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Berries as a potential transmission vehicle for taeniid eggs

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ABSTRACT

Potential role of wild forest berries as a transmission vehicle for taeniid eggs was examined using non-zoonotic *Taenia laticollis* eggs as a model. The berries studied were bilberries (*Vaccinium myrtillus*) (1 m^2 plot, $n = 10$) and lingonberries (*Vaccinium vitis-idaea*) (1 m^2 plot, $n = 11$). The plots in the managed forest were evenly sprayed with 30,000 or 60,000 *T. laticollis* eggs suspended in water, and berries were collected 24 h after spraying. The berries were rinsed with water, and the water was sieved through a 1-mm and a 63-μm sieve to remove coarse material and through a 20-μm sieve to collect possible eggs. A small proportion of the sieved material was examined by microscopy after treatment with fluorescent Calcofluor White stain, which binds to eggshell chitin. In the recovery tests in artificially spiked samples, the detection limit was 5 eggs in 100 g of commercial frozen bilberries and lingonberries. Taeniid eggs were detected in all of the 10 experimentally contaminated bilberry samples and in 10 of 11 lingonberry samples. The sieved debris was also analyzed for *T. laticollis* DNA using semi-quantitative PCR. All samples were positive in quantitative SYBR Green real-time PCR using a *T. laticollis*-specific primer pair amplifying a short fragment of mitochondrial NADH dehydrogenase subunit 1 gene. This indicates that forest berries contaminated in shrubs contained *T. laticollis* eggs, and that berries can serve as a vehicle for taeniid eggs and may pose a possible risk to humans.

1. Introduction

Echinococcus multilocularis is a zoonotic tapeworm of great public health importance. The natural definitive hosts are canids and the intermediate hosts are mainly rodents. Humans can acquire the infection as accidental intermediate hosts. In humans, alveolar echinococcosis caused by *E. multilocularis* is a life-threatening disease. Infection in humans can occur by accidental ingestion of parasite eggs, which are highly resistant to environmental factors [1]. There is a concern that forest berries could carry *Echinococcus* infections to humans [2,3]. It has been suggested that parasite eggs may attach to the surface of the berries and infect humans consuming them raw. Recently, research has been directed to the risk of *E. multilocularis* contamination of foodstuffs, mainly fruits, berries and vegetables [3,4]. Plants growing near the ground and consumed raw, such as berries and lettuce, could be particularly potential transmission vehicles. They are relatively difficult to wash properly. In theory, eggs could spread from the feces of definitive hosts directly onto berries or be transported by insects or water drops [5,6]. In Germany, a statistically significant relationship has been noted between alveolar echinococcosis and chewing grass and eating unwashed strawberries [2], but in a similar study in France, no significant relationship was found between alveolar echinococcosis and eating raw wild berries [7]. Recent study in France showed high occurrence of *E. multilocularis* DNA in dog and fox fecal material collected from kitchen gardens in areas highly endemic to *E. multilocularis* [8].

Echinococcus eggs lose their infectivity at temperatures below −70 °C, but are resistant to freezing at −20 °C, a standard temperature of household freezers [9]. Some studies indicate that the consumption of contaminated food or water may play a role in human echinococcosis [2,10]. *Echinococcus multilocularis* DNA has been isolated from fruits, vegetables, and mushrooms in the endemic areas in Poland using conventional nested PCR [3]. *Taenia* spp. and *Echinococcus granulosus* sensu lato DNAs were detected from the commercial vegetables and fruits in Switzerland using a multiplex PCR and sequencing [4]. The long incubation period before development of clinical signs of alveolar echinococcosis makes it difficult to determine the potential vehicles in epidemiological studies [11]. Moreover, the low prevalence of human alveolar echinococcosis complicates studies [12]. WHO has included alveolar and cystic echinococcosis in the Neglected Tropical Disease list with a globally estimated disease burden of 2–5 × 10^7 DALY (Disability Adjusted Life Years) [13]. After detection of *E. multilocularis* in Sweden in 2011 [14], the authorities have encouraged good hand hygiene when handling foodstuffs, and people have been informed that boiling is the only effective way in households to inactivate *E. multilocularis* eggs

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Table 1
Calcofluor White staining and real-time PCR results for berry samples from plots experimentally contaminated with eggs of *T. laticollis*. The mass of the berry harvest from the plots ranged from 20 g to 309 g (average 124 g). Mean quantification cycle (Cq) values were between 25.70 and 32.48 (average 29.08).

