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ORIGINAL PAPER

Evaluation of an automated magnetic bead-based DNA extraction and real-time PCR in fecal samples as a pre-screening test for detection of *Echinococcus multilocularis* and *Echinococcus canadensis* in coyotes

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Abstract

Efficient and sensitive diagnostic tools are essential for the study of the eco-epidemiology of *Echinococcus* species. We evaluated an automated magnetic bead-based DNA extraction commercial kit followed by qPCR (MB-qPCR), for the detection of *Echinococcus multilocularis* and *Echinococcus canadensis* in coyote (*Canis latrans*) fecal samples. The diagnostic sensitivity was determined by validating the method against the scraping, filtration, and counting technique (SFCT) for samples collected in Canada. From the 60 samples tested, 27 out of 31 SFCT positives samples for *Echinococcus* cestodes were positive in the MB-qPCR for *E. multilocularis*, with a sensitivity of 87.1% (95% CI 70.2 to 96.4%). Two samples were also positive for *E. canadensis* in the MB-qPCR and confirmed by morphological identification of adult worms. The agreement of the MB-qPCR and the SFCT was statistically significant with a kappa value of 0.67 (95% CI 0.48–0.85; p value < 0.001). The magnetic bead-based DNA extraction followed by qPCR proved to have a sensitivity comparable to the SFCT to detect *E. multilocularis*. Although the diagnostic sensitivity for *E. canadensis* was not estimated, MB-qPCR identified *E. canadensis* cases previously overlooked when using SFCT. We propose a combination of molecular and morphological identification using the MB-qPCR and the SFCT to detect both parasites, allowing for a more efficient large-scale surveillance, and detecting co-infections of *Echinococcus* species that can be difficult to identify when only based on morphology.

Keywords *Echinococcus multilocularis* · *Echinococcus canadensis* · Coyote · Real-time PCR · Coprodiagnosis · Magnetic beads

Introduction

Echinococcus multilocularis and *Echinococcus canadensis* are two important, but often neglected, zoonotic parasites of public health concern (FAO/WHO 2014; Thompson 2015), being the etiological agents of alveolar (AE) and cystic echinococcosis (CE), respectively. Humans get infected by accidentally ingesting embryonated eggs excreted in feces of definitive hosts, mainly wild carnivores such as foxes (*Vulpes vulpes*), coyotes (*Canis latrans*), and wolves (*Canis lupus*), but also dogs. These parasites are present in the Northern hemisphere, including most provinces in Canada (Massolo et al. 2014; Davidson et al. 2016; Deplazes et al. 2017). Although human cases of AE are considered rare in North America, recent reports of an autochthonous case of human AE (Massolo et al. 2015), along with high prevalence in urban coyotes (Catalano et al. 2012; Massolo et al. 2014), multiple aberrant cases of liver infections (canine AE) in dogs (Peregrine et al. 2012; Skelding et al. 2014), and the presence of co-infections with both parasites in wolves in the subarctic region (Schurer et al. 2013), make the understanding of the transmission of these parasites a priority. To estimate the prevalence of these parasites in their definitive hosts and to

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understand their eco-epidemiology, and thus the potential risk of human infection, reliable and efficient diagnostic tools are required, particularly when sampling large populations over wide geographical areas.

