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

Maria A. Santa\(^a\), Sonya A. Pastrana\(^b\), Claudia Klein\(^b\), Padraig Duignan\(^c\), Kathreen Ruckstuhl\(^a\), Thomas Romig\(^d\), Alessandro Massolo\(^c,e,f,*\)

\(^a\) Department of Biology, University of Calgary, Alberta, T2N 1N4, Canada
\(^b\) Department of Veterinary Clinical Diagnostic Sciences, Faculty of Veterinary Medicine, University of Calgary, Alberta, T2N 4G6, Canada
\(^c\) Department of Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Alberta, T2N 4G6, Canada
\(^d\) Institute of Zoology/Parasitology, University of Hohenheim, 70593, Stuttgart, Germany
\(^e\) Ethology Unit, Department of Biology, University of Pisa, 56126, Pisa, Italy
\(^f\) UMR CNRS 6249 Chrono-environnement, Université Bourgogne Franche-Comté, 25030, Besançon, France

**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 (*Odocoileus* 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...
Table 1
Primer sequences designed for a partial sequence of the nad2 gene of Echinococcus multilocularis and for the coxl gene of Echinococcus canadensis used to detect infections in coyotes and foxes from Alberta, Canada.

| Target species          | Primer/Probe | Oligonucleotide sequence (5'-3') | Amplicon size |
|-------------------------|--------------|---------------------------------|---------------|
| Echinococcus multilocularis | Nad234_F     | TTGTGTGGCTGTGAATATGTTGAT        | 126 bp        |
|                         | Nad234_R     | CATATAATGGAAACACAAACTCTCA       |               |
|                         | Nad234_P     | FAM-CTGGTATTTGCTCT-MGB-NFQ      |               |
|                         | Cox143_F     | ATGAGGTTGTTGGTCTTGATAGG         | 143 bp        |
| Echinococcus canadensis | Cox143_R     | ACAATCTCAACCACCAAGCA            |               |
|                         | Cox143_P     | FAM-TTGTTGGTGGATTAT-MGB-NFQ     |               |

* 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., 2003).

 Morphological identification of Echinococcus spp. worms can be labour-intensive and difficult. At present, molecular tools are commonly used to identify species and genetic 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 (SFCFT) 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 μg 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 coxl 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 hydrolysise 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 hydrolysise 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
obtain a higher volume and concentration. The sequences obtained were aligned and compared with nucleotide databases using NCBI Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov). The samples that could not be sequenced were tested in a SYBR™ green assay evaluating the melt curve and by agarose gel electrophoresis confirming the presence of single PCR product of expected size. Reactions were carried out in a final volume of 10 µL using Power SYBR™ Green PCR Master Mix (Applied Biosystems, US) 5 µL, 500 nM forward and reverse primers, 3 µL of H2O and 1 µL of DNA. We performed reactions using a StepOnePlus Real-Time PCR System (Applied Biosystems, US) using the default thermocycler program for the standard quantification curve and dissociation curve using SYBR™ green reagents. PCR products were resolved on 1.5% agarose gels containing 0.5 µg/mL ethidium bromide. Additionally, we reanalyzed the aliquots of Echinococcus spp. that were positive for co-infection in qPCR, to try to identify and compare both species morphologically (Jones and Pybus, 2008) and validate the molecular results.

2.3.2. Analytic specificity and sensitivity of qPCR
Primers and probes were tested for specificity in silico using the NCBI Primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Additionally, the specificity was assessed with DNA extracted from single adult worms of E. multilocularis (North American and European genotypes), E. canadensis (G10 genotype), Taenia crassiceps and Taenia pisiformis collected from wild canids in Alberta. The identity of the species used for this test was confirmed through sequencing.

The method detection limits (MDLs) and the efficiency of PCR assay were evaluated based on a standard curve of 8 ten-fold serial dilutions of pure DNA from one adult worm each from E. multilocularis and E. canadensis, ranging from 2 ng/µL to 2 × 10^-7 ng/µL. The initial DNA concentration was measured with Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, US). Seven replicates were performed from each ten-fold dilution. The last point of the DNA range representing 7 out of 7 positive trials was considered the limit of E. multilocularis and E. canadensis DNA detection and accurate quantification.

3. Results
3.1. Analytic specificity and sensitivity of qPCR
The specificity of the primer pair for E. multilocularis (Nad234) was 100% according to in silico analysis and when tested with DNA from E. canadensis and Taenia spp. The primers for E. canadensis (Cox143) also revealed no amplification of DNA from E. multilocularis and Taenia spp. For in silico analysis, Cox143 forward and reverse primers had also a perfect match with E. ortleppi, but presented two mismatches within the probe which may prevent unspecific fluorescent signal.

Based on the standard curve of ten-fold serial dilutions the limit of detection of E. multilocularis with Nad234 primers was 35 cycles corresponding to a DNA concentration of 2 × 10^-5 ng/µL. Beyond this limit, DNA was considered to be detectable but not quantifiable. The efficiency of PCR assay (E = 10^(-1/slope)) was 95%. The limit of detection for E. canadensis with Cox143 primers was 34 cycles corresponding to 2 × 10^-4 ng/µL. Based on the standard curve the efficiency was 99% (Fig. 1).

