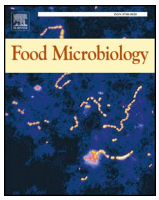
Food Microbiology 89 (2020) 103447

Contents lists available at ScienceDirect

Food Microbiology

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



Comparative evaluation of UNEX-based DNA extraction for molecular detection of *Cyclospora cayetanensis, Toxoplasma gondii,* and *Cryptosporidium parvum* as contaminants of berries

Tamirat Tefera Temesgen[a,*], Alessandra Barlaam[b], Kristoffer R. Tysnes[a], Lucy J. Robertson[a]

[a] *Laboratory of Parasitology, Department of Paraclinical Sciences, Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Adamstuen Campus, P.O. Box 369 Center, 0102, Oslo, Norway*

[b] *Department of Agriculture Science, Food and Environment, University of Foggia, 71122, Foggia, FG, Italy*

ARTICLE INFO

Keywords:
UNEX
DNeasy PowerSoil
Foodborne parasites
Toxoplasma
Cyclospora
Cryptosporidium
DNA extraction
Fresh produce
Berries

ABSTRACT

The potential public health impact of foodborne parasites (FBP) transmitted via contaminated fresh produces indicates the necessity for robust and reliable laboratory methods for their detection and identification on this infection vehicle. Standardization of methods for detection of common FBP in fresh produce is to be expected and ensuring that the DNA extraction approach is most appropriate for the FBP of interest and for the matrix being analyzed is also important. Therefore, the aim of the present study was to compare the efficacy of two commercially available DNA extraction procedures, the UNEX-based method and DNeasy PowerSoil kit in the detection of three protozoan parasites, *C. cayetanensis, C. parvum,* and *T. gondii,* on contaminated berries. Oocysts of each parasite were spiked into the pellets of raspberry and blueberry washes. The spiked pellets were then randomly assigned to DNA extraction using either the PowerSoil or UNEX method, with DNA extraction with both methods performed by two independent analysts. The detection rate when berry washes were spiked with 20 oocysts of *C. cayetanensis, T. gondii,* and *C. parvum* was 95%, 85%, and 40%, respectively, when using the PowerSoil kit; whereas the equivalent results using the UNEX method were 55%, 60%, and 5%, respectively. In addition, significantly lower C_q values were achieved for each parasite in the samples spiked with 500 oocysts when the PowerSoil kit was used. Possible reasons for these results are discussed, and include the composition of both the beads and the buffers in each method.

1. Introduction

Foodborne parasites (FBP) remain a significant public health threat throughout the world, affecting people's well-being and national economy (Devleesschauwer et al., 2017). Among the FBP, are the protozoa *Toxoplasma gondii, Cyclospora cayetanensis,* and *Cryptosporidium parvum*. Although these parasites have different epidemiologies, lifecycles, and transmission routes, all are infectious to humans and can be transmitted via their oocysts contaminating fresh produce, including berries (Bouwknegt et al., 2018).

The potential public health impact of FBP transmitted via contaminated fresh produces indicates the necessity for robust and reliable laboratory methods for their detection and identification on this infection vehicle. The lack of commercially available antibodies that can be used in the detection of most FBP, with the exception of oocysts of *Cryptosporidium* species and cysts of *Giardia duodenalis*, means that molecular detection methods are the approach of choice for the analysis of fresh produce for contamination with FBP.

As a result of repeated outbreaks of cyclosporiasis (https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/foodborneoutbreaks.html) associated with contaminated fresh produce, such as raspberries and cilantro, the U.S. Food and Drug Administration (FDA) has developed and implemented a laboratory method for the detection of *C. cayetanensis* from various types of fresh produces (Murphy et al., 2018). This technique, commonly known as the bacteriological analytical manual chapter 19b (BAM 19b), is a molecular method based on the detection of *C. cayetanensis* DNA extracted from a concentrated suspension of eluate following washing of the suspect fresh produce using a standard procedure using specified detergents.

Given the importance of detection of FBP, other molecular methods have also been developed for detection of *C. cayetanensis* (Lalonde and Gajadhar, 2008; Temesgen et al., 2019a), *T. gondii* (Lalonde and Gajadhar, 2016; Temesgen et al., 2019b), and *Cryptosporidium* spp. (Iqbal et al., 2015; Lalonde and Gajadhar, 2016) as contaminants of

* Corresponding author.
E-mail addresses: temesgen.tamirat@nmbu.no, nagaakoo@gmail.com (T.T. Temesgen).

https://doi.org/10.1016/j.fm.2020.103447
Received 15 October 2019; Received in revised form 5 December 2019; Accepted 22 January 2020
Available online 26 January 2020

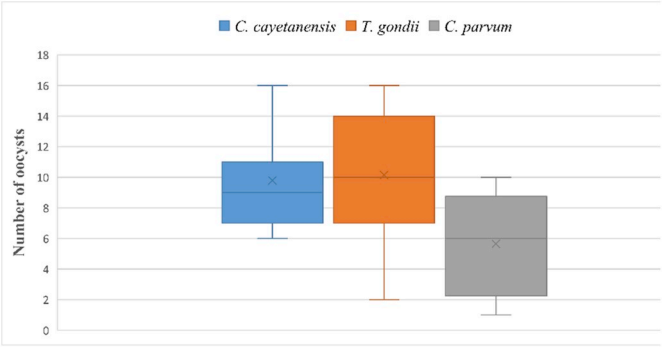


Fig. 1. Distribution of the oocyst counts from 10 independent spikes of *C. cayetanensis*, *T. gondii,* and *C. parvum*.

fresh produce. All these methods are based on the detection of specific target genes after extracting the genomic DNA of the parasites from fresh produce washes.

