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SHORT COMMUNICATION

First morphological and molecular analysis of *Eucoleus boehmi* like eggs in dogs from Argentina

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Abstract
The canid parasites *Eucoleus aerophilus* (syn. *Capillaria aerophila*) and *Eucoleus boehmi* (syn. *Capillaria boehmi*) parasitize the lower and the upper respiratory tract, respectively. Reports and descriptions of these nematodes are scarce in Argentina**,** possibly due to misdiagnosis of morphologically similar trichuroids eggs, and the lack of knowledge about the species of *Eucoleus* in this geographical area. Scanning electron microscopy is a useful tool for identification of *E. boehmi* eggs based on the characteristics of the shell structure which differentiate between species. Molecular analysis complements morphological identification. Until now, there are no studies based on the analysis of *E. boehmi* eggs in Argentina. The aim of the present work was to study by morphological, morphometric, and molecular analysis, eggs attributable to *E. boehmi* isolated from dogs naturally infected in Mar del Plata city, Argentina. Eggs isolated from two dog fecal samples were analyzed by light and scanning electron microscopy. A fragment of mitochondrial DNA (mtDNA) of the cytochrome c oxidase subunit I gene (*cox1*) from eggs was sequenced, and phylogenetic analysis was performed in this study. According to morphological results based on the wall surface ultrastructure, the eggs studied were assigned to *E. boehmi*. Molecular analysis supported the morphological identification. The divergence of 9–12% with the European isolated could suggest a new geographical genetic variation of *E. boehmi*, but also question the possible existence of cryptic species. This is the first characterization of *E. boehmi* eggs in dogs from Argentina.

Keywords *Eucoleus boehmi* eggs · Scanning electron microscope · *cox 1* gene · Argentina

Introduction

The Capillariidae family (Neveu-Lemaire 1936) is a very large group of nematode parasites (Order: Trichinellida) whose taxonomy has not been fully defined as yet. There are about 300 species described within this group parasitizing a wide range of vertebrate hosts (fishes, amphibians, reptiles, birds, and mammals) (Moravec et al. 1987). *Eucoleus* (Dujardin 1845) belongs to this family and includes parasites of respiratory organs, mucosa of the esophagus, mouth cavity, and stomach of birds and mammals (Moravec et al. 1987). The canid parasites *Eucoleus aerophilus* (syn. *Capillaria aerophila*) and *Eucoleus boehmi* (syn. *Capillaria boehmi*) parasitize the lower and the upper respiratory tract, respectively (Campbell and Little 1991). Studies based on these nematodes have recently increased due to their emergence in several European and North American countries (Conboy 2009; De Liberato et al. 2009; Traversa et al. 2010; Di Cesare et al. 2012a; Magi et al. 2012).

Eucoleus aerophilus (Creplin 1839) has a widespread distribution in North and South America, Europe, Russia, Asia, North Africa, and Australia. This species has been reported in several countries from Europe infecting canids, felids, and mustelids. Occasionally, it may cause infection in humans (Lalošević et al. 2008; Traversa et al. 2010; Nithikathkul et

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al. 2011). Adult stages live embedded under the epithelium of trachea, bronchi, and bronchiole. This lungworm may cause intense respiratory distress characterized by irritation, increased mucosal secretion, and constriction of the lumen of the respiratory passages. The symptoms can be rhinitis, tracheitis, bronchitis, and bronchopneumonia (Traversa et al. 2010; Di Cesare et al. 2012a). Animals become infected by ingesting larvated eggs, but the ingestion of earthworms could act as a facultative intermediate or paratenic hosts (Radman et al. 1986).

Eucoleus boehmi (Supperer 1953) has been reported in dogs from Italy, Portugal, and USA and in red foxes from Italy, Denmark, and Austria (De Liberato et al. 2009; Magi et al. 2012; Al-Sabi and Kapel 2013; Clark et al. 2013; Veronesi et al. 2014; Alho et al. 2016; Hodžić et al. 2016; Cervone et al. 2017). This parasite is found in the nasal turbinates, frontal and paranasal sinuses of canids. Despite a relevant pathogenic potential, *E. boehmi* is a neglected and underestimated cause of upper respiratory disease. Animals with high parasite burden may show varying clinical signs, such as cough, sneezing, reverse sneezing, wheezing, epistaxis, mucopurulent nasal discharge with blood, and hypo-or anosmia (Conboy 2009; Traversa et al. 2010; Veronesi et al. 2014; Di Cesare et al. 2015; Alho et al. 2016). Moreover, infection with this parasite has been recognized as a potential cause of chronic meningoencephalitis and consequently convulsive seizures in dogs (Clark et al. 2013). The life cycle of *E. boehmi* is still unknown, but several studies have shown that animals become infected by ingestion of eggs containing the infective larvae or by ingestion of infected earthworms as in *E. aerophilus* (Radman et al. 1986; Campbell and Little 1991; Conboy 2009).

