Food Research International 125 (2019) 108636

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

A novel multiplex real-time PCR for the detection of *Echinococcus multilocularis*, *Toxoplasma gondii*, and *Cyclospora cayetanensis* on berries

Tamirat Tefera Temesgen*, Lucy Jane Robertson, Kristoffer Relling Tysnes

Laboratory of Parasitology, Department of Food Safety and Infection Biology, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Adamstuen Campus, P.O. Box 369, 0102 Oslo, Norway

ARTICLE INFO

Keywords:
Fresh produce
Contamination
Method development
Limit of detection (LoD)
Molecular detection
Foodborne parasites (FBP)
Food testing
Intra-laboratory validation

ABSTRACT

Foodborne parasites (FBP) are of major public health importance and warrant appropriate detection and control strategies. Most of the FBP considered for risk-ranking by a panel of experts are potentially transmitted *via* consumption of contaminated fresh produce, including berries. In this study we focused on the potential of three FBP, namely *Echinococcus multilocularis*, *Toxoplamsa gondii,* and *Cyclospora cayetanensis,* as contaminants of berries. Surveys to assess these parasites as contaminants of fresh produce in general, and berries in particular, are scanty or non-existent mainly due to the lack of optimized laboratory methods for detection. The aim of the present study was to develop and evaluate a novel multiplex qPCR for the simultaneous detection of *E. multilocularis, T. gondii,* and *C. cayetanensis* from berry fruits.

The efficiency and linearity of each channel in the multiplex qPCR were within the acceptable limits for the range of concentrations tested. Furthermore, the method was shown to have good repeatability (standard deviation ≤ 0.2 C_q) and intermediate precision (pooled standard deviation of 0.3–0.6 C_q). The limit of detection was estimated to 10 oocysts for *Toxoplasma* and *Cyclospora*, and 5 eggs for *Echinococcus* per 30 g of raspberries or blueberries. In conclusion, evaluation of the present method showed that the newly developed multiplex qPCR is highly specific, precise, and robust method that has potential for application in food-testing laboratories.

1. Introduction

Food safety is a major concern for the global community, with an estimated 600 million cases of foodborne illnesses occurring annually (FAO/WHO, 2019). Foodborne illnesses could be due to infection (biological risk) or toxicity (chemical risk) resulting from consumption of contaminated food. The infection could result from consuming food contaminated with parasites, bacteria, or viruses. This is particularly true for foods that are consumed fresh and raw, such as fruits and vegetables.

A multi-criteria risk ranking of 25 food-borne parasites, in terms of their importance for Europe, has been conducted. Alveolar echinococcosis, toxoplasmosis, trichinellosis, cystic echinococcosis, and cryptosporidiosis are diseases caused by those parasites listed as 'top five' priority (Bouwknegt et al., 2018). Among these five prioritized parasites, four can be transmitted *via* contaminated fresh produce. As *Echinococcus multilocularis* and *Toxoplasma gondii* were ranked as the top two parasites, it was natural to focus on these in our study. Although *Cyclospora cayetanensis* was not ranked among the top five in Europe, we chose to include it due to the many recent outbreaks, particularly in USA, in which transmission *via* contaminated fresh produce has been implicated.

E. multilocularis has a confined distribution in the northern hemisphere, including central and northern Europe, northern Asia, and North America (Torgerson & Budke, 2003) and has become the number one priority on the list of European risk-ranking of the foodborne parasites (Bouwknegt et al., 2018). Humans acquire the infection *via* the faecal-oral route by accidental ingestion of the eggs, either by consuming contaminated food or *via* contact with the faeces of the infected definitive hosts. The prevalence of *E. multilocularis* among red foxes in European countries varies widely, ranging from below 1% to > 60% (Eckert & Deplazes, 2004). The prevalence of *E. multilocularis* in the red foxes has increased in endemic countries (Combes et al., 2012), and the parasite has also been reported from areas previously considered free. The parasite was found in Denmark in 2000 (Kapel & Saeed, 2000), then more recently in Sweden in 2011 (Osterman Lind et al., 2011), and has also been found in Arctic foxes of Svalbard in Norway (Fuglei et al., 2008). Estimates of new cases of alveolar

Abbreviations: C_q, Quantification cycle; LoD, limit of detection; MGB, minor groove binder; NTC, no template control; SD, standard deviation; S_p, pooled standard deviation

* Corresponding author.
E-mail address: temesgen.tamirat@nmbu.no (T.T. Temesgen).

https://doi.org/10.1016/j.foodres.2019.108636
Received 19 April 2019; Received in revised form 20 August 2019; Accepted 21 August 2019
Available online 23 August 2019

echinococcosis in Western and Central Europe showed a range of 170–200 per year, with the incidence rising during this century (Baumann et al., 2019), and the highest numbers of cases being reported from France, Germany, Switzerland, Lithuania, and Poland (Conraths & Deplazes, 2015). Detection of *E. multilocularis* DNA on raspberries (4 out of 20 samples tested) from Poland has been reported (Lass, Szostakowska, Myjak, & Korzeniewski, 2015).

T. gondii is a coccidian parasite that infects all warm-blooded animals and has a cosmopolitan distribution. Felids are the only definitive hosts, harbouring the sexual stage of the parasite and the oocysts are released to the environment in their faeces. Transmission to humans can happen by several means including: consumption of undercooked meat that contains the bradyzoites or ingestion of food or water contaminated with sporulated oocysts, blood transfusion or organ transplantation, and vertical transmission from mother to foetus (https://www.cdc.gov/dpdx/toxoplasmosis/index.html). The seroprevalence of *T. gondii* differs between countries and age groups. The IgG positivity rate among pregnant women was approximately 9% in Norway (Findal et al., 2015) and about 49% in Germany (Wilking, Thamm, Stark, Aebischer, & Seeber, 2016).

Unsporulated oocysts shed in the faeces of cats sporulate in the environment within 1 to 5 days, depending on temperature, before being infective for the next host. The oocysts are robust and remain viable for a long time in the environment. *Toxoplasma* has an infectious dose as low as 1 oocyst, which makes it a significant public health risk (VanWormer, Fritz, Shapiro, Mazet, & Conrad, 2013).

C. cayetanensis is a foodborne parasite that causes cyclosporiasis, a gastrointestinal illness commonly expressed as watery diarrhoea. *C. cayetanensis* is believed to have a direct life cycle with humans as the only hosts. Humans acquire the infection through the consumption of contaminated water or food. Unsporulated oocysts are released into the environment with the faeces of infected people. Given favourable environmental conditions, *i.e.* a temperature between 22 and 30 °C (Smith, Paton, Mtambo, & Girdwood, 1997), it takes approximately a week or two for sporulation, to enable the parasite to infect another host.