All steps of the protocol must be optimized to ensure that the sensitivity of the method is as high as possible, without compromising specificity and robustness, and there has been focus on improving PCR protocols. However, the method used for the DNA extraction from the wash water is also important, and the kits used have varied across the published protocols. A limited number of studies have been conducted to compare the efficacy of the different commercially available DNA isolation kits for isolating the DNA of these FBP. Shields et al. (2013) compared FastDNA SPIN Kit for soil (FastDNA), UltraClean™ Soil DNA Isolation Kit, QIAamp DNA Mini Stool Kit, and UNEX-based DNA extraction for the isolation of DNA in the detection of *C. cayetanensis* and *C. parvum* on fresh produce. Although no significant difference between kits was found for *C. cayetanensis,* for *C. parvum* the detection rate was higher when the UNEX method was used. Another recent study showed that UNEX-based DNA extraction outperformed FastDNA kit when used in the detection of *C. cayetanensis* in stool samples (Qvarnstrom et al., 2018). The UNEX buffer is a guanidinium isothiocyanate-based lysis buffer that was developed by the U.S. Centers for Disease Control and Prevention (CDC) for the effective simultaneous extraction and recovery of DNA and RNA from a range of microbes that may be transmitted by contaminated water, and has been found to be effective for DNA extraction from *Cryptosporidium* oocysts and *Giardia* cysts (Hill et al., 2015), and has since been made commercially available.

Steps towards standardization of methods for detection of common FBP in fresh produce should be expected and ensuring that the DNA extraction approach is most appropriate for the FBP of interest and for the matrix being analyzed is also important. Therefore, the aim of the present study was to compare the efficacy of two DNA extraction approaches, the UNEX-based DNA extraction approach (Microbiologics, USA) and DNeasy PowerSoil kit (Qiagen, Norway), for the detection of three protozoan parasites, *C. cayetanensis, C. parvum,* and *T. gondii,* on contaminated berries.

2. Methods and materials

2.1. Sample preparation

2.1.1. Parasites

Oocysts of *C. cayetanensis* and *T. gondii* were processed and stored as described elsewhere (Temesgen et al., 2019a). *C. parvum* oocysts were purchased from Bunch Grass Farm (Idaho, USA). Oocyst concentrations for each species were estimated using KOVA® Glasstic® Slide 10 Microscope Slide (VWR, Norway) and appropriate dilutions were performed to obtain two stock solutions for each parasite, Dilution-A with approximately 10 oocysts/μL and Dilution-B with 1 oocyst/μL. Ten samples of Dilution-A, containing an estimated 10 oocysts/μL, were further counted using Glastics KOVA slide for determining the precision of the estimate, and thus the distribution of the number of oocysts in each spike. The stock suspension of *C. parvum* had a lower concentration of oocysts than the other parasites (Fig. 1) and therefore corrections were made before spiking the berry-wash matrices.

2.2. 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 0.1% Alconox™ (Alconox Inc., NY USA) was added. The berries were washed according to Temesgen et al. (2019a) and the final pellets, about 250 μL, were stored in the refrigerator until ready for spiking with oocysts of *C. cayetanensis, T. gondii,* and *C. parvum.*

2.3. Spiking

Spiking was conducted at the same time for all samples to avoid any bias due to variation in the number of oocysts due to storage. The spiking of berries is illustrated in Fig. 2. Forty samples (20 raspberry pellets and 20 blueberry pellets) were spiked with 20 oocysts of each parasite species and another 20 raspberry samples were spiked with 500 oocysts of each parasite species, giving a total of 60 spiked samples for analysis. The spiking was performed by the same person for all the samples. The spiking volume was 20 μL from Dilution-B of the oocysts to obtain a spike of 20 oocysts, and 50 μL from Dilution-A to obtain a spike of 500 oocysts. In addition, one raspberry pellet was spiked with 10^4 oocysts of each species so that a standard curve could be included. An additional sample of each berry pellet was processed without spiking to serve as a negative control. The spiked pellets were stored at −20 °C until DNA extraction.

2.4. DNA extraction

The two methods for DNA extraction, DNeasy PowerSoil kit (Qiagen, Norway) and UNEX-based DNA extraction (Microbiologics, USA), were compared for their efficacy using qPCR as the detection tool. The spiked pellets were randomly assigned to the two kits for DNA extraction protocols using a random-number generating system in Microsoft Excel (Microsoft® Office Excel® 2010). Two analysts were, similarly, randomly assigned to perform the analyses, with each analyst analyzing a total of 30 samples, 15 using the DNeasy PowerSoil kit and 15 using the UNEX-based DNA extraction approach. After DNA extraction with either kit, the DNA was stored at −20 °C until qPCR analysis.

