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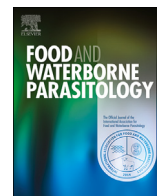
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A new SYBR green real-time PCR assay for semi-quantitative detection of *Echinococcus multilocularis* and *Echinococcus canadensis* DNA on bilberries (*Vaccinium myrtillus*)

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ABSTRACT

Berries and vegetables are potential transmission vehicles for eggs of pathogenic parasites, such as *Echinococcus* spp. We developed a SYBR Green based semi-quantitative real-time PCR (qPCR) method for detection of *Echinococcus multilocularis* and *Echinococcus canadensis* DNA from berry samples. A set of primers based on the mitochondrial NADH dehydrogenase subunit 1 (*nad1*) gene was designed and evaluated. To assess the efficacy of the assay, we spiked bilberries (*Vaccinium myrtillus*) with a known amount of *E. multilocularis* eggs. The detection limit for the assay using the NAD1_88 primer set was 4.37×10^{-5} ng/ μ l of *E. multilocularis* DNA. Under artificial contamination of berries, 50 *E. multilocularis* eggs were reliably detected in 250 g of bilberries. Analytical sensitivity of the assay was determined to be 100% with three eggs. As an application of the assay, 21 bilberry samples from Finnish market places and 21 bilberry samples from Estonia were examined. Previously described sieving and DNA extraction methods were used, and the samples were analyzed for *E. multilocularis* and *E. canadensis* DNA using semi-quantitative real-time PCR and a melting curve analysis of the amplified products. *Echinococcus* DNA was not detected in any of the commercial berry samples. This easy and fast method can be used for an efficient detection of *E. multilocularis* and *E. canadensis* in bilberries or other berries, and it is applicable also for fruits and vegetables.

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1. Introduction

International trade of fresh berry products has expanded, and a greater knowledge of food borne pathogens, including zoonotic parasites, is required by consumers and authorities (Tefera et al., 2018). Taeniid cestodes of the genus *Echinococcus* are significant food-borne zoonotic pathogens at a global scale (Develesschauer et al., 2017). Alveolar echinococcosis, caused by *Echinococcus multilocularis*, is a zoonosis of great public health importance. In humans, alveolar echinococcosis can lead to severe symptoms and even fatal consequences because of the invasive, metastatic growth of the metacestode cysts. The

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definitive hosts of *Echinococcus* spp. are carnivores, mainly canids, such as fox, wolf and dog, and the intermediate hosts are mainly herbivores, including ruminants, camels, pigs, horses and rodents. Definitive hosts get infected by ingesting larval metacestode cysts. Intermediate hosts get infected by ingestion of parasite eggs. Humans can acquire the infection as intermediate hosts, but are often called accidental or aberrant, or dead-end hosts, because they do not normally participate in the parasite lifecycle as a prey of definitive hosts. Infective eggs are resistant to environmental factors such as low temperatures (Veit et al., 1995). It is shown that berries are possible transmission vehicles for taeniid eggs (Malkamäki et al., 2019), which may infect humans consuming berries raw. Vegetables, berries and fruits often have a complex surface structure that enables the attachment of the eggs. Because of the taste and nutritional value, these foodstuffs are often consumed raw (Tefera et al., 2018). The risk of *E. multilocularis* contamination of potential transmission vehicles, such as fruits, berries and vegetables, has been studied recently (Alvarez Rojas et al., 2018; Federer et al., 2016; Kern et al., 2004; Lass et al., 2015). Eggs can spread to foodstuff from feces of definitive hosts directly or be transferred, for example, by birds or insects (Thompson and McManus, 2001; Torgerson et al., 1995). It has been suggested that contaminated foodstuff are the most important infection route to humans for *E. multilocularis* (Alvarez Rojas et al., 2018). In some countries, the prevalence of *E. multilocularis* in foxes is high even in urban environments (Deplazes et al., 2004; Vuitton et al., 2015). In highly endemic areas for alveolar echinococcosis, a high occurrence of *E. multilocularis* DNA has been shown in carnivore feces in kitchen gardens (Pouille et al., 2017). *Echinococcus multilocularis* DNA has been isolated from fruits, vegetables, and mushrooms in endemic areas using conventional nested PCR (Lass et al., 2015). *Echinococcus granulosus* sensu lato (s.l.) DNA was detected from commercial vegetables and fruits using multiplex PCR and sequencing (Federer et al., 2016). *Echinococcus multilocularis* eggs have also been isolated from soil samples (Knapp et al., 2014; Umhang et al., 2017). For detection of different *Echinococcus* species, a number of conventional and multiplex PCR and different probe-based qPCR protocols have been developed and published (Isaksson et al., 2014; Knapp et al., 2014; Lass et al., 2015; Macchiaroli et al., 2015; Schurer et al., 2019; Trachsel et al., 2007). To the authors' knowledge, there is no published SYBR Green based qPCR assay for detection of *E. multilocularis*. In this study, we investigated a set of primers to develop a sensitive SYBR Green based qPCR assay for detection of *Echinococcus* DNA from berries. It can also be applied to investigation of other foodstuff when SYBR Green chemistry is used.

