Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2021:11

Detection and viability assessment of foodborne parasites of public health importance on berries

Påvisning og vurdering av levedyktighet av matbårne parasitter med betydning for folkehelsen på bær

Tamirat Tefera Temesgen

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“In all science, error precedes the truth, and it is better it should go first than last”

Hugh Walpole (1884 – 1941)

To my wife (Lalise) and our twin boys (Jaasiel and Hanniel)

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List of abbreviations

BAM 19b	Bacteriological analytical manual chapter 19b
CI	Confidence interval
COWP7	*Cryptosporidium* oocyst wall protein 7
C_q	Quantification cycle
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organisation
FBP	Foodborne parasites
FDA	Food and Drug Administration
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
GOI	Gene of interest
GRA6	Dense granule protein 6
HSP70	Heat-shock protein 70
ISO	International Organization for Standardization
ITS-1	Internal transcribed spacer 1
LMS	Lectin magnetic separation
LoD	Limit of detection
MGB	Minor groove binder
MIQE	The minimum information for publication of quantitative real-time PCR experiments
mRNA	Messenger ribonucleic acid
MSB	Menadione Sodium bisulphite
NASBA	Nucleic acid sequence-based amplification
PCA	Principal component analysis
PCR	Polymerase chain reaction
PMA-PCR	Propidium monoazide polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism

RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SD	Standard deviation
UGDH	Uridine diphosphate glucose 6-dehydrogenase
WHO	World Health Organization

Summary

Foodborne parasites (FBP) are public health concern throughout the world causing significant effects on the health and wellbeing of people. Despite the tremendous impact of FBP, the relative attention paid to avert their transmission has been rather scanty, and there is a lack of standardized methods that could be used for risk assessment.

A wide range of parasites are potentially transmitted via contaminated fresh produce, including berries. These FBP shows diversity in many ways, including their biological makeup, life cycle, and pathogenicity. The physical properties of the transmission stages also differ, which makes development of a universal method for detection challenging.

Despite the growing number of laboratory methods developed for the detection of foodborne pathogens, the case of parasites is still lagging compared to bacteria and viruses. The lack of standard methods developed for detection of most FBP means that there is very little surveillance of berries for parasite contamination. Furthermore, there are no validated methods to assess the viability of parasites identified as contaminants of berries. The 'gold standard' method for assessing the infectivity of parasites is by using animal bioassay. However, this is time consuming, labour intensive, ethically challenging, and not applicable for parasites that are host specific.

The present PhD project was aimed at developing novel methods for the detection and viability assessment of parasite contaminants of berries. The study focused on molecular methods, i.e. quantitative PCR (qPCR) for detection and reverse transcription qPCR (RT-qPCR) for viability assessment. The study resulted in the development of a novel molecular method for the simultaneous detection of *Echinococcus multilocularis, Toxoplasma gondii,* and *Cyclospora cayetanensis* as contaminants of berries. Surveillance of berries for parasite contaminants using the methods developed showed that berries sold in the market stores of Norway were contaminated with *Toxoplasma* (3 %) and *Cyclospora* (< 1 %). No *E. multilocularis* was detected.

Furthermore, the study also investigated the removal efficiency of different berry washing techniques. The results of the investigation showed that washing the berries under running water for 1 min could remove at least 80 % of contaminating parasites except

Cyclospora cayetanensis. The use of salad spinner showed better removal efficacy and using one-part vinegar to 3-part water for washing the berries were even more effective in removing parasites.

Efforts were made to develop methods for viability assessment of *T. gondii and C. cayetanensis*. But due to lack of availability of viable oocysts of these parasites, *Cryptosporidium parvum* was used as a surrogate instead. Therefore, a novel RT-qPCR method was developed for the assessment of viability of *Cryptosporidium* oocysts. The novel RT-qPCR could be employed to evaluate the inactivation efficacy of different treatments that could be used by the fresh produce industry.

In conclusion, novel methods that could be used in the surveillance of berries for parasite contamination were developed, evaluated, and applied. The development of novel RT-qPCR for viability assessment of *Cryptosporidium* oocysts paved the way for developing similar methods for *T. gondii* and *C. cayetanensis*.

Sammendrag (Norwegian summary)

Matbårne parasitter (MBP) er en utfordring for folkehelsen over hele verden og forårsaker betydelige effekter på menneskers helse og velvære. Til tross for de enorme samfunnsmessige innvirkningene MPB kan ha så har innsatsen for å hindre deres overføring vært sparsom, og det er foreløpig mangel på standardiserte metoder som kan brukes til risikovurdering.

Et bredt spekter av parasitter kan potensielt overføres via kontaminerte ferske råvarer, inkludert bær. Disse er forskjellig på mange måter, inkludert deres biologiske sammensetning, livssyklus og evne til å forårsake sykdom. De fysiske egenskapene til overføringsstadiene er også forskjellige, noe som gjør det utfordrende å utvikle universelle påvisningsmetoder.

Til tross for et økende antall laboratoriemetoder som har blitt utviklet for påvisning av matbårne patogenere, så er det relativt få metoder som fokuserer på parasitter, sammenlignet med bakterier og virus. Mangelen på standardiserte metoder rettet mot påvisning av de fleste MBP har ført til at det er veldig lite overvåking av bær for kontaminering med parasitter. Videre er det ingen validerte metoder for å vurdere levedyktigheten til parasitter som kan overføres med bær. Infeksjonsstudier med forsøksdyr har så langt vært gullstandarden for å vurdere parasittenes evne til å forårsake infeksjon. Denne fremgangsmåten er imidlertid tid- og arbeidskrevende, etisk utfordrende og ikke aktuelt for parasitter som er spesifikke for mennesker.

Dette PhD-prosjektet var rettet mot å utvikle nye metoder for påvisning og levedyktighetsvurdering av parasitter som kan overføres med bær. Studien fokuserte på molekylære metoder, dvs. kvantitativ PCR (qPCR) for påvisning og revers transkripsjon qPCR (RT-qPCR) for levedyktighetsvurdering. Dette resulterte i en ny molekylær metode for samtidig påvisning av *Echinococcus multilocularis*, *Toxoplasma gondii* og *Cyclospora cayetanensis* som forurensninger av bær. Metoden ble deretter brukt i en studie der bær som var tilgjengelig på det norske markedet ble analysert. Studien avdekket kontaminering av bær med to av parasittene, henholdsvis *T. gondii* (3 %) og *C. cayetanensis* (< 1%), mens ingen av prøvene testet positivt for *E. multilocularis*.

Videre ble også effektiviteten til å fjerne parasitter fra bær ved hjelp av ulike vaskemetoder sammenlignet. Resultatene fra forsøkene viste at skylling av bærene under rennende vann i 1 minutt kunne fjerne minst 80 % av parasittene unntatt *C. cayetanensis*. Ved bruk av en salatspinner eller en fortynnet eddikløsning så kan man for øvrig fjerne betraktelig flere parasitter fra overflaten til bær.

Et av hovedmålene med dette prosjektet har vært å utvikle metoder for vurdering av levedyktig av *T. gondii* og *C. cayetanensis*. Men på grunn av mangel på levedyktige oocyster (overføringsstadiet) til disse parasittene, ble *Cryptosporidium parvum* brukt som et surrogat, og den nyutviklede RT-qPCR-metoden for vurdering av levedyktigheten er derfor basert på *Cryptosporidium*-oocyster. Prosjektet resulterte i utviklingen av en ny metode basert på komparativ omvendt transkripsjon qPCR (RT-qPCR) for vurdering av levedyktigheten til *Cryptosporidium*-oocyster. Den nye metoden kan brukes til å evaluere effektiviteten til ulike deaktiveringsprosesser som kan anvendes i ferskvarebransjen.

Avslutningsvis ble de nye påvisningsmetodene, som skal kunne brukes til overvåking av bær for parasittkontaminering, evaluert og anvendt. Utviklingen av en ny RT-qPCR metode for vurdering av levedyktighet av *Cryptosporidium*-oocyster kan bane vei for utvikling av lignende metoder for *T. gondii* og *C. cayetanensis*.

List of papers

Paper I

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

Authors: Tamirat T. Temesgen, Alessandra Barlaam, Kristoffer R. Tysnes, Lucy J. Robertson

Published: Food Microbiology 2020; 89, 103447.

Paper II

A New Protocol for Molecular Detection of *Cyclospora cayetanensis* as Contaminants of Berry Fruits

Authors: Tamirat T. Temesgen, Kristoffer R. Tysnes, Lucy J. Robertson

Published: Frontiers in Microbiology 2019;10

Paper III

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

Authors: Tamirat T. Temesgen, Lucy J. Robertson, Kristoffer R. Tysnes

Published: Food Research International 2019; 125, 108636.

Paper IV

A novel comparative Reverse transcription qPCR method for assessing the viability of oocysts of *Cryptosporidium*: a potential tool for inactivation efficacy trials

Authors: Tamirat T. Temesgen, Kristoffer R. Tysnes, Lucy J. Robertson

Status: submitted to Water Research

Related publications not included in the thesis

Paper I

Parasite contamination of berries: Risk, occurrence, and approaches for mitigation

Authors: Tamirat Tefera, Kristoffer R. Tysnes, Kjersti S. Utaaker, Lucy J. Robertson

Published: Food and Waterborne Parasitology, 2018, 10: 23-38

Paper II

Multiplex qPCR analysis of strawberries from Bogota, Colombia for contamination with three parasites

Authors: Carolina O. Pineda, Tamirat T. Temesgen, Lucy J. Robertson

Published: Journal of Food Protection, 2020, 83(10):1679-1684

Paper III

Contamination of fresh produce sold on the Italian market *with Cyclospora cayetanensis* and *Echinococcus multilocularis*

Authors: Alessandra Barlaam, Tamirat T. Temesgen, Kristoffer R. Tysnes, Laura Rinaldi, Nicola Ferrari, Anna R. Sannella, Giovanni Normanno, Simone M. Cacciò, Lucy J. Robertson, Annunziata Giangaspero

Status: submitted to Food Microbiology

1. Introduction

1.1. Background

Food is essential to life and people's awareness of the importance of a healthy diet has increased the consumption of fresh produce including fresh berries and hence the increasing production of berries worldwide (Tefera et al. 2018). Some of the health benefits of berries include prevention of chronic non-communicable diseases, such as diabetes, cardiovascular diseases and cancer (Skrovankova et al. 2015). However, consumption of fresh berries may be an important risk factor for foodborne infections, such as parasites, as a result of contamination.

Foodborne parasites (FBP) are a public health concern throughout the world, causing significant effects on the health and wellbeing of people. Although the transmission of FBP could be prevented, they still infect millions of people every year and causes diseases that ranges from mild to severe, and even deaths. Estimates showed that more than 90 million people were ill due to FBP and led to more than 50 thousand deaths in 2010. Furthermore, more than 7 million healthy life years were lost as a result (Torgerson et al. 2015).

The impact posed by FBP is multifaceted and could range from the sustained morbidity and mortality by individuals to affecting the socio-economy of countries. For instance, import of raspberries from Guatemala was restricted by the U.S. Food and Drug administration (FDA) during the late 1990s and by Canada later in 2000 in the wake of cyclosporiasis outbreak linked to raspberries from Guatemala. This led to loss amounted to around US$20 million (cumulative from 1996-2001) to the Guatemalan farmers (Cruz-Castillo et al. 2006). Not only this, repeated outbreaks associated with certain produce may affect people's confidence in a food that is of importance in a healthy diet. Despite the tremendous impact from FBP, the attention paid to its prevention and control has been rather scanty.

The reason for the little attention paid to FBP is probably multifactorial and include the lack of awareness among many physicians, the lack of laboratory methods for detection

and legislation demanding for screening produces for FBP, and challenges related to sources attribution due to long incubation period of many, but not all, parasitic diseases and short shelf-life of fresh produces (Robertson 2019). Without standardized methods to study FBP, researchers, the industry and other stakeholders are unable to provide the knowledge and insight that are needed to mitigate FBP outbreaks in the future.

Considering the significance of FBP in food safety concerns pertaining to consumption of fresh berries, a project on "Detection and inactivation of parasites on berries; development and implementation of food-safety tools for the industry" (PARABERRY) was funded by the Research Council of Norway (project no. 267430). PARABERRY aimed at providing the industry with knowledge on parasites potentially transmitted by fresh berries and approaches to reduce this risk. The project has five work packages (WP): WP1 concerned with development of analytical methods for surveillance purposes; WP2 conducting the surveillance of berries for parasite contamination; WP3 aimed at developing methods for viability assessment; WP4 designed to evaluate the approaches to removal and inactivation of transmission stages of the parasites; WP5 comprising method validation and cost-benefit analyses. This thesis is based on work conducted in WP1, 2, 3, and 4.

A wide range of parasites are potentially transmitted via contaminated fresh produce including berries. These parasites belong to different categories, i.e. protozoa, cestodes (tapeworms), trematodes (flukes), and nematodes (round worms). These FBP shows diversity in many ways which includes their biological makeup, life cycle, and pathogenicity. The physical properties of the transmission stages also differ, which makes development of a universal method for detection challenging (Tefera et al. 2018).

Three parasites, *Echinococcus multilocularis, Toxoplasma gondii,* and *Cyclospora cayetanensis* were in focus in PARABERRY. This selection was based on the following five criteria: i) Likelihood of transmission via fresh produce; ii) Potential severity of infection; iii) Documented cases/outbreaks; iv) Endemic spread in countries from which produce is imported to Norway; v) Documented evidence to survive freezing. In this thesis, *Cryptosporidium parvum* was included in addition to the three parasites in focus in PARABERRY.

1.2. *Echinococcus multilocularis* as a foodborne parasite

Echinococcus multilocularis is a zoonotic tapeworm responsible for alveolar echinococcosis (AE). It is limited in its distribution, being only confined to 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 prioritisation of FBP (Bouwknegt et al. 2018).

