

2.2.3. Diagnostic efficiency of a semi-nested PCR specific for Eucoleus böhmi

Each fecal sample collected from dogs either positive or negative for *E. böhmi* (Tables 1 and 2) was subjected to a flotation technique as previously described to concentrate parasite elements **(Traversa et al., 2004).** An aliquot of 200 μl of supernatant for each sample was stored at -20 °C prior to molecular analysis. All supernatants were subjected to three freeze/thaw cycles (liquid nitrogen for 5′ and at 95 °C for 5′) and then to the genomic DNA extraction using a QiAamp DNA stool Mini Kit (Qiagen Gmbh, Germany). Stages of common canine endoparasites were processed for DNA extraction using GeneAll® ExgeneTM Tissue SV mini kit (GeneAll®, Biotechnology). All DNA extracts were subjected to the semi-nested PCR as in section 2.2.2. All generated amplicons were sequenced and sequences analyzed as above. Sequences obtained in this study have been deposited in GenBank database (GenBank accession numbers KR186213-KR186215).

3. Results

Neither insertions nor deletions in any of the sequences generated were found at the analysis of the 344 bp-long cox1 region from the single adults of E. böhmi (n. 18) and of the internal~324 bp-long amplicons generated from the faecal samples.

The molecular analysis of all PCR products confirmed (i.e., homology of ~99–100%) their identity as *E. böhmi* when compared with each other and with cox1 sequences obtained from adult nematodes previously identified at the species level and genetically characterized (Sections 2.2.1 and 2.2.2). Twelve out of the 14 faecal samples collected (85.14%, 95% IC 67.38–100%) from dogs with nasal eucoleosis and other trichurids scored positive (Table 1) using the semi-nested PCR for the expected amplicon. Two samples of dogs diagnosed with nasal eucoleosis were negative at the semi-nested-PCR, possibly due to a high presence of PCR-inhibitors. No PCR products were obtained when DNA extracted from faeces of dogs negative for *E. böhmi* or positive for other endoparasites, and from common dog parasites. when undertaken to the seminested assay.

The here assessed DNA-based assay proved to be able to provide the molecular diagnosis of nasal eucoleosis in naturally infected animals, despite a <100% sensitivity. A high specificity (100%) was achieved as species-specific amplicons were generated from faecal samples from dogs infected only by *E. böhmi* and also from samples containing *E. böhmi* eggs and eggs shed by closely-related trichuroids (i.e., *E. aerophilus*) and other common dog endoparasites (Tables 1 and 2).

4. Discussion

The direct observation of adult *E. böhmi* in situ via rhinoscopy has inherent hindrances rendering its applicability in clinical practice truly troublesome (**Veronesi et al., 2014**). Thus, a diagnosis may be usually achieved through confirmatory copromicroscopical findings based on the morphological and morphometric analysis of eggs in faecal samples. Despite this is crucial in confirming a clinical suspicion, the identification of the *E. böhmi* eggs may be problematical.

Eggs of *E. böhmi* in canine samples are at risk of misdiagnosis with those of the canine intestinal whipworm *T. vulpis* and the respiratory trichuroid *E. aerophilus*, which present overlapping features (**Traversa et al., 2010**). In fact, being a neglected parasite, in routine clinical practice, *E. böhmi* is not taken into account as a cause of respiratory distress and trichuroid eggs are mostly accounted to *T. vulpis*, due to the common wrong misconception that *T. vulpis* is the only nematode shedding barrel-shaped eggs in dog faeces (**De Liberato et al., 2009; Traversa et al., 2009, 2010; Di Cesare et al., 2012a,b; Traversa and Di Cesare, 2014**). In addition, cases of mixed infections by respiratory *E. böhmi* and *E. aerophilus* may occur more frequently than expected, especially where these parasites are endemic. This scenario is even more complicated by the occurrence, in the same animal, of the ubiquitous intestinal *T. vulpis* (**Di Cesare et al., 2012a,b; Veronesi et al., 2014**).

Since ‘80s cases of “nasal capillariosis” were attributed to *E. aerophilus* which, however, infect trachea, bronchi and bronchioles of animals (Evinger et al., 1985; King et al., 1990; Traversa et al.,

2010). Thus, it is clear that the closely-related species *E. aerophilus* (and likely *T. vulpis*) has been often mistaken with *E. böhmi* and misidentified in cases of nasal eucoleosis of dogs (**Campbell and Little, 1991; Conboy, 2009**).