2. Materials and methods

2.1. Parasites

We used adult *E. multilocularis* worms and *E. canadensis* and *E. granulosus* sensu stricto (s.s.) protoscolices to obtain eggs and/or DNA. *Echinococcus* species and genotypes were confirmed by conventional PCR of partial COX1 gene and sequencing. In addition, specimens of other taeniid or cyclophyllid species were used to verify the specificity of the method. Species, genotypes and their origins are listed in Table 1. Adult parasites were stored in 70% ethanol, and the metacestodes were stored frozen at -20°C or in ethanol.

2.2. DNA extraction from metacestodes and adult parasites

DNA was extracted from approximately 20 mg of parasite tissue or 10 protoscolices using a Tissue and Hair Extraction Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The incubation time was 2 h at 56°C with 50 μl of incubation buffer. After this, 100 μl of lysis buffer and 7 μl of resin were added. The sample was vortexed for 3 s, and 3 washing steps with 100 μl of washing buffer were done. DNA was diluted in a final volume of 50 μl with elution buffer. After extraction, the DNA was column-purified with a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher scientific, USA), and stored at -20°C until real-time qPCR.

Table 1

Species, genotypes, their origins and the qPCR results in SYBR Green semi-quantitative PCR reaction of the parasites used in this study.

Species	Isolate origin	c DNA ng/ μl	Cq mean	T _m °C mean	Nad1_88 qPCR results
<i>Echinococcus multilocularis</i>	Hungary, <i>Vulpes vulpes</i>	9	19.35 (± 0.05)	78.3–78.4	+
<i>Echinococcus multilocularis</i>	Norway, <i>Microtus levis</i>	12	21.51 (± 0.06)	78.3–78.5	+
<i>Echinococcus canadensis</i> genotype G10	Finland, <i>Rangifer tarandus</i>	28	17.91 (± 0.08)	78.8–78.9	+
<i>Echinococcus canadensis</i> genotype G10	Finland, <i>Rangifer tarandus</i>	20	25.05 (± 0.02)	78.8–78.9	+
<i>Echinococcus canadensis</i> genotype G10	Finland, <i>Rangifer tarandus</i>	14	25.33 (± 0.09)	78.9–79.0	+
<i>Echinococcus granulosus</i> s.s. genotype G1	East Europe, human	25	25.00 (± 1.66)	79.1	+
<i>Echinococcus granulosus</i> s.s. genotype G1	Romania, <i>Ovis aries</i>	11	32.70 (± 1.71)	78.9–79.0	+
<i>Hydatigera kamiyai</i>	Finland, <i>Felis catus</i>	16	N/A	–	–
<i>Versteria mustelae</i>	Finland, rodent	25	N/A	–	–
<i>Taenia laticollis</i>	Finland, <i>Lynx lynx</i>	14	N/A	–	–
<i>Taenia lynciscapreoli</i>	Finland, <i>Lynx lynx</i>	12	N/A	–	–
<i>Moniezia</i> sp.	Finland, <i>Bos taurus</i>	40	N/A	–	–
<i>Mesocestoides</i> sp.	Finland, <i>canis familiaris</i>	24	34.63 (± 0.27)	78.0	–

2.3. Primer design and qPCR assay

PCR primers are designed with Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) for the molecular detection of *E. multilocularis*. The complete mitochondrial genome of *E. multilocularis* is available in GenBank with the accession number AB018440. The target chosen for this purpose is a part of the mitochondrial NADH dehydrogenase subunit 1 (*nad1*) gene. The forward sequence of the selected primer pair named EmNAD1_88 is 5'-TTGTGTGCTGGTTGGGGTAG-3' and the reverse sequence is 5'-AACAAAGCTTCAAACCTAACAGACC-3', and the product size is 88 bp. In in-silico analysis in Primer-BLAST, the designed primers did not show any relevant cross reactivity with the gene sequences of other database organisms than *E. multilocularis*, *E. canadensis*, *E. granulosus* s.s., *E. equinus* and *E. ortleppi*. Among other cestodes, *Mesocostoides* sp. showed closest similarity with two to four nucleotide differences.

