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Detecting co-infections of *Echinococcus multilocularis* and *Echinococcus canadensis* in coyotes and red foxes in Alberta, Canada using real-time PCR

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ABSTRACT

The continued monitoring of *Echinococcus* species in intermediate and definitive hosts is essential to understand the eco-epidemiology of these parasites, as well to assess their potential impact on public health. In Canada, co-infections of *Echinococcus canadensis* and *Echinococcus multilocularis* based on genetic characterization have been recently reported in wolves, but not yet in other possible hosts such as coyotes and foxes. In this study, we aimed to develop a quantitative real-time PCR assay to detect *E. multilocularis* and *E. canadensis* and estimate the occurrence of co-infections while inferring about the relative abundance of the two parasites within hosts. We tested DNA extracted from aliquots of *Echinococcus* spp. specimens collected from intestinal tracts of 24 coyote and 16 fox carcasses from Alberta, Canada. We found evidence of co-infections of *E. multilocularis* and *E. canadensis* in 11 out of 40 (27%) samples, with 8 out of 24 (33%) in coyote samples and 3 out of 16 (19%) in red fox samples. DNA concentrations were estimated in three samples with Cq values within the range of the standard curve for both parasites; two of them presented higher DNA concentrations of *E. multilocularis* than *E. canadensis*. The use of qPCR aided detection of co-infections when morphological discrimination was difficult and quantification of DNA for samples within the standard curve. This is the first molecularly confirmed record of *E. canadensis* in coyotes and the first evidence of co-infections of *E. multilocularis* and *E. canadensis* in coyotes and red foxes.

1. Introduction

The genus *Echinococcus* currently includes at least nine species of parasites (reviewed in Romig et al., 2017) some of which have a cosmopolitan distribution and represent an important concern for animal and public health. As a typical taeniid cestode, *Echinococcus* spp. cycle between two mammalian hosts which may include both domestic and wild animals. Carnivores act as definitive hosts, harbouring the adult worms in the intestine and shedding the parasite's embryonated eggs into the environment, while a wide variety of intermediate hosts develop larval stages in their viscera upon ingestion of eggs. Humans can be affected as aberrant hosts and develop echinococcosis disease by accidental ingestion of embryonated eggs (Thompson, 2017).

In Canada, only two *Echinococcus* species have been confirmed so far: *Echinococcus multilocularis* and *Echinococcus canadensis* (G8 and G10 genotypes) (Davidson et al., 2016) which are causative agents of human alveolar echinococcosis (AE) and cystic echinococcosis (CE), respectively. Previously, *E. canadensis* was classified as a subspecies of *Echinococcus granulosus* (Sweatman and Williams, 1963) but was later recognized as a cryptic species within the *E. granulosus* (sensu lato) complex (Romig et al., 2015). *E. canadensis* is widely distributed across Canada with mainly wolves (*Canis lupus*), but also coyotes (*Canis latrans*) and dogs as definitive hosts, whereas intermediate hosts include cervids such as moose (*Alces alces*), elk (*Cervus canadensis*), caribou (*Rangifer tarandus*) and deer (*Odocoilecus* spp.) (Sweatman, 1952; Rausch, 2003; Romig et al., 2015).

The historical distribution of *E. multilocularis* in Canada encompasses two disjunct geographic areas: Northern Tundra Zone and North Central Region, both involving different predator-host communities (Eckert et al., 2001). However, recent findings of the parasite in

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Table 1
Primers and hydrolysis probes designed for a partial sequence of the *nad2* gene of *Echinococcus multilocularis* and for the *cox1* gene of *Echinococcus canadensis* used to detect infections in coyotes and foxes from Alberta, Canada.

Target species	Primer/Probe[a]	Oligonucleotide sequence (5′–3′)	Amplicon size
Echinococcus multilocularis	Nad234_F	TTGTTGAGCTATGTAATAATGTGTGGAT	
	Nad234_R	CATAAATGGAAACAAACCAAACTTCA	126bp
	Nad234_P	FAM-CTGTGCTATTAGTACTC-MGB-NFQ	
Echinococcus canadensis	Cox143_F	ATGAGGTGTTGGGTTCGTATAGG	143bp
	Cox143_R	ACAATCATCAACCCAACGCA	
	Cox143_P	FAM-TTGGTTTGGTGGATTATT-MGB-NFQ	

[a] F: forward; R: reverse; P: probe.

areas previously considered as nonendemic suggest an expansion of its geographic range (Schurer et al., 2013b, 2016; Gesy et al., 2014). This parasite circulates primarily through smaller canids as definitive hosts: foxes (*Vulpes* spp.) and coyotes; whereas intermediate hosts include several species of small mammals, mostly small rodents such as *Peromyscus*, *Myodes* and *Microtus* species (Holmes et al., 1971; Eckert et al., 2001; Liccioli et al., 2013). Domestic species such as dogs can be involved in semi-synanthropic cycles; however, the lifecycle of the parasite is considered primarily sylvatic (Eckert et al., 2001).