There have been frequent outbreaks of cyclosporiasis associated with contamination of fruits and vegetables, mostly reported from the USA. Contaminated raspberries and/or blackberries were implicated in the cyclosporiasis outbreaks of 2000, 2001–2002, and 2008 in different states of the USA (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html).

Surveys on contamination of berries with the parasites mentioned above are scanty, partly due to lack of standardized laboratory methods for analysis. Various methods have been developed for the detection of foodborne parasites from fresh produce. These include methods based on fluorescence microscopy for *Cyclospora* (Robertson, Gjerde, & Campbell, 2000), nested PCR for *Echinococcus* (Lass, Szostakowska, Myjak, & Korzeniewski, 2017), conventional PCR for *Cyclospora* (Lalonde & Gajadhar, 2008), real-time PCR melting-curve analysis for *Eimeria* as surrogate for coccidia (Lalonde & Gajadhar, 2016), and TaqMan probe qPCR for *Cyclospora* (Murphy, Lee, & da Silva, 2017). Each of these methods followed different protocols for the sample processing, which makes it difficult for comparison, and tend to be directed towards detection of just a single parasite genus.

As analysts may wish to analyse berries for all three of these parasites, a multiplex qPCR was considered a suitable approach. Here we describe the development and evaluation of a novel multiplex qPCR for the detection of *E. multilocularis, T. gondii,* and *C. cayetanensis* on berry fruits.

2. Material and methods

2.1. Sample preparation

2.1.1. Target parasites and surrogates

The eggs of *E. multilocularis* were kindly provided by Prof. Peter Deplazes, University of Zurich, Switzerland. Unsporulated oocysts of *C. cayetanensis* in faeces were kindly provided by Dr. Kristin Elwin, Public Health Wales Health Protection Division, UK. The faecal sample containing the *Cyclospora* oocysts was washed twice with 0.5% sodium dodecyl sulfate (SDS) and the oocysts isolated using saturated salt flotation. The oocysts were suspended in distilled water and then stored in the refrigerator. Oocysts of *T. gondii* from a previous project were also used; the details of the oocyst strain and origin are described elsewhere (Harito, Campbell, Prestrud, Dubey, & Robertson, 2016). The oocysts of *T. gondii* that had been stored in 2% H_2SO_4 were washed with water three times before proceeding to DNA extraction. The number of oocysts from all parasites were estimated using KOVA® Glasstic® Slide 10 Microscope Slide (VWR, Norway). These oocysts were used for evaluation of the performance characteristics of the developed method as applied on the berry matrix.

Oocysts of *Eimeria mitis* were isolated from chicken faeces, *Cystoisospora canis* from canine faeces, and *Cryptosporidium parvum* from stool samples from calves. These samples had all been submitted for diagnostic analysis at the Parasitology Laboratory, Faculty of Veterinary Medicine, Norwegian University of Life Sciences. After repeated washing steps in water, the oocysts were isolated by saturated salt flotation and stored refrigerated. The eggs of *Taenia crassiceps* were isolated from the worms collected from fox intestine and kindly provided by Dr. Relja Beck, Croatian Veterinary Institute, Croatia. These parasites were used for evaluating the specificity of the proposed multiplex qPCR.

2.1.2. Berry matrices

Sample matrices were prepared from store-bought raspberries and blueberries. About 30 g of each berry type were weighed into plastic boxes to which 200 ml of 1% Alconox™ (Alconox Inc., NY USA) was added. The boxes were then placed on an automatic shaker (Heidolph Vibramax 100); raspberry samples were shaken at 300 rpm for 10 min, whereas blueberry samples were shaken at 600 rpm for 10 min to facilitate the detachment of parasite stages from the berry surfaces. The rotational speeds were varied due to the differences in berries (raspberries are more fragile and therefore a lower speed was used to preserve intactness and minimize release of inhibitory components into the eluate).

The wash solution was then transferred into four 50 ml tubes for concentration by centrifugation at 1690 × *g* for 10 min and the supernatant removed by vacuum suction (Nalgene® Polypropylene Vacuum Pump Aspirator, Thermo Scientific), leaving 10 ml of the sediment. The pooled sediment was centrifuged at 3803 × *g* for 10 min with a deceleration brake set at 6 (on a scale of 0–9, to minimize disturbance of the pellet when the centrifuge was stopped) and about 1.5 ml of the sediment further concentrated down to 250 µl by centrifugation at 13,000 × *g* for 5 min.

2.2. Isolation of DNA

DNA was isolated from the parasite species using DNeasy PowerSoil Kit (Qiagen, Norway) following the manufacturer's instructions with slight modifications. Briefly, 250 µl of the sample containing the parasites were subjected to bead-beating to break the oocyst walls and facilitate the release of DNA, using FastPrep-24 5G™ High Speed Homogeniser (MP Biomedicals, France) in two cycles of 4 m/s for 60 s. The lysate was then centrifuged at 10,000 × *g* for 1 min at room temperature, and 500 µl of the supernatant used for the subsequent step in the protocol. The final elution volume was 50 µl. Samples were stored at −20 °C until further analysis. A plasmid containing the 12 s rRNA gene of *E. multilocularis* was kindly provided by Dr. Mats Isaksson, National Veterinary Institute, Sweden. The plasmid was used for preparing standard curves and assessing the precision of the new method due to the limited availability of the eggs of *E. multilocularis*.

2.3. Real-time PCR (qPCR) assay

2.3.1. Primers and probe design

The oligos used for the detection of *E. multilocularis* in this study have been described elsewhere (Isaksson et al., 2014). The primers have been designed to amplify a product of 77 base pairs (bp) from the 12 s rRNA region of the genome of *E. multilocularis*.

The primers Tox-9F, Tox-11R and probe Tox-TP1 for detection of *T. gondii* have been previously described elsewhere (Opsteegh et al., 2010), but in our study were slightly modified at the 3′ end of the probe. The 5′ end of the probe (Toxo-TP1) was labelled with Cy5 and the 3′ end was modified by MGBEQ instead of BHQ1. The primers have been designed to amplify a product of 162 bp from the 529 bp repeat of *Toxoplasma*. Detection of this target has been reported to be of greater sensitivity compared with detection of the B1 gene (Edvinsson, Lappalainen, & Evengård, 2006).

The primers and probe for *C. cayetanensis* have been previously described (Temesgen, Tysnes, & Robertson, 2019). The 5′ of the probe was labelled by a fluorescent dye HEX and the 3′ end by MGBEQ.

Reverse-phase cartridge (RP1) purified primers were purchased from Sigma Aldrich and the MGB probes were obtained from Eurofins. The sequences of oligos used in this study are presented in Table 1.