A real-time qPCR assay was performed in white 96-well plastic plates in a final reaction volume of 20 µl that consisted of 10 µl of 2 X SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA), 1 µl of 5 µM primer solution (final concentration 250 nM) containing both forward and reverse primers (custom primers from Bio-Rad Laboratories), 5 µl of template DNA and 4 µl of nuclease-free water. Cycling conditions were as follows: an initial activation step of 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing and elongation at 60 °C for 30 s using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The melting curve was constructed from 65 °C to 95 °C at 0.5 °C increments with a dwell time of 5 s at each temperature.

Real-time qPCR reaction conditions were optimized for *E. multilocularis* using the gradient PCR method with eight annealing temperatures ranging from 54 °C to 64 °C. The optimal annealing temperature was 59.5 °C. Melting curve analysis showed a distinct melting point (T_m) of 78 °C consistently specific for the amplicon without non-specific products or primer dimers. Products were also tested by running them in a 2% agarose gel stained with ethidium bromide. In the agarose gel, one distinct band was visible at the expected molecular weight (88 bp); no other bands were detected.

To obtain the calibration curve and the detection limit, a ten-fold dilution series of *E. multilocularis* DNA was prepared. Concentrations from 4.37 ng/µl to 4.37×10^{-10} ng/µl were prepared to determine the limit of detection. The detection limit was determined as the point with the lowest DNA concentration for which all six replicates showed positive amplification. The readout of the reaction with melting temperatures of 78 ± 0.5 °C and a Cq value below the Cq of the detection limit (34.31) was used to confirm a positive reaction. Each sample was amplified in six replicates and the average Cq value and standard deviation were calculated. The amplification was analyzed using Bio-Rad CFX Maestro software. Standard curve was constructed also for *E. canadensis* (concentrations to 28 ng/µl to 28×10^{-6} ng/µl) and *E. granulosus* s.s. (concentrations to 5.64 ng/µl to 5.64×10^{-6} ng/µl).

2.4. Specificity

To check the specificity of PCR amplification for the gene fragment of the *nad1* gene, we tested *Echinococcus*, other taeniid species and other cyclophyllid cestodes listed in Table 1. The qPCR was performed in four replicates for each sample. The additional melting curve was constructed with 0.1 °C increment between readings to *Echinococcus* samples listed in Table 1. The readout of the reaction with melting temperatures of 78 ± 0.5 °C and a Cq value below the Cq of the detection limit (34.31) was used to confirm a positive reaction. Positive reactions were confirmed by sequencing.

2.5. Analytical sensitivity of the assay with the known number of eggs

To assess the sensitivity of the test, defined numbers of *E. multilocularis* eggs were studied. To obtain the *E. multilocularis* eggs, caudal segments of gravid *E. multilocularis* worms were sliced with a scalpel blade under stereomicroscope in a Petri dish, a drop of Milli-Q water was added on top of the segment, and eggs were retrieved with micropipette and collected to Eppendorf tubes. The number of eggs was one, three, five and ten, number of samples per amount of eggs was four. The qPCR was performed in four replicates for all the samples. DNA extracted from adult *E. multilocularis* worm was used as a positive control.

2.6. Analysis of bilberries artificially spiked with *E. multilocularis* eggs

To assess the efficacy of the sieving method, DNA extraction and real-time PCR, gradually decreasing quantities of *E. multilocularis* eggs were added to samples of 250 g of Finnish bilberries from the batch previously tested negative for parasite DNA by qPCR method described in this study. Berries grown in western Finland were purchased from the marketplace as a fresh product. Berries were stored at -20 °C and thawed at room temperature before spiking. The number of eggs spiked to 250 g of berries was 0 (n = 1), 1 (n = 5), 5 (n = 5), 10 (n = 5), and 50 (n = 5). Eggs were retrieved under a stereo microscope and pipetted onto the berries. Washing of the berries and DNA extraction of the debris was performed as in a previous study (Malkamäki et al., 2019). Briefly, the berry samples with spiked eggs were washed on an automatic shaker shaking berries in a 0.04% Tween 20 washing solution for 30 min at 50 rpm at room temperature. The washing solution and berries were sieved with three sieves of the size 200 (diameter) x 50 mm (height) with different mesh sizes; 1 mm and 63 µm sieves to remove larger particles and 20 µm to retain taeniid eggs. The material from the smallest mesh size sieve was collected and centrifuged for 10 min at 670×g. Then the pellet was transferred to a 2 ml Eppendorf tube and the sample was further concentrated by