In general, the diagnosis of *Echinococcus* spp. infections in definitive hosts is performed using molecular, morphological, or immunological techniques (Eckert et al. 2001; Conraths and Deplazes 2015). However, the sensitivity of these methods varies depending on the stage of infection and the same test parameters are not applicable for different host species and for high or low endemic areas (Eckert 2003). The sedimentation and counting technique (SCT), used for the identification of adult worms in the intestine of definitive hosts, was considered the reference standard, but its proposed high sensitivity has been debated (Maas et al. 2016; Wahlström et al. 2016). This method is also time-consuming, requires well-preserved carcasses, and the sensitivity can decrease when worm burdens are low (Karamon et al. 2010). Several modifications of this technique have been proposed aiming to reduce the processing time. One of them, the scraping, filtration, and counting technique (SFCT), has a comparable sensitivity and increases significantly the efficiency of the process (Gesy et al. 2013), but its processing time is still a limiting factor in large-scale surveillance studies (i.e., counting can take up to 98 min per sample), and it does not allow for an efficient and accurate quantitative assessment of co-infections with different *Echinoccocus* species. As an alternative, several coprodiagnosis methods using molecular tools have emerged in the last years trying to obtain good-quality DNA from fecal samples while avoiding PCR inhibition. For example, a semi-automated magnetic capture probe-based DNA extraction using a specific probe for *E. multilocularis* and real-time PCR on fox fecal samples showed comparable sensitivity and specificity to the SCT while decreasing the processing time (i.e., ~ 48 samples per day) (Isaksson et al. 2014; Conraths and Deplazes 2015). However, accessible commercial kits for DNA extraction (without specific probe) have not been tested using similar automated tools nor in areas co-endemic for *E. canadensis* and *E. multilocularis*. Therefore, we aimed to evaluate an assay using a magnetic bead-based DNA extraction commercial kit and real-time PCR after freeze-thawing of fecal samples (MB-qPCR) to detect *E. canadensis* and *E. multilocularis* in fecal samples. Our final goal was to use a rapid, sensitive, and efficient technique to screen fecal samples by PCR prior to performing the SFCT to reduce processing time allowing for a more efficient large-scale surveillance of *Echinococcus* parasites. This approach combines the advantages of both techniques allowing a fast coprodiagnostic coupled with the isolation of worms for molecular characterization.

Materials and methods

Experimental design and sampling

We tested our MB-qPCR method using a case-control comparison blinded design in which each sample was its own control: we compared the results obtained with the tested method vs. a modification of the considered "gold standard" method or the best performing methodology (i.e., SFCT; sensitivity of 91% and negative predictive value of 97%), the results of which were blinded for the person that performed the MB-qPCR testing.

Our methodology was tested on 60 coyote fecal samples from 60 different gastrointestinal (GI) tracts collected from carcasses of road-killed and trapped coyotes in Canada between 2012 and 2016. These GI tracts were previously screened during the respective year of collection for the presence of *Echinococcus* spp. worms using the SFCT (Gesy et al. 2013) with some modifications (Santa et al. 2018). The intestines were scraped and the content was filtrated using sieves of decreasing mesh (1 mm, 250 μm, and 75 μm). After collecting the filtrated of each sieve, 25% of the final volume was analyzed to determine the worm burden. On the very same GI tracts, fecal samples were collected from the rectum and stored at − 20 °C. Overall, we used 31 positive and 29 negative samples based on the morphological identification of *Echinococcus* spp. adult worms. In all the 31 positive samples, only *E. multilocularis* adult worms were clearly identified and co-infections were not detected in this initial screening based on morphology.

DNA extraction

Total DNA extraction was performed from fecal samples using an automated magnetic bead-based protocol to decrease sample processing time. The Mag-Bind Stool DNA 96 Kit (Omega Bio-Tek, USA) was used following the manufacturer's instructions to extract DNA from 200 mg of feces with an additional step of five freeze-thaw cycles as described by Klein et al. (2014) to liberate DNA from the eggs. After the lysis process, the KingFisher Flex magnetic particle processor (Thermo Fisher Scientific, USA) was used to process the samples in an automated way. Water was used as a negative extraction control. Elution was performed in 50 μl of elution buffer, and extracts were maintained at − 20 °C until further use.