Such hindrances have greatly impacted on our knowledge on the geographic distribution of the parasite. In fact, the vast majority of information on the presence of *E. böhmi* from the nasal cavity of naturally infected dogs come from necroscopic reports (**Campbell and Little, 1991; Schoning et al., 1993**), while only few reports have described the presence of the adult stages in living symptomatic dogs (**Evinger et al., 1985; Baan et al., 2011; Veronesi et al., 2013**).

Thus, the molecular assay here proposed may indeed contribute obtaining new epidemiological insights on this little-known parasite.

From the epidemiological perspective, although *E. böhmi* is considered an occasional parasite, recent reports have suggested a rise in the infection of companion dogs in both the Americas and Europe (**Piperisova et al., 2010; Baan et al., 2011; Di Cesare et al., 2012a,b; Magi et al., 2012; Clark et al., 2013; Veronesi et al., 2013, 2014**).

A recent trial carried to evaluate the efficacy of a parasiticide formulation in the treatment of nasal eucoleosis showed an infection rate of 6.6% in ~300 dogs from Italy (**Veronesi et al., 2014**). Therefore, the possibility that *E. böhmi* is another respiratory nematode of companion animals which is potentially emerging in several territories (**Traversa et al., 2010; Veronesi et al., 2014**) should be taken into a proper account.

Changes in the phenology of wildlife is a compelling factor that is currently spurring an overflow of respiratory parasites from wild reservoirs to domestic hosts. For instance, this is the case of *Echinococcus multilocularis* (**Hegglin et al., 2015**), *A. vasorum* (**Elsheikha et al., 2014**), *Troglostrongylus brevior* (**Traversa and Di Cesare, 2014**), and *E. aerophilus* (**Di Cesare et al., 2014**). Interestingly, the use of a similar PCR assay provided a genetic make-up of *E. aerophilus* in different hosts and countries, showing that some parasite populations are shared between wild and domestic animals (**Di Cesare et al., 2014**). Similar studies for *E. böhmi* would be insightful for elucidate patterns of transmission in endemic areas.

This molecular assay would also assist in filling gaps into the biology of the parasite. An appropriate knowledge of *E. böhmi* life cycle, including timing and modalities of the exogenous development and the biological role of earthworms and/or paratenic hosts, is crucial toward efficacious control measures to prevent common re-infections and frequent recurrence of the disease after anthelmintic treatments (**Baan et al., 2011; Veronesi et al., 2013**).

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Table 1
PCR positivity (+) or negativity at a species-specific semi-nested PCR of individual fecal samples collected from 14 naturally infected dogs diagnosed infected by *Eucoleus böhmi* and other trichurids at the microscopic examinations of faeces.

Sample	PCR	Other parasites
1	+	–
2	+	–
3	+	–
4	–	*Eucoleus aerophilus*
5	+	–
6	+	*Trichuris vulpis*
7	+	*Eucoleus aerophilus*
8	+	–
9	+	–
10	–	–
11	+	–
12	+	*Eucoleus aerophilus*
13	+	–
14	+	–

Table 2
Individual fecal samples collected from 20 naturally infected dogs negative for *E. böhmi*, but positive for other common parasites in both single and mixed infections.

Sample	**Nematodes identified**
1	Ancylostomatids
2	Ancylostomatids
3	Ancylostomatids
4	Ancylostomatids, *Eucoleus aerophilus*
5	Ancylostomatids, *Eucoleus aerophilus*
6	*Eucoleus aerophilus*
7	*Eucoleus aerophilus*, *Trichuris vulpis*
8	*Eucoleus aerophilus*, *Trichuris vulpis*
9	*Toxocara canis*
10	*Toxocara canis*
11	*Toxocara canis*
12	*Toxocara canis*
13	*Toxocara canis*, Ancylostomatids
14	*Toxocara canis*, Ancylostomatids
15	*Toxocara canis*, Ancylostomatids

16	*Toxocara canis*, Ancylostomatids
17	*Toxocara canis*, Ancylostomatids
18	*Toxocara canis*, Ancylostomatids
19	*Toxocara canis*, *Trichuris vulpis*
20	*Toxocara canis*, *Trichuris vulpis*