2.3.2. qPCR conditions

The PCR was performed in a 0.3 ml PCR plate without skirt (Multiply®, Sarstedt, Norway). The qPCR was performed in a total of 20 µl volume that included 2 µl of template, 10 µl of 2× KiCqStart® Probe qPCR ReadyMix™, low ROX™ (Sigma-Aldrich, Norway), and appropriate concentrations of each primer and probe for the three parasites. The detailed reaction conditions and relevant concentrations are presented in Table 1. The reaction mix was subjected to initial denaturation at 95 °C for 3 min followed by 45 cycles of denaturation at 95 °C for 15 s and combined annealing and extension at 60 °C for 30 s. ROX was used as a reference dye against which the target fluorescence data were normalized.

2.4. Method evaluation

The method's performance characteristics were evaluated, including: specificity, efficiency, linearity, limit of detection (LoD), repeatability, intermediate precision, and robustness.

2.4.1. Specificity

The specificity of the primers and probes for *T. gondii* and *C. cayetanensis* were evaluated *in silico* using the NCBI nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) against coccidia, while the oligos for *E. multilocularis* were 'blasted' against *Echinococcus* genus.

The specificity of the assay was then investigated *in vitro* against DNA extracted from related parasites that included *E. mitis, C. parvum, C. canis,* and *T. crassiceps* (Section 2.1.1). In every reaction, the no template control (NTC) sample was included.

2.4.2. Efficiency and linearity

In order to evaluate the efficiency and linearity of the assay, a calibration curve was prepared using tenfold serial dilutions of the DNA of the parasites as follows. A mixture containing the DNA of the three parasites was prepared to include approximately 20,000 oocysts of *Toxoplasma* and *Cyclospora* as well as 5×10^6 copies of *Echinococcus* plasmid (diluted from the stock). Then a 10-fold serial dilution of the mixture was performed (for three tubes). The efficiency of the qPCR was determined automatically by the Mx3005P QPCR Systems Software (Agilent Technologies, US). The linearity of the method was assessed by the coefficient of determination (r^2) automatically calculated by the Mx3005P software, with $r^2 \geq 0.98$ considered acceptable.

Table 1
The oligos and the triplex qPCR setup used in the present study.

Parasite	*C. cayetanensis*	*T. gondii*	*E. multilocularis*
Forward primer (5′→3′)	CyITS1_TT-F ATGTTTTAGCATGTGGTGTGGC	Tox-9F AGGAGAGATATCAGGACTGTAG	EmMGB_F GTGCTGCTYATAAGAGTTTTTG
Reverse primer (5′→3′)	CyITS1_TT-R GCAGCAACAACAACTCCTCATC	Tox-11R GCGTCGTCTC GTCTAGATCG	EmMGB_R CTATTAAGTCCTAAACAATACCATA
Probe (5′→3′)	CyITS1_TT-P HEX-TACATACCCGTCCCAACCCTCGA-MGBEQ	Tox-TP1 Cy5-CCGGCTTGGCTGCTTTTCCT-MGBEQ	EmMGB_P FAM-ACAACAATATTCCTATCAATGT-MGBEQ
Primers conc.	0.5 µM	0.5 µM	0.4 µM
Probe conc.	0.15 µM	0.25 µM	0.13 µM
Amplicon size	141 bp	163 bp	77 bp
Reference	(Temesgen et al., 2019)	(Opsteegh et al., 2010)	(Isaksson et al., 2014)

Table 2
Experimental design for testing the robustness of the new assay.

Factor	Setup of the experiments					
	Test-1	Test-2	Test-3	Test-4	Test-5	Test-6
Master mix type	−1	−1	−1	1	1	1
Primer concentration	−1	1	−1	1	1	−1
Probe concentration	1	−1	−1	1	1	−1
Super mix volume	1	−1	1	−1	1	−1
Annealing temperature	−1	−1	1	1	1	−1

Key		
Sign used	−1	1
Master mix type	PerfeCTa qPCR ToughMix	KicqStart
Primer concentration *T. gondii*	0.4 µM	0.5 µM
Probe concentration *T. gondii*	0.2 µM	0.25 µM
Primers concentration *C. cayetanensis*	0.4 µM	0.5 µM
Probe concentration *C. cayetanensis*	0.12 µM	0.15 µM
Primers concentration *E. multilocularis*	0.32 µM	0.11 µM
Probe concentration *E. multilocularis*	0.4 µM	0.13 µM
Super mix volume	17.1 µl	18.9 µl
Annealing temperature	59 °C	61 °C

2.4.3. Comparison between the triplex assay and the respective simplexes

The C_q values of the Triplex qPCR assay were compared with the C_q values of each of the simplex qPCR counterparts. A difference of ≤ 1 C_q was considered acceptable for the Triplex qPCR to proceed further.

2.4.4. Inhibition

Inhibition from the berry matrices was tested by using a tenfold serial dilution of isolated DNA. Blueberry matrix spiked with approximately 10^4 oocysts of *C. cayetanensis* and *T. gondii* and 50 eggs of *E. multilocularis* was subjected to DNA extraction as mentioned in Section 2.2. The efficiency of the qPCR was used as an indicator of the presence or absence of inhibition.

2.4.5. Precision

2.4.5.1. Repeatability. The repeatability of the assay was estimated by using two levels of DNA concentration prepared as follows: i) approximately 1000 oocysts of *C. cayetanensis*, 500 oocysts of *T. gondii*, and plasmid containing approximately 10^6 copies of *E. multilocularis* 12 s rRNA gene ii) approximately 20 oocysts of *C. cayetanensis*, 10 oocysts of *T. gondii*, and plasmid containing approximately 20,000 copies of *E. multilocularis* 12 s rRNA gene. The repeatability of the assay was then expressed as the standard deviation of C_q from 12 replicates of each level. The two levels were chosen to represent: i) excess number of parasites that is easily detectable and ii) the concentration close to the LoD of the method (where poor repeatability would be expected).

2.4.5.2. Intermediate precision. The intermediate precision of the assay was also evaluated by varying the analyst and the day of analysis. Three levels of template concentration were analysed by two different analysts. Analyst-A conducted the Triplex qPCR on day-1 and day-2, and Analyst-B conducted the assay on day-2 simultaneously with, but independently from, Analyst-A, enabling both between-day and between-analyst comparisons. The three levels of template concentration included sample 1: approximately 1000 oocysts of *C. cayetanensis*, 500 oocysts of *T. gondii*, and plasmid containing approximately 10^6 copies of *E. multilocularis* 12 s rRNA gene; sample 2: 10-fold dilution of sample 1; sample 3: 100-fold dilution of sample 1. Each level was run in 10 replicates and the intermediate precision was expressed as the pooled standard deviation (S_p) of each level of concentration.