2.4.1. DNeasy PowerSoil kit

The pellets were subjected to the DNA extraction protocol provided by the manufacturer with slight modifications. Briefly, the lysis step was enhanced using a bead-beater (FastPrep 24G, MP Biomedicals, France), in which the 'Powerbead' tubes containing the pellets and appropriate volume of C1 solution were subjected to 2 cycles of bead-beating at a speed of 4 m/s for 60 s with 45 s pause between cycles. The final elution volume was 50 μL in the elution buffer (C6 solution) provided with the kit.

2.4.2. UNEX-based DNA extraction

UNEX-based extraction was performed according to the protocol previously described (Qvarnstrom et al., 2018). Briefly, in a lysing matrix E tube (MP Biomedicals), which contains a mixture of 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead, about 500 μL of sample was mixed with 600 μL of UNEX buffer (Microbiologics, USA) and 60 μL of proteinase K (Qiagen, Norway) and briefly vortexed. The tubes were then incubated at 56 °C for 15 min, followed by 1 cycle of bead-beating (FastPrep 24G, MP Biomedicals) at 6 m/s for 60 s as previously described (Qvarnstrom et al., 2018). The lysate was

then centrifuged at 13,000 × *g* for 1 min and the supernatant passed through MB spin columns (Qiagen, Norway). The spin column was then washed with 500 μL of absolute alcohol (200 proof, VWR) and further washed with 500 μL of 70% alcohol following the instructions provided with the UNEX buffer. The DNA was finally eluted into 50 μL of RNase-free water before storage.

2.5. Real-time PCR (qPCR) for C. cayetanensis, T. gondii, and C. parvum

The extracted DNA were analyzed for *C. cayetanensis* and *T. gondii* using an already published protocol (Temesgen et al., 2019b), whereas analysis for *C. parvum* followed an in-house method. Briefly, the oligos used for detecting *C. cayetanensis* were CyITS1_TT-F (ATGTTTTAGCA TGTGGTGTGGC), CyITS1_TT-R (GCAGCAACAACAACTCCTCATC), and CyITS1_TT-P (HEX-TACATACCCGTCCCAACCCTCGA-MGBEQ), and the oligos for detecting *T. gondii* were Tox-9F (AGGAGAGATATCAGGACT GTAG), Tox-11R (GCGTCGTCTC GTCTAGATCG), and Tox-TP1 (Cy5-CCGGCTTGGCTGCTTTTCCT-MGBEQ).

The primer pair and probe used for detection of *C. parvum* were designed to amplify a product of 92 bp from a target gene coding for thioredoxin peroxidase (GenBank accession number XM_001388017) using primer BLAST and Geneious Software. The oligos were TrxPx328F (5′-AGCAAGAACTATGGTGTACTTCTC-3′), TrxPx419R (5′-ACTTCAGAACGAACAACACCCT-3′), and TrxPx353P (FAM-AGGAA GAAGGTATTGCTCTCAGAGGT-MGBEQ).

The qPCR primers were used at a final concentration of 500 nM and the probes had a final concentration of 250 nM for Tox-TP1 and TrxPx353P, and 150 nM for CyITS1_TT-P in a reaction volume of 20 μL. KicqStart probe qPCR ready mix low ROX (Sigma Aldrich, Norway) was used and the reaction mix was heated at 95 °C for 3 min followed by 50 cycles of denaturation at 95 °C for 15 s, and a combined annealing and extension at 60 °C for 1 min. Each sample was run in triplicate and the standard curve was included in every run.

As the samples had been randomly assigned to each DNA extraction protocol, it was not known until after the lab analysis had been completed which sample had been extracted with each protocol.