Berry Species	Number of eggs sprayed on the plot (x 10^3)	Identi- fication of the plot	Mass (g)	Rain (dry, slight, moderate)	Micros-copy result	Real-time PCR mean Cq value (SD)	Real-time PCR mean starting quantity (ng/μl)
Bilberry	30	$A_{bilberry}$	178	Dry	+	27.67 (0.42)	4.78×10^{-3}
	30	$B_{bilberry}$	228	Dry	+	27.76 (0.53)	4.55×10^{-3}
	60	$C_{bilberry}$	210	Dry	+	28.72 (0.33)	2.29×10^{-3}
	30	$D_{bilberry}$	134	Dry	+	28.88 (0.00)	2.02×10^{-3}
	30	$E_{bilberry}$	92	Dry	+	30.12 (2.46)	1.55×10^{-3}
	30	$F_{bilberry}$	120	Dry	+	26.96 (0.17)	7.57×10^{-3}
	60	$G_{bilberry}$	225	Dry	+	26.75 (0.75)	9.42×10^{-3}
	30	$H_{bilberry}$	309	Slight	+	27.80 (0.12)	4.27×10^{-3}
	30	$I_{bilberry}$	145	Slight	+	30.00 (0.07)	9.30×10^{-4}
	30	$J_{bilberry}$	109	Slight	+	28.89 (0.10)	2.01×10^{-3}
Lingon berry	60	$A_{lingonberry}$	103	Dry	+	30.75 (0.17)	5.60×10^{-4}
	30	$B_{lingonberry}$	95	Dry	+	28.05 (0.20)	3.60×10^{-3}
	30	$C_{lingonberry}$	165	Dry	+	29.13 (0.72)	1.80×10^{-3}
	30	$D_{lingonberry}$	79	Moderate	+	27.18 (0.67)	6.92×10^{-3}
	30	$E_{lingonberry}$	113	Moderate	+	32.35 (0.12)	1.80×10^{-4}
	30	$F_{lingonberry}$	105	Moderate	+	30.58 (0.55)	7.57×10^{-3}
	30	$G_{lingonberry}$	76	Slight	+	31.99 (0.45)	9.42×10^{-3}
	30	$H_{lingonberry}$	40	Slight	+	32.48 (0.12)	1.70×10^{-4}
	30	$I_{lingonberry}$	26	Slight	−	29.88 (0.02)	1.01×10^{-3}
	30	$J_{lingonberry}$	20	Slight	+	29.14 (0.02)	1.69×10^{-3}
	60	$K_{lingonberry}$	26	Slight	+	25.70 (0.08)	18.37×10^{-3}

[15]. However, the Swedish authorities do not wish to alarm people unnecessarily and have stated that it is not necessary to wash or boil berries to avoid of *E. multilocularis* infection [15].

The hypothesis of this study was that forest berries can serve as a vehicle for taeniid eggs and thereby *Echinococcus* infection. In this study, non-zoonotic *Taenia laticollis* eggs were used as a model. These taeniid eggs do not cause a public health risk. Tapeworms of *Echinococcus* species and *Taenia* species belong to the same family, Taeniidae, and their eggs are comparatively similar in appearance, structure, and physical characteristics [16]. Therefore, a method developed for the detection of taeniid eggs could also be applicable for the detection of *Echinococcus* eggs. Lingonberries (*Vaccinium vitis-idaea*) and bilberries, also called European blueberries (*Vaccinium myrtillus*), in managed forest were selected as model foodstuffs in this experiment. Their shrubs are low-growing and thus theoretically easily exposed to carnivore feces when rainwater splashes from the ground and can therefore potentially become contaminated with parasite eggs.