The pooled standard deviation (S_p) for each channel in the triplex qPCR was calculated using the following formula.

$$S_p = \sqrt{\frac{(n_1-1)S_1{}^2 + (n_2-1)S_2{}^2 + (n_3-1)S_3{}^2 + \ldots + (n_k-1)S_k{}^2}{(n_1-1)+(n_2-1)+(n_3-1)+\ldots+(n_k-1)}}$$

where:

- n_1 is the total number of replicates for sample 1, $S_1{}^2$ is the variance of C_q for sample 1
- n_2 is the total number of replicates for sample 2, $S_2{}^2$ is the variance of C_q for sample 2
- n_3 is the total number of replicates for sample 3, $S_3{}^2$ is the variance of C_q for sample 3
- n_k is the total number of replicates for sample k, $S_k{}^2$ is the variance of C_q for sample k

2.4.6. Limit of detection (LoD)

To estimate the limit of detection (LoD) of the method, two levels of spikes (each in triplicate) were performed on the blueberry and raspberry samples. The first spike included 50 oocysts of *T. gondii* and *C. cayetanensis* each, and 10 eggs of *E. multilocularis*. The second spike included 10 oocysts of *T. gondii* and *C. cayetanensis* each, and 5 eggs of *E. multilocularis*. These levels were chosen based on preliminary experiments (data not shown) that indicated that DNA directly isolated from five oocysts of *C. cayetanensis* could be detected, but not from two oocysts. It was also shown that DNA directly isolated from 1 egg of *E. multilocularis* could be detected with the method. The Triplex qPCR assay was run in duplicates for each spike.

2.4.7. Robustness

The robustness of the Triplex qPCR was assessed after introducing small, but deliberate, changes into various factors of the assay, including the commercially available master mixes, concentrations of primers and probe, annealing temperature, and volume of the super mix (containing all reagents except template). An experimental design that enables detection of the main effects was used (Table 2). Twelve replicates of the sample and negative control were included per experimental setup (the six different combinations of the different factors).

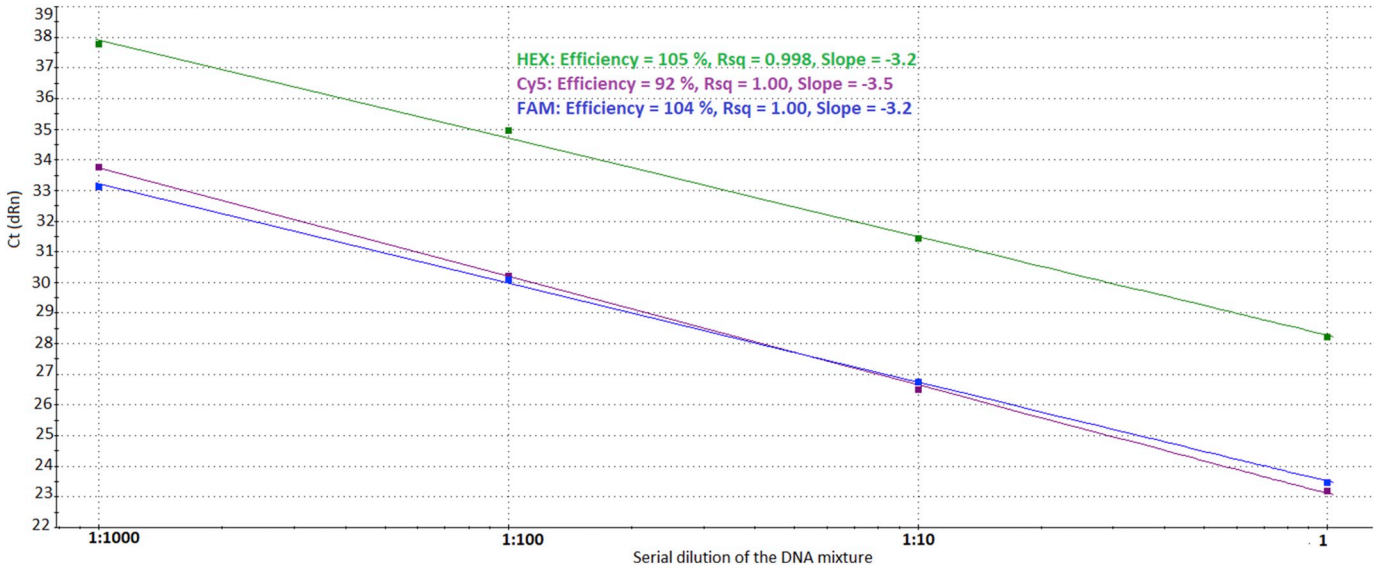

Fig. 1. Calibration curve prepared for the Triple qPCR using a mixture of the three parasites. Note: Fluorescent channel representations HEX for *Cyclospora*, Cy5 for *Toxoplasma,* and FAM for *Echinococcus*.

Table 3
Comparison of the C_q values obtained with the triplex assay and its respective simplex assays.

Samples	*Toxoplasma*		*Echinococcus*		*Cyclospora*	
	Triplex	Simplex	Triplex	Simplex	Triplex	Simplex
Sample 1	21.7	21.6	22.7	22.4	26.3	27.3
Sample 2	25.3	25.1	26.2	25.6	29.7	30.6
Sample 3	28.5	28.2	29.4	28.9	32.9	34.0
NTC	No C_q	No C_q	No C_q	No C_q	No C_q	No C_q

3. Results

3.1. Specificity

3.1.1. In silico test

The *in silico* evaluation showed the primer pairs would only amplify from the target parasites, as no cross-reactivity with DNA sequences from other parasites, even those that are closely related, was found.

3.1.2. In vitro test

The *in vitro* test of specificity showed that the primer pairs did not amplify DNA from four parasites: *E. mitis, C. parvum, C. canis,* and *T. crassiceps.*

3.2. Efficiency and linearity

The triplex qPCR showed good efficiency and linearity for the range of concentrations tested for the three parasites, with assay efficiency of 104%, 92%, and 105%, for *Echinococcus, Toxoplasma*, and *Cyclospora*, respectively. The linearity of the assay was also shown to be in agreement with the performance of an ideal qPCR assay (Fig. 1). The efficiency and linearity of the method were not affected by the changes made to threshold line setting between 0.1 and 0.5, indicating that the results are reliable.

3.3. Comparison between the triplex assay and the respective simplexes

The C_q values obtained with the triplex qPCR were compared with those from the simplex counterparts and no significant difference was observed. As shown in Table 3, the C_q values from the triplex assay are in agreement with their respective simplex assays. Although, there was a larger difference between the *Cyclospora* assays, the biggest difference observed was 1.1, which could be due to variations in the templates due to the concentration of DNA. The first attempt to develop the triplex qPCR was performed with ordinary (non-MGB) probes, and the use of MGB probe apparently improved the overall performance of the triplex qPCR and respective simplex qPCR for *Cyclospora* (results not shown).

The amplification plot for the triplex qPCR and its simplex counterpart for *Toxoplasma* (Cy5 channel) is presented in Fig. 2. It is interesting that the two assays showed a slight difference in the fluorescence intensity, with the simplex assays showing relatively higher intensities.