qPCR conditions

Species-specific primers and hydrolysis probes *Nad234* and *Cox143* previously developed and tested were used to detect *E. multilocularis* and *E. canadensis* positive samples (Santa

et al. 2018). The targets included a 126 bp region of the *nad2* gene for *E. multilocularis* and 143 bp region of *cox1* gene for *E. Canadensis*, allowing the detection of different genotypes. The analytic specificity of the primers and probes was assessed in silico using NCBI Primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and with DNA extracted from *Echinococcus* species present in Canada (*E. multilocularis* and *E. canadensis G8,G10*) and *Taenia* species collected from wild canids in Alberta (*Taenia crassiceps* and *Taenia pisiformis*) (Santa et al. 2018). We included samples of *E. canadensis* G6, G7 to confirm the amplification of all the genotypes. Additionally, we tested the primers and probes with *Echinococcus* species from Europe (*E. granulosus* s.s., and *E. ortleppi*) to confirm the specificity. The presence of PCR inhibitors was assessed through the use of an internal amplification control (IAC plasmid) as described by Deer et al. (2010) with some modifications of the plasmid preparation as described by Klein et al. (2014). The primers and probes used in this study are summarized in Table 1. Duplex reactions were carried out for each target along with the IAC in a final volume of 10 μl containing: 5 μl of TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, US), 450 nM of each primer (forward and reverse *Nad234*/IAC or *Cox143*/IAC), 125 nM of each hydrolysis probe, 100 copies of the IAC plasmid, and 1 μL of total extracted DNA. Reactions were carried out using the StepOnePlus Real-Time PCR System (Applied Biosystems, USA) with the following conditions: 95 °C for 2 min, 40 cycles of 95 °C for 1 s, and 60 °C for 20 s. Each sample was amplified in duplicate and the average Cq was calculated. The threshold for detection of PCR inhibitors was set at 34 cycles based on the average Cq value for 100 copies/μL of IAC plasmid run in 7 replicates without DNA extract (Santa et al. 2018). 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 this case, Cq values were normalized with the IAC Cq average as described by Knapp et al. (2014) to account for the lower efficiency of PCR. For total inhibited samples, when IAC was not detected, qPCR was performed again at 1:10 and 1:100 dilutions in order to overcome the inhibitory effect. Water was included as a negative control for qPCR reactions. The method detection limit (when DNA is detectable and quantifiable with 99% confidence) for *E. multilocularis* and *E. canadensis* using *Nad234* and *Cox143* primers was set at Cq 35 and 34, respectively, based on the standard curve of tenfold dilutions of DNA (Santa et al. 2018). Beyond this limit, DNA was considered detectable but not quantifiable. Positive samples for *E. multilocularis* in qPCR but negative in SFCT were sequenced to validate the molecular results. The sequences obtained were aligned and compared with nucleotide databases using NCBI Nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov). To confirm the positive cases for *E. canadensis* in MB-qPCR, we reanalyzed the aliquots of *Echinococcus* spp. worms, attempting to identify this species morphologically (Jones and Pybus 2001).

Statistical analysis

The diagnostic sensitivity and negative predictive value of the MB-qPCR were calculated using the SFCT as a reference due to similarity with the considered gold standard method SCT. The 95% confidence intervals estimate for sensitivity were calculated using Clopper-Pearson (exact) method (Vollset 1993). Confidence intervals for the negative predictive value were calculated according to Mercaldo et al. (2007). Cohen's kappa coefficient was used to test the significance of agreement between the two diagnostic techniques. Non-parametric Spearman's rank correlation was calculated to measure the strength of the relation between the Cq values in the MB-qPCR and the number of worms detected by the SFCT. The mean and range of Cq values were also calculated according to SFCT worm burden. Statistical analysis was performed using SPSS (version 22.0).