Life cycle

The parasite follows a complex life cycle involving wild canids and domestic dogs as its definitive hosts and rodents and lagomorphs as the intermediate hosts based on predator-prey relationship. Red foxes (*Vulpes vulpes*), Arctic foxes (*Vulpes lagopus*), coyotes (*Canis latrans*), jackals (*C. aureus*), wolves (*C. lupus*), dogs (*C. familiaris*), and raccoon dogs (*Nyctereutes procyonoides)* are known definitive hosts contributing to the life cycle of the parasite. The hosts differ according to geographical locations. For example, red foxes and voles are the main hosts maintaining the life cycle of the parasite in Europe, whereas dogs appear to be the main transmitters of the parasite in parts of China (Baumann et al. 2019). In addition, the parasite infects other hosts that don't contribute to the maintenance of its life cycle, which are commonly referred to as dead-end hosts. Such hosts include humans and other primates where the infection results in fatal condition if left untreated. Some dead-end hosts, such as pigs and horses, apparently do not develop the disease from the infection (EFSA 2018).

Transmission and epidemiology

Humans acquire the infection *via* the faecal-oral route by accidental ingestion of the eggs, either by consuming contaminated food or water or *via* contact with the faeces of the infected definitive hosts (Figure 1). According to a recent systematic review and meta-analysis on the source attribution of human echinococcosis, contact with dogs and consumption of contaminated water were statistically significantly attributed to the alveolar echinococcosis cases (Torgerson et al. 2020). However, there was no statistically sufficient evidence for the consumption of contaminated food and contact with foxes as

the source of infection of the AE cases, probably due to lack of data (Torgerson et al. 2020).

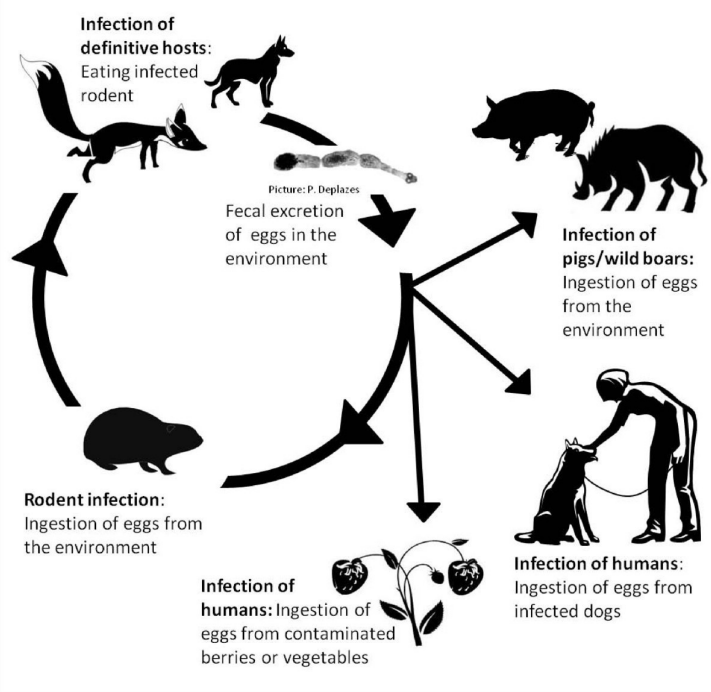


Figure 1. Life cycle of *E. multilocularis* (source: (Wahlström et al. 2011)).

The prevalence of *E. multilocularis* among red foxes in European countries shows huge variation, ranging from 1 % to > 60 % (Eckert & Deplazes 2004). Furthermore, the number of worms per fox could be highly variable, according to spatial variation and season, ranging from 1 to more than 10,000 (Otero-Abad et al. 2017) and the distribution is highly aggregated. In the Netherlands, a survey conducted between 2002-2003 on the positive samples showed a worm count ranging from 1 to 1,000 (Takumi et al. 2008).

According to the study done on naturally infected foxes, the number of eggs produced per worm ranged from 158 to 210 with a mean of 178 eggs (Thompson & Eckert 1982). This means that about 178 eggs produced per worm is potentially released to the environment, in the faeces of infected foxes, which ensures its transmission to the intermediate hosts. The eggs are known to survive harsh environmental conditions such

as freezing temperatures (Table 1). The adult worms could survive in the definitive host for about 5 months (CFSPH 2020).

Table 1. Survival of *E. multilocularis* eggs at different freezing temperatures (Robertson et al. 2012)

Temperature	Duration of storage	Post-freezing Infectivity
-18°C	240 days	Yes
-27°C	54 days	Yes
-30°C	24 hours	Yes
-50°C	24 hours	Yes
-70°C	96 hours	No
-80°C to -83°C	48 hours	No

The global multicriteria-based ranking of FBP showed that *E. multilocularis* is ranked 3rd next to *T. solium* and *E. granulosus*. However, the European multicriteria-based ranking of FBP showed that *E. multilocularis* ranked first, but the rank varied between the different parts of Europe (Figure 2), but was ranked highest in both Eastern Europe and Northern Europe (including Norway).

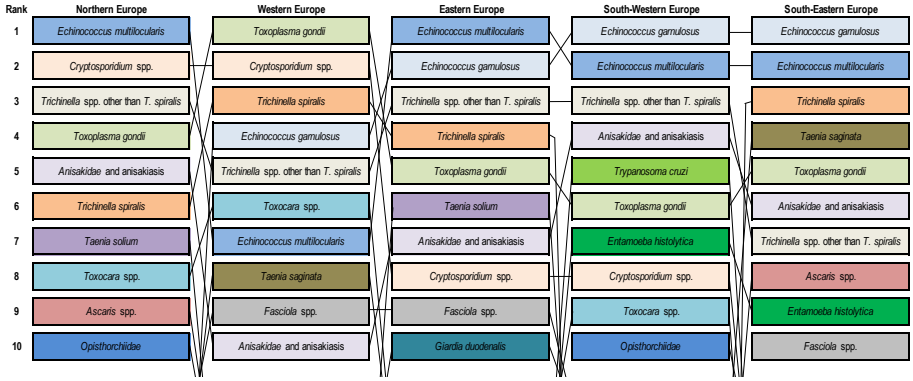


Figure 2. Comparison of the FBP ranks in different parts of Europe (Source: (Bouwknegt et al. 2018))

In Europe, the parasite used to be limited to certain areas such as southern Germany, Eastern France and parts of Switzerland and Austria until 1990s. But later, in addition to

the increased prevalence in those endemic areas (Combes et al. 2012), the parasite expanded its distribution to previous non-endemic countries such as Denmark, Poland, Romania, and Slovenia (Oksanen et al. 2016). This could be due to the successful oral vaccination against fox rabies that resulted in extended distribution of foxes to the urban areas (Deplazes et al. 2004).

Mainland Norway, but not Svalbard, is considered free of *E. multilocularis*. The parasite was found more recently in neighbouring Sweden in 2011 (Osterman Lind et al. 2011), and has also been found in Arctic foxes (*V. lagopus*) of Svalbard in Norway (Fuglei et al. 2008). It was suggested that the life cycle of *E. multilocularis* could be maintained by the foxes and sibling voles (*Microtus levis*), which were believed to have been accidentally introduced to Spitsbergen from Eastern Europe probably in animal fodder transported by sea freight (Davidson et al. 2012).

Following the increasing trend of this infection, the European Union adopted Commission Delegated Regulation (EU) No 1152/2011 of 14 July 2011 regarding preventive health measures for the control of *E. multilocularis* infection in dogs and hence decreasing the risk of human infection (Oksanen et al. 2016). The annual surveillance reports from Norway in the last decade showed that no *E. multilocularis* was detected from the faecal samples collected from wild canids, mainly the red foxes (*Vulpes Vulpes*) and grey wolves (*Canis lupus*) (Hamnes et al. 2020).

Data on the extent of contamination of fresh produce with *E. multilocularis* are minimal, but data from Poland suggest such contamination might be extensive; one study reports over 23 % of fresh produce being contaminated with *E. multilocularis.* The report showed that 20 % (4/20) of raspberries analysed in the study were contaminated with *E. multilocularis* DNA (Lass et al. 2015). The high prevalence of contamination reported for raspberries, which are elevated from ground level, raised questions on the accuracy of the report among other researchers (Robertson et al. 2016). But the authors argued that the findings indicate that *E. multilocularis* was present in the environment and consumers should be advised to wash berries before consumption (Lass et al. 2016).

Pathogenesis

Alveolar echinococcosis is a chronic disease, fatal if left untreated, caused by the metacestode of *E. multilocularis*. Humans are infected by accidental ingestion of the eggs of the parasite via different routes, such as the consumption of contaminated berries. Following infection, it could remain asymptomatic for 5-15 years and is then manifested by a range of symptoms. The metacestode develops in various organs but mostly in the liver, leading to a primary tumour-like lesion. The larval stage then could metastasize to other organs, such as brain and lung. The symptoms may vary based on the site of the lesion, but generally include weight loss, abdominal pain, signs of hepatic disease such as jaundice and anaemia accompanied by portal hypertension (Kantarci et al. 2012; Kern 2010).

The course of infection with *E. multilocularis* presents a challenge in the control of the parasite because the onset of symptoms usually presents 5 years, or more, after the infection occurred. This hampers source attribution (determining how the person became infected), and collection of data for risk assessment and design of targeted control strategies. Foodborne *E. multilocularis* infection has never been definitely proven in people, possibly due to the long incubation period of AE. However, a case report from the North-eastern France showed that AE in non-human primate (*Macaca fascicularis*) was probably as a result of ingesting food contaminated with foxes' faeces (Brunet et al. 2015). There have been other reports of AE cases in captive primates, including western lowland gorillas from a zoo in Switzerland (Wenker et al. 2019).

The treatment options for AE cases included surgery and chemotherapy. The treatment is challenging, because surgical treatment is usually not appropriate and chemotherapy is long-term, potentially life-long (Eckert & Deplazes 2004). Surgery may lead to complete cure, but diffuse and undetected parasites necessitate postsurgical chemotherapy for at least 2 years (Eckert & Deplazes 2004).

1.3. *Toxoplasma gondii* as a foodborne parasite

T. gondii is a protozoan in the phylum Apicomplexa, known to infect possibly all warm-blooded animals and has a worldwide distribution. The name *Toxoplasma* was coined from a Greek word 'toxon' meaning 'arc' due to the crescent shape of the parasite. It is estimated that at least one-third of the world's population has been exposed to the parasite. The prevalence of *T. gondii*, however, shows wide variations between countries and within countries. Generally, the prevalence is high in Latin American and tropical African countries whereas countries such as in Northern Europe and North America have low prevalence (Robert-Gangneux & Dardé 2012).

Although most of the infections remain asymptomatic, it is the causative agent of toxoplasmosis. The risk group includes immunocompromised people and pregnant women. Most epidemiological studies have been focused on pregnant women because of the risk of congenital toxoplasmosis- that occurs due to transmission of *T. gondii* from mother to foetuses. A recent metanalysis of studies conducted on seroprevalence of *T. gondii* among pregnant women indicated a global IgM seroprevalence of 1.9 %, whereas the global IgG seroprevalence was 32 % (Bigna et al. 2020). However, the prevalence varied widely across different countries as shown in Figure 3. The IgG seroprevalence of *T. gondii* among pregnant women in Norway has been estimated as 9.3 % (Findal et al. 2015).

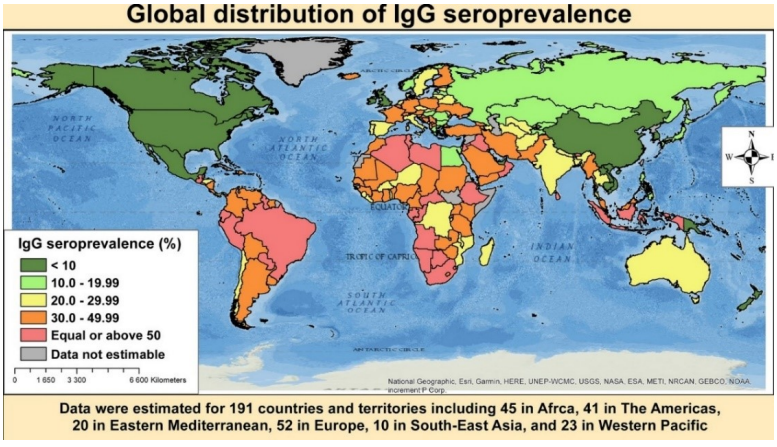


Figure 3. The global distribution of IgG seroprevalence among pregnant women (source: (Bigna et al. 2020)).

Life cycle and transmission

T. gondii has a complex life cycle that involves felids as the only definitive hosts, harbouring the sexual stage of the parasite and the oocysts are released to the environment in their faeces. The parasite has three infectious developmental stages including the sporozoites, tachyzoites (rapidly dividing stage), and bradyzoites (slowly dividing stage). The sporozoites are formed within mature oocysts (the environmental stage); the tachyzoites rapidly multiply in the host cells until the cells rupture; and the bradyzoites slowly divide within the tissue cysts. Cats are infected either by ingestion of the tissue cysts (e.g., from mice and birds) or the sporulated oocysts (e.g., contact with faeces from other cats or contaminated food or water) and then the parasite reproduces sexually to form the oocysts that are released in millions to the external environment in their faeces. Intermediate hosts acquire the infection via consumption of food or water contaminated with the sporulated oocysts, or by ingestion of tissue cysts. All warmed blooded animals can act as intermediate host.

Cats have a very short period of oocyst excretion (between 4 and 13 days after infection) and, after a primary infection, are considered immune. However, during the period of oocyst shedding, huge number of oocysts (≥ 20 million) are released in their faeces to the external environment (Dubey 1995).

It takes about a week (1-5 days) for the oocysts, released to the environment in the faeces of infected cats, to sporulate and become infective for the next host. The sporulated oocysts contain two sporocysts (Figure 4) each containing four sporozoites. It has been shown that the oocysts are very robust and could survive the extremely harsh conditions in the environment (Shapiro et al. 2019). The low infectious dose, as low as 1 oocyst, means that the parasite poses significant public health risk (VanWormer et al. 2013).