Reports and descriptions of these nematodes are scarce in Argentina. This could be related to the misdiagnosis due to confusion with morphologically similar trichuroid eggs and the lack of knowledge about the species of *Eucoleus* in this geographical area. Epidemiological studies about zoonotic parasites have reported eggs assigned to *E. aerophilus* in dog feces and soil samples, based on the morphology of eggs by light microscopy (LM) (D' Oliveira de Podestá 1969; Venturini and Radman 1985; Soriano et al. 2010; Dopchiz et al. 2013). Radman et al. (1986) tested experimentally the life cycle of this parasite, obtaining adult worms through the infection of dogs with larvated eggs and the administration of infected earthworms. In Mar del Plata city, studies have reported the presence of eggs assigned to this parasite in dog fecal samples (Madrid et al. 2005; Lavallén et al. 2011). With regard to *E. bohemi* in Argentina, a unique case of respiratory capillariosis attributable to this parasite has been reported. Eggs were isolated from feces and nasal discharge of a dog and characterized by LM (González et al. 2014).

The morphological characters are very similar for both *E. aerophilus* and *E. boehmi*. They differ in the target organ during host infection and in the features of the superficial sculpture of the eggs (Campbell and Little 1991; Moravec 2000). In that sense, specific diagnosis of these parasites can be challenging due to the similar morphology and overlapping size of eggs. Besides, the difficulty in recovering adult specimens and their variable clinical presentations cause the misdiagnosis of these capillariids, thus underestimating their real worldwide prevalence (Alho et al. 2016).

Because of diagnosis problems, molecular tools provide a useful method for identification of capillariid eggs complementing morphological identification. The analysis of a fragment of mitochondrial DNA (mtDNA) of the *cytochrome c oxidase subunit I* gene *(cox1)* has produced genomic information to identify *E. aerophilus* and *E. boehmi* in European countries (Di Cesare et al. 2014, 2015).

Until now, there are no studies based on the analysis of *Eucoleus* in Argentina. The aim of the present work was to study by morphological, morphometric, and molecular analysis, eggs attributable to *Eucoleus* isolated from dogs naturally infected in Mar del Plata city, Buenos Aires province, Argentina.

Materials and methods

Eucoleus like eggs were isolated from two dog fecal samples to develop morphological and morphometric analysis and molecular studies. These fecal samples were previously studied by coproparasitological methods, as part of an epidemiological research about zoonotic parasites in relation to socio-environmental conditions in a peripheral neighborhood from Mar del Plata city (Lavallén 2016). In this study, a total of 306 dog fecal samples had been recovered from the yard of 108 households, taking into account the number of dogs present in each one.

Morphological and morphometric analysis

A fraction of both samples stored in 10% *v*/*v* formaldehyde were processed through the Ritchie modified sedimentation technique (Méndez 1998). Briefly, 2 ml of the fecal sample was sieved through a 300-μm mesh and transferred to a 15-ml tube. Then, the samples were washed twice with 8 ml of 10% *v*/*v* formaldehyde shaking the samples energetically and centrifuged at 1030×*g* for 3 min. A final wash was performed with 6 ml of formaldehyde 10% and 2 ml of ethyl ether followed by centrifugation. Pellets were observed by LM equipped with micrometric eyepiece (Arcano XSZ 107), at 10× and 40× magnifications. Twenty-seven eggs were morphologically and morphometrically characterized. The rest of the pellet was washed twice in 0.1× PBS and distilled water followed by centrifugation at 1030×*g* for 3 min. This pellet was examined by LM and the eggs observed were manually isolated by Pasteur pipettes and transferred to a 30-μm mesh sieve. The sieve was mounted on a metallic slide and covered with gold particles (100 Å) and examined by SEM (Jeol-JSM-6460LV). Differential diagnosis of *Eucoleus* sp. eggs was based on egg size, shape and color, plug shape, embryo, and the surface structure of the outer egg shell (Campbell and Little 1991; Moravec 2000).

