



# Survey and first molecular characterization of *Echinococcus granulosus* sensu stricto (G1) in Pampas fox (*Lycalopex gymnocercus*) in Buenos Aires province, Argentina



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## ABSTRACT

Echinococcosis is a zoonosis caused by tapeworms of the genus *Echinococcus*. *Echinococcus granulosus* sensu lato (s. l.) has a world-wide distribution and its transmission is primarily maintained in a synanthropic cycle with dogs as definitive hosts and livestock species as intermediate hosts. However, many wild canids also function as definitive hosts for *E. granulosus* s. l. Echinococcosis in humans is mainly caused by *E. granulosus* sensu stricto (s. s.) G1 genotype. In the present work, we expanded the epidemiological study on echinococcosis reported cases in Pampas fox (*Lycalopex gymnocercus*) to provide a prevalence estimate for rural areas of southern Buenos Aires province, Argentina. Ninety-five whole intestines were analyzed using the sedimentation and counting technique with a result of 83 foxes (87.37%) harboring at least one helminth species. *E. granulosus* s. l. adults were found in one Pampas fox (1.05%). These adult helminthes were *E. granulosus* s. s. (G1) according to the genotyping analysis of a 450-bp region of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene.

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## 1. Introduction

Tapeworms of the genus *Echinococcus* (phylum Platyhelminthes, class Cestoda) are important parasites of mammals that cause life-threatening diseases called echinococcosis. The adult worm lives in the small intestine of suitable domestic and wild carnivores (canids, felids and hyenids) and their eggs are excreted with the faeces of these animals with the subsequent contamination of the environment. Susceptible intermediate host species (ungulate and rodent) ingest infective eggs, after which a larval stage develops. Humans are an aberrant or “dead-end” host that does not play a role in the natural cycle of the parasite. The larval stage may cause severe illness and even death in the intermediate host (Jenkins et al., 2005).

Based on recent molecular and phylogenetic evidence, the *Echinococcus* genus comprises nine valid species including *E. granulosus* sensu stricto (*E. granulosus* s. s. genotypes G1–G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6–G10), *E. felidis*, *E. multilocularis*, *E. oligarthrus*, *E. shiquicus*, and *E. vogeli* (e.g., Nakao et al., 2007; Thompson, 2008). All the studies that used several molecular markers have contributed to define genetic variation in *Echinococcus* genus. A marker widely used is a 450 bp fragment of the cytochrome c oxidase subunit 1 (*cox1*) gene (Bowles et al., 1992).

*Echinococcus* species/genotypes have marked differences regarding host infectivity and specificity, geographical distribution, zoonotic potential, development, and pathogenicity. *Echinococcus granulosus* s. s. and *E. multilocularis* are the most clinically relevant species involved in human infections causing cystic echinococcosis (CE) and alveolar echinococcosis (AE), respectively (McManus, 2013).

Several species of *Echinococcus* are distributed along the Americas. *Echinococcus multilocularis* is present in Alaska, Canada, and much of the North-Central and Midwestern United States (Kazacos,

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2003; Moro and Schantz, 2006). The sylvatic transmission cycle is maintained between coyotes and foxes, which act as definitive host species, and a number of small rodents such as voles, mice, and muskrats, which serve as intermediate host species (Catalano et al., 2012). With *E. canadensis* (G8–G10), other sylvatic cycles occur in North America, where the parasite is primarily transmitted between wolves and large wild ungulate (cervid) species (Kazacos, 2003). Domestic dogs may become infected through accidental consumption of fertile hydatid cysts within the viscera of affected cervids and serve as source of infection to humans (Himsworth et al., 2010). *E. granulosus* s. l. is highly endemic in many areas of South America (Cardona and Carmena, 2013; Alvares Rojas et al., 2014). The infection is essentially maintained in domestic cycles and involves dogs and a number of livestock species (Cardona and Carmena, 2013; Carmena and Cardona, 2013); however, there are scarce data regarding the role of wildlife animals in the ecology of *Echinococcus* (e.g., Aguilera, 2001; Zanini et al., 2006). Regarding Neotropical species of *Echinococcus*, *E. vogeli* and *E. oligarthrus* are indigenous to the humid tropical forests in central and northern South America. Both species are primarily transmitted in sylvatic cycles with bush dogs and wild cats as definitive host species, and pacas and agouties as main intermediate hosts, respectively (D'Alessandro and Rausch, 2008).