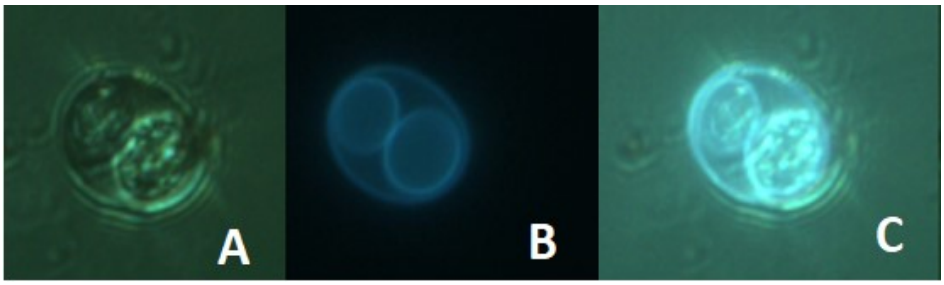


Figure 4. Sporulated oocyst of *T. gondii* A) Nomarski microscopy B) Autofluorescence microscopy C) Merger of Nomarski and autofluorescence microscopy

Transmission to humans may be via several routes including the consumption of undercooked meat that contains the bradyzoites; ingestion of food or water contaminated with sporulated oocysts; transfusion of blood or transplantation of organ containing the tissue cysts, and vertical transmission from mother to foetus (Figure 5). It has also been shown that infection could be acquired through consumption of milk containing the tachyzoites (Koethe et al. 2017).

The global risk-ranking of FBP conducted by the panel of experts appointed by the FAO and WHO indicated that *T. gondii* is placed in the top 4 list next to *T. solium, E. granulosus, and E. multilocularis*. The European risk-ranking of FBP shows that *T. gondii* is placed second to *E. multilocularis* (Bouwknegt et al. 2018).

Various studies have investigated the potential for foodborne transmission of *T. gondii* via contaminated berries. Among these are the studies from Bogota, Colombia, which showed that 6 out of 120 strawberry samples (5 %) were positive for *T. gondii* (Pineda et al. 2020) and a report from Portugal showing 3 out of 7 samples of berry fruits (strawberry, raspberry and blueberry) positive for *T. gondii* (Marques et al. 2020).

There have been reports of outbreak of foodborne toxoplasmosis, reported mainly from Brazil (Meireles et al. 2015). However, most of the outbreaks were linked to game meat consumption. A case-control study of an acute toxoplasmosis outbreak in São Paulo, Brazil showed a significant association with consumption of green vegetables (Ekman et al. 2012).

Pathogenesis

Most immunocompetent people infected with *T. gondii* remain asymptomatic, and this is probably a major reason why outbreaks of infection are rarely identified. However, individuals may develop symptoms like those of the flu such as headache, fever, fatigue, and lymphadenitis, in a varying range of severity, that usually resolves within a few weeks. Although rare, some cases include serious symptoms including ocular manifestations such as loss of vision. Primary infections acquired during pregnancy may lead to congenital toxoplasmosis and result in severe pathological consequences for the foetus, including abortion, still-birth, hydrocephalus or microcephalus and intracerebral calcification (EFSA 2018).

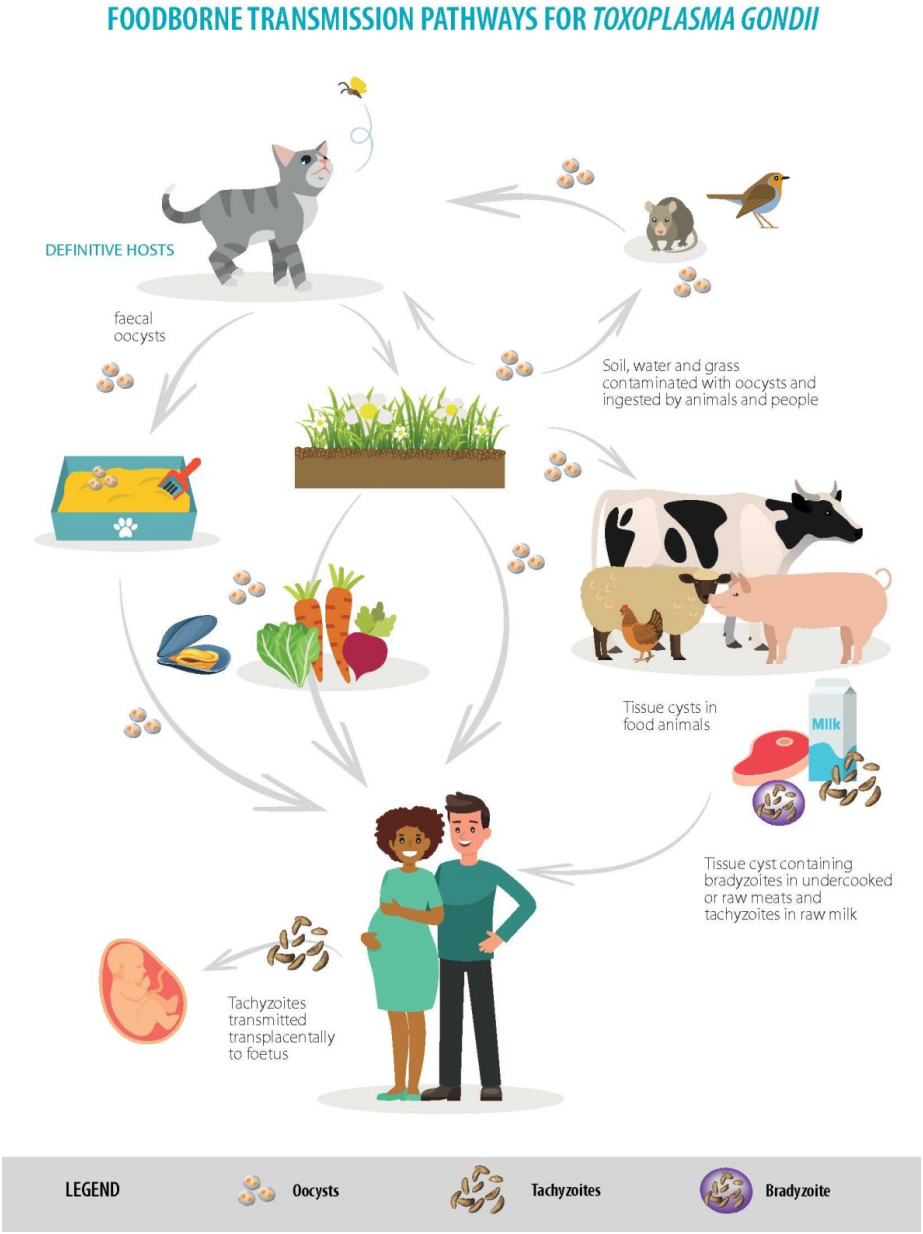


Figure 5. Food-borne transmission pathways for *Toxoplasma gondii* (source: (EFSA 2018))

Once humans are infected with the sporulated oocysts, the sporozoites invade the intestinal epithelium and disseminate throughout the body, where they form tissue cysts in any nucleated cells and remain dormant. Acute toxoplasmosis may cause heart failure, pneumonia, and encephalitis. The dormant cysts (bradyzoites) can later reactivate and actively multiply causing chronic toxoplasmosis. The pathogenesis of toxoplasmosis is due to the propagation of tachyzoites (the actively dividing stage) throughout various organs (virtually all) of the body. Toxoplasmosis could be fatal in immunosuppressed people as in HIV/AIDS patients that might be due to impaired cellular immune response. It has been shown that oocysts-induced infection results in clinically more severe disease than bradyzoites-acquired infection (Jones & Dubey 2010).

Chronic toxoplasmosis is most frequently manifested with neurological disease in patients with CD4 count < 100 cells/µL (Gandhi 2019). Moreover, it has been shown that latent toxoplasmosis is associated with mental health disorders such as autism, schizophrenia, attention deficit hyperactivity disorder, obsessive compulsive disorder, antisocial personality disorder, learning disabilities, and anxiety disorder (Flegr & Horáček 2020).

Apart from the immune status of humans, the parasite's virulence, which varies across the different genotypes of the parasite, affects the clinical symptoms. Three different genotypes (type I, type II, and type III) were previously described and their virulence in mice showed that type I strain was linked to severe toxoplasmosis, whereas the virulence of type II and type III were graded as low and medium, respectively (Wang et al. 2013). Most human infections are associated with type II strain. The three clonal lineages compose more than 95 % of *T. gondii* strains (Howe & Sibley 1995).

From more recent studies, it could be understood that frequent exchange of strains between hosts and sexual recombination, the predominant route of reproduction, might have led to complex variation of genetic composition. The recombinant strains could be of type I/II, type I/III, and type II/III (Herrmann et al. 2012; Minot et al. 2012).

Moreover, other strains have been found to be distinct to the three types and their recombinants and are referred to as atypical strains. These atypical strains are highly polymorphic, virulent and could be fatal even in immunocompetent individuals (Carme et al. 2009). The diversity of strains is associated with the geographic locations, where type

II is mostly reported in Europe and North America. The atypical strains are mostly reported from Africa and South American countries (Stajner et al. 2013).

The treatment of toxoplasmosis may vary based on the patient receiving the treatment, with a common approach being pyrimethamine and sulphadiazine combination treatment plus supplementary folinic acid to decrease the toxic effect of pyrimethamine. The treatment could be a lifelong medication for immunosuppressed people (Konstantinovic et al. 2019).

1.4. *Cyclospora cayetanensis* as a foodborne parasite

Cyclospora cayetanensis is a coccidian parasite responsible for cyclosporiasis, a gastrointestinal illness commonly expressed as watery, and sometimes explosive, diarrhoea. Cyclosporiasis has been reported from many countries and is endemic in those where the climate allows sporulation of the oocysts. *C. cayetanensis* is believed to have a direct life cycle that involves only humans, but there is uncertainty as to whether non-human primates represent hosts (Giangaspero & Gasser 2019). The oocyst of *C. cayetanensis*, like those of *T. gondii*, is characterized by its robust, double-layered walls that shows autofluorescence under UV-light. The oocysts contain two sporocysts each containing two sporozoites.

Life cycle and transmission

Humans are infected by the sporulated oocysts of *C. cayetanensis* through consumption of contaminated food and or water. After ingestion of oocysts, the sporozoites are released and invade the intestinal epithelium, where both asexual and sexual reproduction occurs. The sexual reproduction, fusion of the macrogametocytes and microgametocytes, results in the formation of unsporulated oocysts that exit the host through faeces. In the external environment, sporulation of the oocysts occurs within a week or two, given favourable conditions, and hence ready to infect another host (Figure 6).

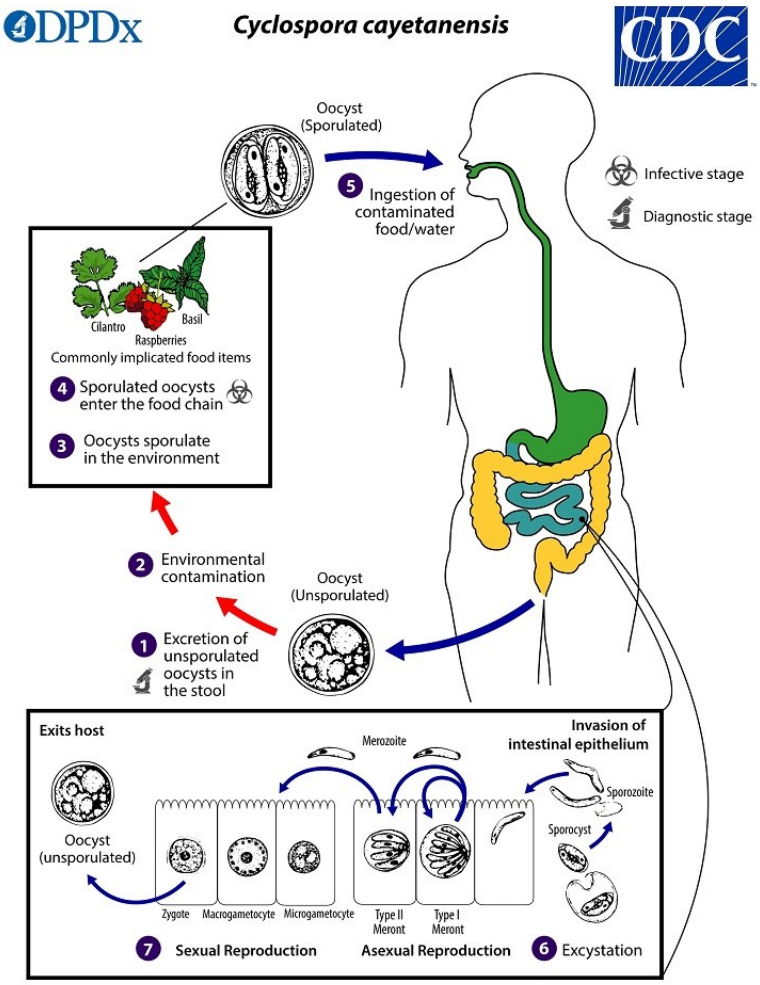


Figure 6. Life cycle of *C. cayetanensis*

The requirement for sporulation in the environment means that, unlike *Cryptosporidium parvum*, another coccidian parasite that is shed fully sporulated, direct person-to-person transmission is unlikely. As oocysts require a temperature between 22 and 30 °C to sporulate (Almeria et al. 2019; Smith et al. 1997), it is unlikely for *C. cayetanensis* to become endemic in countries like Norway where the temperature is rarely so high for an extended period.

Epidemiology

The parasite has a worldwide distribution and it is endemic in tropical and sub-tropical regions. In those endemic regions, the transmission of *Cyclospora* shows seasonality that has no consistency with time of the year, temperature, and rainfall. A high incidence of *C. cayetanensis* was associated with the warm period of maximal rainfall in some countries (e.g., Guatemala, Honduras, Mexico, Nepal). However, in other regions such as Peru and Turkey, drier and hotter months of the year were associated with high incidence (Almeria et al. 2019).

People living in non-endemic regions could be infected during travelling to the endemic regions. However, in USA, many cases of cyclosporiasis have been diagnosed in people without a travel history. In most of the outbreak investigations, infection has been linked to consumption of certain fresh produce including raspberries, blackberries, mesclun lettuce, snow peas, salads, cilantro, and basil (Almeria et al. 2019; Giangaspero & Gasser 2019). There have been frequent outbreaks of cyclosporiasis associated with contamination of fruits and vegetables, mostly reported from the USA (Table 2).