3.4. Inhibition

Evaluation of berry matrices for potential inhibition of the qPCR showed that introducing the berries matrices at the DNA extraction step resulted in no signs of inhibition.

3.5. Precision

3.5.1. Repeatability

Another important feature of a given analytical method is the precision. The precision of the assay was evaluated for both its repeatability and intermediate precision. The results confirmed that the triplex qPCR had a high degree of repeatability at both concentrations tested. The standard deviation of the C_q value was ≤ 0.2 (Table 4).

3.5.2. Intermediate precision

The intermediate precision of the C_q values obtained with the triplex qPCR was good, with a pooled standard deviation of 0.3, 0.6, and 0.4 for *Toxoplasma, Cyclospora,* and *Echinococcus*, respectively. The results of experiments on intermediate precision are presented graphically in Fig. 3.

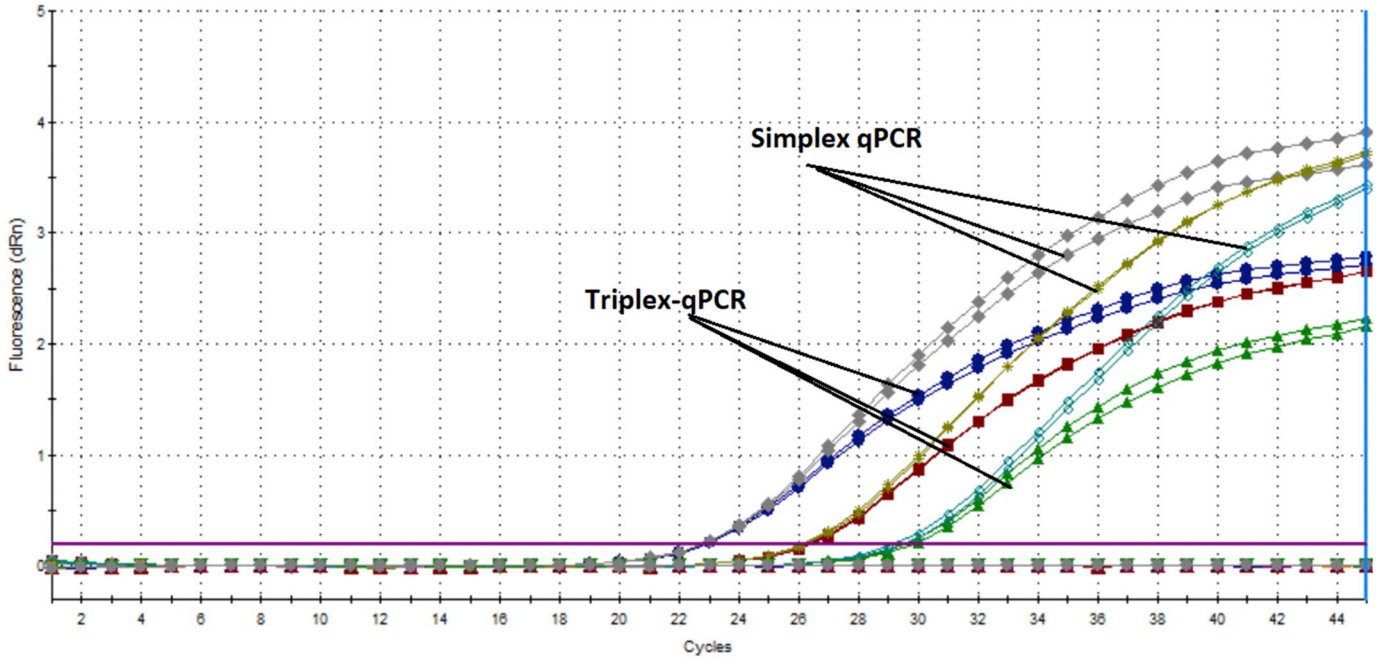

Fig. 2. Amplification plot of the triplex qPCR and simplex assay for *T. gondii* (Cy5 channel).

Table 4
Repeatability of the Triplex qPCR presented as means and standard deviations calculated from 12 replicates of each level of concentration tested.

Sample	*Toxoplasma*	*Echinococcus*	*Cyclospora*
	Mean C_q ± SD	Mean C_q ± SD	Mean C_q ± SD
Level A	23.2 ± 0.1	24.4 ± 0.1	29.5 ± 0.1
Level B	29 ± 0.2	30.2 ± 0.1	34.9 ± 0.2
NTC	No C_q	No C_q	No C_q

3.6. Limit of detection (LoD)

The LoD of the triplex qPCR was estimated to be 10 oocysts for *Toxoplasma* and *Cyclospora,* and 5 eggs for *Echinococcus* from 30 g berries (Table 5). The assay could detect DNA isolated directly from 1 egg of *Echinococcus* and 5 oocysts of *Cyclospora,* but could not detect DNA isolated from 2 oocysts of *Cyclospora* (result not shown).