2. Materials and methods

2.1. Parasite eggs used for contamination

We used adult *T. laticollis* parasites to obtain eggs and DNA in this study. *Taenia laticollis* worms were collected from the intestines of lynx (*Lynx lynx*) carcasses submitted by the authorities for necropsy at the Finnish Food Safety Authority Evira in 2016–2017 and identified morphologically. Eggs were harvested by mincing adult parasites in a blender, and after sieving (2 mm, 60 μm and 20 μm), they were cleaned by repeated sedimentation and centrifugation with 300 $\times g$ for 10 min in water, and stored in water at −20 °C. Before freezing, every batch of egg samples was evaluated under the stereomicroscope to ensure that immature eggs or other material wasn't apparent. Adult parasites used to obtain positive DNA controls were stored in 70% ethanol. The contamination doses of *T. laticollis* eggs (60,000 or 30,000 eggs) were prepared from the suspension of the eggs in distilled water. The egg concentration of the suspension was determined by the quantitative modified McMaster flotation method using $MgSO_4$ (saturated solution). A total of 2.6 ml of $MgSO_4$ solution was mixed with 0.4 ml of egg suspension and both chambers of the McMaster slide were filled fully with Pasteur pipette. Then the slide was left standing for 5 min to allow the eggs to float to the surface of the flotation solution. The McMaster chambers were examined and eggs counted under laboratory microscope at 100× magnification in two replicates to obtain the amount of eggs per milliliter. Contamination doses of 30,000 and 60,000 eggs were prepared and stored in Falcon tubes, frozen at −20 °C, and thawed at room temperature before preparing the final contamination suspension at the research site, where tap water was added to the egg dose to obtain final volume of 200 ml, suspension was carefully mixed and loaded in the single used spraying can.

2.2. Recovery test

To assess the efficacy of the sieving method, DNA extraction, Calcofluor White method and real-time PCR, a known quantity of *T. laticollis* eggs was added to 100 g of commercial Finnish frozen bilberries and lingonberries. Berries were thawed at room temperature before spiking. The DNA extracted from adult *T. laticollis* was used as a positive control. The number of eggs spiked to 100 g of berries was 5 ($n = 3$), 10 ($n = 1$), 50 ($n = 1$), 100 ($n = 1$), and 500 ($n = 1$). Eggs were retrieved under a stereo microscope and pipetted onto the berries. The samples were treated in the same way as those described for the experimental contamination of plots as described below.

2.3. Experimental contamination of berry plots

Experimental contamination of berries with *T. laticollis* eggs in the field condition was conducted in forests with the landowner's permission in autumn 2017. The number of the plots was 10 for bilberries in a single geographical location (Sastamala 61.34 N, 22.92 E, Finland) and 11 for lingonberries in four different locations (Heinola 61.21 N, 26.05 E, Kihniö 62.20 N, 23.18 E, Muonio 67.96 N, 23.68 E, and Sastamala 61.34 N, 22.92 E, Finland). The research plots were selected where berry density of the particular berry species was the greatest, measured 1 × 1 m and were marked with signs (in Finnish, English and Thai, because of many professional Thai berry-pickers) and bright-colored ribbons. Plots were identified with a letter from A to J for each berry species (Table 1). Before contamination, approximately one third to one half of the berries within each plot were picked as a control sample. The weather conditions (temperature, rain, wind) were recorded at the time of contamination and subsequent 24 h. Plots were contaminated by spraying as evenly as possible 30,000 (17 plots) or 60,000 (4 plots) eggs suspended in 200 ml water on the plot with single use spraying can at

one squirt every 10 × 10 cm from a height of approximately 1 m. The spraying method and distances were determined by a preliminary experiment using colored water on a white absorbent surface of 1 m^2 from the height of 1 m. The spray can was shaken during spraying to prevent the sedimentation of the taeniid eggs. After 24 h, all the berries from the plot were picked by gloved hand and stored in plastic bags at −20 °C until analysis.

2.4. Washing of the eggs from berry samples

All the berry samples (corresponding to the mass reported in a Table 1) were washed in a bucket with 5 l of tap water containing 0.04% Tween 20 and shaken on an automatic shaker for 30 min at 50 rpm at room temperature. After this, the water was strained through three sieves of the size 200 × 50 mm with different mesh sizes; 1 mm and 63 μm sieves to remove coarse debris and 20 μm to retain taeniid eggs [17]. The sludge that remained on the sieve with the 20 μm mesh size was collected into a 10 ml glass tube with an automatic pipette by flushing the sieved material at the corner of the sieve with tap water and centrifuged for 10 min at 670 ×*g*. The supernatant was removed, and the pellet was transferred to a 2 ml Eppendorf tube. To further concentrate the sample, it was centrifuged for 15 min at 9000 ×*g* (Eppendorf centrifuge 5415 D, Germany). The supernatant was removed from the tube by pipetting. The pellet was used for further analysis immediately or stored at 4 °C for a maximum of 24 h. Between the sieving of separate samples, the sieves were washed and cleaned in an ultrasonic cleaner at 27 °C for 5 min at the maximum power setting. The cleanliness of the sieves was carefully monitored by microscopy and additional DNA extraction and qPCR for the centrifuged flushing water from sieve surface.