Pathogenesis

Cyclosporiasis is a self-limiting intestinal illness among immunocompetent people and shows symptoms such as watery diarrhoea, loss of appetite, abdominal cramps, weight loss, nausea, fatigue, and low-grade fever (Ortega & Sanchez 2010). However, it can cause severe chronic diarrhoea in immunocompromised people (Almeria et al. 2019). In endemic areas, asymptomatic infections are common. But if a person ill from cyclosporiasis is left untreated, the clinical symptoms may persist for several weeks or months (Thapa & Basnyat 2017).

The standard treatment for cyclosporiasis include administration of antibiotic, trimethoprim-sulphamethoxazole (TMP-SMX), which is considered effective for both immunocompetent and immunocompromised patients. Ciprofloxacin and nitazoxanide are alternative treatments for patients allergic to sulphonamides (Almeria et al. 2019).

Table 2. Outbreaks of cyclosporiasis linked to consumption of berries and berry products

Year (month)	Place	No. of cases	Type of berry	Reference
1995	Florida, U.S.	38	Raspberries	(Huang et al. 1995)
1996	U.S. and Canada	1465	Raspberries	(Herwaldt et al. 1997)
1996	Boston, United States	57	Berry dessert	(Fleming et al. 1998)
1996	U.S. (Multiple states) and Ontario, Canada	850	Raspberries	(Palumbo et al. 2013)
1997	U.S cruise ship (departure Florida)	220	Raspberries	(CDC 1997)
1997	U.S. (Multiple states) and Ontario, Canada	1012	Raspberries	(Palumbo et al. 2013)
1998	Ontario, Canada	192	Raspberries	(Palumbo et al. 2013)
1998	Ontario, Canada	221	Raspberries (garnish)	(CDC 1998)
1999	Florida, U.S.	94	Most likely berries in a fruit salad.	(Strausbaugh & Herwaldt 2000)

1999	Ontario, Canada	104	Blackberries, raspberries, strawberries	Anonymous, 2000
May 2000	Georgia, U.S.	19	Raspberries and or blackberries (suspected)	(CDC 2015)
Jun 2000	Pennsylvania, U.S.	54	Raspberries	(CDC 2015)
Dec. 2001-Jan 2002	Vermont, U.S.	22	Raspberries (likely)	(CDC 2015)
Jul 2008	California, U.S.	45	Raspberries and/or blackberries (likely)	(CDC 2015)
2009	Connecticut, U.S.	8	Raspberries and blackberries	(CDC 2015)

1.5. *Cryptosporidium parvum* as a foodborne pathogen

Cryptosporidium parvum is a coccidian parasite responsible for cryptosporidiosis, a gastrointestinal disease manifested as watery diarrhoea, nausea, vomiting, fatigue, and other signs and symptoms. The incubation period could range between two to ten days with a mean of seven days. *C. parvum* belongs to the phylum Apicomplexa, subclass coccidia, family *Cryptosporidiidae*, and the genus *Cryptosporidium*.

Life cycle and transmission

C. parvum is a zoonotic parasite that infects both animals and humans, unlike *Cryptosporidium hominis* in which humans are usually the only host. Humans acquire the infection through consumption of food and or water contaminated with the sporulated oocysts, or directly from contact with the faeces of infected people or animals (Figure 7). The sporulated oocysts contain four sporozoites that invade the intestinal epithelium. The transmission is mainly via faecal-oral route as a result of contact with contaminated food or water used for drinking or recreational purpose (incidental swallowing of water in lakes, rivers and swimming pools). Person-to-person transmission is possible because the oocysts shed in faeces are immediately infectious. The parasite has a wide range of hosts, mainly young ruminants.

Epidemiology

Cryptosporidiosis is a worldwide public health problem, irrespective of wealth status and has both medical and veterinary importance. Anyone can be infected given exposed to the risk factors. It is more common among children under 2 years, travellers, and people working with animals (e.g., farmers and veterinarians). It is considered as the leading cause of diarrhoea morbidity and mortality in children younger than 5 years (Khalil et al. 2018).

The prioritisation of *Cryptosporidium* as an FBP is hugely different in the various regions of Europe. It is considered as the second to top priority in Northern and Western Europe whereas it ranked 8th for Eastern and South-Western Europe. However, the parasite is not in the top 10 priority FBP for South-Eastern European countries such as Bulgaria, Croatia,

Serbia, Greece, and Turkey (Bouwknegt et al. 2018), which likely reflects the endemicity of other parasites that are considered more important.

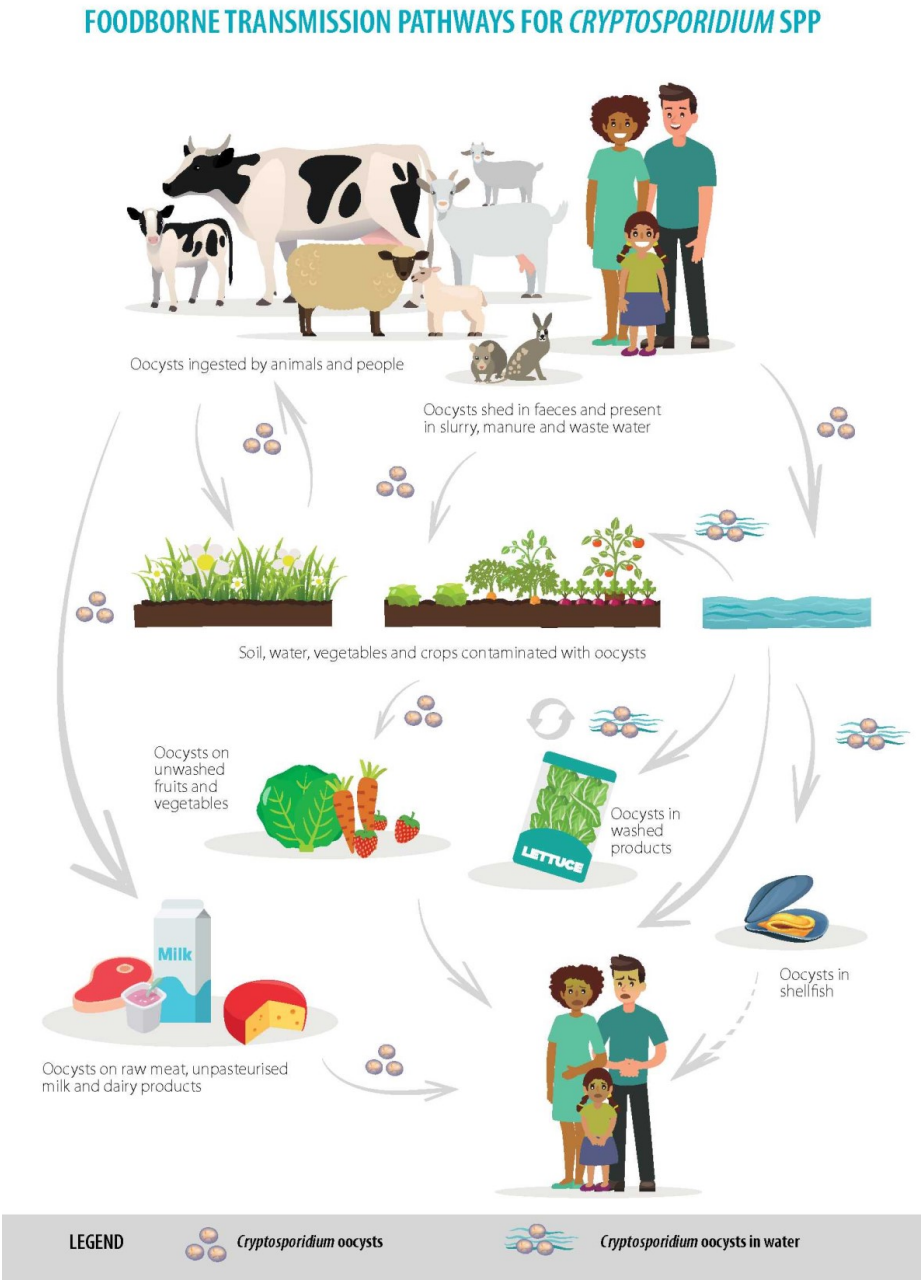


Figure 7. Life cycle of *Cryptosporidium* spp. (Source: (EFSA 2018))

Cryptosporidiosis is a notifiable disease in many countries, especially developed countries. Outbreaks of cryptosporidiosis have frequently been attributed to water, both drinking water and recreational water. It is considered as the leading cause of outbreaks of diarrhoea linked to water contamination in the U.S (Gharpure et al. 2019).

Although water is the most common route of transmission, other routes have also been described. For instance, apple cider has been indicated as the source of cryptosporidiosis outbreaks in the U.S. (Blackburn et al. 2006) and more recently in Norway (Robertson et al. 2019).

Pathogenesis

It has been known that *Cryptosporidium* infections can be asymptomatic or cause self-limited diseases. However, they could also cause severe diarrhoea that lasts for 2 or more weeks, the symptoms being worse in immunocompromised individuals. The watery diarrhoea, which is typical of cryptosporidiosis, could sometimes be profuse and prolonged and thereby result in critical illness due to dehydration and wasting (Bouzid et al. 2013).

The treatment of cryptosporidiosis may be affected by the immune status of the patient. Nitazoxanide is the only FDA-approved drug for the treatment of cryptosporidiosis in immunocompetent individuals whereas the drug's effectiveness among immunosuppressed patients is not clear (Amadi et al. 2009).

1.6. Berries as vehicles of infection

Studies have shown that various transmission stages of parasites can stick to the berries' surfaces, which means that berries could be a potential vehicle for the FBP. For example, it was shown that mice fed with raspberries and blueberries spiked with the oocysts of *T. gondii* developed acute infection (Kniel et al. 2002). The potential of wild forest berries as a vehicle for transmission of taeniid eggs has been investigated by spraying specified plots with taeniid eggs and collecting the berries after 24 h- which was then examined for detection of the parasite using qPCR (Malkamäki et al. 2019).

Many parasite transmission stages show a sticky nature that might help them attach to different hosts or transmission vehicles thereby maintaining their life cycle. Tapeworm eggs, such as those of *Taenia* spp. and *Echinococcus* spp., are very sticky and resilient as well (Erickson 2017). *Ascaris* eggs are also known to be sticky (Quilès et al. 2006). According to one study on the infectivity of *Cryptosporidium* after spiking apples, it was not possible to attain complete removal of the oocysts by using the elution methods commonly used for detection purposes (Macarisin et al. 2010). Using scanning electron microscopy, Macarisin et al., (2010) revealed a filamentous matrix between the parasite and the apple surface, but it was not clear if this was derived from the oocyst or from the apple. The oocysts of *C. cayetanensis* are believed to be even more sticky than *Cryptosporidium* oocysts, probably due to their specific adhesins (Ortega & Shields 2015).

In addition to the sticky nature of the parasites' transmission stages, the form of some berries enables the adherence of parasites. One study showed that raspberries retained more oocysts of *T. gondii* than blueberries- in which mice fed with raspberries inoculated with just 10 oocysts were infected while it was not observed for the blueberries counterpart (Kniel et al. 2002). This was speculated to be due to the hairy projections on the surface of raspberries that contributed to greater retainment of the parasites than the smoother surfaces of blueberries (Kniel et al. 2002).

Few studies have been conducted to assess the parasite contamination of berries and showed the relevance of berries as potential vehicle for transmission of FBP. A study conducted in Bogota, Colombia, indicated that 5 % (6 out of 120) strawberry samples tested positive for *T. gondii* DNA and 1 sample tested positive for *C. cayetanensis* DNA (Pineda et al. 2020).

1.7. Detection tools for parasites on berry matrices

Parasite contaminants maybe detected either directly, by visualisation of the parasite stages using microscopy, or indirectly, by amplifying the DNA of the parasite, using molecular techniques such as qPCR.

Various methods have been published in connection with analysis of berries for parasite contaminants (Table 3). However, those methods tend to be limited, in that they focus

only on particular parasites or berry matrices. In addition, there has been no standardised methods, except for *Cryptosporidium* and *Giardia*, for which there is ISO standard method (ISO 18744:2016). It is a microscopy-based method that relies on the specific monoclonal antibodies designed to bind with the oocysts of *Cryptosporidium* and *cysts* of *Giardia* for both isolation and detection, thereby increasing sensitivity and specificity by reducing the background debris in the washing eluates.

Table 3. Overview of methods used for the detection of FBP on berries

Parasite detected	**Method**	**Matrix**	**References**
Cryptosporidium spp. and *G. duodenalis*	ISO18744:2016	Berry fruits	(Cook et al. 2006; ISO 2016)
C. cayetanensis	Microscopy after LMS	Raspberries	(Robertson et al. 2000)
C. cayetanensis	qPCR	Raspberries	(Murphy et al. 2018)
C. cayetanensis	Nested PCR	Berries	(Resendiz-Nava et al. 2020)
E. multilocularis	Nested PCR	strawberries, raspberries, blueberries & cowberries	(Lass et al. 2017)
*Eimeria**	qPCR	blackberries, blueberries, cranberries, raspberries strawberries	(Lalonde & Gajadhar 2016)

*used as surrogate for coccidia of public health importance

In the absence of such monoclonal antibodies or equivalent methods for the other FBP, due to the considerable amount of debris in the eluates, microscopy would be likely to have low sensitivity if used as a detection tool. Therefore, molecular methods, especially those based on real-time PCR or equivalent, could be more sensitive as standard methods.

1.8. Viability assessment tools for parasites

Ideally, methods to detect parasites should have low limits of detection (i.e., detect as few parasite contaminants of berries as possible). However, to obtain information about whether the parasites that are detected are also potentially infective, it would be even more useful to have a method that could differentiate inactivated parasites from the viable ones. Due to the difficulty of cultivating most parasites in the lab, it has been challenging to develop sensitive methods that could determine the viability of the parasites. *In vivo* tests, i.e., animal bioassays, are considered the gold standard method for the viability assessment of parasites. Other techniques that have been used for viability tests include cell culture, *in vitro* excystation, sporulation, vital dye inclusion/exclusion, egg hatching, electrorotation, propidium monoazide PCR (PMA-PCR), nucleic acid sequence based amplification (NASBA), mRNA detection using reverse transcription qPCR (RT-qPCR), and fluorescence *in situ* hybridisation (FISH).