Molecular analysis

Eggs were obtained from fractions of both samples stored at − 20 °C. Egg isolation and molecular analysis were performed according to Petrigh et al. (2015). Briefly, the eggs were isolated manually by LM using a capillary tube and preserved in 2 μl of sterile 1× PBS at − 20 °C until use. A pool of each sample containing 6–13 eggs was processed for molecular analysis. DNA was extracted according to Petrigh et al. (2015) with modifications. Briefly, eggs were immersed in 50 μl of compatible lysis-PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.8, 0.4% *v/v* Nonidet P-40, 0.8% *v/v* Tween 20, 2.5 mM $MgCl_2$). The samples were digested with 400 μg/ml of Proteinase K (Biobasic) for 2 h at 56 °C and then at 37 °C overnight and boiled for 15 min for Proteinase K inactivation. Sterile ultrapure water was added to dilute proteinase K in the samples and to reduce PCR inhibitory concentrations. Subsequently, samples were centrifuged at 6700×*g* for 5 min. The supernatants were kept at − 20 °C until use. A negative control of DNA extraction was included.

A ~ 344 bp fragment of *cox1* gene (Di Cesare et al. 2012a) was used for molecular identification of capillariid eggs. PCR reaction was performed in 25 μl containing 2 μl of DNA sample, 200 μM of each dNTP (Thermo Scientific), 0.4 μM of each primer (Cox1NEMF 5′-CCTGAGGTTT ATATTYTWRTT-3′ and Cox1NEMR 5′-CCTG TTARRCCTC CRATACT-3′) (Di Cesare et al. 2012a), 0.65 units of GoTaq DNA Polymerase (Promega) in 1× Go Taq Buffer green and 3 mM of $MgCl_2$. The PCR conditions were as follows: an initial denaturation step (94 °C for 3 min), 40 cycles at 94 °C for 1 min (denaturation), 50 °C for 1 min (annealing), 72 °C for 1 min (extension), and 72 °C for 10 min (final extension). The negative control was included in all PCR experiments.

Specific DNA fragments were sequenced and chromatograms were analyzed using BioEdit v7.2.0 (copyright © 1997–2013, Tomm Hall, Ibis Biosciences). The consensus sequences obtained were compared with the GenBank sequences by using the BLASTN algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the National Center for Biotechnology Information (NCBI, USA).

Phylogenetic analysis of 229 bp *cox1* fragments obtained in this work, and *E. boehmi* and *E. aerophilus* sequences from GenBank, was performed by the neighbor joining test (MEGA 7 program) (Kumar et al. 2016). *Trichinella spiralis* *cox1* sequence (Accession number-AN-: KF241631.1) was used as outgroup.

Results and discussion

The morphological and morphometric analysis of the 27 eggs evidenced features and dimensions attributable to capillariid nematodes. The eggs were ellipsoidal, bipolar plugged, and clear to golden in color. The polar plugs were asymmetrically positioned in relation to the axis of the eggs, the multicellular embryo did not fill the eggs, and the shell was pitted giving a porous appearance. The mean length of the eggs was 60.7 ± 3.3 μm, while the mean width was 30.1 ± 1.5 μm (Fig. 1). According to the observed features and the available morphological and morphometric identification keys, eggs from dogs were assigned to the *Eucoleus* genus (Magi et al. 2012; Al-Sabi and Kapel 2013; Alho et al. 2016).

Taxonomy of capillariids based on morphological features is controversial. Diagnosis through egg morphometry is especially difficult; hence, misdiagnosis among different trichuroid species could occur (Moravec 2000; Conboy 2009). Because the size range reported in the literature was markedly overlapped between *E. aerophilus* and *E. boehmi*, other morphological features should be used to differentiate between them (Al-Sabi and Kapel 2013). The ornamentation of the eggs, such as thickness and structure of the surface layer, is frequently species specific (Moravec 2000). In that sense, SEM is useful to highlight detailed characteristics of the egg shell structure which differentiate between species (Magi et al. 2012). Eggs of *E. boehmi* show several pits of different sizes, which are generally round to oval and irregularly scattered, which differ from the network of anastomosing ridges and bridges of *E. aerophilus* eggs (Campbell and Little 1991; Conboy 2009; Traversa et al. 2010; Magi et al. 2012). In the present study, microscopic images obtained with SEM confirmed the pitted appearance of the shell, which is a distinctive characteristic of *E. boehmi* eggs (Fig. 2). Therefore, the morphological characters of eggs studied in the present work were similar to those described for eggs of *E. boehmi* from Europe (De Liberato et al. 2009; Traversa et al. 2010; Magi et al. 2012; Al-Sabi and Kapel 2013; Hodžić et al. 2016; Cervone et al. 2017). Results of molecular analysis showed an identical 344-bp fragment of *cox1* gene to eggs isolated from both dog fecal samples. BLASTN analysis showed a 91 and 88% of identity with two sequences of adult stage of *E. boehmi* (AN: KX027314.1 and KR186215.1). An identity range of 87–89% was reached between *cox1* sequences from this work and *E. aerophilus* sequences (different haplotypes) from GenBank. Until now, 15 haplotypes have been reported in *E. aerophilus* from Europe and one from Canada with an intraspecific nucleotide difference ranging from 0.4 to 5.1% (Di Cesare et al. 2014). However, haplotypes have not been