In Argentina, *E. granulosus* s. l. is present in several wild definitive hosts as culpeo fox (*Lycalopex culpaeus*), gray fox (*L. griseus*) and Pampas fox (*L. gymnocercus*) (Szidat, 1963; Blood and Lelijveld, 1969; Schantz et al., 1972; Zanini et al., 2006). Furthermore, *Echinococcus* hydatid cysts assigned to *E. vogeli* have been recently confirmed in a paca from the province of Misiones in the North-eastern corner of the country (Vizcaychipsi et al., 2013).

*Lycalopex gymnocercus* (Fischer, 1814), commonly called the Pampas fox (Lucherini and Vidal, 2008), is the most abundant wild canid from South America; it inhabits grasslands and open woodlands as well as areas highly modified by extensive ranching and agricultural activities (Lucherini et al., 2004). This canid is an omnivorous predator with an opportunistic behavior and its dietary items vary according to seasonal availability and geographic location (Farias and Kittlein, 2008).

In the present work, we expanded the epidemiological study on echinococcosis in *L. gymnocercus* reported by Scioscia et al. (2013) to provide a prevalence estimate for rural areas of southern Buenos Aires province, Argentina. For this purpose, by using molecular tools, we genotyped *E. granulosus* s. l. adults detected in one Pampas fox.

## 2. Materials and methods

The study was conducted in rural areas located in seven departments of southern Buenos Aires province, Argentina, which encompassed the ecoregions El Espinal (southwest—El Caldén sub-region) and La Pampa (southcentral and southeast) (Burkart et al., 1999) (Fig. 1). The study area is currently dominated by cattle farming and agricultural activities and it is home to high densities of Pampas foxes.

Ninety-five (44 females and 51 males) whole intestines from roadkilled *L. gymnocercus* and other dead animals provided by licensed hunters from 2010 through 2013 were analyzed. Sixty-one of these Pampas foxes were part of a previous study (Scioscia et al., 2013). In this work, we include other 34 Pampas foxes. All intestines (from the 95 Pampas foxes) were examined and fecal content from the rectum was sampled for coproparasitological and molecular analyses. The sample collection and transport was allowed by the Ministerio de Asuntos Agrarios and Dirección de Flora y Fauna of Buenos Aires Province. All analyzed Pampas foxes were adults.