Results

Twenty-seven out of 31 SFCT positive samples were positive in the MB-qPCR for *E. multilocularis*, with a sensitivity of 87.1% (95% CI 70.2 to 96.4%) (Table 2). Two of these samples were positive for co-infection with *E. canadensis* with Cq values of 32.19 and 35.11 for this parasite. Single infections of *E. canadensis* were not detected. Due to the low prevalence of *E. canadensis* in this set of samples, and an uncertain "true infection status" for this parasite, the diagnostic sensitivity and negative predictive value were only estimated for *E. multilocularis*. In coyotes with more than 150 worms, MB-qPCR was positive in 22 out of 23 cases (95.7%). In coyotes with less than 150 worms, MB-qPCR was positive in 5 out of 8 cases (62.5%) (Table 3). From these five positive cases, two had the lower count of worms in SFCT (eight worms). Six out of 29 SFCT negative samples were positive in MB-qPCR (Table 2). The negative predictive value was calculated as 85.19% (95% CI 69.34 to 93.60%). PCR inhibitors were detected in 24 of the 60 samples with a range between 1.55 and 3.03 cycles above the IAC average (34 Cq) and a mean of inhibition of 2.1 cycles (moderate inhibition). Total inhibition was present in four samples which overcame after 1:10 DNA dilution. From those samples, only one was positive for *E. multilocularis* after dilution.

From the six samples positive for *E. multilocularis* in qPCR but negative in SFCT, we could sequence only four samples due to the low DNA concentration. The sequences

Table 1 Sequences of primers and probes used in this study

Target	Primer/Probe	Oligonucleotide sequence (5′–3′)	Reference
Echinococcus multilocularis	Nad234 forward	TTGTTGAGCTATGTAATAATGTGTGGAT	
	Nad234 reverse	CATAAATGGAAACAAACCAAACTTCA	Santa et al. (2018)
	Nad234 probe	FAM-CTGTGCTATTAGTCTC-MGB-NFQ	
Echinococcus canadensis	Cox143 forward	ATGAGGTGTTGGGTTCGTATAGG	
	Cox143 reverse	ACAATCATCAACCCAACGCA	Santa et al. (2018)
	Cox143 probe	FAM-TTGGTTTGGTGGATTATT-MGB-NFQ	
Internal amplification control (IAC)	IAC forward IAC reverse	CTAACCTTCGTGATGAGCAATCG GATCAGCTACGTGAGGTCCTAC	Deer et al. (2010)
	IAC probe	VIC-AGCTAGTCGATGCACTCCAGTCCT CCT-MGB-NFQ	

obtained were ~ 80 base pairs long, and the percentage identities were 100% for *E. multilocularis* (European-like strains). The two cases of co-infection with *E. canadensis* in qPCR were confirmed based on morphology identification after reanalyzing the aliquots of *Echinococcus* worms and identifying some adult *E. canadensis* specimens, although a precise worm burden could not be determined due to the high presence of fragmented worms. The analytic specificity of the primers and probes was also confirmed with the negative results on *E. granulosus* s.s. and *E. ortleppi* DNA.

The agreement of the MB-qPCR and the SFCT was statistically significant with a kappa value of 0.67 (95% CI 0.48–0.85; p value < 0.001). In 10 out of 60 samples, the tests disagreed. We found a negative correlation ($r_s = 0.4$; $p < 0.05$) between the number of worms and the Cq-values in the 31 samples that were positive only for *E. multilocularis* in MC-qPCR. The mean and range of Cq-values for *E. multilocularis* positive samples according to the number of worms are summarized in Table 3.

Discussion

The aim of this study was to evaluate a diagnostic method using automated magnetic bead-based DNA extraction and real-time PCR (MB-qPCR) to detect *E. canadensis* and *E. multilocularis* in coyote fecal samples, to be used as a pre-screening test prior to performing the SFCT. The proposed MB-qPCR method proved to be an efficient and sensitive way to detect *E. multilocularis* DNA using small fecal samples and overcoming the presence of PCR inhibitors. Although the diagnostic sensitivity for *E. canadensis* was not estimated, the finding of two positive cases confirms the apparently underestimated presence of co-infections recently reported in coyotes and foxes (Santa et al. 2018) allowing to identify *E. canadensis* cases non detected based solely on morphological identification.