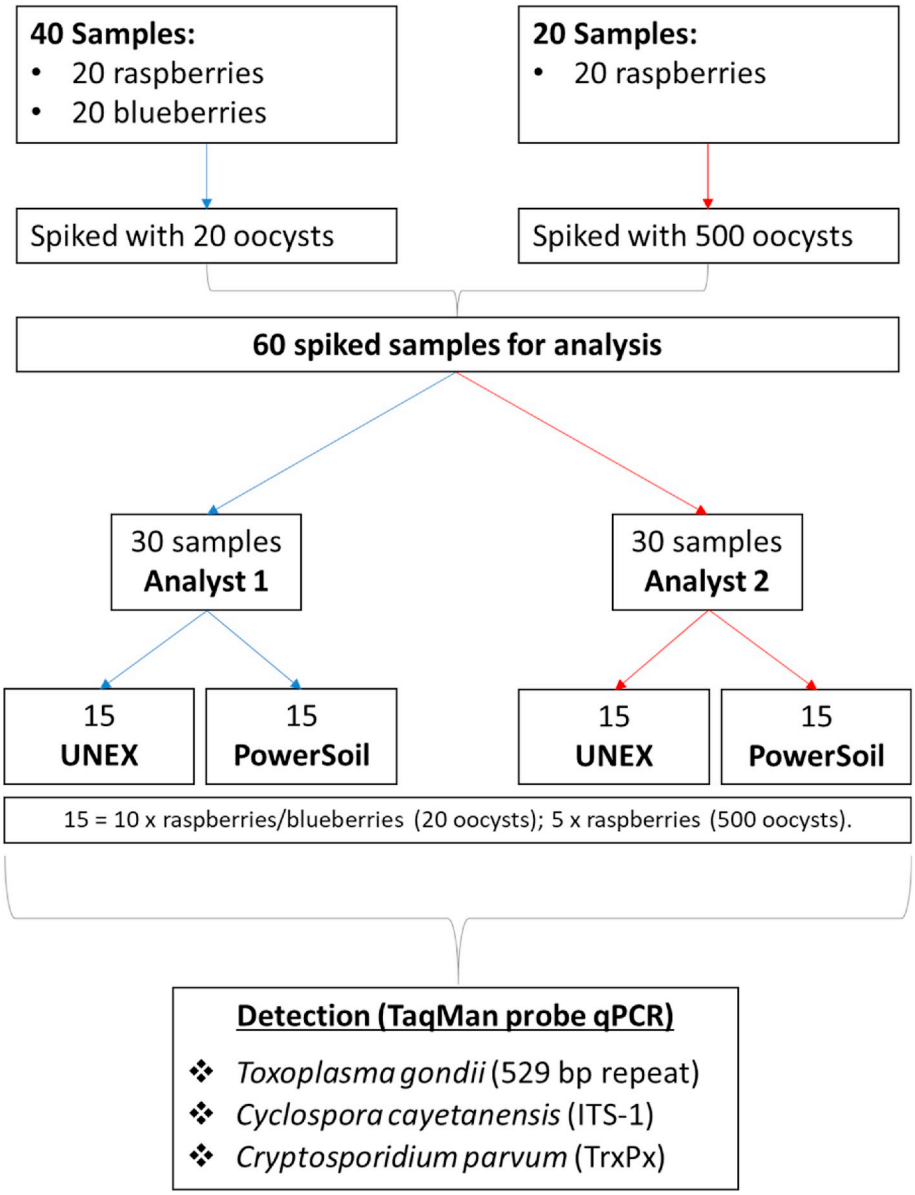


Fig. 2. The experimental design for the comparison of DNeasy PowerSoil kit with UNEX-based DNA extraction of *C. cayetanensis, T. gondii,* and *C. parvum* as contaminants of berries.

2.6. Additional experiments

Preliminary results led us to two further experiments. Firstly, we decided to investigate whether inhibition was an issue. To determine this, the DNA templates from selected samples were 4-fold diluted and then subjected to the qPCR. These were 5 samples spiked with 20 oocysts (2 from blueberry matrix and 3 from raspberry matrix) and 5 from the raspberry samples spiked with 500 oocysts.

In addition, we speculated that the two cycles of bead-beating in the PowerSoil kit may have affected the results. Therefore, duplicate water samples were spiked with about 1000 oocysts of each parasite, and DNA was extracted using the two approaches, but using the same bead-beating cycles for both methods (that used for PowerSoil; see section 2.4.1.).

2.7. Statistical analysis

Detection rate was calculated for both methods of DNA extraction from the samples spiked with 20 oocysts as the number of positive samples divided by the total number of samples spiked with 20 oocysts and multiplied by 100. Differences in detection rate between the samples according to the method of DNA extraction, inter-analyst variation, and berry matrices were determined using Fisher's exact test. For this purpose, results were converted to categorical data (negative and positive qPCR) and presented using contingency tables. For the samples spiked with 500 oocysts, the mean C_q values were compared using the student's t-test. to determine the sensitivity of detection associated with each of the methods of DNA extraction.

3. Results

3.1. Precision of the spiking experiment

The precision of the spikes was evaluated by counting the number of oocysts from 10 independent dilutions of oocysts containing an estimated 10 oocysts/μL. The results of the experiment showed a standard deviation from mean of 3 oocysts/μL for both *C. cayetanensis* and *C. parvum,* and 5 oocysts/μL for the spikes of *T. gondii* (Fig. 2).

3.2. qPCR results

The results of the qPCR analysis are categorized according to the parasite species selected for the present study, i.e. *C. cayetanensis, T. gondii,* and *C. parvum* and presented in the following sections. The linearity and efficiency of the qPCR assays used for the comparative evaluation of the DNA extraction methods was in the acceptable range (Fig. 3).

3.2.1. Cyclospora cayetanensis

3.2.1.1. Samples spiked with 20 oocysts. The findings of the qPCR analysis for *C. cayetanensis* indicated that, in our experiments, DNeasy PowerSoil kit provided superior results. The detection rate for 20 oocysts of *C. cayetanensis* using DNeasy PowerSoil kit was calculated as 95% (95% CI: 76, 99), with 19 out of 20 samples determined as positive for *C. cayetanensis*. However, the detection rate for 20 oocysts of *C. cayetanensis* using the UNEX approach was calculated as 55% (95% CI: 34, 74), with only 11 out of 20 samples determined as positive for *C. cayetanensis* (Table 1). The difference between positive results according to the two extraction methods was statistically significant ($p = 0.004$). There was no significant difference in the results between the two analysts who performed the DNA extraction ($p = 0.716$). The mean C_q value of the positive samples with DNA extracted using the DNeasy PowerSoil kit was 37.7, ranging from 36.5 to 39.2. For the UNEX-based protocol, the mean C_q of the positive samples was 38.1, ranging from 36.8 to 39.6.

In addition, the detection rate was also compared between the sample matrices (raspberries and blueberries). The findings indicated that the overall detection rates were not significantly different, being 80% for blueberries and 70% for raspberries ($p = 0.48$).