centrifuging it for 15 min at $9000\times g$ (Eppendorf centrifuge 5415 D, Germany). DNA was extracted from received debris using a Tissue and Hair Extraction Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions with slight modifications to adapt the protocol for the sieving debris: After incubation the sample was vortexed for 5 s and centrifuged for 10 s and then transferred to a DNA isolation tube and $150\ \mu\text{l}$ of lysis buffer and $14\ \mu\text{l}$ of resin were added. The DNA was column-purified with a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The qPCR was performed in four replicates for all the samples. DNA extracted from an adult *E. multilocularis* worm was used as a positive control.

2.7. Analysis of commercial bilberry samples

We purchased fresh bilberry samples from marketplaces from Finland ($n = 21$) and Estonia ($n = 21$) during summer 2018. Finnish samples were from several wholesalers originating from 16 different localities mainly from southern, eastern and central Finland. The origin of the Estonian market berries is not known. Samples were stored frozen at $-20\ ^\circ\text{C}$ and thawed at room temperature prior to washing and sieving of the berries. Prior to washing, 250 g of berries were weighed for examination. DNA extraction was performed as described in section 2.5. The qPCR was performed, and the melting curve was constructed as described in this study.

3. Results

3.1. Sensitivity of the real-time qPCR method

A standard curve was constructed with a ten-fold dilution series of the template DNA from an adult *E. multilocularis* worm, and a linear relationship was defined between the logarithmic DNA concentration and mean quantification cycle (Cq) values. The determined efficiency was 102.8%. The correlation coefficient (R^2) of the standard curve was 0.999 (slope -3.26) (Fig. 1). The detection limit of the assay was determined to be Cq 34.31 (± 0.25) obtained with a DNA concentration of 4.37×10^{-5} ng/ μl . This was the lowest concentration that showed positive amplification with all six replicates. In the next lower concentration, 4.37×10^{-6} ng/ μl , mean Cq value was 38.71 (± 0.25), while sample type negative controls were showing amplification in a same level, mean Cq value being 38.70 (± 0.42), thus Cq values over 34.31 were considered negative. A standard curve was also constructed for *E. canadensis* genotype G10. The efficiency was 98.2% and the correlation coefficient (R^2) of the standard curve 0.998 (slope -3.37). For *E. granulosus* s.s. genotype G1, the efficiency was very high, 349.4%.

3.2. Specificity

Among the *Echinococcus* species tested, *E. granulosus* s.s. and *E. canadensis* were amplified using the *E. multilocularis* NAD1_88 qPCR method. Results are presented in Table 1. The melt curves of different *Echinococcus* species constructed with $0.1\ ^\circ\text{C}$ increment between readings showed slightly distinct melting points between *E. multilocularis* (Tm $78.3\text{--}78.5\ ^\circ\text{C}$) and *E. canadensis* (Tm $78.8\text{--}79.0\ ^\circ\text{C}$). (Fig. 2). The melting point of *E. granulosus* s.s. (Tm $78.9\text{--}79.1\ ^\circ\text{C}$) was overlapping with melting point of *E. canadensis*. Other cestode samples tested were negative.

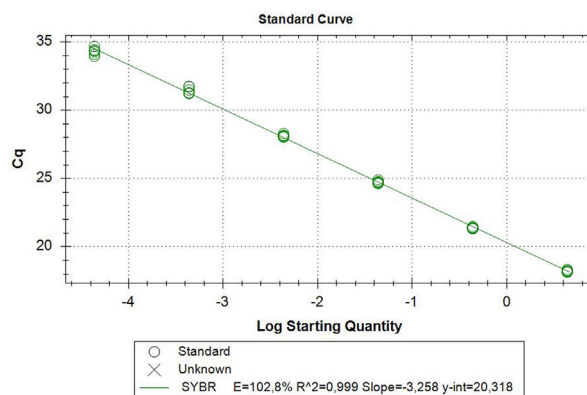


Fig. 1. A standard curve of the SYBR Green qPCR reaction. Data represents six replicates of each reaction. A ten-fold serial dilutions are linear between Cq values and logarithmic quantity of *E. multilocularis* DNA.