Bioassay

The basic concept of bioassay method is based on the inoculation of the infective stages into animals to detect infection. Different animal models are used for the experiments depending on the parasite biology. Among the animals used are calves, pigs, lambs, neonatal mice, Swiss Webster mice, and Mongolian gerbils.

Animal bioassay has been extensively used for the viability/infectivity assessment of *C. parvum* (Fayer et al. 2003; Li et al. 2006; Quilez et al. 2005; Slifko et al. 2002), *T. gondii* (Dumètre et al. 2008; Esmerini et al. 2010; Lindsay et al. 2008), and *E. multilocularis* (Federer et al. 2015; Kniel et al. 2002; Veit et al. 1995; Woolsey et al. 2015).

The main advantage of bioassays is that they provide a dependable assessment of the effectiveness of a specific inactivation treatment. However, this approach has several drawbacks and is not applicable for all parasites. Some of the limitations include it is time consuming, labour intensive, costly, and ethically challenging. Moreover, some parasites don't have hosts other than humans (e.g., *C. cayetanensis*) making it non-applicable for such parasites.

Cell culture

To overcome the challenges of bioassay, cell culture has been used for various parasites including *T. gondii* and *C. parvum.* Cell culture involves the process by which cells are grown *in vitro* under controlled conditions. The complexity of life cycle followed by parasites makes it difficult to apply cell culture. It is more complex and difficult to cultivate helminths than obligate intracellular protozoan parasites such as *Toxoplasma, Cyclospora,* and *Cryptosporidium* (Ahmed 2014).

Cryptosporidium has been successfully tested for infectivity using cell culture of human enterocytes (HCT-8), which is considered the model of choice for oocyst infectivity study (Rousseau et al. 2018). Another human cancer cell line (COLO-680N) has been suggested for long-term and sustainable culturing of infective oocysts (Miller et al. 2018), which could be employed for viability tests.

The biology of *T. gondii* indicates that any nucleated cell could support the multiplication of the parasite. However, not many options have been investigated for cell culture of the parasite. The most widely used methods include human foreskin fibroblasts (HFF) and murine L20B fibroblasts (Rousseau et al. 2018). This approach has been used for assessment of the efficacy of various treatments such as chlorine, ozone, UV, heating, and hydrogen peroxide (Rousseau et al. 2018).

The challenges of cell culture technique include that it requires highly skilled personnel, maintaining strictly aseptic conditions, and the cost and efforts required are considerable (Arango et al. 2013).

***In vitro* excystation and egg-hatch test**

For coccidian parasites such as *Toxoplasma, Cyclospora,* and *Cryptosporidium*, it has generally been assumed that *in vitro* excystation could be a good indicator of the viability of the oocysts, because excystation results in the release of sporozoites that eventually infect the cells. *In vitro* excystation tests involve the treatment of oocysts with conditions that resemble *in vivo* situations in a susceptible host; taking in to account for example, body temperature and pH (Campbell et al. 1992).

The application of this approach seems quite limited due to poor correlation with the infectivity bioassays during assessment of the efficacy of different treatments against *Cryptosporidium* (Black et al. 1996; Korich et al. 1990; Quilez et al. 2005).

The viability of helminth eggs could be assessed by using the egg hatch test, a test that allows visualization of egg development during hatching. It has been used for the assessment of anthelmintic resistance of *Fasciola hepatica* and shown a good correlation with *in vivo* tests (Ceballos et al. 2019). On the other hand, a study conducted on *Echinococcus granulosus* eggs showed that the hatching rate was not significantly different among the viable and inactivated ones (Moazeni & Rakhshandehroo 2012). The viability of *Taenia saginata* eggs has been evaluated using this approach. However, the egg hatch test was further complemented with *in vitro* activation of oncospheres, using artificial intestinal fluid, to evaluate embryo motility and or secretory globules as the viability indicator (Bucur et al. 2019).

Vital dye exclusion/inclusion tests

Vital dyes are dyes or stains that are capable of staining cells without affecting their normal function. The dye exclusion tests are based on the membrane integrity of the parasites, such that the dyes stain only those parasites with compromised membrane integrity, an indirect indicator of nonviability. Some of the vital dyes that have been used for such tests include eosin and propidium iodide (PI) (Bingham et al. 1979; Campbell et al. 1992).

A method based on PI exclusion test was used to test the viability of *C. parvum* oocysts recovered from experimentally contaminated oysters (Freire-Santos et al. 2002). In another study, eosin staining of the oncosphere of *E. granulosus* was used to assess the viability of the eggs (Moazeni & Rakhshandehroo 2012). The same study reported that egg hatching test by itself did not show any significant difference between viable and inactivated eggs.

The limitation of dye exclusion test includes that membrane integrity does not necessarily indicate the viability status of the parasite, i.e. a parasite with an intact membrane may

nevertheless be non-viable. This means that the test could underestimate the efficacy of certain treatments (Rousseau et al. 2018).

As a remedy to the shortcomings of dye exclusion tests, a combination of dye exclusion and inclusion tests have also been designed. A notable example of the dye inclusion test is the use of 4',6-diamidino-2-phenylindole (DAPI), a dye that can go through the intact membrane and stain the nuclei (Campbell et al. 1992). Fluorescein diacetate, which penetrates a viable cell and results in fluorescence upon cleavage by intracellular enzymes such as esterase, was used in combination with PI for viability tests of *Giardia muris* cysts (Schupp & Erlandsen 1987). However, it was reported that cysts of *G. duodenalis* that stained with PI also stained with fluorescein diacetate (Smith & Smith 1989). This shows that the results might not be reliable, and enzymes may still be active when the parasite's transmission stage is inactivated.

Viability qPCR (v-qPCR)

Viability qPCR (v-qPCR) is a modification of a qPCR protocol that enables differentiation of viable and inactivated parasites. It exploits the principle of vital dye exclusion tests, in which viable cells remain impermeable to dyes such as EMA and PMA. Given the relative toxicity of EMA and its poor selectivity for inactivated cells, PMA has been recommended for v-PCR. More recently, PMAxx has been developed by Biotium, Inc. to improve the selectivity for inactivated cells (https://biotium.com/technology/microbiology/pma-for-viability-pcr/).

v-PCR relies on the characteristic covalent bond between the vital dyes and DNA of the parasites following photoactivation. This means that the vital dyes penetrate the non-viable cells and form covalent bonds with the DNA, thereby preventing amplification during PCR. Several studies reported the use of PMA-qPCR for the assessment of viability of oocysts of *Cryptosporidium* (Alonso et al. 2014; Brescia et al. 2009; Liang & Keeley 2012), whereas only one study reported attempts to develop v-PCR for *T. gondii* (Rousseau et al. 2019). The v-PCR for *T. gondii* was not successful according to the study. One issue seems to be that despite working effectively on clean parasite suspensions, it is less successful when the parasites are in matrices.

Reverse transcription qPCR (RT-qPCR)

Another approach to assessing the viability of parasites is based on detection of mRNA, which indicates that cell is metabolically active and thus viable. Different target genes have been suggested for different parasites for the purpose of identifying the viable parasite stages from the inactivated ones. Some of the target genes used include HSP70, COWP, β-tubulin, and amyloglucosidase.

Several studies have been conducted to develop a RT-qPCR method for *C. parvum*. Various authors have followed different approaches to selecting the target genes that could be used as the viability marker. HSP70 has been commonly used for viability assessment of *C. parvum* and often the protocol includes heat induction (45°C for 20 min) for improving the sensitivity of the method (Bajszár & Dekonenko 2010; Garcés-Sanchez et al. 2013; Travaillé et al. 2016).

On the other hand, another study argued that the ideal viability marker should not be altered by an external stressor and hence suggested the use of CP2 over HSP70 (Lee et al. 2008). Others have suggested using the ratio of mRNA to DNA for assessment of viability following heat-induction (Garcés-Sanchez et al. 2013).

Although several efforts have been made to develop useful methods for *Cryptosporidium*, few studies considered *Toxoplasma*. Two target genes, SporoSAG and ACT1 have been investigated for *T. gondii* (Villegas et al. 2010). SporoSAG is a surface glycoprotein that is highly expressed on the surface of sporozoites and ACT1 is expressed in both sporulated and unsporulated oocysts (Rousseau et al. 2018). Villegas et al., (2010) reported that the difference in C_q values between untreated control and samples treated with 10 % formalin was huge ($\Delta C_q \approx 8.99$), whereas no significant difference was observed for oocysts heated at 80°C for 1h. However, such differences might reflect the effect of formalin on the RNA extraction and / or the RT-qPCR, rather than any difference in viability. This is supported by a lack of infection in mice from oocysts heated at 80°C for 2 min (Travaillé et al. 2016).

Results from these different studies suggest that RT-qPCR based methods could be a rapid, sensitive, and reliable estimate of the viability of parasites, but the approach to

selecting the target genes, the experimental setup, and the interpretation of results must be considered carefully.

One approach, with the potential to produce more reliable results, would be to induce gene expression in response to stressors and compare the relative quantity of the gene of interest between the test and control samples (such that genetic upregulation being a proxy for viability). Thus, a more informative and appropriate indication of the metabolic activity of the parasite would be to use comparative RT-qPCR that targets inducible gene/s but uses reference gene/s (as normaliser) for comparative evaluation. This approach would not be hampered by the constraints (e.g. overestimation of viability) indicated in previous RT-qPCR approaches.

Other molecular techniques

Other molecular techniques such as FISH and NASBA have also been used for viability assessment of *Cryptosporidium*. However, the results of these methods showed inconsistency with the results of infectivity tests (Rousseau et al. 2018). Moreover, it seems that the stability of RNA has been underestimated and hence the methods detect both live and experimentally inactivated oocysts (Hønsvall & Robertson 2017; Smith et al. 2004).

1.9. Knowledge gaps

Despite the growing number of laboratory methods developed for detection of foodborne pathogens, parasites are still lagging behind in comparison with bacteria and viruses. This includes the availability of standard methods that could help in source attribution during outbreak investigation. So far, there has only been a microscopy-based standardised method for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* from contaminated fresh produce, commonly referred to as ISO 18744:2016 (ISO 2016).

Considering the robustness of transmission stages of parasites, such as *Toxoplasma, Cyclospora,* and *Echinococcus,* compared with some other pathogens, it would be inappropriate to use the commonly used indicator organism *Escherichia coli* as a marker of contamination of berries. This marker indicates only recent faecal contamination, but parasites transmission stages can remain infectious long after *E. coli* has been inactivated.

Thus, there is a clear need for the development and validation of novel methods for the detection of those parasites on contaminated berries.

Due to the lack of attention and standardized methods with acceptable sensitivity and specificity for detection of most FBP, there is very little surveillance of berries for parasite contamination. In Norway, only two studies have been conducted to assess parasite contamination of berries on the Norwegian market- the first was conducted between 1999-2001 (Robertson & Gjerde 2001) and more recently between 2015 and 2016 (Johannessen et al. 2017). Furthermore, these studies were limited to detection of *Giardia* and *Cryptosporidium.* Development of standardised methods for detection of a broader range of parasites transmission stages on berries is therefore warranted.

Furthermore, there are no validated methods to assess the viability of parasites identified as contaminants of fresh produce, including berries. Due to the aforementioned limitations of using animal bioassay assays, it would be highly useful to have molecular methods that could differentiate viable parasites from the inactivated ones and could be used for investigating whether novel treatments are effective at inactivating parasite contaminants of berries.

Due to the delicate nature of berries and the reduced shelf-life once washed, producers usually don't wash berries. Therefore, consumers are expected to wash the berries prior to consumption, and this might be more effective way of infection prevention. However, the efficacy of how people wash berries before consumption in removing the parasite contaminants from the berries surface has not been investigated.

2. Objectives of study

The main objective of the present PhD project was to develop and validate analytical methods for the detection and viability assessment of parasite contaminants of berries.

Specific objectives

Embedded in the heart of PARABERRY, my PhD project was designed to answer the following research questions

- Can we develop and optimise method/s for the detection of *E. multilocularis, T. gondii,* and *C. cayetanensis* as contaminants of berries?
- Are berries sold in the Norwegian market contaminated with foodborne parasites?
- How effective are the methods we normally use to wash berries, prior to consumption, in removing potential parasite contaminants?
- Can we differentiate viable parasites from the inactivated ones?

3. Summaries of individual papers

The scientific papers published in peer-reviewed international journals, as outputs of the experimental works of my PhD project, are summarized in this section.

Paper I

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

Summary

In this paper, we compared the efficacy of two DNA extraction approaches, the UNEX-based method and DNeasy PowerSoil kit, for DNA extraction of parasites on berries. Three protozoan parasites namely, *Cyclospora cayetanensis, Toxoplasma gondii,* and *Cryptosporidium parvum* were spiked into berry matrices prior to DNA extraction. This study was of importance to my PhD project because, as contamination levels are expected to be low, it is essential that every step in the sample processing for detection of the parasites be optimal for low numbers of parasites. The results of the experiments showed that 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 DNeasy PowerSoil kit; whereas the equivalent results using the UNEX-based 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. Furthermore, it was shown that the DNeasy PowerSoil kit provided inhibition-free DNA template for the qPCR assay. That means there would be no need of dilution of the DNA template in attempt to overcome matrix related PCR inhibition.

Paper II

A New Protocol for Molecular Detection of *Cyclospora cayetanensis* as Contaminants of Berry Fruits

Summary

Is it worth having an alternative method for detection of *C. cayetanensis* on contaminated berries to the standard methods currently available? Initial experiments on the standard method at the time that the project started, i.e. the Bacteriological analytical manual chapter 19b (BAM 19b) showed nonspecific amplification when the method was tested on DNA isolated from the related parasite, *Toxoplasma.* Therefore, the experiments described in this paper were aimed at developing a new, more-specific assay for detection of *C. cayetanensis* as a contaminant of berry fruits. This new assay, targeting the internal transcribed spacer 1 (ITS-1) region, was tested on three different berry matrices: raspberries, blueberries, and strawberries. The new assay showed good efficiency (102 %), linearity (r^2 = 0.999), repeatability, i.e., standard deviation (SD) of 0.2 C_q (95 % CI: 0.2, 0.3) and specificity for *Cyclospora*, with no cross-reactivity with various related coccidia (*Toxoplasma gondii, Eimeria mitis, Cystoisospora canis,* and *Cryptosporidium parvum*) when tested *in vitro*. The new method showed no effect of matrix inhibition. However, the method was also improved by incorporating an internal control as a duplex in order to monitor PCR inhibition due to sample matrix components to improve the usability of the new method in laboratories using different kit for DNA extraction. The duplex assay also showed acceptable efficiency (100 %) and linearity (r^2= 0.99). The results showed that the new assay has potential for standard use in food testing laboratories.