Morphological identification of *Echinococcus* spp. worms can be labour-intensive and difficult. At present, molecular tools are commonly used to identify species and genotypic variations and can be applied to fecal samples collected in the environment. However, microscopic screening of intestinal content to identify and count *Echinococcus* worms is still the most accurate way to estimate the intensity of infection with a high sensitivity and specificity (Gesy et al., 2013; Conraths and Deplazes, 2015). Nevertheless, in areas co-endemic for *Echinococcus* species, difficulties in morphological identification in early stages of infection could lead to misdiagnosis (Conraths and Deplazes, 2015).

The recent findings of the first *E. multilocularis* infections in wolves in Canada, including mixed infections with *E. canadensis* (Schurer et al., 2013b), led us to the hypothesis that co-infections with these parasites could also be present in other definitive hosts. Therefore, we aimed to develop a real-time PCR assay to be used for detection of mixed infections of *E. multilocularis* and *E. canadensis,* to estimate the occurrence of co-infections in coyotes and red foxes in Alberta, Canada. We also aimed to assess the feasibility of using quantitative real-time PCR (qPCR) results to infer about the relative abundance of the two species within hosts.

2. Materials and methods

2.1. Collection of host and parasite samples

We used aliquots of *Echinococcus* spp. specimens collected from intestinal tracts of 24 coyote and 16 fox carcasses of road-killed and trapped animals collected between 2012 and 2016, from rural and urban areas in Alberta, Canada. Intestinal tracts were collected upon post-mortem examination, frozen at −80 °C for 72 h to inactivate eggs and then stored at −20 °C upon further processing. Carcasses and intestines were processed during the same year of collection. The intestines were examined for the presence of *Echinococcus* spp. using the scraping, filtration and counting technique (SFCT) described by Gesy et al. (2013) with the following modifications: intestinal content was filtered through sieves of decreasing mesh size (1 mm, 250 µm and 75 µm), and 25% of each aliquot was analyzed to determine the worm burden. The total filtrate from the 250 µm and 75 µm sieves was collected in a final volume of 50 ml of 70% ethanol for further extraction of DNA from the worm population per host (mixed worm samples).

2.2. DNA extraction

Total DNA extraction was performed from mixed worm samples per host using 5 ml of the 50 ml aliquot that represents approximately 10% of the worm population per host. The E.Z.N.A stool DNA kit (Omega bio-tek, US) was used to prepare bulk DNA lysates to remove PCR inhibitors commonly found in intestinal content samples. After careful mixing, five milliliters of the sample, which included worms and the intestinal content that passed through the sieves, were centrifuged to remove the ethanol (5 min, 13,000 × *g*). Tubes were left open for an additional 20 min for evaporation to occur and the remaining pellet was used for DNA extraction (up to 200 mg per extraction) following the manufacturer's instructions. If needed, more than one extraction was performed per sample according to the amount of pellet and then combined in only one DNA extract. The final elution volume was 100 µL and following extraction, DNA was stored at −20 °C until use.

2.3. Real-time PCR on worm populations

2.3.1. qPCR conditions

To evaluate the occurrence of *E. multilocularis* and *E. canadensis* co-infections and quantify the DNA from each, we designed species-specific primers and hydrolysis probes (supplied by Applied Biosystems, US) from highly polymorphic regions of the mitochondrial *nad2* gene for *E. multilocularis* (GenBank accession number AB018440.2) and *cox1* gene for *E. canadensis* (GenBank accession number AB745463.1) using Primer3 software (http://primer3.ut.ee/) (Table 1). Simplex qPCR assay was performed for each target in a final volume of 10 µL containing 5 µl TaqMan Fast Advanced master mix (Applied Biosystems, US), 900 nM forward and reverse primer respectively, 300 nM of hydrolysis probe and 1 µL of DNA extract. We performed reactions in duplicates using a StepOnePlus Real-Time PCR System (Applied Biosystems, US) with the following conditions: 95 °C for 2 min, 40 cycles of 95 °C for 1 s, and 60 °C for 20 s. Additionally, we performed a duplex qPCR with an internal amplification control (IAC) to assess the presence of PCR inhibitors as described by Deer et al. (2010) with modifications of plasmid preparation as described by Klein et al. (2014). For this duplex qPCR, each final 10 µL reaction mixture contained 450 nM of each primer (forward and reverse Nad234/IAC), 125 nM of each hydrolysis probe, 100 copies of the IAC plasmid and 1 µL of total extracted DNA. The threshold for detection of PCR inhibitors was calculated as 34 cycles based on the average Cq value for 100 copies/µL of IAC plasmid run in 7 replicates without DNA extract. PCR inhibitors were considered present when the IAC Cq value of the sample was over 1.5 cycles greater than the IAC Cq average (Klein et al., 2014). In these cases, Cq values were normalized with the IAC Cq average as described by Knapp et al. (2014) to account for the lower efficiency of PCR. If the IAC was not detected, the samples were diluted in ten-fold dilutions to overcome inhibition.

The 143bp and 126bp amplicons obtained from samples positive for both *E. canadensis* and *E. multilocularis* were sequenced with the respective forward primer to validate the results. For samples with low concentration of qPCR product, we performed classical PCR in order to