2.5. Calcofluor White method

After concentration of the fine debris from the samples, Calcofluor White (Sigma-Aldrich, USA) staining was performed to visualize taeniid eggs. Calcofluor White stain binds non-specifically to chitin in the egg wall. A small portion of the extracted debris (25 μl; the total amount of the obtained sludge varied from 30 to 300 μl) was applied onto a glass slide and 50 μl of Calcofluor White stain and 50 μl of 10% potassium hydroxide was added to the top of the sample. The sample was then covered with a cover slip and, after one to ten min of incubation at room temperature, was examined in a dark room under a fluorescence microscope (Leica DM4000 B) using 350 nm for UV light excitation at 100× to 400× magnification [18]. Eggs that were detected with this method were also observed under light microscopy to see the typical morphological characteristics of taeniid eggs, such as striation of the embryophore and larval hooks [19]. Samples were classified as positive or negative for taeniid eggs.

2.6. DNA extraction

DNA was extracted from sieving sludge using a Tissue and Hair Extraction Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions with some modifications to adapt the protocol for the sieving sludge. The incubation time was 2 h at 55 °C with 200 μl of incubation buffer. The sample was vortexed for 5 s and centrifuged for 10 s and then transferred to a DNA isolation tube and 150 μl of lysis buffer and 14 μl of resin were added. Then, the protocol was continued according to the manufacturer's instructions. After extraction, the DNA was column-purified with a OneStep™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions. The DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher scientific, USA) for samples and a Qubit 3.0 fluorometer (Thermo Fisher, France) for reference DNA from an adult worm, and stored at −20 °C until real-time qPCR.

2.7. Primer design targeting the nad1 gene and qPCR assay

PCR primers were designed with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) for the molecular detection of *T. laticollis*. The complete mitochondrial genome of *T. laticollis* is available in GenBank with the accession number AB731727 [20]. The target chosen for this study is a part of the mitochondrial DNA region NADH dehydrogenase subunit 1 (*nad1*) gene. The forward sequence of the selected primer pair is 5′-TCACAGTTTCGTAAGGGTCCAAAT-3′ (position 100–123) and the reverse sequence is 5′-CCAACTAACAACAACACCCC AGT-3′ (position 248–226), with a product size of 149 bp.

A real-time PCR 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), 1 μl of template DNA and 8 μl of nuclease-free water. Cycling conditions were as follows: an initial activation step of 98 °C for 3 min, followed by 40 cycles of denaturation at 98 °C for 15 s and annealing and elongation at 58 °C for 30 s using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Samples were amplified in duplicate and the mean Cq value was calculated. 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.

PCR reaction conditions were optimized using the gradient PCR method with eight annealing temperatures ranging from 54 °C to 64 °C. The optimal annealing temperature was 58 °C. To determine the distinct melting point of this specific DNA region, DNA from an adult *T. laticollis* was used as a template. PCR products using a species-specific primer set were identified in melting curve analysis showing a distinct melting point (T_m) of 77 °C consistently specific for the amplicon without non-specific products or primer dimers (Fig. 1). 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 (149 bp); no other bands were detected.

To obtain the calibration curve and the detection limit, a ten-fold dilution series was prepared. Concentrations from 4.58 ng/μl to 4.58×10^{-4} ng/μl were made as duplicates, and to determine the detection limit, additional concentration points from 4.58×10^{-3} ng/μl to 4.58×10^{-7} were prepared with six replicates to assess the precision of the technique. 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 77 ± 0.5 °C and a Cq value below the Cq of the detection limit (36.14) was used to confirm a positive reaction. Each sample was amplified in duplicate and the average Cq value and standard deviation were calculated. The amplification was analyzed using Bio-Rad CFX Maestro software.