3.2.1.2. Samples spiked with 500 oocysts. All samples spiked with 500 *Cyclospora* oocysts were considered positive using both extraction methods, except for with one sample for which the DNA had been extracted with UNEX. Comparison of the sensitivity associated with the two extraction methods was therefore possible for these samples, with the negative sample excluded from the calculation. The mean ± standard deviation C_q value obtained from the samples extracted using PowerSoil kit was 34.3 ± 0.5, whereas for UNEX extracted samples it was calculated to be 36.1 ± 0.8 (Table 2; $p < 0.001$). No significant difference between the two analysts who performed the extraction was found ($p = 0.23$).

3.2.2. Toxoplasma gondii

3.2.2.1. Samples spiked with 20 oocysts. The detection rate of *T. gondii* from samples spiked with 20 oocysts of *T. gondii* was 85% (95% CI: 64, 95) and 60% (95% CI: 39, 78) for PowerSoil and UNEX, respectively (Table 1). Although more positive samples were identified when the PowerSoil method of DNA extraction had been used, the observed difference was not statistically significant (Table 3; $p = 0.15$). The mean C_q value of the positive samples with DNA extracted using the DNeasy PowerSoil kit was 37.9, ranging from 35.6 to 39.5. For the UNEX-based protocol, the mean C_q of the positive samples was 38.0, ranging from 35.6 to 39.7.

Although detection rate among the sample matrices was higher in blueberries, with a detection rate of 85%, as compared with 60% from raspberries, this difference was not statistically significant ($p = 0.08$).

3.2.2.2. Samples spiked with 500 oocysts. All samples spiked with 500 *Toxoplasma* oocysts were considered positive using both extraction methods. Comparison of the sensitivity of detection associated with both methods of DNA extraction, by comparing mean C_q values showed that when the PowerSoil kit was used the mean C_q value was 33.6, which was significantly lower than the equivalent value of 36.2 associated with the UNEX extraction protocol (Table 2; $p < 0.001$). No difference in results was found between the analysts ($p = 0.9$).

3.2.3. Cryptosporidium parvum

3.2.3.1. Samples spiked with 20 oocysts. At spikes of 20 *Cryptosporidium* oocysts, the detection rate was low for both methods of extraction. The detection rate of samples spiked with 20 oocysts of *C. parvum* when the DNeasy PowerSoil kit had been used was 40%, which is significantly higher ($p = 0.02$) than when the UNEX-based method had been used, for which only 5% of samples were found to be positive (Table 3). No significant differences between detection rates in the two berry matrices were observed ($p = 0.13$), nor were any differences due to analyst detected ($p = 1$). The C_q values for the positive samples extracted using the DNeasy PowerSoil kit ranged from 38.3 to 39.7. With the UNEX-based method, only one sample was positive with a C_q value of 38.6.

3.2.3.2. Samples spiked with 500 oocysts. Among the samples spiked with 500 *C. parvum* oocysts, 8 samples out of 10 that were extracted using DNeasy PowerSoil kit were found positive, and 6 of the 10 samples subjected to UNEX-based method. Comparison of the mean C_q values obtained in these positive samples (Table 2), showed a statistically significant difference, with a mean value of 34.9 for samples extracted with the PowerSoil kit which is significantly lower than the mean value of 37 for the UNEX-based method ($p = 0.005$). For these samples also, no differences were found between the analysts or between the different berry matrices.