Table 2 Results of the analysis with MB-qPCR and SFCT for *E. multilocularis* on 60 samples from coyotes collected in Alberta, Canada

		SFCT		TOTAL
		+	–	
MB-qPCR	+	27	6	33
	–	4	23	27
TOTAL		31	29	60

Factors influencing diagnostic sensitivity and specificity

The diagnostic sensitivity of the MB-qPCR to detect *E. multilocularis* was comparable with the SFCT and with techniques using magnetic capture probe-based DNA extraction and qPCR (Isaksson et al. 2014; Maas et al. 2016), even taking into account that the DNA extraction commercial kit used in this study does not use a specific probe to capture solely *E. multilocularis* DNA. From the four positive SFCT cases that could not be detected by MB-qPCR, 2 of them had less than 100 worms and the other 2 had a moderate level of inhibition, which can be related with a lower likelihood of detection. For example, Al-Sabi' et al. (2007) reported a lower sensitivity of copro-DNA PCR during the pre-patent period and low patent period, when the parasite material is less abundant. Therefore, the stage of infection can strongly affect the sensitivity of the diagnostic methods (Conraths and Deplazes 2015). Additionally, one of the major challenges of using a copro-PCR test is to overcome the presence of inhibitory substances such as tannic acid, calcium, or humic acid which may prevent amplification (Opel et al. 2010). This might be overcome by using DNA extraction kits designed for stool samples or by diluting the extracted DNA samples to reduce the concentration of the inhibitory substances (Waits and Paetkau 2005; Knapp et al. 2014). Nonetheless, pre-treatment to disrupt the taeniid egg's keratin layer is always necessary and

Table 3 Average and range of Cq values for *E. multilocularis* positive samples according to the number of worms

SFCT (worm burden)	Number of samples	MB-qPCR positive	*Em* Cq-values average (range)
0	29	6	32.30 (25.62–36.94)
1–150	8	5	31.03 (27.30–33.77)[a]
151-2000	9	8	26.50 (20.74–30.91)[a]
2001-20,000	7	7	27.33 (18.86–32.54)
> 20,000	7	7	26.98 (22.54–33.49)
Total samples	60	33	

Em Echinococcus multilocularis

[a] Positive samples for *E. canadensis* were excludedr

usually, only up to 0.2 g of fecal sample can be used with these commercial kits (Al-Sabi' et al. 2007). Although this low amount of sample seems insufficient to detect low-grade infections, we detected DNA in samples with low worm burden (8 worms) and obtained similar sensitivity compared with techniques that used 0.5 g up to 1 g of fecal sample (Knapp et al. 2016; Maas et al. 2016).

Compared to other extraction methods of DNA from fecal samples, the addition of the freeze-thawing cycle can significantly increase PCR sensitivity (i.e., 20%) by mechanically disrupting the chitinous eggshell and liberating DNA without adding chemicals that can affect the PCR performance (Klein et al. 2014). Additionally, the freeze-thawing cycle helped to overcome the effects of PCR inhibitors due to a higher amount of DNA liberated. Despite detecting inhibitors in 24 of the 60 samples, the PCR performance was not heavily affected. Indeed, only one sample out of 4 with total inhibition of IAC came out positive to *E. multilocularis* after dilution and was confirmed as positive in the SFCT.

The diagnostic specificity is usually evaluated in populations with low prevalence in order to avoid an overestimation of positives cases (false positives) in areas of high prevalence such as Canada (Eckert 2003). Using MB-qPCR, we detected 6 positive cases for *E. multilocularis* that were negative with SFCT; however, it is unlikely that all these cases were indeed false positives. First, the species-specific primers *Nad234* showed no cross-reaction with other *Taenia* species commonly found in wild canids in Canada (as confirmed by the sequencing of the qPCR amplicons), neither with species of *E. granulosus* complex. Second, it should be considered that the PCR is able to detect DNA from various sources that include not only eggs but also cells or tissue fragments from worms and inclusive DNA from infected but non-infectious rodents consumed by the definitive host (Isaksson et al. 2014). Third, the considered gold standard method and similar methods might not detect the parasite when worm burden is low (Karamon et al. 2010), due to the concomitant presence of immature or fragmented worms and intestinal villi that can make morphological detection and identification very difficult (Gesy et al. 2013). Therefore, we cannot rule out there were positive cases of *E. multilocularis* infections not detected by the SFCT. On the other hand, if the test is used to evaluate only the presence or absence of *Echinococcus* in a specific area, the detection of cases of infected but non-infectious animals would not be a critical factor, as both cases will reflect the presence of the parasite in the environment (Wahlström et al. 2016).