3. Results

3.1. Sensitivity of the real-time qPCR method

A standard curve was constructed with a 10-fold dilution series of the template DNA from an adult worm, and a linear relationship was defined between the logarithmic DNA concentration and Cq values. The correlation coefficient (R^2) of the standard curve was 0.999 (slope − 3.441) (Fig. 2). The detection limit of the assay was determined to be Cq 36.14 (± 0.59) obtained with a DNA concentration of 4.58×10^{-5} ng/μl. This was the lowest concentration that showed positive amplification with all six replicates. In the next dilution step, 4.58×10^{-6} ng/μl, only 2/6 replicates showed positive amplification, and in the lowest concentration, 4.58×10^{-7} ng/μl, all samples were negative.

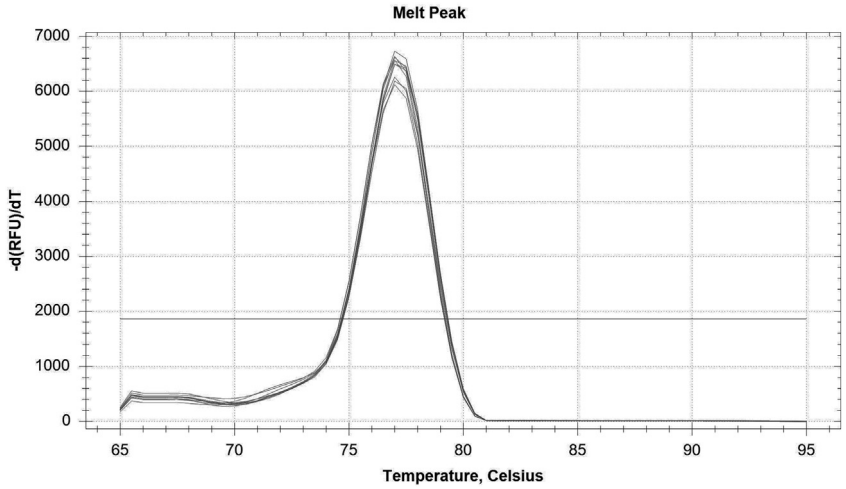

Fig. 1. Melting curve analysis of SYBR Green real-time PCR products targeting the mitochondrial *nad1* gene in the DNA of an adult *T. laticollis*. The melting point is 77 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. The outcome of the recovery test

In epifluorescence microscopy with Calcofluor White staining of spiked recovery test samples, the detection limit was 50 eggs per 100 g of berries for both bilberry and lingonberry. Taeniid eggs were detected in all the recovery test samples spiked with 50, 100, and 500 eggs. No eggs were detected in any of the samples spiked with 5 or 10 eggs.

In real-time PCR, all the spiked samples showed DNA amplification. Mean quantification cycle (Cq) values were between 30.90 and 32.82 with five eggs in blueberries and between 30.20 and 34.24 with lingonberries. The sensitivity of the complete method was 100% with five eggs in 100 g of berries. The standard deviation (SD) of the Cq values varied between 0.071 and 1.54. None of the negative control berry samples with no spiked eggs were positive in real-time qPCR.

3.3. Microscopic and molecular detection of T. laticollis eggs in experimentally contaminated forest berries

In total, 20 out of 21 experimentally contaminated berry samples (Table 1) were positive for taeniid eggs in epifluorescent microscopy with Calcofluor White staining (Fig. 3). Only one lingonberry sample with a very small sample size (26 g) was negative for eggs in microscopy.

All the samples ($n = 21$) from the contaminated research areas tested positive for *T. laticollis* DNA in quantitative real-time PCR based on the mitochondrial *nad1* gene. Cq values varied between 25.70 and 28.72 in berries from the plots contaminated with 60,000 eggs and between 26.96 and 32.48 in berries from the plots contaminated with 30,000 eggs (Table 1). All the control samples from the plots, collected before contamination, were negative in real-time qPCR. Taeniid eggs were not detected in any of the control samples in Calcofluor White staining.

4. Discussion

In this study, we investigated whether taeniid eggs can attach to the surface of berries in simulated natural conditions and whether they can be detected with a fluorescent stain and using a molecular method. Until recently, there has been little evidence of fresh foodstuffs serving as potential sources of infection with taeniids, particularly with *Echinococcus*. Recent works have suggested that fresh products could serve as a vehicle for taeniid eggs [3,4]. The surface of the berries used in this study is relatively smooth, especially of lingonberries, which have a smooth, shiny, waxy epicarp. Thus, the berry surface is probably not as susceptible to parasite egg attachment as in the case of plants or berries with a soft or coarse outer layer.