Paper III

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

Summary

It is good to have methods designed specifically for each parasite for detection and/ or quantification on contaminated berries. However, since FBPs have similar routes of transmission, it would be useful to have a multiplex approach-to detect and/or quantify multiple parasites without compromising the key performance characteristics of each method. With this aim, the experiments described in this paper were conducted to evaluate and validate the novel multiplex qPCR for detection of the three parasites that were central to the PARABERRY project: *E. multilocularis, T. gondii,* and *C. cayetanensis*. The results of the study showed that 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 (SD ≤ 0.2 C_q) and intermediate precision (pooled SD 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.

Paper IV

A novel comparative reverse transcription quantitative PCR method for assessing the viability of *Cryptosporidium* oocysts: a potential tool for inactivation efficacy trials

Summary

In this paper, a novel approach to assessing the viability of *Cryptosporidium* oocysts is presented. It describes the scientific steps followed to develop the RT-qPCR method, and includes the search and identification of inducible target genes and validation of the selected targets using the RT-qPCR. Although RT-qPCR has been previously explored as a tool for viability assessment of *Cryptosporidium* oocysts, the novelty of the approach described here lies in the use of RNA sequencing (RNA-Seq) analysis for the identification of inducible target genes and exploiting the 18s rRNA gene as a normaliser in the relative quantification of gene expression among samples exposed to an oxidative stressor.

To identify the most suitable targets for viability assessment, *C. parvum* oocysts were subjected to different stressors before total RNA extraction. Bioinformatic analysis of the RNA-Seq data identified several differentially expressed genes in the samples exposed to heat shock and oxidative stress due to xanthine oxidase catalysed reactions. Target genes were then pragmatically selected for developing RT-qPCR methods using the xanthine oxidase catalysed reaction as the induction approach. Preliminary experiments investigating six different target genes selected for the RT-qPCR method development showed that all were upregulated following oxidative stress. Among these the Thioredoxin and COWP7 genes were selected for further evaluation of the RT-qPCR on mixtures of viable and inactivated oocysts, as well as on oocysts that had been exposed to various treatments, such as freezing and chlorination, that may have an inactivation effect on *Cryptosporidium* oocysts. Evaluation of the RT-qPCR with samples that included mixtures of inactivated and viable oocysts in different proportions provided promising results, with the gene expression level proportionally related to the number of viable oocysts in the sample.

The inactivation efficacy of freezing at -20°C was time dependent, with longer storage duration resulting in increased inactivation; although oocyst viability was not affected by freezing for 2 h, complete inactivation was seen after 24 h. Chemical treatment of oocysts

with 0.2 and 4 mg/L free chlorine for 30 min at room temperature did not affect the viability of the oocysts.

The results of the study show that this approach could be used for testing the efficacy of different inactivation treatments. The RT-qPCR method offers a quick and cost-effective alternative to viability assessment compared with mouse infectivity and cell culture assays. However, before use as a standard method, this approach should be validated against the gold standard bioassay (mouse infectivity test).

4. Materials and methods

4.1. Parasites

Various parasites were employed for the development of novel methods for detection and viability assessment of parasites on berries. These included the parasites selected as the focus of the present study, i.e., *E. multilocularis, T. gondii,* and *C. cayetanensis*, and others that were employed for the development and evaluation of the novel methods, including *Eimeria mitis, Cystoisospora canis, Cryptosporidium parvum,* and *Taenia crassiceps*. In particular, the viability work concentrated on *C. parvum* oocysts as these are commercially available in high quantities.

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 sulphate (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 et al. 2016). Additional oocysts of *T. gondii* were kindly provided by Prof. Břetislav Koudela, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. 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. Purified oocysts of *C. parvum* IOWA strain were purchased from Bunch Grass Farm (Idaho, USA).

Oocysts of *E. mitis* were isolated from chicken faeces and *C. canis* from canine faeces. 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 *T. 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 newly developed methods.

4.2. DNA and plasmids

Purified DNA from *C. cayetanensis* oocysts was kindly provided by Dr. Ynes Ortega, University of Washington, USA. Plasmid containing the 12s rRNA gene of *E. multilocularis* was kindly provided by Prof. Peter Deplazes, University of Zurich, Switzerland. Purified DNA from *E. granulosus* was also kindly provided by Prof. Laura Rinaldi, University of Naples Federico II, Italy.

4.3. Berries

Two sources of berries were used in this study, the groceries and the distributors (importers). Berries bought from groceries were used for the development of the novel methods presented in this thesis, whereas the berries obtained from the distributors were used for surveillance. The distributors involved in this study were industrial partners in the PARABERRY project, BAMA and Coop Norge.

4.4. Spiking studies

"Spiking" in the context of this project means that the berries or berry matrices are artificially contaminated with certain number of the parasites' transmission stages. Spiking experiments were employed for the development and evaluation of the novel methods in this project. These experiments were used for estimating the limit of detection (LoD) of the new methods and as well as for assessing the matrix effect on the performance of the methods. Moreover, this approach was used in the comparative evaluation of DNA extraction kits. The numbers of oo/cysts were estimated by using KOVA® Glasstic® Slide 10 Microscope Slide (VWR, Norway). The eggs of *E. multilocularis* were received in predefined numbers. Based on the purpose of the study, either the berries or berry washes were spiked with the parasites of interest.

4.4.1. Spiking of berries

In order to estimate the LoD of the whole method, i.e., starting from the washing of berries up to the molecular detection of the parasites using qPCR, berries were spiked with 10 and 50 oocysts of *T. gondii* and *C. cayetanensis*. In addition, 5 and 10 eggs of *E. multilocularis* were also included in the spiking study.

4.4.2. Spiking of berry washes

The berry washes were spiked with the infective stages of each parasites prior to DNA extraction to assess whether there were inhibitory effects from the matrices during qPCR detection. For this purpose, the spiked pellets were subjected to DNA extraction and two-fold and four-fold dilution of the DNA extracts were included in the qPCR run. The spiking of the berry washes was also used to compare the efficacy of commercially available DNA extraction kits (Paper I). For the comparison of DNA extraction kits, 20 and 500 oocysts of *C. cayetanensis, T. gondii,* and *C. parvum* were spiked into the berry washes.

4.5. Sample processing

The sample processing for molecular detection of parasites on berry surfaces requires a multi-step procedure that includes three main steps: i) elution of the transmission stage of the parasites by using appropriate washing protocol; ii) concentration of the eluted parasites by centrifugation to the final sample volume that is appropriate for the specific DNA extraction kit requirement; and iii) extraction of genomic DNA so that the sample is ready for detection using appropriate molecular assay.

4.5.1. Elution of parasites' stages on the surfaces of berries

Washing of the appropriate amount of berries for detaching potential parasite contaminant is the first step in the multi-step protocol for detecting the parasites using a suitable tool. There are differences in the selection of washing solutions according to different publications. Among the most commonly used washing solutions are 0.1 - 1 % Alconox, 1M Glycine buffer (pH 5.5), and various detergents such as Triton X-100, Tween 20, and Tween 80 solutions. It is worth noting that different parasites attach differently to the berries' surfaces; some are stickier than others and hence there are differences in the efficacy of removal of different parasites. Attempts have been made in comparing the efficacy of various washing solutions and it has been reported that 0.1 % Alconox outperformed the other alternatives for *Cryptosporidium* and *Cyclospora* oocysts (Shields et al. 2012).

In the experiments presented here, 200 ml of 0.1 % Alconox was added to a plastic box containing about 30 g of berries. The box was closed and put on a shaker (VibraMax) with

a setting of 600 rpm for blueberries and strawberries, but at a lower setting of 300 rpm for raspberries due to their greater fragility, for 10 min.

4.5.2. Concentration of parasites' stages

4.5.2.1. Centrifugation concentration

The elution step is followed by a concentration step in which the 200 ml of eluate is concentrated down to the volume that is appropriate for the pre-selected detection method. Regarding concentration approaches, the centrifugation concentration is the most commonly applied method for most parasites, except for *Cryptosporidium* and *Giardia* for which an additional purification step, immunomagnetic separation, may be used. In this study, only concentration by centrifugation was employed.

Briefly, the eluted solution was carefully transferred to four 50 ml conical tubes and then centrifuged at 1690 × g for 10 min. The supernatants, except 10 ml, were removed by vacuum suction from each tube and then combined into single tube after the tubes are vortex mixed. An additional 10 ml of distilled water was used for rinsing.

The tube was then centrifuged again at 3,803 × g for 10 min, setting the brake at 6 (0-9 scale). The supernatant was removed except about 1.5 ml of the sediment, which was then transferred to a microcentrifuge tube after vortex mixing. The microcentrifuge tube was then spun at 13,000 × g for 5 min. The supernatant was removed and the remaining 250 µL sediment was used for DNA extraction.

4.5.2.2. Lectin magnetic separation (LMS)

Apart from the widely used centrifugation concentration, other approaches have been used for different parasites. One example is the application of immunomagnetic separation, which uses specific monoclonal antibodies on paramagnetic beads. This has been in use and commercially available for the concentration and purification of *Cryptosporidium* and *Giardia* spp., and is part of the ISO Method (ISO, 2016) mentioned previously. Another technique that could be applied relies on the exploitation of the surface-binding properties of parasites with lectins, carbohydrate-binding proteins. It is worth noting that lectins are, unlike monoclonal antibodies, not specific to a parasite immunological epitope against which antibodies can be raised but bind to specific carbohydrate moieties found on the surface of the parasite stages. Thus, their binding is

based on the biochemical structure of the parasite cell wall. In the present study, a panel of 13 different lectins were employed for testing the binding of *Eimeria* oocysts and *Taenia* eggs (Table 4).

Table 4. A panel of lectins was tested for their binding to oocysts of *Eimeria mitis* and eggs of *Taenia crassiceps*

No.	**Lectin**	**Abbreviation**
1	Concanavalin A	CON A
2	*Dolichos biflorus* agglutinin	DBA
3	Peanut agglutinin	PNA
4	*Ricinus communis* agglutinin I	RCA I
5	Soybean agglutinin	SBA
6	*Ulex europaeus* agglutinin I	UEA I
7	Wheat Germ agglutinin	WGA
8	*Griffonia simplicifolia* lectin I	GSL I
9	*Lens culinaris* lectin	LCA
10	*Phaseolus vulgaris Erythroagglutinin*	PHA-E
11	*Phaseolus vulgaris* Leucoagglutinin	PHA-L
12	*Pisum sativum* agglutinin	PSA
13	Wheat Germ agglutinin, succinylated	SWGA

4.5.3. DNA extraction

Several kits are commercially available for the extraction of DNA from various matrices and that could be applicable for the investigations described here. In this study, a preliminary comparison test was conducted on four kits that included QiaAmp DNA stool mini kit (Qiagen, Norway), DNeasy PowerSoil kit (Qiagen, Norway), FastDNA spin kit for soil (MP Biomedicals, France), and DNeasy PowerFood microbial kit (Qiagen, Norway). The preliminary tests compared extraction of DNA from 10,000 and 1,000 oocysts of *T. gondii* run in triplicate, and 500 and 100 oocysts extracted in 6 replicates. The kits' instructions were followed with slight modifications for the DNeasy PowerSoil kit that included the use of bead-beating (2 cycles of 4 m/s for 60 s; 45 s pause between the cycles) to facilitate the break opening of oocysts instead of just vortex mixing.

The DNeasy PowerSoil kit was further compared to the UNEX-based nucleic acid extraction, which had previously been found optimal for the extraction of DNA from the oocysts of *C. cayetanensis* (Qvarnstrom et al. 2018). For the comparison study, two levels of spiking of pellets from berry washes (with 20 and 500 oocysts of *T. gondii* and *C. cayetanensis* as well as *C. parvum)* were used and spiked samples randomly assigned to the one of the two extraction methods and two separate analysts (myself and a visiting PhD student). Briefly, a total of 60 samples were included in the study, 40 samples spiked with 20 oocysts and 20 samples with 500 oocysts. Each analyst analysed a total of 30 samples, 15 using the DNeasy PowerSoil kit and 15 using the UNEX-based approach.

4.6. Techniques for detection of parasites

4.6.1. Microscopy

The KOVA Glasstics slide was employed for estimating the number of parasites in suspension. Briefly, appropriate volumes of homogenous suspensions of parasites were deposited into the slide chamber for counting the parasites in the grids. The parasites found in the grids, lying on the two diagonals of the chamber, were counted and the number of parasites per microliter calculated from these numbers.

Fluorescence microscopy was employed for visualisation of the binding of fluorescein-labelled lectins to the parasites. The Leica DMLB Fluorescence microscope equipped with fluorescein isothiocyanate (FITC) filter setting (emission at 490 nm and excitation at 525 nm) was used and examinations were done at 200 × and 400 x magnifications.

4.6.2. Molecular techniques

4.6.2.1. Real-time PCR (qPCR)

Stratagene Mx3005P of Agilent was used for the qPCR assays. Various detection chemistries were employed for the development and evaluation of the new methods. SYBR green-based qPCR assays were used for the assessment of the specificity of the qPCR products by exploiting the added advantage of melt-curve analysis at the end of the PCR, which produce a dissociation curve with a specific melting temperature (T_m). Probe-based qPCR assays rely on the specific hydrolysis probes that are designed for binding to

particular sequences in target genes. The 3' ends of each probe were modified to improve the stability of binding by including the minor groove binder (MGB).