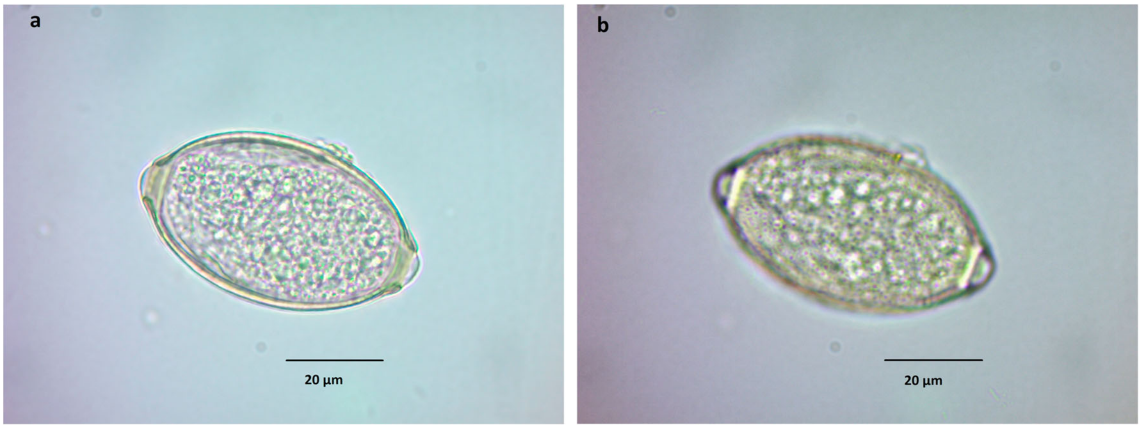

Fig. 1 Egg of *Eucoleus boehmi* at light microscopy: **a** the polar plugs are asymmetrically positioned and there is a space between the multicellular embryo and the shell. Original magnification 100×/scale bar: 20 µm; **b** by fine adjustment focusing on the outer shell plane, the surface gives a porous appearance. Original magnification 100×/scale bar: 20 µm

described yet in *E. boehmi* isolates. Even though ultramicroscopy images obtained for Argentinian *Eucoleus* eggs shell showed a pattern similar to *E. boehmi* eggs, the existence of cryptic species has been reported in nematodes (Anderson et al. 1998; Blouin 2002). Also, a 10–20% range of variation between closely related congeneric species of nematodes has been observed (Blouin 2002). The 9–12% of divergence between *Eucoleus cox1* sequences from the present work and European *E. boehmi* isolates could be due to geographical variation; a divergence of 10% or more could question whether they are the same species (Blouin et al. 1998). *Eucoleus* eggs characterized in this work create a question as to which species of *Eucoleus* circulate in canines of this region. On the other hand, phylogenetic analysis showed a well-supported separation in two clades of *E. aerophilus* and *E. boehmi cox1* sequences. Sequences from eggs of this work grouped with the *E. boehmi* clade, suggesting that eggs isolated from Argentinian dog feces are closely related with *E. boehmi* (Fig. 3). Other mitochondrial genes or the whole mitochondrial genome must be analyzed to confirm this preliminary phylogenetic analysis.