The morphological identification of *Echinococcus* parasites in case of co-infections could be affected by the stage of development of the parasite, the worm burden, and the quality of the sample (Conraths and Deplazes 2015; Santa et al. 2018). Particularly in coyotes, the presence of mostly immature *E. canadensis* worms or a possible reduced development of the species could make more difficult to distinguish from *E. multilocularis* (Holmes 1961; Santa et al. 2018). The use of MB-qPCR aided to identify two *E. canadensis* infections that were not detected during the SFCT, although these cases were confirmed after re-analyzing the aliquots of worms. Therefore, the use of MB-qPCR as a pre-screening test before performing SFCT could help to detect mixed infections in co-endemic areas. However, the estimation of the diagnostic sensitivity and diagnostic specificity to detect *E. canadensis* in coyote fecal samples is still necessary using different approaches in the absence of a gold standard method to know true infection status of the animals. As an example, latent class analysis has been used to evaluate the sensitivity and specificity of different tests to detect *E. multilocularis* infections (Maas et al. 2016; Wahlström et al. 2016; Otero-Abad et al. 2017). Wahlström et al. (2016) used this analysis to assess the sensitivity and specificity of a semi-automated magnetic capture probe-based DNA extraction and real-time PCR technique finding no significant differences compared to the SCT test. Nonetheless, Maas et al. (2016) tested a similar magnetic capture and PCR method that evidenced a higher sensitivity than SCT and IST (Intestinal Scraping Technique), although the uncertainties in sensitivity estimates were wide for all the tests evaluated. The latent class analysis could be used as a complementary approach to assess the performance of the MB-qPCR when comparing two populations from areas with low and high prevalence.

Correlation between worm burden and Cq value

Previous studies have shown a negative correlation between the worm burden and the Cq value (Isaksson et al. 2014), or with the concentration of DNA in the fecal sample (Knapp et al. 2014). We observed the same negative correlation; however, the variation of the range of Cq values according to SFCT worm burden was higher than expected. This could be related to the fact that there is not a perfect correlation between the number of eggs in the feces and the worm burden in the intestine. The irregular shedding of eggs during the patency period added to additional sources of parasite DNA such as disintegrated worms in the feces could significantly affect the Cq value (Conraths and Deplazes 2015). In fact, Knapp et al. (2014) found that the DNA concentration from one adult worm was 400 times higher than that of one egg. Therefore, the Cq should be used only as an indicator of the worm burden (Isaksson et al. 2014).

Conclusions

The magnetic bead-based DNA extraction and real-time PCR proved to have a sensitivity comparable to the SFCT to detect *E. multilocularis* and could be a valuable tool to identify undetected co-infections with *E. canadensis* based on morphological diagnosis, with the advantage of increasing throughput processing fecal samples in an automated way. Nevertheless, the kind of study and the parameters that need to be determined are key for choosing the most appropriate technique. Indeed, necropsy techniques are still necessary to determine a precise worm burden and to collect adult worms for genetic studies. Here, we propose a combination of molecular and morphological identification using the MB-qPCR to identify positive intestine samples to *E. multilocularis* and *E. canadensis*. Hence, it would be possible to save time in studies focused not only on the prevalence but also the genetics of the parasite, host-parasite interactions, or the study of co-infections of *Echinococcus* species that can be difficult to identify only based on morphology.

Future research should be focused on assessing the diagnostic specificity of MB-qPCR in low prevalence areas and the use of latent class analysis comparing two populations in the absence of a perfect gold standard method.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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