The qPCR was used for the detection and or quantification of *E. multilocularis, T. gondii, C. cayetanensis, C. parvum, G. duodenalis.*

qPCR for E. multilocularis, T. gondii, and C. cayetanensis

For the detection and or quantification of *E. multilocularis, T. gondii, and C. cayetanensis,* a multiplex qPCR developed and evaluated in this study was employed. The detailed protocol for the multiplex qPCR is described in Paper III. Briefly, the qPCR was performed in a total volume of 20 µL that included appropriate concentrations of oligos, and thermal cycling condition as described in Table 5.

qPCR for Cryptosporidium spp. and Giardia duodenalis

For the comparative evaluation of different washing techniques in removing *Cryptosporidium* and *Giardia* from contaminated berries, qPCR protocols that had been previously described for the detection and quantification of *Cryptosporidium* spp. (Elwin et al. 2020; UKWIR 2020) and *Giardia duodenalis* (Verweij et al. 2004) were used with slight modifications.

The qPCR protocol for *Cryptosporidium* was performed in a total volume of 25 µL that included 5 µL of template DNA, 0.6 µM of each primer and 80 nM of the Probe. The oligos included forward primer JF1/2 which was prepared in 1:1 mixture of JF1 (5'-AAGCTCGTAGTTggatTTCTG-3') and JF2 (5'-AAGCTCGTAGTTaatcTTCTG-3'), the reverse primer JR (5'-TAAGGTGCTGAAGGAGTAAGG-3'), and the probe JT2 (5'-TCAGATACCGTCGTAGTCT-3').

For *Giardia*, 0.4 µM of each primer Giardia-80F (5'-GACGGCTCAGGACAACGGTT-3') and Giardia-127R (5'-TTGCCAGCGGTGTCCG-3'), and 0.16 µM of the probe Giardia-105T (FAM-5'-CCCGCGGCGGTCCCTGCTAG-3'-MGBEQ) and 5 µL of the DNA template was combined in a total volume of 25 µL.

Table 5. The oligos and the multiplex 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
Primer 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	Paper II	(Opsteegh et al. 2010)	(Isaksson et al. 2014)
Thermal profile	95°C for 3 min 1× 95°C for 15 s 45× 60°C for 30 s 45×		

4.6.2.2. Nested PCR RFLP

Nested PCR protocol was employed for the genotyping of samples found to be positive for *T. gondii.* The protocol was adapted from previously published protocol (Khan et al. 2005; Su et al. 2010). Briefly, the samples detected as positive for *T. gondii* were subjected a nested PCR targeting the dense granule protein 6 (GRA6) and the surface antigen 2 (SAG2) genes. The nested PCR was initially designed to be followed the restriction fragment length polymorphism (RFLP) assay. However, as the nested PCR showed non-specific amplifications, the RFLP was not attempted.

4.7. Method development and validation: for detection purposes

Prior to developing a new method, already published methods were tested for potential application in the detection of the parasites from berry matrices. Following the suboptimal performance of the FDA-approved method (Murphy et al. 2017) for the detection of *C. cayetanensis* from fresh produce and berries, also known as BAM 19b, we needed to develop a new method. It was found that the BAM 19b primers and probe cross-reacted with *Toxoplasma gondii* DNA. There were minor differences in the qPCR setup in our lab, but these could not be considered as the reason for the cross-reactivity. The differences were associated with the way in the probe was quenched, as the probe was ordered from a different provider without the internal quencher and a different master mix to that used in BAM 19b was used.

Therefore, a new method with improved specificity was developed for the detection of *C. cayetanensis* targeting the internal transcribed spacer-1 (ITS-1) gene. The ITS-1 gene was selected due to its high degree of inter-species variation that indicates it could be a suitable target gene for improved specificity. Subsequently, this newly developed qPCR was multiplexed with the already available qPCR protocols for *T. gondii* (Opsteegh et al. 2010) and *E. multilocularis* (Isaksson et al. 2014). This multiplex qPCR for 3 parasites would enable the surveillance to be as efficient as possible.

The development of new qPCR methods for the detection of *E. multilocularis, T. gondii, and C. cayetanensis* as contaminants of berries followed a systematic approach as described in the flowchart (Figure 8). The first step was testing the *in-silico* specificity of

the oligos designed and or selected from published articles by using the basic local alignment search tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and further testing with the Geneious software, version 11.1.4. Next, a series of optimisations and evaluations of the methods was performed. Among the key performance characteristics evaluated were linearity, efficiency, *in vitro* specificity, precision, limit of detection, and robustness. In addition, the effect of berry matrices on the qPCR inhibition was evaluated.

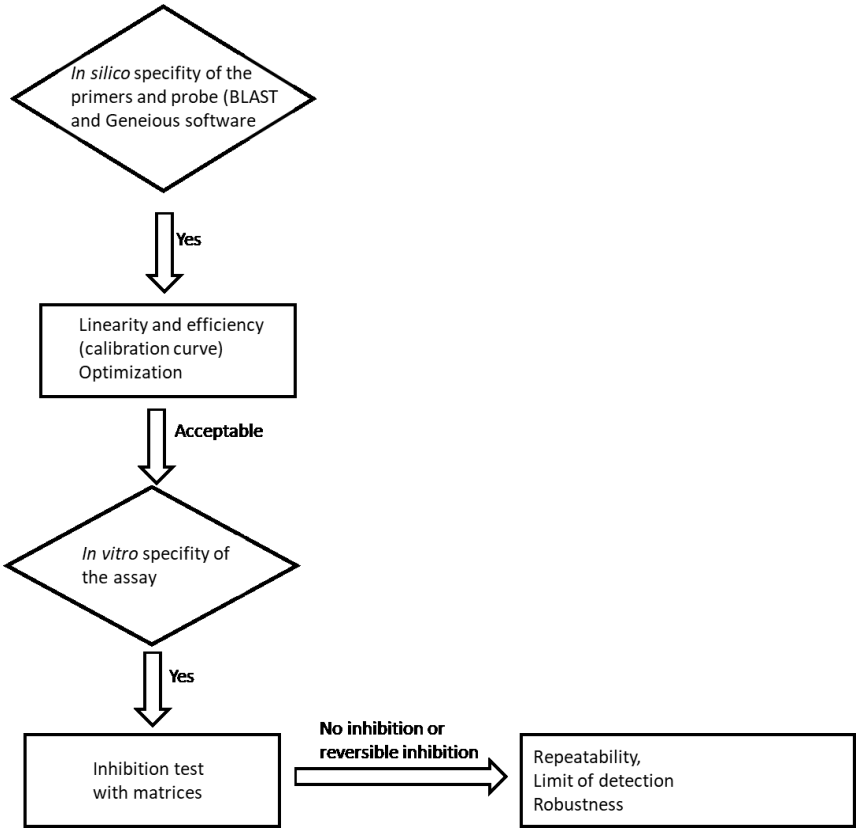


Figure 8. A flowchart of the method evaluation steps followed in the present study

Linearity and efficiency

In order to evaluate the rest of the key performance characteristics, it was necessary to verify that the qPCR conditions were optimal. The best indicator for the optimality of the conditions is reflected by the linearity and efficiency of the method. The setup required for this test was preparing a standard curve that encompassed at least four concentration

levels prepared by 10-fold serial dilution of template DNA. According to the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines, the acceptable ranges of values for linearity is $r^2 \geq 0.98$ (ideally 1) and for efficiency it is between 90-110 % (ideally 100 %) (Bustin et al. 2009). Significant deviation from the acceptable ranges means that the conditions needed further optimisation, which could involve adjustment of the concentration of the oligos and/or tweaking the thermal profile.

***In vitro* specificity**

The newly developed methods were tested against related parasites to ensure a lack of cross-reactivity of primers and or probes. For this purpose, the DNA isolated from *E. mitis, C. parvum, C. canis, Echinococcus granulosus,* and *T. crassiceps* were used as templates; the first three parasites for cross reactivity with *Cyclospora* or *Toxoplasma* primers/probes, and the last two parasites for cross reactivity with the *E. multilocularis* primers/probes.

Limit of detection

The minimum number of parasites that the new methods could detect was estimated by spiking the pellets of berries with 2, 5, 10, and 100 oocysts of *C. cayetanensis* and *T. gondii*, 1, 5, and 10 eggs of *E. multilocularis*. Based on the results from the pellets spiking, 30 g of berries were spiked with 10 oocysts of *C. cayetanensis* and *T. gondii* and 5 eggs of *E. multilocularis* and processed for molecular detection using the newly developed qPCR.

Precision

The precision of the methods was evaluated by using two indicators, repeatability and intermediate precision. To test the repeatability of the methods, replicate analysis of three samples representing low, medium, and high concentrations of DNA were performed under the same conditions (i.e., the same analyst and the same day). The intermediate precision was evaluated by including changes regarding analyst and day of analysis.

Robustness

To best address the objective of the robustness test, the factors that might affect the performance of the new methods were intentionally altered from the optimal setup. The changes included decreasing the concentration of primers and probes by 20 %, changing the super mix volume by ± 5 %, changing the annealing temperature by ± 1°C, and testing additional commercially available master mix.

4.8. Surveillance of berries for parasite contamination

4.8.1. Sampling of berries

The berries used in the survey were provided by both Coop Norge and BAMA, the PARABERRY project industry partners that import fresh produce, including berries, and distribute these and Norwegian-grown produce to the market. Three main types of berries were considered in the present study: blueberries, strawberries, and raspberries. The sampling period was between August 2019 and November 2020.

The distributors that provided the berries were asked to diversify the origin of berries in those available from their stock so that the survey could be representative of those berries on the Norwegian market and available to consumers. The exporting countries included Colombia, Peru, and Chile from South America; Morocco, South Africa, Zimbabwe, and Egypt from Africa; Portugal, Netherlands, Poland, Spain, and Belgium from Europe. In addition, berries produced in Norway were also included in the sampling plan.

Regarding the sample size, possible scenarios were considered for calculating (see formula below) the minimum number of samples required for the survey. For imported berries, the estimated proportion of contaminated berries was considered 20 % for *E. multilocularis* (Lass et al., 2015) and 9.7 % for *T. gondii* (Lass et al., 2012), the desired level of precision at 3 %, and assuming a 95 % confidence level, the minimum number of samples was estimated to 683.

$$n = z^2 * p(1-p)/e^2$$

Where:

- n is the minimum sample size,
- z is the Z score related to the confidence level,
- p is the estimated proportion, and
- e is the desired precision of the estimate.

4.8.2. Analysis of berries

The samples were processed as described in section 4.5. and analysed for contamination with the parasites of interest including *E. multilocularis, T. gondii, C. cayetanensis* using the newly developed multiplex qPCR (Paper III).

An attempt was made to further analyse *Toxoplasma*-positive samples in order to identify the specific genotypes that might be associated with severe toxoplasmosis as described in section 4.6.2.2.

4.9. Removal of parasites from berries' surfaces

The comparison of removal efficiency of 3 different washing methods was conducted on 100 g of blueberries and raspberries spiked with *C. cayetanensis* ($\sim 5\times10^5$ oocysts), *C. parvum* ($\sim 2\times10^6$ oocysts), and *G. duodenalis* ($\sim 3\times10^5$ cysts). The procedure for each washing method was adapted from online sources https://www.wikihow.com/Clean-Blueberries and https://www.wikihow.com/Clean-Raspberries. The experimental design for this study is presented in Figure 10.

The spiked berries were kept at room temperature for 3 h and then overnight in the refrigerator. The protocols for the 3 washing methods selected for this study are described below. After washing, samples with the different methods, all samples were transferred to plastic boxes and washed with 0.1 % Alconox according to 4.5.1. DNA was isolated with PowerSoil as described in 4.5.3 and the number of parasites remaining on the berries was estimated by qPCR according to 4.6.2.1. In addition to spiked berries, unspiked berries were included to ensure that the berries were not contaminated prior to spiking. Parasites

were also spiked into pellets from washed berries to assess the recovery following the Alconox washing.

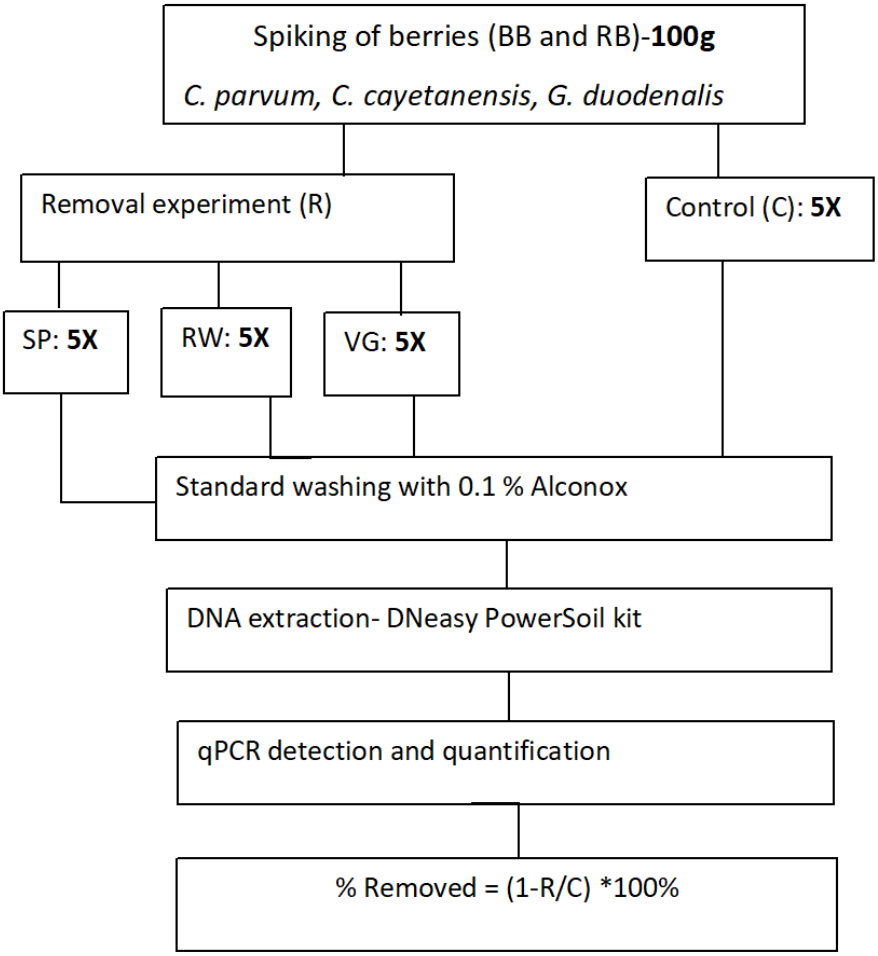


Figure 10. The experimental design for comparative evaluation of the removal efficiency of 3 methods for washing berries prior to consumption. Key: BB- blueberry, RB- raspberry, RW- running water, VG- vinegar, SP- salad spinner.