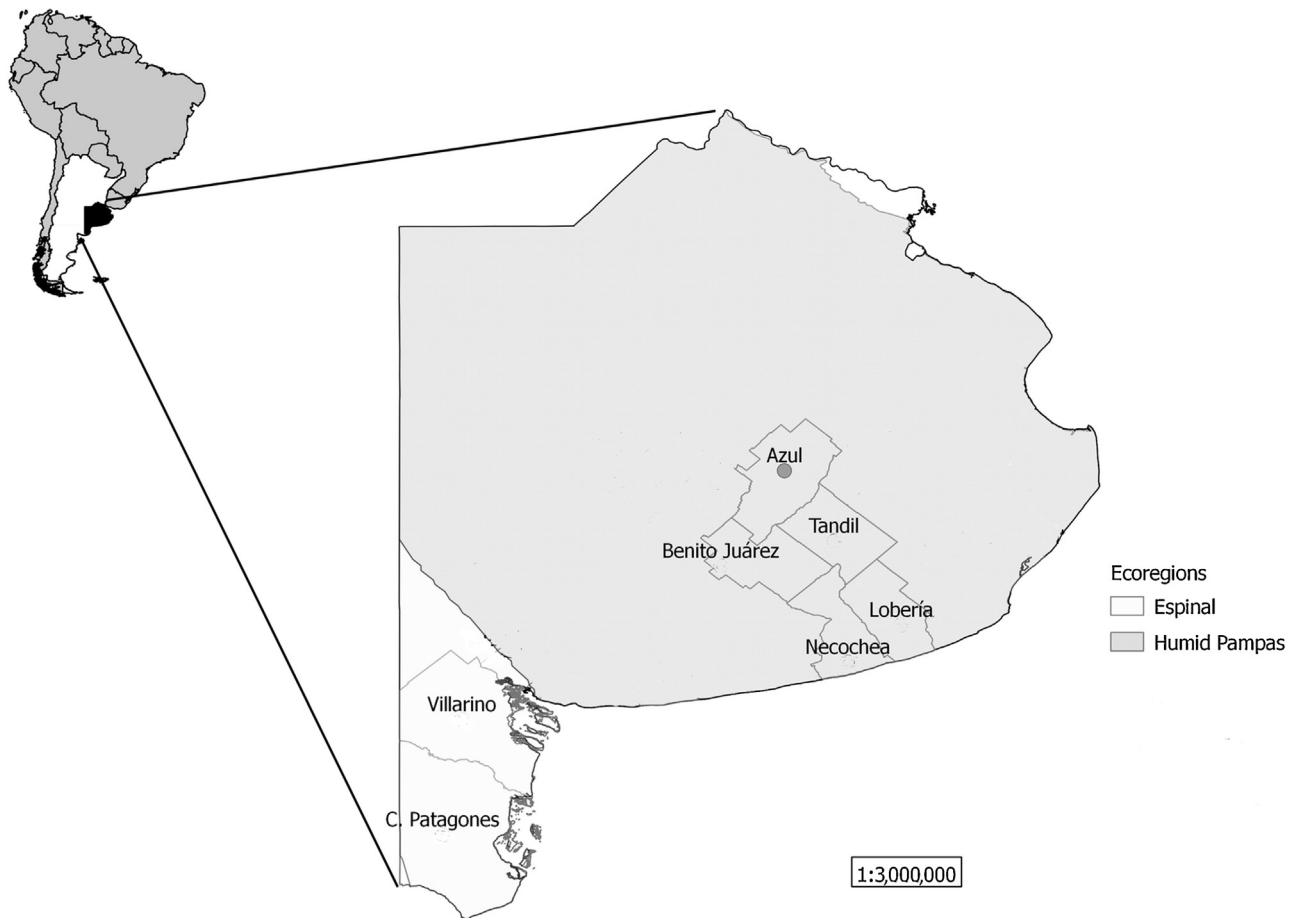
All samples were kept at  $-20^{\circ}\text{C}$  for at least 1 month prior to processing in order to inactivate *Echinococcus* spp. eggs. The examination of the intestinal content was performed using the sedimentation and counting technique (SCT) described by Eckert et al. (2001) with modifications described by Scioscia et al. (2013). All *Echinococcus* spp. were washed in saline solution and then some samples were preserved in ethanol 70% for molecular studies. The remaining samples were preserved in formol 3% until stained with hydrochloric carmine, dehydrated in a series of alcohols ( $70^{\circ}$ ,  $85^{\circ}$ ,  $96^{\circ}$  and  $100^{\circ}$ ), cleared in beechwood creosote and mounted in Canada balsam. The morphometric data were expressed in micrometers. Small fecal aliquots were separated for Copro-ELISA test (Pierangeli et al., 2010; Scioscia et al., 2013). The remainder of each sample was concentrated by Ritchie sedimentation (Young et al., 1979) and Sheather flotation (Benbrook and Sloss, 1965) methods and subsequently examined microscopically (by duplicate) for taeniid egg identification (Soulsby, 1987).