The samples analyzed for *Eucoleus* also evidenced eggs that were attributed to *Trichuris vulpis* in one sample, and to *Calodium hepaticum* (syn. *Capillaria hepatica*) in the other one. *Trichuris vulpis* is one of the most recognized nematode parasite of canines. The eggs are brown-yellowish, symmetrical in shape with prominent plugs with a ring-like thickening at the base, a smooth surface and larger than the capillariid eggs (Campbell 1991; Traversa et al. 2011). Although the morphological features are substantially different from *Eucoleus* eggs, it is a common misconception that this parasite is the only nematode shedding ellipsoidal shaped eggs in dog feces (De Liberato et al. 2009; Traversa et al. 2010). Even more, the risk of misidentification is high in the presence of mixed infections (Di Cesare et al. 2012b). The eggs with features compatible with *C. hepaticum* were yellowish-brown, barrel shaped, with shallow polar plugs and radial striations (Grigonis and Solomon 1976). This parasite infects the

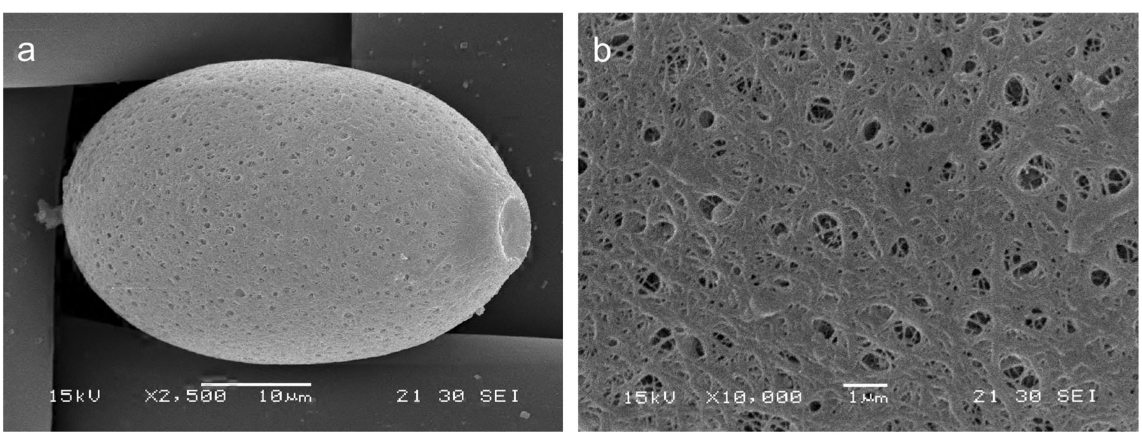

Fig. 2 Egg of *E. boehmi* at scanning electron microscopy: **a** overall view of the outer shell with irregularly distributed small pits. Original magnification 2500×/scale bar: 10 µm. **b** Detail of the dense network with a fine mesh, surrounding the small pits. Original magnification 10,000×/scale bar: 1 µm