Taenia laticollis, used here as a model, is a non-zoonotic tapeworm that is very common in the Eurasian lynx (*Lynx lynx*) in Finland [21]. This parasite was chosen as a model organism because all tapeworms of the family Taeniidae have eggs that are basically similar in their

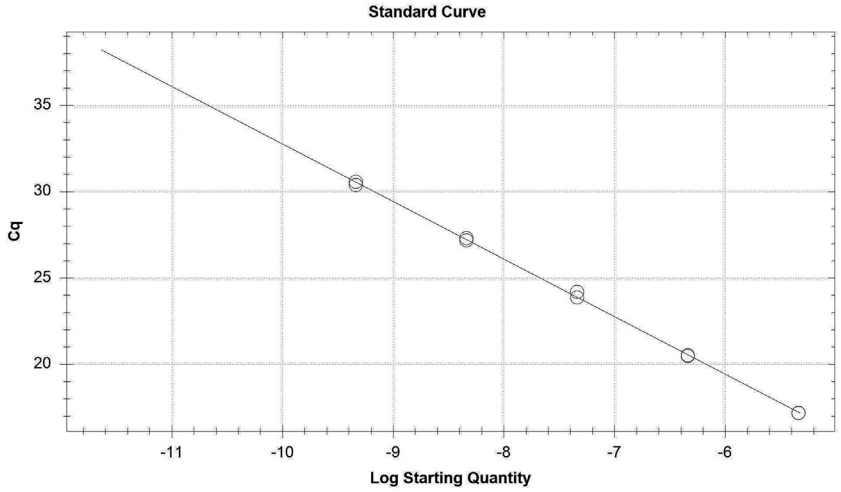

Fig. 2. A standard curve showing linearity in the SYBR Green real-time PCR reaction between Cq values and the log starting quantity of 10-fold dilutions of template DNA from an adult *T. laticollis* ($R^2 = 0.999$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

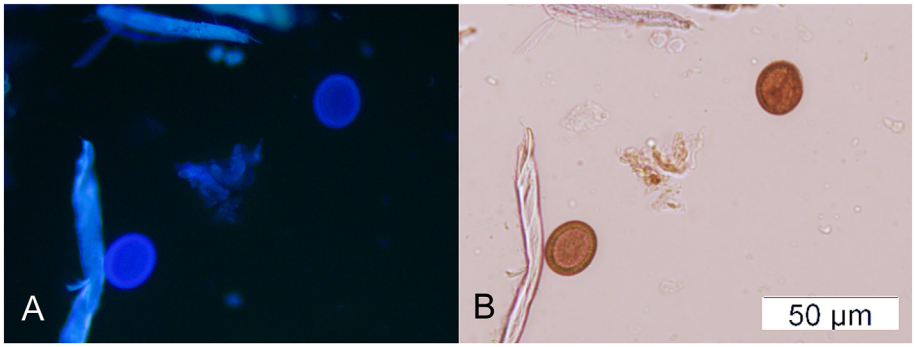

Fig. 3. Eggs of *T. laticollis* extracted from a contaminated bilberry plot: A) stained with Calcofluor White and viewed with fluorescence microscopy; B) viewed with light microscopy. The purpose of the Calcofluor White staining was to ease the visibility of the parasitic eggs from other debris material in larger scale.

physical and structural characteristics [16], but *T. laticollis* did not pose an occupational risk in the laboratory or for the occasional berry picker ignoring the warning signs at the experimental sites. Because we performed the contamination of the berries in natural conditions in forests, it was also important not to introduce eggs of alien species into the Finnish environment.

Research areas were selected with known owners, one in northern Finland and the others in the central-southern part of the country. The plots were established in areas having the greatest local density of berries; however, some of the research plots did not contain many berries, as can be seen from the variable mass of the berry samples (Table 1). In the plots containing less than 30 g of berries, the total berry surface area exposed to the egg spray was relatively small. The plots were sprayed as evenly as possible following a pre-designed spraying pattern. The weather conditions were somewhat variable, from dry to moderate rain, and the temperature was between 15 and 25 °C and 5–13 °C at the time of collection of the blueberries and lingonberries, respectively. Despite these variable conditions and sample sizes, all the samples tested were positive in real-time PCR and only one of the samples from the contaminated lingonberry plots was negative for taeniid eggs in Calcofluor White staining.