Three different adult samples from *E. granulosus* s. l. were individually processed for molecular analysis. DNA was extracted according to Petrih and Fugassa, (2013) with some modifications. Briefly, proglottids were immersed in 25  $\mu\text{l}$  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  $\text{MgCl}_2$ ). The samples were digested with 400  $\mu\text{g}/\text{ml}$  of Proteinase K (Biobasic) for 2 h at  $56^{\circ}\text{C}$  and then at  $37^{\circ}\text{C}$  overnight (maximum activity at  $37^{\circ}\text{C}$ ). Subsequently, these samples were boiled for 15 min for Proteinase k inactivation and 75  $\mu\text{l}$  of sterile ultrapure water was added. Finally, they were centrifuged at 4000g for 5 min and the supernatants were kept at  $-20^{\circ}\text{C}$  until use. A negative control of DNA extraction was included. A 450-bp region of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene was amplified using already available primers: JB3 5' TTTTGGGCATCCTGAGGTTTAT 3' and JB4, 5' TAAAGAAA-GAACATAATGAAAATG 3' (Bowles et al., 1992). PCR was performed in 25  $\mu\text{l}$  final volume containing 2  $\mu\text{l}$  of DNA sample, 200  $\mu\text{M}$  of each dNTP (ThermoScientific), 0.4  $\mu\text{M}$  of each primer and 0.65 units of Go Taq DNA Polymerase (Promega) in 5X Go Taq Buffer containing 1.5 mM of  $\text{MgCl}_2$ . The PCR conditions were as follows: an initial denaturation step ( $94^{\circ}\text{C}$  for 3 min), 40 cycles at  $94^{\circ}\text{C}$  for 45 s (denaturation),  $50^{\circ}\text{C}$  for 45 s (annealing), and  $72^{\circ}\text{C}$  for 45 s (extension), and  $72^{\circ}\text{C}$  for 10 min (final extension). A negative control was included in all PCR experiments. Triplicates of the specific fragment were sequenced in forward and reverse sense (JB3 and JB4, 5 primers) and chromatograms were analyzed using BioEdit v7.2.0 (copyright © 1997–2013, Tom 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).

## 3. Results

In the present work, 83 Pampas foxes (87.37%) had at least one intestinal helminth species in the postmortem exam. In the small intestine, the overall prevalences of nematodes, cestodes, and trematodes were 57%, 40% and 46%, respectively. In addition, 5% of the large intestines contained only nematodes.

All fecal samples (34) were negative to Taeniidae eggs by using concentration techniques and Copro-ELISA. However, ten adults of the genus *Echinococcus* were collected in small intestine of one Pampas fox male from Azul departament corresponding to La Pampa ecoregion (1.05% of prevalence) (Fig. 1). The internal structures of the proglottids could not be observed because of the advanced state of disintegration caused by long time freezing. The external morphology (length 3 mm) and hook measures ( $25 \times 12.5 \mu\text{m}$ ,  $n=2$ ) coincided with *E. granulosus* s. l. species.



**Fig. 1.** Sampling sites in Buenos Aires Province, Argentina. The circle indicates the departament where *Lycalopex gymnocercus* infected with *Echinococcus granulosus* sensu stricto was found.

A 450-bp specific fragment of the *cox1* gene was amplified and sequenced in the three parasite samples. The 431-bp consensus sequence obtained was identical in all analyzed *E. granulosus* adults. A BLASTN analysis showed  $\geq 99\%$  of identity with sequences of *E. granulosus* s. s. G1 genotype. The sequence was submitted to the NCBI (AN: KT446001.1) as the first nucleotide sequence of *E. granulosus* s. s (G1) isolated from Argentinean Pampas fox.

#### 4. Discussion

In this study, ten adult parasites found in the small intestine of one male of *L. gymnocercus* from Buenos Aires, Argentina, were morphologically identified as *E. granulosus* s. l. The state of conservation of the samples did not allow the evaluation of the gravidity of the proglottids; however, the third largest proglottid was morphologically similar to a typical gravid proglottid of *E. granulosus* s. l. (Eckert et al., 2001).

The SCT is the gold standard method to detect *Echinococcus* spp. in intestinal content (Eckert, 2003). This technique allows the detection of worm burdens up to a single parasite. Therefore, the diagnostic sensitivity and specificity of SCT are close to 100% (Hofer et al., 2000). Although the infected animal had low burdens in this study, we cannot ruled out cestode losses during freezing and thawing periods (procedure necessary for the deactivation of the infectious eggs) (Geszy et al., 2013).