3.2. qPCR on worm populations
All the 40 mixed worm samples were positive for E. multilocularis with Cq values ranging from 11.28 to 37.15. DNA from both parasites E. canadensis and E. multilocularis was detected 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. Cq values for E. canadensis ranged from 20.53 to 38.54 (Table 2). There was no disagreement between replicates from each host and parasite.

Only three samples presented some grade of inhibition and the Cq values were normalized accordingly. In one sample showing total inhibition, the IAC was detected after a 1:10 dilution but the sample was positive only to E. multilocularis. For the other two samples showing partial inhibition, only one was positive for co-infection.

The sequences obtained were ~90 long, and the percentages identities were 100% for E. canadensis G10 and E. multilocularis (European-like strains). Within these sequences, there were at least 10 SNPs to differentiate between E. multilocularis and E. canadensis, and 3 SNPs to identify E. canadensis G10 and G8 strains. For the samples that could not be sequenced, the melt curve and the band on agarose gel corresponded to the targeted amplicon. After reanalyzing aliquots of positive samples for co-infections in qPCR, we identified morphologically E. multilocularis adult worms from ~1800 µm to ~3100 µm long. In only a single sample from a coyote were we able to identify E. ca-
dadensis adult worms, which were ~2300 µm long.

DNA concentrations of E. canadensis and E. multilocularis in positive samples for co-infection were estimated using the standard curve calculated for each target and the Cq values. Considering the limits of detection for each target, only three samples had Cq values under these limits for both parasites, two of them presenting higher DNA concentrations of E. multilocularis than E. canadensis (Table 2).

4. Discussion
This study represents the first evidence of co-infections of E. multilocularis and E. canadensis in red foxes and coyotes and the first molecularly confirmed record of E. canadensis infection in coyotes after morphological identification of adult worms. Red foxes have been shown to occasionally act as definitive hosts for E. granulosus s.s. in different locations such as Great Britain and Australia (Thompson and Smyth, 1975; Jenkins and Morris, 2003). In North America, Rausch and Schiller (1951) reported Echinococcus granulosus s.l. in red and arctic foxes in northern Alaska. This is most likely due to scavenging on domestic and wild host carcasses, although they do not seem to play a significant role in transmission (Carmena and Cardona, 2014; Thompson, 2017). There is no reason to doubt that the presence of E. canadensis in red foxes in Alberta is due to scavenging behavior. Although red foxes mainly prey on small animals such as rodents, which are the intermediate host to E. multilocularis, they are generalists and opportunistic predators that could feed on cervid carcasses infected with E. canadensis cysts. Availability of ungulate carcasses is likely influenced by prey abundance, seasonality, hunting pressure and presence of large carnivores (Theberge and Wedeles, 1989; Needham et al., 2014), and so could be the encounters with E. canadensis. For example, post-mortem surveys of cervid hosts and molecular characterization of hydatid cysts indicated a higher prevalence of E. canadensis in moose than in caribou, elk, or white-tailed deer (Schurer et al., 2013a). At the same time, it has been suggested that higher parasite intensity in moose may increase the level of predation by wolves and human hunters, due to reduced lung function, making these infected carcasses more accessible to scavengers (Joly and Messier, 2004).

Echinococcus granulosus s.l. has been reported in coyotes in Canada (Alberta, Manitoba, and Ontario); it can be assumed that these parasites have indeed been E. canadensis, but in the absence of genotype identification retrospective proof is not possible (Freeman et al., 1961; Holmes and Podesta, 1968; Samuel et al., 1978). Recent studies failed to detect any species of the E. granulosus s.l complex in coyotes (Davidson et al., 2016). Although mixed infections of E. canadensis and E. multilocularis were found in wolves in Western Canada (Schurer et al., 2013b), the present study is the first evidence of co-infections by these parasites in coyotes and foxes. In this study, all the cases positive for E. canadensis were identified as genotype G10, which could be related to an apparently higher abundance of G10 over G8 in Canada, according to molecular characterization in both intermediate and definitive hosts (Davidson et al., 2016). Indeed, Schurer et al. (2016), found a prevalence of 6% G10-E. multilocularis co-infections in wolves, and an
overall prevalence of G8 and G10 genotypes of 6% and 24%, respectively. Interestingly, G8-E. multilocularis co-infections were not found. The detection of mixed infections could be limited by the techniques of intestinal screening and the number of adult worms processed per host (Schurer et al., 2013b). Additionally, morphological identification of the parasites could be affected by the level of autolysis and the stage of development of worms (Conraths and Deplazes, 2015). The use of qPCR allowed for the rapid and sensitive detection of mixed infections using DNA extracted from worm subpopulations per host instead of only using a few worms. Using this approach, E. canadensis DNA could be detected in samples where morphological discrimination was not possible or the low burden of worms did not allow for detection.