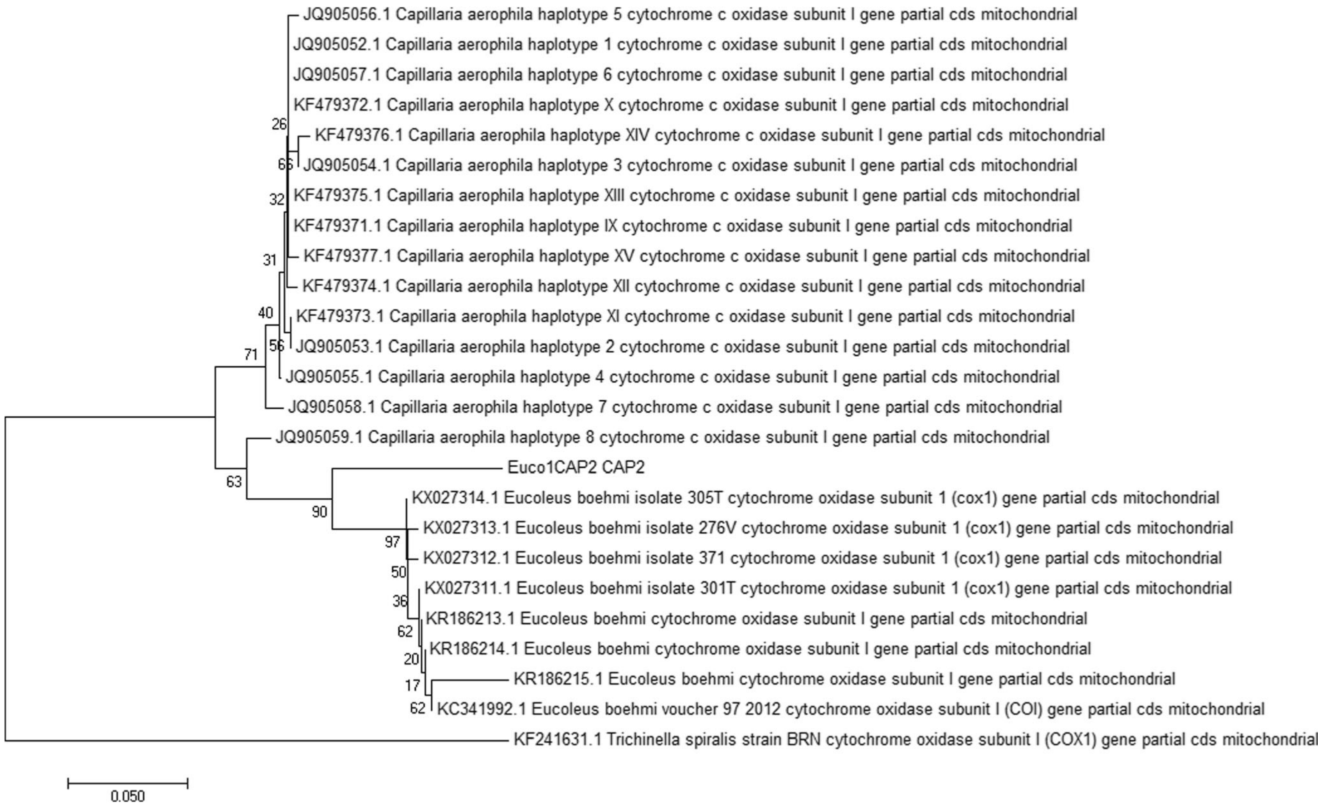

Fig. 3 Phylogenetic analysis based on *cox1* gene fragment. The evolutionary history was inferred using the neighbor-joining method. Bootstrap test of 1000 replicates. The evolutionary distances were computed using the maximum composite likelihood method. Outgroup: *Trichinella spiralis* (AN: KF241631.1)

hepatic parenchyma principally of rodents, and other mammals such as carnivores and humans (Piazza et al. 1963). In dogs, it could cause spurious infections through the ingestion of the definitive host and the release of eggs in the feces (Queiroga Gonçalves et al. 2012). The presence of these trichuroids added to the similar features between *E. aerophilus and E. boehmi* eggs could generate confusion in the coproparasitological specific diagnosis (Traversa and Di Cesare 2014; Di Cesare et al. 2012b). In that sense, the presence of respiratory capillariids in canids was likely to be underestimated in previous studies developed in Argentina.

The epidemiological study of Lavallén (2016) that included the fecal samples analyzed showed that families who raised farm animals such as pigs, practiced poor hygiene habits, among other precarious habitational characteristics. This situation could lead to the presence of small rodents. The finding of *C. hepaticum* in one of the dog fecal samples would be due to a spurious infection after eating small rodents, carcasses, or infected liver of mammals (Lubinsky 1956). However, from the overall sampling of 306 analyzed feces in the study area, only 2 samples presented eggs attributable to *C. hepaticum*, while 84 samples showed eggs assigned to the *Eucoleus* genus (Lavallén 2016). Furthermore, the fact that the definitive host reported to *E. boehmi* were domestic and wild canids (Campbell and Little 1991; Hodžić et al. 2016; Cervone et al. 2017), would indicate that *E. boehmi* was causing a true infection.

The awareness regarding the possible presence of different species of capillariid nematodes in dogs is essential for an accurate diagnosis (Magi et al. 2012). The infection caused by these parasites is not generally associated to clinical signs in dogs (Di Cesare et al. 2015), which would explain the existence of a unique case of nasal capillariosis assigned to *E. boehmi* in Argentina. Although these parasitoses are considered uncommon, veterinarians should include these diseases in the differential diagnosis of cardiopulmonary affections (Traversa et al. 2010; Magi et al. 2012; Cervone et al. 2017).

Based on ultrastructural features of the wall surface, the eggs studied in this work were assigned to *E. boehmi*. Molecular data closely associated these eggs with the *Eucoleus* genus with a 91–88% of identity with European *E. boehmi* isolates. The assignment of these *Eucoleus* eggs to *E. boehmi* must be confirmed with further studies. In this sense, morphological and molecular studies will be performed with a higher number of egg samples isolated from domestic and wild canids from this region.

The present work reported the first morphological and molecular analysis of *E. boehmi* eggs in dogs from Argentina. To improve taxonomic classification of *E.*

aerophilus and *E. boehmi* from Argentinian dogs, a morphologic and genetic analysis of the adult stage is necessary.

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