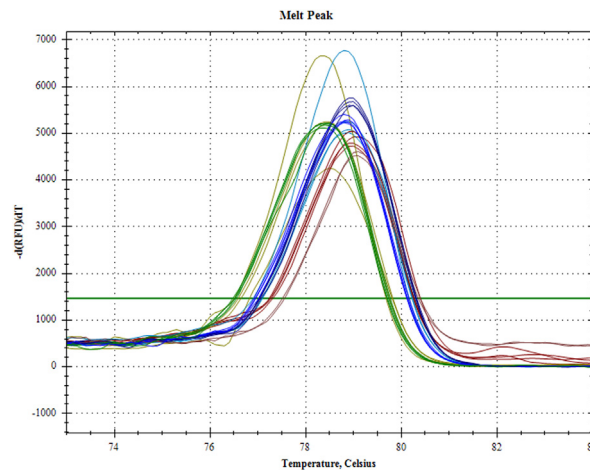


Fig. 2. Melting curve analysis constructed with 0.1 °C increment to two *E. multilocularis* (green), three *E. canadensis* (blue) and two *E. granulosus* s.s. (red), isolates in four replicates.

3.3. The outcome of the qPCR of a known amount of *E. multilocularis* eggs

The analytical sensitivity of the assay with only eggs was 100% with three eggs, Cq values were between 26.31 and 31.47, with standard deviation of 0.4–0.1. With five eggs, one out of four samples was negative. Others had a Cq values between 28.15 and 32.8, standard deviation being 0.08–0.3. Result with 10 eggs was Cq 28.00–28.37 and the standard deviation was from 0.05 to 0.11. With one egg, all the samples were negative.

3.4. The outcome of the qPCR of artificially spiked *E. multilocularis* eggs in bilberries

Mean quantification cycle (Cq) values were between 30.90 and 31.35 with 50 eggs in bilberries. The standard deviation of the Cq values varied between 0.04 and 0.19. The sensitivity of the complete method was 100% with 50 eggs in 250 g of berries. With ten eggs, only one of six samples had Cq value under the limit of detection (34.31), having Cq value of 33.45 (± 0.41). With five eggs, also one of six samples was positive with Cq value 33.40 (± 0.26). The negative control bilberry sample with no spiked eggs was negative in real-time qPCR.

3.5. Analysis of commercial berry samples

Application of the optimized qPCR method was performed with commercial berry samples. All of the 42 bilberry samples tested were negative in real-time qPCR with NAD1_88 primers for detection of *E. multilocularis* and *E. canadensis* DNA. The readout of the reaction with melting temperatures of 78 ± 1.0 °C and a Cq value below the detection limit (34.31) was used to confirm a positive reaction.

4. Discussion

Recent works have suggested that fresh products could serve as a vehicle for taeniid egg (Federer et al., 2016; Lass et al., 2015; Malkamäki et al., 2019). Direct detection of *Echinococcus* eggs is far from a routine procedure and currently, there is no standardized methodology for the detection of taeniid eggs in food samples (Alvarez Rojas et al., 2018). The SYBR Green real-time PCR protocol described here was developed to target the *nad1* gene of *Echinococcus* spp. The method is able to initially differentiate *E. multilocularis* and *E. canadensis* due to slightly distinct melting points of the qPCR product of these organisms. Melting points were distinct, but near each other, 78.3–78.5 °C for *E. multilocularis* and 78.8–79.0 °C for *E. canadensis*. There is a possibility that melt curves this close can be overlapping, since it is not uncommon to have 0.5 °C variation in the melting point (Ririe et al., 1997). The melting points of *E. canadensis* qPCR products were partly overlapping with melting point of the qPCR products of *E. granulosus* s.s., that was 78.9–79.1 °C. *E. granulosus* s.s. and *E. canadensis* both belong on a same group *Echinococcus granulosus* s.l. complex. For the confirmation of the species, sequencing would be preferred for the positive samples detected with this assay, especially in geographic areas where more than one species of *Echinococcus* coexist. In specificity testing, *Mesocestoides* sp. DNA was amplified with only slightly higher Cq value than the determined detection limit, and had the same melting temperature as *E. multilocularis*. This could be considered a potential source of false positive result. However, the amount of *Mesocestoides* DNA used for testing was much higher than it could be in real DNA extracts from berry samples. For the confirmation of the species, sequencing would be preferred for the positive amplifications detected

with this assay, especially in geographic areas where more than one species of *Echinococcus* coexist, and in areas considered free from the species detected.