The use of molecular tools to analyze the 431-bp fragment of *cox1* gene allowed us to identify species and genotype of *E. granulosus* s. l. adults found in this study as, *E. granulosus* s. s. G1. This finding represents the first report of this genotype in a wild

carnivore from South America. In addition, this finding becomes even more relevant with the identification of the genotype (G1), because it is most commonly associated with human infections (Alvares Rojas et al., 2014). Also, this report coincides with most genotypes isolated from sheep in Buenos Aires province and even in domestic definitive and intermediate host from Argentina (Andresiuk et al., 2013; Cucher et al., 2015).

Several studies were carried out in Argentina with the purpose of characterizing the genetic variants involved in different *E. granulosus* s. l. life cycles. A total of six genotypes are circulating in livestock (G1 in sheep, cattle, goat and pig; G2 in sheep and cattle; G3 in sheep; G5 in cattle; G6 in goats and cattle; and G7 in pigs). Regarding the molecular epidemiological scenario in the definitive hosts, four genotypes (G1, G5, G6 and G7) were found in dogs. These data show that at least the most widely distributed and frequent genotypes are developing the complete life cycle in our country. The genotypes identified in patients were G1, G2, G5 and G6 and G2 and G6 were the most commonly detected genotype (Cucher et al., 2015).

*E. granulosus* s. s. is essentially maintained in domestic cycles involving dogs and sheep as the principal intermediate host, although the infection also occurs in a wide array of livestock and herbivorous wildlife species worldwide (Cardona and Carmena, 2013). According to Carmena and Cardona 2013, 2014, the infection of a wild definitive host in Argentina is directly related to the livestock CE assuming that access to sheep viscera containing fertile hydatid cysts cause the acquisition of the infection in these animals. Diet studies of the same foxes of this work showed an opportunistic diet, where murine rodents were the most common

item in the food habit (Canel, 2014). Therefore, *E. granulosus* adults found in one Pampas fox was probably acquired by feeding of abandoned carcass from sheep, goat or cow. Moreover, due to the low prevalence found in this study it would seem that this population of Pampas foxes are not frequently fed livestock.

With the aim to increase the sensitivity of *E. granulosus* detection, other non-invasive diagnostic techniques such as copro-parasitologic analysis and Copro-ELISA were applied. However, no positive results were found in the 34 fecal samples analyzed with Taeniidae eggs. These results were expected considering the few adult worms found in necropsy. Deplazes et al. (1992) reported that the diagnostic sensitivity of Copro-ELISA is closely dependant on the *Echinococcus* worm burden in natural and experimental infections. In only one of eight wild canids naturally infected with 3–70 *E. granulosus* worms Copro-ELISA was positive.

Sylvatic hosts may be the potential risk of infection to domestic animals and humans (Carmena and Cardona, 2013); especially the potential public health risk that infected foxes may be for the environmental contamination with infective eggs in urban and peri-urban areas. In addition, some life history features of *E. granulosus* make the infection of foxes with *E. granulosus* s. s. a potential threat to human health. Indeed, *E. granulosus* shows a high resistance of the infective forms in the environment (eggs), diverse egg dispersion mechanisms, large residence time of adults inside the infected carnivores intestine (6–24 months) and high egg production and shedding through the faeces per adult parasite (Sánchez Acedo, 2002). On the other hand, infection may be detrimental to other wild animals that may act as intermediary hosts. For this reason, the identification of wildlife species that are susceptible to infection with *E. granulosus* in different regions is crucial (Barnes et al., 2012). Our data confirmed that *L. gymnocercus* can be considered as definitive host for *E. granulosus* s. s. (G1) in southern of Buenos Aires province, Argentina. Despite the low prevalence found, the significance of this finding lies in the abundance of this fox and synanthropic position in the strongly modified ecosystems of the region.

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