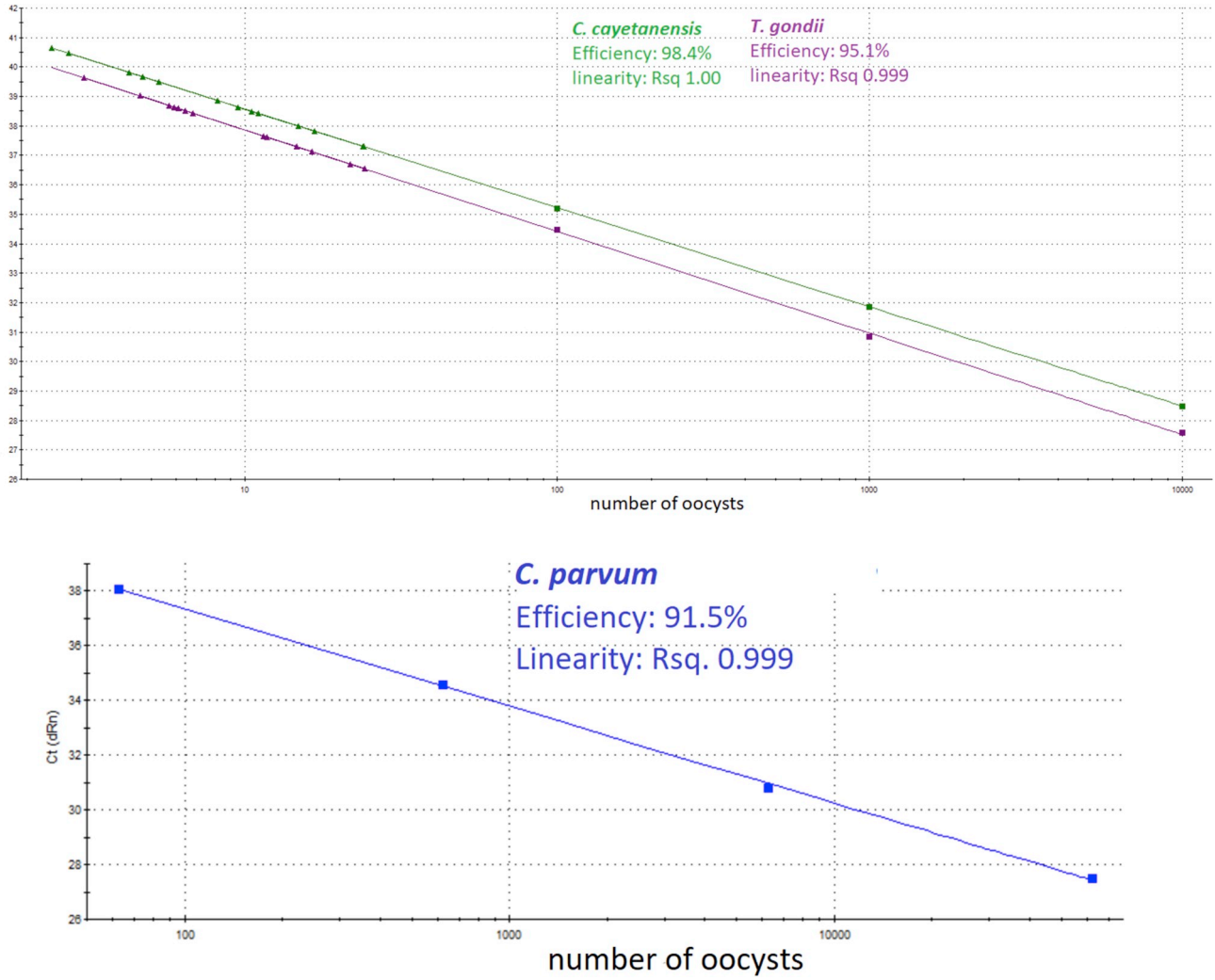


Fig. 3. The standard curves of the qPCR assays used for the detection of *C. cayetanensis, T. gondii, and C. parvum.*

3.3. Additional experiments

The analysis of the 4-fold diluted templates, both from the spikes of 20 oocysts and 500 oocysts, indicated that there was no sign of inhibition.

When we used the same bead-beating parameters for both methods (i.e., 2 cycles of bead-beating with 4 m/s for 60 s with 45 s pause between cycles), we did not find an improvement in results using the UNEX method. Indeed, the results were actually poorer than when the bead-beating factors were as initially used and as described in the procedure by Qvarnstrom et al. (2018); a single cycle of bead-beating 6 m/s for 60 s.

4. Discussion

The present study compared the efficacy of PowerSoil kit with UNEX-based DNA extraction for the detection of *C. cayetanensis, T. gondii,* and *C. parvum* as contaminants of berries, using TaqMan probe qPCR. The overall findings of the study were that more positive samples and with lower Cq values were identified in samples for which the PowerSoil kit had been used than for samples for which the UNEX-based protocol for extraction of DNA had been used. This pattern was seen for all three coccidian parasites and for both berry matrices, indicating superior performance of the PowerSoil kit in these matrices. The absence of a significant difference between the two analysts, as well

Table 1
Summary of the performance of DNeasy PowerSoil kit and UNEX-based DNA extraction for the detection of *C. cayetanensis, T. gondii,* and *C. parvum* using TaqMan probe qPCR from berries spiked with 20 oocysts of each parasite.

Matrix	DNeasy PowerSoil kit						UNEX-based DNA extraction					
	C. cayetanensis		T. gondii		C. parvum		C. cayetanensis		T. gondii		C. parvum	
	Pos. (%)	Neg	Pos. (%)	Neg	Pos. (%)	Neg	Pos. (%)	Neg	Pos. (%)	Neg	Pos. (%)	Neg
Raspberries (n = 10)	9 (90)	1	8 (80)	2	2 (20)	8	5 (50)	5	4 (40)	6	NA	10
Blueberries (n = 10)	10 (100)	NA	9 (90)	1	6 (60)	4	6 (60)	4	8 (80)	2	1 (10)	9
Detection rate (n =20)	**19 (95%)**		**17 (85%)**		**8 (40%)**		**11 (55%)**		**12 (60%)**		**1 (5%)**	

NA- Not applicable.

Table 2
Comparison of DNeasy PowerSoil kit and UNEX-based DNA extraction using the mean C_q values obtained from raspberry matrices spiked with 500 oocysts of *C. cayetanensis, T. gondii, and C. parvum.*

Factors		Mean C_q ± SD	p value (*t*-test)
C. cayetanensis			
Extraction method	PowerSoil (n = 10)	34.3 ± 0.5	<0.001[a]
	UNEX (n = 9)	36.1 ± 0.8	
Analyst	Analyst-I (n = 9)	34.8 ± 0.9	0.234
	Analyst-II (n = 10)	35.4 ± 1.2	
T. gondii			
Extraction method	PowerSoil (n = 10)	33.6 ± 0.5	<0.001[a]
	UNEX (n = 10)	36.2 ± 1.2	
Analyst	Analyst-I (n = 10)	34.8 ± 1.9	0.901
	Analyst-II (n = 10)	34.9 ± 1.3	
C. parvum			
Extraction method	PowerSoil (n = 10)	34.9 ± 0.5	0.005[a]
	UNEX (n = 4)	37 ± 0.7	
Analyst	Analyst-I (n = 8)	35.7 ± 1.2	0.374
	Analyst-II (n = 6)	35.2 ± 0.9	

[a] Indicates significance.