This method is easy and fast to perform, and it has a good efficacy for detection of *E. multilocularis* and *E. canadensis* and relatively low costs when comparing to the probe-based real-time qPCR. In amplification of *E. granulosus* s.s., efficiency was constantly too high, thus the method cannot be recommended for detection of this parasite. High efficiency can be due to amplification of unspecific products i.e. generation of excimers. SYBR Green can bind to any double stranded DNA and this offers a possibility to develop primers for assays that can detect several pathogenic organisms with distinct melting points in a melt-curve analysis. This lowers the diagnostic costs and makes the assay more applicable and practicable for screening purposes compared to probe-based assays that are highly specific. The reagents for SYBR Green assay are also cheaper and primers do not require labels.

The semi-quantitative method developed was able to detect the *E. multilocularis* DNA concentration of 4.37×10^{-5} ng/ml of DNA in a 5 µl of reaction solution. Theoretically, the approximate amount of DNA in single *E. multilocularis* egg is 0.008 ng (Gottstein and Mowatt, 1991). In this study, the qPCR result of 50 eggs in 250 g of bilberries was detected reliably. Analytical sensitivity with plain *E. multilocularis* eggs as a sample was 100% with 3 eggs. With 5 eggs one sample showed a negative result probably due to a laboratory error, as the eggs are easily lost when picked up one by one. From samples of one egg, only one sample showed amplification, with Cq value slightly over the detection limit of the assay. In theory, only one egg should be detected. It is obvious that the washing and sieving method is not able to retrieve all parasitic eggs. Using higher quantities of the berries would be more difficult to sieve and potentially reduce sensitivity. Experience from preliminary studies shows that freezing is also lowering sensitivity, but is often necessary for practical reasons. In addition, the detection method should be applicable for frozen berries, which are common commercial berry products. For practical use, it would be advisable to examine multiple samples from the batch rather than sieve larger quantities of berries. Another justification for multiple small samples is that egg contamination of the berries is originating from feces of carnivorous definitive host as a point contamination before harvesting.

Limit of detection was determined to be Cq 34.31 in this study. Because Cq values above this determined cutoff value are interpreted as negative results, there is a possibility of not detecting a small amount of DNA in a sample. Examples of the late non-specific amplification with corresponding melting curves are provided as additional (supplementary data Fig. S1 and S2) figure. Also, the presence of inhibitory substances can lead to false-negative qPCR results. For these reasons, an additional step of inhibitor removal as well as inhibitor tolerant SYBR Green Supermix were used (Malkamäki et al., 2019).

For testing the application of the assay, sieving, DNA extraction and qPCR with developed primer pair EmNAD1_88 was performed for 21 bilberry samples from Finland and 21 bilberry samples from Estonia. In this study, *Echinococcus* DNA was not detected in commercial bilberries purchased from the marked places around Finland and Estonia. Negative finding was not surprising because of the rareness of *Echinococcus* species and the small sample size. Finland is one of the few countries in Europe claiming to be free from *E. multilocularis* (Commission Delegated Regulation (EU) No 1152/2011; Wahlström et al., 2015) and treatment against *E. multilocularis* is required for dogs before entry to Finland. In Estonia, *E. multilocularis* is endemic (Moks et al., 2005).

In conclusion, SYBR Green based real-time qPCR is an effective method for semi-quantitative detection of *E. multilocularis* and *E. canadensis* DNA from the berries, that can be preliminary differentiated due slightly different melting point of the qPCR product in a melting curve analysis. Analytical sensitivity of the assay was 100% with three eggs. The sensitivity of the complete method was 100% with 50 eggs in 250 g of bilberries. Recovery tests with the *Echinococcus* eggs were performed only for *E. multilocularis* eggs due to availability of the eggs. The development of fast and cost-effective testing methods can advance the control of food borne echinococcosis in endemic areas, especially in northern boreal and arctic zones where *E. canadensis* and *E. multilocularis* are present in sylvatic life cycles and, on the other hand, berry picking has a long tradition and economic significance.

Ethical standards

The study was performed in compliance with current Finnish national laws and regulations.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fawpar.2019.e00068>.

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