Interestingly, no E. canadensis co-infection was ever found in coyotes and foxes using the intestinal scraping investigation, despite a considerable number of animals that have been examined in recent years (Liccioli et al., 2012; Gesy et al., 2014). This could be due to a reduced development of the species that may make it more difficult to distinguish from E. multilocularis. Indeed, Holmes (1961) compared E. granulosus adult worms from coyotes and wolves and found that worms in coyotes were smaller and only 5% contained normally developed ova. From all the positive cases to co-infection, we could only identify E. canadensis worms in one coyote where the differences in size were more than 500 μm compared to E. multilocularis worms and the uterus shape was clearly distinguishable. Additionally, the size of these E. canadensis worms only reached ∼2300 μm, while it could reach up to 11 mm, as described for E. granulosus s.l. species (Jones and Pybus, 2008). On the other hand, the detection of DNA in foxes could possibly result from the consumption of cysts without the establishment of a true infection, as they are generally considered less suitable hosts for E. granulosus s.l. The presence of immature worms that established (but would never shed eggs) in a marginally suitable host would also lead to positive PCR results and can therefore not be ruled out. Additional studies (e.g. on the presence of E. canadensis eggs in fox faeces) will be needed to draw definite conclusions on the host role of foxes for E.

![Fig. 1. Standard curve for qPCR assays to detect E. canadensis and E. multilocularis using Cox143 and Nad234 primers/probes, respectively.](image)

### Table 2
Positive samples for co-infection of Echinococcus canadensis (Ec) and Echinococcus multilocularis (Em) from coyotes and foxes from Alberta, Canada.

| Host        | Sample ID | Em Cq | Ec Cq | DNA Em (ng/μL)   | DNA Ec (ng/μL)   | Worm burden |
|-------------|-----------|-------|-------|-----------------|-----------------|--------------|
| Vulpes vulpes | F7-2016   | 11.28 | 38.39 | ne              | ne              | 12,200       |
|             | F9-2016   | 26.82 | 35.26 | 4.55 × 10⁻³     | ne              | 40           |
|             | F13-2016  | 29.74 | 36.26 | 6.35 × 10⁻⁴     | ne              | 40           |
| Canis latrans | 5-2016    | 11.22 | 36.61 | ne              | ne              | 91,144       |
|             | 13-2016   | 24.06 | 33.50 | 2.93 × 10⁻²     | 3.05 × 10⁻⁴     | 6276         |
|             | 14-2016   | 35.08 | 20.53 | 1.73 × 10⁻⁵     | 2.35            | 260          |
|             | 17-2016   | 36.63 | 31.34 | ne              | 1.35 × 10⁻³     | 404          |
|             | 27-2016   | 37.15 | 23.99 | ne              | 2.16 × 10⁻¹     | 608          |
|             | 35-2016   | 14.52 | 38.45 | ne              | ne              | 6628         |
|             | 48-2016   | 21.68 | 36.81 | 1.46 × 10⁻¹     | ne              | 500          |
|             | 51-2016   | 21.84 | 28.72 | 1.31 × 10⁻¹     | 8.25 × 10⁻³     | 728          |

ne: not estimated (values out of the range of the standard curve).

a Cq values normalized.
b Samples with Cq values under limit of detection for both targets.
The use of qPCR allowed a quantitative estimation of the DNA of each parasite, but only for samples with Cq values within the range of the standard curve. A significant negative correlation between the Cq value and the worm burden of E. multilocularis was found in previous studies designed to detect DNA of E. multilocularis in fecal samples (Isaksson et al., 2014; Knapp et al., 2014). Nevertheless, the Cq value was suggested as an indicator, but not as a precise quantification of the worm burden (Isaksson et al., 2014). In our case, DNA concentrations might be used as an indicator of the parasites relative abundance in samples were differences are notably high. However, we could only calculate DNA concentrations for both parasites in three samples, and validation of results through morphological identification and counting of E. canadensis worms was not possible. Therefore, these results are not conclusive, and many limitations exist for the comparison of the abundance of these parasites based solely on qPCR, including differences in size, stage of infection and presence of gravid worms. As an alternative, qPCR could be used as a complementary test to detect mixed infections in co-endemic areas, helping to avoid a possible overestimation of worm burden of one or another Echinococcus species via morphological discrimination. Additionally, this assay could be used on fecal samples to monitor environmental contamination by E. multilocularis and E. canadensis eggs. According to the standard curve, up to $2 \times 10^{-4}$ and $2 \times 10^{-6}$ ng/µL of DNA could be detected for each parasite respectively. Therefore, the high sensitivity of the test would allow for the detection of DNA from only one egg ($5 \times 10^{-3}$ ng) (Knapp et al., 2014), while overcoming the presence of inhibitors.

The monitoring of Echinococcus species in intermediate and definitive hosts is essential to study the distribution and prevalence of these parasites, as well to assess the potential impact on public health, hosts is essential to study the distribution and prevalence of these parasitoses and Wildlife 7 (2018) 111–115

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