Table 3
Statistical test of significant difference in the detection rate from berries spiked with 20 oocysts of *C. cayetanensis, T. gondii, and C. parvum.*

Factors		No. analyzed	Positive	Negative	Fisher's exact test
C. cayetanensis					
Extraction method	PowerSoil	20	19	1	0.008[a]
	UNEX	20	11	9	
Sample matrix	Raspberry	20	14	6	0.72
	Blueberry	20	16	4	
Analyst	Analyst-I	20	16	4	0.72
	Analyst-II	20	14	6	
T. gondii					
Extraction method	PowerSoil	20	17	3	0.15
	UNEX	20	12	8	
Sample matrix	Raspberry	20	12	8	0.15
	Blueberry	20	17	3	
Analyst	Analyst-I	20	14	6	1
	Analyst-II	20	15	5	
C. parvum					
Extraction method	PowerSoil	20	8 (40)	12	0.019[a]
	UNEX	20	1 (5)	19	
Sample matrix	Raspberry	20	2 (10)	18	0.127
	Blueberry	20	7 (35)	13	
Analyst	Analyst-I	20	4 (20)	16	1
	Analyst-II	20	5 (25)	15	

[a] Indicates significant difference.

as no differences between the berry matrices, indicates that the difference was due to the technical efficacy of the methods.

As both methods are proprietary, it is difficult to determine which parameters may result in the differences seen here. Our speculation that the difference might also be due to differences in the bead-beating parameters was not reflected in our results from the additional experiments that we performed. It should be noted that the additional experiment with two cycles of bead beating with the UNEX-based kit (such that the beating parameters more similar to those of the PowerSoil kit) were performed in water rather than in the berry matrix; as both kits were used with water rather than berry matrix in this experiment the comparison remains valid. Furthermore, in addition to the bead-beating parameters potentially having an effect, the difference could be due to the materials from which the beads are made, as these are not the same in both methods. In the study by Hill et al. (2015), beads made of 0.2 mm and 0.5 mm zirconium oxide had better performance than glass beads, although the difference was not statistically significant. The lysing matrix E tube used in our study for the UNEX-based method, contained a mixture of 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead. In contrast, the 'powerbead tube' used in Qiagen's DNeasy PowerSoil kit contains 0.7 mm crushed garnet beads, the sharp edges of which may be superior at cracking open the oocysts walls.

A similar study conducted by Shields et al. (2013) showed that UNEX had a better performance as compared with FastDNA SPIN Kit for soil (FastDNA), UltraClean™ Soil DNA Isolation Kit, and QIAamp DNA Mini Stool Kit for detection of *C. parvum*, but that there was no significant difference for *C. cayetanensis*. Another study reported that the UNEX-based method had better performance for *C. cayetanensis* detection from stool sample when compared with FastDNA SPIN kit (Qvarnstrom et al., 2018). The protocol for UNEX-based method was not identical in both these previous publications. For example, in the experiments by Shields et al. (2013), the sample-proteinase K mixture was incubated at room temperature for 15 min, but in the work of Qvarnstrom et al. (2018) it was incubated at 56 °C for 15 min. Such differences are not minor, and could make a significant difference on the disruption of the oocysts' walls, which in turn could affect the recovery of nuclear DNA.

Although the findings of the present study are not directly comparable, it could be concluded that PowerSoil kit outperformed UNEX and, by extrapolation, may be expected to provide superior results to the other methods mentioned above and which have been found in previous studies to be generally inferior to the UNEX approach. Indeed, a pilot study in our lab showed that the DNeasy PowerSoil kit provided better results than FastDNA SPIN kit for soil, DNeasy PowerFood Microbial kit, and QIAamp DNA stool mini kit for the detection of *T. gondii* (Temesgen, unpublished data). It is also of interest to note that a recent study on extraction of DNA from *Ascaris* eggs found that isolation of target DNA using the PowerSoil DNA extraction kit resulted in better results (greater sensitivity) than 5 other kits tested, although a UNEX approach was not used (Amoah et al., 2019). The authors suggest that both the mechanical disruption (bead-beating steps) and superior inhibitor removal technology contributed to this higher performance. However, in our study, we did not detect any signs of qPCR inhibition and thus the comparison was not compromised by inhibitory effects from berry matrices. This agrees with the report by Shields et al. (2013) who reported that there was no inhibition from samples extracted by UNEX, although the same study reported the presence of inhibitors in the DNA samples extracted using Fast DNA SPIN kit. Thus, the presence of inhibitors can clearly be an issue to consider when selecting the best DNA extraction approach, and different matrices are likely to have different inhibitors in varying quantities. It should be noted that we only investigated inhibition by using dilution DNA template, and use of an endogenous control is probably a preferable approach for investigating this possibility.

In our study, the detection rate varied between the three parasites, with the lowest rate being for *C. parvum* using both methods of DNA extraction. This reduced detection rate probably reflects that the qPCR protocol used has not yet been optimized for use, rather than reflecting a particular problem associated with the DNA extraction methods for this parasite.