3.7. Robustness

Evaluation of the robustness of the triplex qPCR indicated that the

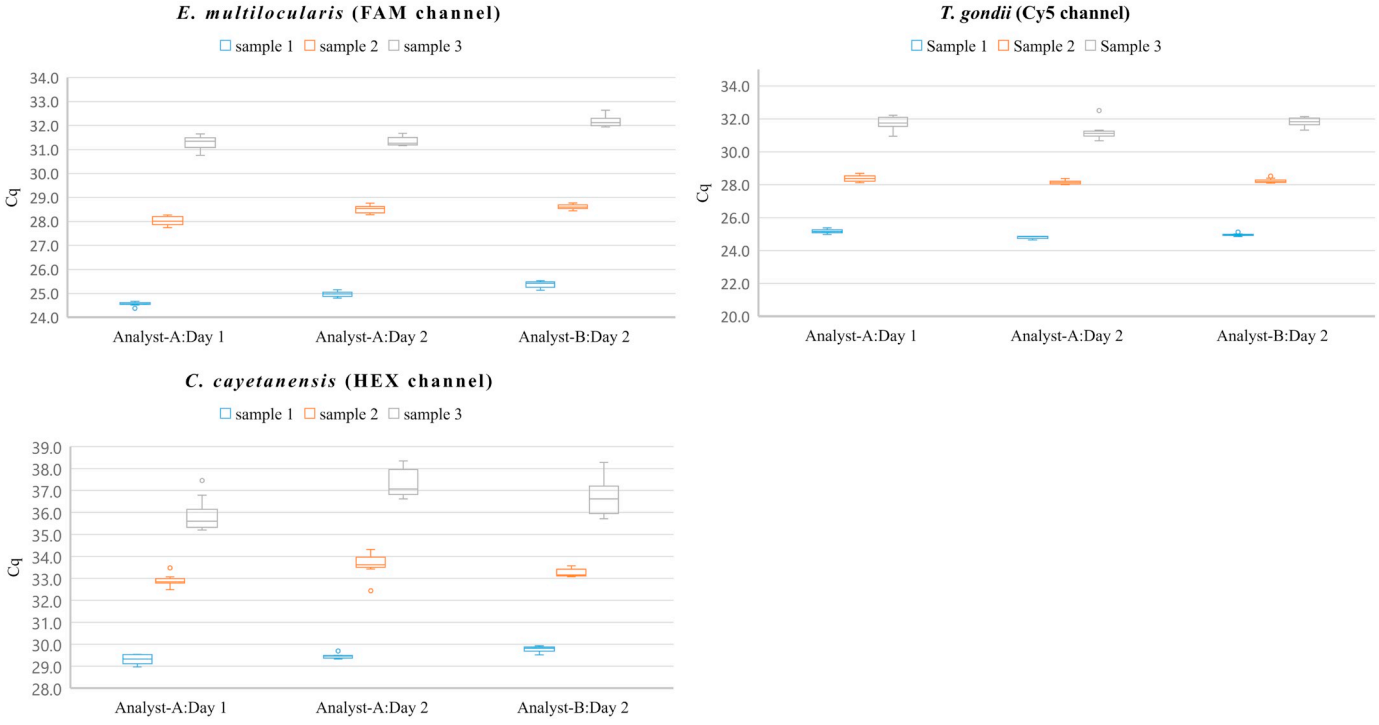

Fig. 3. Graphical representation of the results obtained from the assessment of intermediate precision.

Table 5
Estimation of the LoD of the triplex qPCR using blueberries and raspberries spiked with the three parasites.

Spikes	Replicate	C_q values obtained with the triplex qPCR		
		Toxoplasma	*Cyclospora*	*Echinococcus*
Raspberry				
50 oocysts and 10 eggs	1	33.5	36	38.6
	2	31.6	34.9	No C_q
	3	32.2	36	37.2
10 oocysts and 5 eggs	1	34.4	37.4	37.7
	2	33.9	38.1	38.2
	3	34.8	37.4	No C_q
Blueberry				
50 oocysts and 10 eggs	1	31.7	35.9	36.7
	2	32.4	37.4	34.6
	3	31.4	34.6	No C_q
10 oocysts and 5 eggs	1	32.1	No C_q	No C_q
	2	33.3	38.1	34.8
	3	36.6	37.5	No C_q

Table 6
Mean C_q values obtained under the six different conditions of the experiment.

	Test-1	Test-2	Test-3	Test-4	Test-5	Test-6	STD
E. multilocularis	25.1	24.9	26.3	25.2	25.3	24.7	25.7
T. gondii	25.7	25.5	25.8	26.3	26.4	26.0	25.6
C. cayetanensis	30.7	30.5	31.6	30.6	30.6	30.2	31.2

Note: The conditions for each test are detailed in Table 2.

method was highly robust, such that no significant changes in the performance of the assay were observed following deliberate modification of some of the factors that could affect PCR (Table 6 and Fig. 4). As it can be seen from the figure, no significant change occurred in the mean C_q values nor in the precision of replicate runs. In addition, there was no non-specific amplification from the NTC included in each test condition.

4. Discussion

In the present study, a novel molecular method for simultaneous analysis for three parasites, *E. multilocularis T. gondii, and C. cayetanensis,* as contaminants of berry fruits has been developed and evaluated for use. Although the assay was designed for analysis of berry fruits, due to associations in the literature of these parasites with berries (particularly *Cyclospora* and *E. multilocularis*), it could be perfectly well used with other types of fresh produce that could act as vehicles of infection, such as salad vegetables. Indeed, as berries are known to contain a range of potential inhibitors and are also too delicate for harsh washing procedures to elute the parasites (such as stomaching), this method applied to other fresh produce may be even more sensitive.

The C_q values obtained with the triplex qPCR were similar to those obtained with the simplex set up, particularly for *Toxoplasma* and *Echinococcus,* whereas the C_q values of *Cyclospora* showed a systematic trend in which the triplex qPCR showed approximately 1 C_q less than those obtained with its simplex counterpart. Although the reason why such differences occurred among the assays is not clear, further optimization of conditions for *Cyclospora* might improve the results.