The number of eggs in the suspension used to spray the plots was high, because we wanted to mimic the point contamination situation, where rain spreads feces and parasite eggs from the ground surface onto shrubs, in the whole square meter plot area. Therefore, we sprayed a total of 30,000 eggs on most of the research plots and 60,000 on two bilberry and two lingonberry plots. In an experimental infection study, Kapel et al. [22] infected foxes, raccoon dogs, dogs, and cats, and by mathematically modeling *E. multilocularis* egg excretion dynamics, they estimated that the average biotic potential of foxes and raccoon dogs infected with 20,000 protoscolices each is over 330,000 eggs, with a mean maximum production of about 20,000 eggs daily. In naturally infected foxes, examined by segmental sedimentation and counting technique, the worm burden varies greatly and can be over 100,000 [23]. The quantitative method we developed was able to detect a DNA concentration of 4.58×10^{-5} ng/µl in 1 µl of DNA extract in a reaction solution, which is far less than the approximate amount of DNA in single *E. multilocularis* eggs [24,25].

The detergent Tween 20 was added to enhance the washing solution. The concentration of the Tween 20 was determined by preliminary studies. At a concentration of 0.04% there was no foaming that would disturb the sieving. In this study, all the spiked samples were positive in real-time PCR. Further development of the washing solution should be done in a larger amount of berries and also with a smaller number of eggs, including also only one egg, to determine the real recovery limit of the eggs.

The Cq values differed between bilberries and lingonberries due to differences in the sample sizes and the berry surface area in each research plot. Nevertheless, the methodology used in this study is semi-quantitative and it is important to consider that a proportion of the sieved fine debris was removed for Calcofluor White microscopy before DNA extraction. Thus, the quantitative sensitivity is reduced by loss of some of the eggs. The recovery tests revealed that taeniid eggs could still be detected using real-time PCR, and the washing and sieving steps were thus efficient in concentrating eggs from the samples. The sensitivity of the Calcofluor White staining method is not quite satisfactory, since the amount of sieved sludge was notably greater with commercial frozen berries used in the recovery tests than in berry samples from the experimental plots, regardless of the mass of the berry sample, probably due to the picking method. This needs to be considered when studying the contamination of commercially available berries.

The Calcofluor White staining in this study confirmed that contaminated samples contained entire morphologically detectable taeniid eggs that were still attached to the berry surface 24 h after contamination, not only DNA or fragments of it. Real-time PCR methods are highly sensitive but are also able to detect the non-viable remains of the targeted parasitic eggs if they contain DNA. The infectious dose for *E. multilocularis* in humans is not known. In experimental models with rodents, the susceptibility has varied between host species and even between strains, and the typical infectious dose used in these studies has been 100–1000 eggs when inoculated orally [26,27].

The splashing of contaminated feces or soil with water is not the only route via which taeniid eggs can be transferred to raw foodstuffs. Lawson and Gemmel [28] concluded that blowflies can transfer taeniid eggs to intermediate hosts. There is also evidence that *Taenia hydatigena* has been endemic on an island where definitive hosts are absent, and the taeniid eggs must have dispersed over a long distance [5]. Several studies have detected the taeniid eggs, including *Echinococcus*, in soil [29,30], demonstrating widespread environmental contamination.

In future studies, the spread of taeniid eggs in the environment and to wild forest berries and other foodstuffs should be more precisely evaluated thorough space and time with experimentally contaminated feces of carnivores. The efficacy of different procedures to advance the safety of various raw foodstuffs, such as washing methods, should be assessed, as Wahlström et al. [15] have proposed earlier. The attachment of *E. multilocularis* eggs onto the surface of the berries should also be demonstrated.

5. Conclusion

Taenia laticollis DNA was detected in all the berry samples from experimentally contaminated research plots and intact eggs were found in all except one sample. This indicates that berries can potentially serve as a vehicle for taeniid eggs, most probably also including *E. multilocularis*, and pose a possible risk to humans consuming uncooked berries.

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Ethical standards

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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