Nevertheless, although the performance of the two methods was not significantly different for samples spiked with 20 oocysts of *T. gondii,* the difference was huge between samples spiked with 500 oocysts.

5. Limitations

The present study relied on the theoretical dilutions of oocysts, instead of flow cytometric counts, for estimating the number of oocysts used for spiking. This is likely to have resulted in considerable variation, particularly with lower numbers of oocysts, and this could have a significant impact on detection. We attempted to reduce the impact of such unavoidable bias by increasing the number of independent replicates for the spikes involving 20 oocysts, by investigating the range of spikes using microscopy, and by randomization of samples assigned

to the two methods of extraction, as well as between the two analysts would avert the effect of such bias.

Another potential limitation of the present study is regarding that the parasites were spiked into the concentrates of berry washes rather than onto the berries prior to washing. This spiking approach was chosen as we wanted to have better control of the number of oocysts subjected to each DNA extraction protocol, the relative efficiency of different DNA extraction protocols being the focus of this study, and we wished to avoid any bias due to the washing protocol, especially as the parasites may have not been heterogeneously distributed throughout the pellet. However, by using this approach we deviate from the procedure that would be used during food testing for parasites, and we are unable to include any potential effect of the washing procedures on the parasites that may have affected the performance of the different DNA extraction kits. As there has not been a standardized washing protocol, the choice of washing solutions and concentration techniques vary from one lab to another and this adds to the complexity of the factors to consider. We emphasise that all factors that could potentially affect the performance of the kits should be considered when selecting the most appropriate kit.

6. Conclusion

In conclusion, the present study showed that PowerSoil kit is currently the method of choice for extraction of DNA of coccidian oocyst from berry matrices and detection by using TaqMan probe qPCR protocols.

Declaration of interest

None.

Acknowledgements

The study was funded by the Norwegian Research Council (Project No. 267430). 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 grateful for the support from Professor Annunziata Giangaspero and the University of Foggia regarding the research stay of Alessandra Barlaam at the Norwegian University of Life Sciences. 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.

References

Amoah, I.D., Singh, G., Troell, K., Reddy, P., Stenstrom, T.A., Bux, F., 2019. Comparative assessment of DNA extraction procedures for *Ascaris* spp. eggs. J. Helminthol. 1–7.

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. Euro Surveill. 23 17-00161.

Devleesschauwer, B., Bouwknegt, M., Dorny, P., Gabriël, S., Havelaar, A.H., Quoilin, S., Robertson, L.J., Speybroeck, N., Torgerson, P.R., van der Giessen, J.W.B., Trevisan, C., 2017. Risk ranking of foodborne parasites: state of the art. Food Waterborne Parasitol. 8–9, 1–13.

Hill, V.R., Narayanan, J., Gallen, R.R., Ferdinand, K.L., Cromeans, T., Vinjé, J., 2015. Development of a nucleic Acid extraction procedure for simultaneous recovery of DNA and RNA from diverse microbes in water. Pathogens 4, 335–354.

Iqbal, A., Labib, M., Muharemagic, D., Sattar, S., Dixon, B.R., Berezovski, M.V., 2015. Detection of *Cryptosporidium parvum* oocysts on fresh produce using DNA aptamers. PloS One 10, e0137455.

Lalonde, L., Gajadhar, A., 2008. Highly sensitive and specific PCR assay for reliable detection of *Cyclospora cayetanensis* oocysts. Appl. Environ. Microbiol. 74, 4354–4358.

Lalonde, L., Gajadhar, 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 Waterborne Parasitol. 2, 1–7.

Murphy, H.R., Cinar, H.N., Gopinath, G., Noe, K.E., Chatman, L.D., Miranda, N.E., Wetherington, J.H., Neal-McKinney, J., Pires, G.S., Sachs, E., Stanya, K.J., Johnson, C.L., Nascimento, F.S., Santin, M., Molokin, A., Samadpour, M., Janagama, H., Kahler, A., Miller, C., da Silva, A.J., 2018. Interlaboratory validation of an improved method for detection of *Cyclospora cayetanensis* in produce using a real-time PCR assay. Food Microbiol. 69, 170–178.

Qvarnstrom, Y., Benedict, T., Marcet, P.L., Wiegand, R.E., Herwaldt, B.L., da Silva, A.J., 2018. Molecular detection of *Cyclospora cayetanensis* in human stool specimens using UNEX-based DNA extraction and real-time PCR. Parasitology 145, 865–870.

Shields, J.M., Joo, J., Kim, R., Murphy, H.R., 2013. Assessment of three commercial DNA extraction kits and a laboratory-developed method for detecting *Cryptosporidium* and *Cyclospora* in raspberry wash, basil wash and pesto. J. Microbiol. Methods 92, 51–58.

Temesgen, T.T., Tysnes, K.R., Robertson, L.J., 2019a. A new protocol for molecular detection of *Cyclospora cayetanensis* as contaminants of berry fruits. Front. Microbiol. 10.

Temesgen, T.T., Robertson, L.J., Tysnes, K.R., 2019b. A novel multiplex real-time PCR for the detection of *Echinococcus multilocularis, Toxoplasma gondii*, and *Cyclospora cayetanensis* on berries. Food Res. Int. 125, 108636.