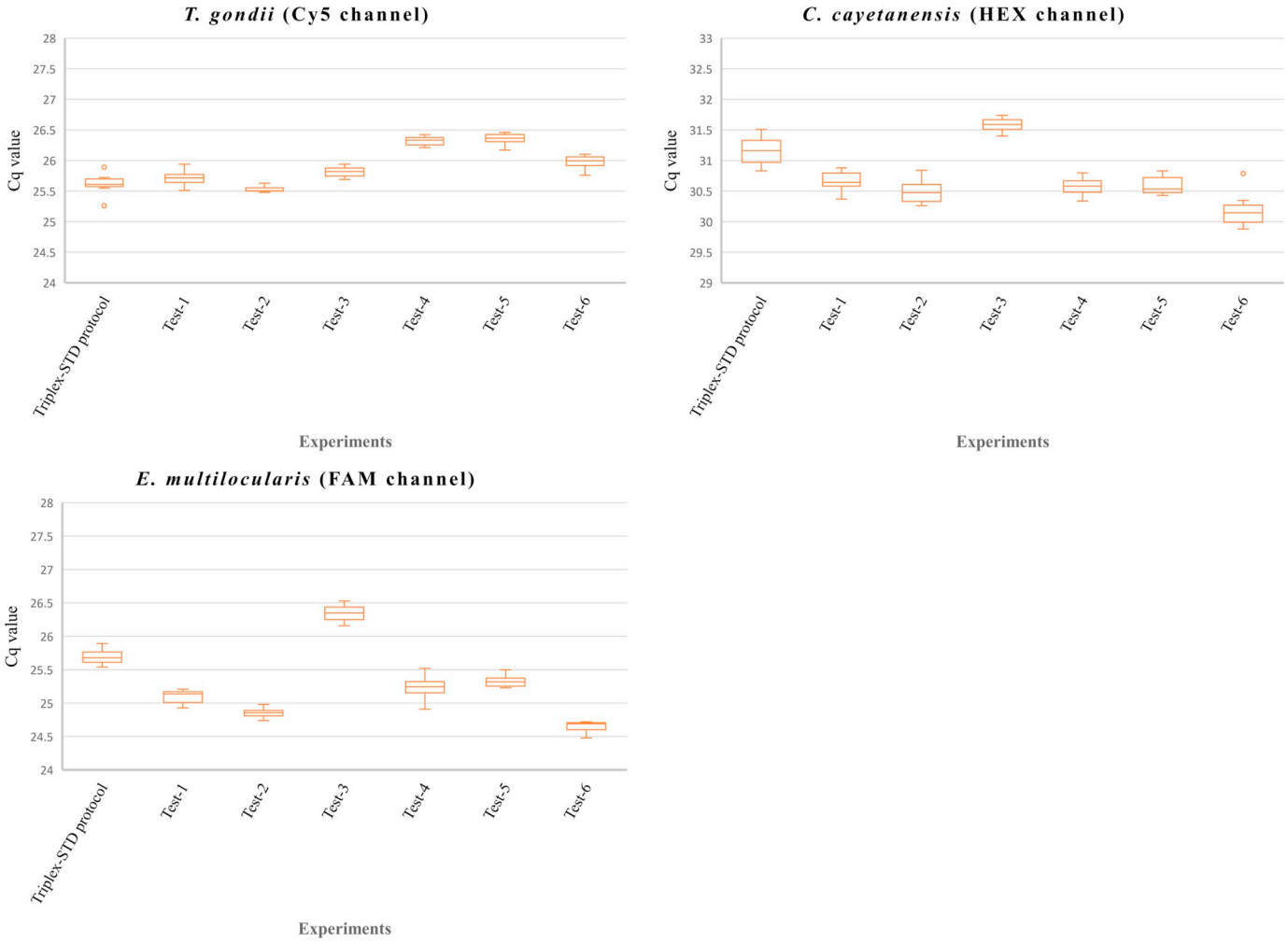

Fig. 4. Investigation of the robustness of the triplex qPCR by making deliberate changes in different factors of the qPCR as described in Table 2.

Nevertheless, the current methodology, as described, appears to be satisfactory for use in screening surveys of berries for these three parasites. In addition to the comparison of the C_q values, the fluorescence intensities of the assays were assessed visually and it was shown that each simplex assay had a relatively higher intensity than its respective result in the triplex assay. This is probably due to the reagents being consumed more quickly in a multiplex assay than the simplex assay (https://www.idtdna.com/pages/education/decoded/article/multiplex-qpcr-how-to-get-started).

In this study, the use of MGB probes significantly improved the multiplexing of the qPCR assays for the three parasites. The rationale for using MGB probe is to enable the use of shorter sequences by increasing the melting temperature (T_m) to maintain the specificity of probe binding to template (https://www.eurofinsgenomics.eu/mgb-probes). The performance of the triplex qPCR was improved with this probe, possibly due to increased sequence specificity and the ability to form a more stable duplex (Kutyavin et al., 2000).

The C_q values obtained with respect to the LoD determination of for *Toxoplasma* and *Cyclospora* were significantly different. The difference observed could be due to the number of gene copies of the targets used in the qPCR. The 529 bp repeat gene of *Toxoplasma* has been estimated to be available in > 300 copies (Reischl, Bretagne, Krüger, Ernault, & Costa, 2003). The huge difference could also be due to the sporulation status of the oocysts used in the experiment. In the present study, none of the *Cyclospora* oocysts were sporulated, whereas all the oocysts of *Toxoplasma* were sporulated.

It is known that various matrices have different components that could inhibit the PCR. Berries contain inhibitors such as polyphenols and polysaccharides (Schrader, Schielke, Ellerbroek, & Johne, 2012). In the present study, no inhibition was observed. This could be due to the efficiency of the DNA extraction kit at removing the inhibitors from the DNA eluate. It has been reported elsewhere that the qPCR could be inhibited due to the berry matrices, such that 4-fold dilution of the template was warranted (Murphy et al., 2017).

The present method showed a high degree of robustness, as demonstrated by the continued level of efficiency despite the introduction of changes introduced to the various conditions of the qPCR. The findings show the potential of the new method for standard use in food testing laboratories. It is noteworthy that robustness testing is often not considered in qPCR method development studies, although it is a very important performance characteristic of a given method. Robustness investigations provide various types of useful information: i) they help in identifying the source/s of variation that can be tweaked for possible optimisation adjustment; ii) they provide preliminary study results before investing in inter-laboratory comparison studies; iii) they provide evidence for the authors' confidence in the performance of the newly developed method; iv) they enable other laboratories to determine whether to proceed in testing the method, despite not having identical equipment and reagents to those of the developing laboratory.

Multiple qPCR approach has considerable advantages that include cost reduction that is highly pronounced as the number of targets detected increases. This could easily be appreciated by the amount of master mixes used and other supplies such as PCR plates, pipette tips, and molecular grade water per assay that would be reduced by at least two-fold depending on the number of targets in the multiplex qPCR. Another important advantage of multiplex qPCR is that less time and less amount of sample is used to obtain more information.

Although multiplex qPCR has the benefit of targeting several parasites simultaneously, and may help reduce costs compared to a simplex approach, it also has some limitations. For example, it might be challenging to design primers and probes such that there would be no cross-reactivity. In addition, there might be competition among the primer pairs, such that one target would be dominantly amplified while the others might be suppressed. However, with appropriate design of primers and probes, complemented by thorough evaluation and optimization of the PCR conditions, multiplex qPCR is a pertinent alternative that could be applied in food testing laboratories.

In conclusion, the evaluation of the present method showed that the newly developed triplex qPCR is a highly specific, precise, and robust method that could be applied in food testing laboratories. Although developed for use in analysis of berries for these parasite contaminants, it may be of equal utility for analysis of other relevant fresh produce such as salad vegetables or herbs.

Declaration of Competing Interest

We declare no conflict of interest.

Acknowledgements

We gratefully acknowledge Dr. Kristin Elwin, Public Health Wales Health Protection Division, UK for providing the oocysts of *C. cayetanensis* used in this study. We are also grateful to Prof. Peter Deplazes, University of Zurich, Switzerland for kindly providing the eggs of *E. multilocularis* and Dr. Mats Isaksson, National veterinary Institute, Sweden for providing *E. multilocularis* plasmid. We are also grateful to Vetle Stigum for his participation in the evaluation of the intermediate precision of the new method. We are also grateful to Dr. Relja Beck, Croatian Veterinary Institute, Croatia for providing the worms containing eggs of *T. crassiceps*. We acknowledge that collaboration within the framework of the European Network for Foodborne Parasites in Europe, COST Action FA1408 facilitated this research by enabling sharing of the parasites used in this study.

Funding

This work was supported by the Norwegian Research Council (grant no. 267430).

References

Baumann, S., Shi, R., Liu, W., Bao, H., Schmidberger, J., Kratzer, W., ... the interdisciplinary Echinococcosis Working Group UIm (2019). Worldwide literature on epidemiology of human alveolar echinococcosis: A systematic review of research published in the twenty-first century. *Infection*. https://doi.org/10.1007/s15010-019-01325-2.

Bouwknegt, M., Devleesschauwer, B., Graham, H., Robertson, L. J., van der Giessen, J. W., & The Euro-FBP Workshop Participants (2018). Prioritisation of food-borne parasites in Europe, 2016. *Eurosurveillance, 23*(9), 17–00161.

Combes, B., Comte, S., Raton, V., Raoul, F., Boué, F., Umhang, G., ... Giraudoux, P. (2012). Westward spread of *Echinococcus multilocularis* in foxes, France, 2005-2010. *Emerging Infectious Diseases, 18*(12), 2059–2062.

Conraths, F. J., & Deplazes, P. (2015). *Echinococcus multilocularis*: Epidemiology, surveillance and state-of-the-art diagnostics from a veterinary public health perspective. *Veterinary Parasitology, 213*(3), 149–161.

Eckert, J., & Deplazes, P. (2004). Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clinical Microbiology Reviews, 17*(1), 107.

Edvinsson, B., Lappalainen, M., & Evengård, B. (2006). Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis. *Clinical Microbiology and Infection, 12*(2), 131–136.

FAO/WHO. The burden of foodborne diseases and the benefits of investing in safe food. (2019). available at: http://www.fao.org/3/CA2809EN/ca2809en.pdf (Accessed March 14, 2019) .

Findal, G., Barlinn, R., Sandven, I., Stray-Pedersen, B., Nordbø, S. A., Samdal, H. H., ... Jenum, P. A. (2015). *Toxoplasma* prevalence among pregnant women in Norway: A cross-sectional study. *APMIS, 123*(4), 321–325.

Fuglei, E., Stien, A., Yoccoz, N. G., Ims, R. A., Eide, N. E., Prestrud, P., ... Oksanen, A. (2008). Spatial distribution of *Echinococcus multilocularis*, Svalbard, Norway. *Emerging Infectious Diseases, 14*(1), 73–75.

Harito, J. B., Campbell, A. T., Prestrud, K. W., Dubey, J. P., & Robertson, L. J. (2016). Surface binding properties of aged and fresh (recently excreted) *Toxoplasma gondii* oocysts. *Experimental Parasitology, 165*, 88–94.

Isaksson, M., Hagström, Å., Armua-Fernandez, M. T., Wahlström, H., Ågren, E. O., Miller, A., ... Juremalm, M. (2014). A semi-automated magnetic capture probe based DNA extraction and real-time PCR method applied in the Swedish surveillance of *Echinococcus multilocularis* in red fox (*Vulpes vulpes*) faecal samples. *Parasites & Vectors, 7*(1), 583.

Kapel, C., & Saeed, I. (2000). *Echinococcus multilocularis*-a new zoonotic parasite in Denmark. *Dansk Veterinærtidsskrift, 83*(8), 14–16.

Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., ...

Hedgpeth, J. (2000). 3′-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research, 28*(2), 655–661.
Lalonde, L. F., & Gajadhar, A. A. (2008). Highly sensitive and specific PCR assay for reliable detection of *Cyclospora cayetanensis* oocysts. *Applied and Environmental Microbiology, 74*(14), 4354–4358.
Lalonde, L. F., & Gajadhar, A. A. (2016). Optimization and validation of methods for isolation and real-time PCR identification of protozoan oocysts on leafy green vegetables and berry fruits. *Food and Waterborne Parasitology, 2*, 1–7.
Lass, A., Szostakowska, B., Myjak, P., & Korzeniewski, K. (2015). The first detection of Echinococcus multilocularis DNA in environmental fruit, vegetable, and mushroom samples using nested PCR. *Parasitology Research, 114*(11), 4023–4029.
Lass, A., Szostakowska, B., Myjak, P., & Korzeniewski, K. (2017). Detection of *Echinococcus multilocularis* DNA in fruit, vegetable, and mushroom samples collected in the non-endemic territory of the Pomerania province and comparison of the results with data from rural areas of the neighbouring highly endemic Warmia-Masuria province, Poland. *Acta Parasitologica, 62*(2), 459–465.
Murphy, H. R., Lee, S., & da Silva, A. J. (2017). Evaluation of an improved U.S. Food and Drug Administration method for the detection of *Cyclospora cayetanensis* in produce using real-time PCR. *Journal of Food Protection, 80*(7), 1133–1144.
Opsteegh, M., Langelaar, M., Sprong, H., den Hartog, L., De Craeye, S., Bokken, G., ... der Giessen, J. V. (2010). Direct detection and genotyping of *Toxoplasma gondii* in meat samples using magnetic capture and PCR. *International Journal of Food Microbiology, 139*(3), 193–201.
Osterman Lind, E., Juremalm, M., Christensson, D., Widgren, S., Hallgren, G., Ågren, E. O., ... Wahlström, H. (2011). First detection of *Echinococcus multilocularis* in Sweden, February to March 2011. *Eurosurveillance, 16*(14), 19836.
Reischl, U., Bretagne, S., Krüger, D., Ernault, P., & Costa, J.-M. (2003). Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. *BMC Infectious Diseases, 3*, 7.
Robertson, L., Gjerde, B., & Campbell, A. (2000). Isolation of *Cyclospora* oocysts from fruits and vegetables using lectin-coated paramagnetic beads. *Journal of Food Protection, 63*(10), 1410–1414.
Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors – Occurrence, properties and removal. *Journal of Applied Microbiology, 113*(5), 1014–1026.
Smith, H. V., Paton, C. A., Mtambo, M. M., & Girdwood, R. W. (1997). Sporulation of *Cyclospora* sp. oocysts. *Applied and Environmental Microbiology, 63*(4), 1631–1632.
Temesgen, T. T., Tysnes, K. R., & Robertson, L. J. (2019). A new protocol for molecular detection of *Cyclospora cayetanensis* as contaminants of berry fruits. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2019.01939 (in press).
Torgerson, P. R., & Budke, C. M. (2003). Echinococcosis – An international public health challenge. *Research in Veterinary Science, 74*(3), 191–202.
VanWormer, E., Fritz, H., Shapiro, K., Mazet, J. A. K., & Conrad, P. A. (2013). Molecules to modeling: *Toxoplasma gondii* oocysts at the human-animal-environment interface. *Comparative Immunology, Microbiology and Infectious Diseases, 36*(3), 217–231.
Wilking, H., Thamm, M., Stark, K., Aebischer, T., & Seeber, F. (2016). Prevalence, incidence estimations, and risk factors of *Toxoplasma gondii* infection in Germany: A representative, cross-sectional, serological study. *Scientific Reports, 6*, 22551.

Web references

CDC. Toxoplasmosis. (). https://www.cdc.gov/dpdx/toxoplasmosis/index (html Accessed April 10, 2019).
CDC. U.S. foodborne outbreaks of cyclosporiasis—2000–2017. (). https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks (html Accessed March 28, 2019).
Eurofins. MGB probes. (). https://www.eurofinsgenomics.eu/mgb-probes (Accessed April 10, 2019).
Intergrated DNA Technologies. Advantages of multiplex qPCR. (). https://www.idtdna.com/pages/education/decoded/article/multiplex-qpcr-how-to-get (started Accessed March 28, 2019).
NCBI. Standard nucleotide BLAST. (). https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome (Accessed June 20, 2019).