Washing under running water (RW)

a) Transfer the spiked berries onto the sieve (strainer).
b) Turn on the cold tap in the sink, and ensure the water stream is gentle. Rinse the berries under the stream of cold water for 1 min (until all the berries are wet) as shown in Figure 11.
c) Shake the strainer to drain out the water.

Figure 11. Direct washing of blueberry under gentle running water (Photo credit: Kristoffer Tysnes)

Washing protocol using vinegar (VG)

a) Mix 1-part vinegar (Eddik 7 % Klar, Eldorado Norge) with 3-parts cold tap water in a bowl of sufficient size to hold the berries
b) Transfer the spiked berries into the bowl.
c) Stir the berries in the bowl for 1 min, swishing the berries around in the bowl by hand (wearing gloves that are discarded after swishing) as shown in Figure 12.
d) Transfer the bowl's contents into a strainer to ensure the excess water drains

e) Turn on the cold tap in the sink, and ensure the water stream is gentle. Rinse the berries under the stream of cold water for 30 s
f) Shake the strainer to drain out the water.

Figure 12. Washing of blueberry using 1-part vinegar and 3-part water followed by rinsing (Photo credit: Kristoffer Tysnes)

Washing protocol using salad spinner (SP)

a) Fill the salad spinner's bowl with cold water with the spinner "cage" inside it.
b) Transfer the spiked berries into the salad spinner.
c) Stir the berries in the cage in the bowl for 1 min, swishing the berries around the bowl by hand (wear gloves that are discarded after swishing).
d) Lift out the cage and empty out the liquid from the bowl as shown in Figure 13.
e) Replace the cage in the bowl.
f) Spin the berries for 10 s twice to remove excess water (different directions)

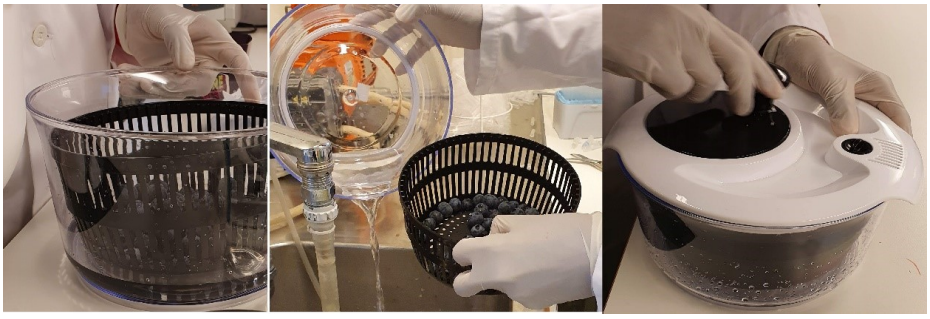


Figure 13. Washing of blueberry using water and salad spinner (Photo credit: Kristoffer Tysnes)

4.10. Method development for viability assessment

Due to lack of access to fresh oocysts of *T. gondii* and *C. cayetanensis*, a surrogate parasite that is readily available from commercial suppliers was chosen for the experimental works related to RT-qPCR method development. *C. parvum* was selected as the surrogate parasite due to the commercial availability of *C. parvum* oocysts and that it is relatively closely related to both *Toxoplasma* and *Cyclospora*. This study was designed to develop a novel RT-qPCR method based on the induction of gene expression following exposure of oocysts to oxidative stress challenges.

The main steps followed in the development of the RT-qPCR method included: a) induction of gene expression as a response to stress; b) identification of differentially expressed genes; c) selection of the potential target genes; d) evaluation of the target genes using RT-qPCR. Figure 9 shows the workflow for the development of a novel RT-qPCR method for viability assessment of *C. parvum.*

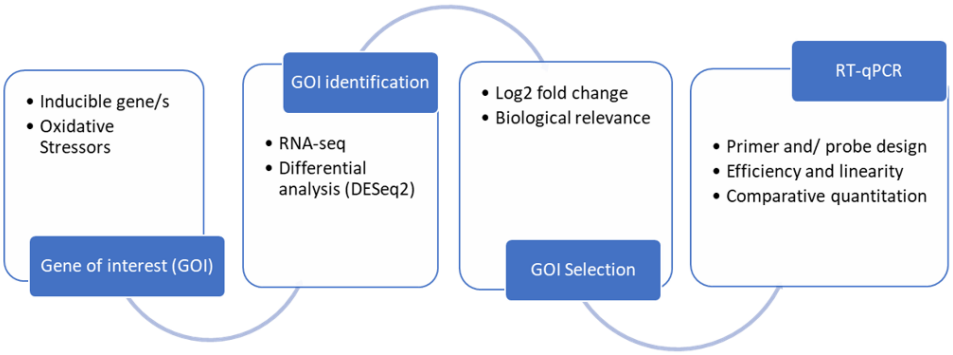


Figure 9. The general workflow implemented for the development and evaluation of methods for viability testing based on RT-qPCR.

4.10.1. Induction of gene expression in *Cryptosporidium* oocysts

Five different gene-expression induction approaches were explored using, in brief: a) two concentrations of menadione sodium bisulphite (MSB); b) an enzymatic reaction composed of xanthine oxidase and hypoxanthine; and c) heat shock at two different temperatures; see Table 6 for details. These approaches were chosen based on either the expected biological effect or because they had previously been used for induction of gene

expression. All chemicals used were purchased from Sigma Aldrich, Norway. Each of the 5 induction groups contained four independent replicates of approximately 10 million *C. parvum* oocysts. Oocysts were pre-washed twice in water before being subject to the different treatments. A control group was stored refrigerated prior to RNA extraction.

Table 6. Description of the gene expression induction approaches explored for RNA-Seq

Treatment	**Brief description**
1M MSB	200 µL of oocysts were mixed with 500 µL of 1 M MSB, vortexed, then held at room temperature for 4 h. The suspension was then washed 3 times with water before total RNA extraction
0.1M MSB	200 µL of oocysts were mixed with 500 µL of 0.1 M MSB, vortexed, then held at room temperature for 4 h. The suspension was then washed 3 times with water before total RNA extraction
Xanthine oxidase and hypoxanthine	200 µL of oocysts were vortexed and 50 µL of xanthine oxidase (20 U/mL) added to the suspension. The suspension was brought up to 500 µL with PBS (pH 8.5) and incubated at 37°C for 10 min. Thereafter, 500 µL of 0.5 mM hypoxanthine was added to the mixture, briefly vortexed and further incubated at 37°C for 30 min with the lids open. The sample was then washed 3 times with water before total RNA extraction.
Heat shock	200 µL of oocysts were incubated at 37°C for 20 min. The sample was then washed 3 times with water before total RNA extraction.
Heat shock	200 µL of oocysts were incubated at 45°C for 20 min. The sample was then washed 3 times with water before total RNA extraction.
Control	Control samples of oocysts were washed five times with water before total RNA extraction.

4.10.2. RNA extraction and quality assessment for RNA-Seq

The RNA extraction protocol for this study was based on RNeasy plus mini kit (Qiagen, Norway) with slight modifications to the lysis approach. Briefly, oocyst lysis was performed in a lysing matrix E tube (MP Biomedicals, France) to which 600 µL of the RLT plus buffer was added, and the tube was subjected to 2 cycles of bead-beating at 4 m/s for 25 s with a 3-min pause on ice. The lysate was centrifuged at 12,000 × g for 2 min and then added to the gDNA eliminator spin column before elution into 55 µL of nuclease-free water and storage at -20°C. RNA quality was assessed using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano kit was used for sample preparation. The RNA integrity number (RIN) produced by the Bioanalyzer provides an indication of the RNA quality; RIN ranges from 1-10, where 10 indicates intact RNA.

Optimisation of the RNA quality

The bead-beating protocol that had been employed for DNA extraction (section 4.5.3), resulted in low quality RNA that could not be used for RNA sequencing (RNA-Seq), and therefore milder bead-beating conditions were tried. Accordingly, the bead-beating duration was reduced from 60 s to 30 s and 15 s. In addition, the lysate was put on ice for 3 min pause in between bead-beating cycles. The quality of the RNA was assessed using the Agilent's Bioanalyzer.

4.10.3. RNA sequencing (RNA-seq)

The RNA samples were sent to the Norwegian Sequencing Centre for sequencing. The RNA library preparation was performed using Truseq stranded RNA prep kit and sequenced in the NovaSeq SP flow cell with the NovaSeq 6000 sequencer. The samples were sequenced in the same flow cell after indexing. The sequencing approach was single-end reads of 100 bp and with sequencing depth of ca. 20 million reads per sample.

4.10.4. RT-qPCR method development

Based on the results from the bioinformatics analysis of the RNA-Seq data (see section 4.11.1.1), primers were designed for selected targets that included thioredoxin (cgd7_4080), HSP70 (cgd4_3270), type-3 malate dehydrogenase (cgd7_470), UDP glucose dehydrogenase (cgd8_920), COWP7 (cgd4_500), and prohibitin (cgd7_4240).

The one-step RT-qPCR was conducted in 20 µL reaction volume composed of 500 nM of each primer, 10 µL of the One-Step SYBR Green Master Mix (Quantabio, Norway), 0.4 µL of the qScript RT-mix, and 2 µL RNA template. The reaction mixes were subjected to reverse transcription at 50°C for 10 min, initial denaturation at 95°C for 5 min, 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 30 s. In addition, a melt-curve analysis of fluorescence data collected during a gradual increase of temperature from 65°C - 95°C was included. The thermocycler used in this study was Stratagene Mx3005P (Agilent, US).

Based on the SYBR green results COWP7 was chosen as the target to develop a probe-based RT-qPCR protocol. The reaction setup for this included 500 nM of each primer, 250 nM of the probe, 10 µL of KiCqStart One-Step Probe RT-qPCR ReadyMix (Sigma Aldrich, Norway), 50 nM of ROX (passive reference dye) and 2 µl template in a final reaction volume of 20 µl.

4.10.5. Evaluation of the RT-qPCR method

The novel RT-qPCR method was evaluated for its applicability in the differentiation of viable and inactivated oocysts using different inactivation treatments. The main steps of the RT-qPCR are summarized in the flow chart presented in Figure 9.

4.10.5.1. Inactivation of Cryptosporidium oocysts

In order to assess the applicability of the RT-qPCR method, physical and chemical inactivation approaches, which have previously been evaluated using mice bioassay (Fayer & Nerad 1996; Travaillé et al. 2016), were used to assess whether the method developed here could be used to evaluate inactivation efficacy. Accordingly, *Cryptosporidium* oocysts were inactivated by heating at 80°C (on heat block) for 3 min and incubated at room temperature for 3 h prior to RNA extraction. Other inactivation tests included heating at 60°C for 2 min, freezing at -20°C for 2 h, 24 h, and 48 h. In addition to thermal inactivation, chemical treatment was assessed by subjecting oocysts to 4 mg/L and 0.2 mg/L free chlorine concentrations for 30 min.

4.10.5.2. Discriminating between viable and inactivated oocysts

The new RT-qPCR method was evaluated for its ability to discriminate between different proportions of viable and inactivated oocysts by analysing samples containing different proportions of untreated oocysts and oocysts subjected to inactivation treatment. In brief, the oocyst mixtures (containing untreated/inactivated oocysts in the following proportions: 0/100, 1/99, 10/90, 100/0 %) were exposed to an inactivation regime as described in section 4.10.5.1. The oocysts were then exposed to xanthine oxidase catalysed oxidative stressor before RNA extraction. RT-qPCR targeting thioredoxin, COWP7, and 18s rRNA was used to determine relative quantity of thioredoxin and COWP7 genes from exposure to the oxidative stressor. Controls that contained the same proportion of viable/inactivated oocysts, but had not been exposed to the oxidative stressor, were included in each run.

4.11. Statistical analysis

The key performance characteristics of the newly developed diagnostic methods were evaluated by using statistical indicators. Repeatability of the qPCR methods were estimated as SD of the C_q values of replicate measurements including 95 % confidence interval (CI). The intermediate precision was estimated as pooled SD of three different levels of concentrations run by two different analysts over 2 days. The efficiency of the qPCR methods and the relative quantitation of genes was automatically calculated by the MxPro software.

The removal efficiency of different washing methods was estimated according to the following formula.

$$\%\, removal\ = \left[1 - \left(\frac{estimated\ no.of\ parasite\ detected\ after\ washing}{estimated\ no.of\ parasite\ spiked}\right)\right] \times 100$$

The median percentage removal of each washing method was presented with its 95 % CI. Quantile regression was performed to evaluate the significance of the differences between each washing methods and parasites.

4.11.1. Bioinformatics analysis

4.11.1.1. Data pre-processing and mapping

The raw sequence data (fastq files) were trimmed for adapter sequences and low quality reads (having phred score < 33) by using trimommatic 0.39 (http://www.usadellab.org/cms/?page=trimmomatic). The trimmed reads were assessed for quality by using FASTQC tool. The trimmed reads were mapped to the reference genome of *C. parvum* IOWA II obtained from CryptoDB release 46 (https://cryptodb.org/common/downloads/release-46/CparvumIowaII/fasta/data/CryptoDB-46_CparvumIowaII_Genome.fasta).

The STAR version 2.5 was employed as the mapping tool (Dobin et al. 2013). In addition, the Salmon tool (Patro et al. 2017) was applied for quasi-mapping of the trimmed reads against the annotated transcript of *C. parvum* IOWA II available from the CryptoDB release 46 (https://cryptodb.org/common/downloads/release-46/CparvumIowaII/fasta/data/CryptoDB-46_CparvumIowaII_AnnotatedTranscripts.fasta).

4.11.1.2. Differentially expressed genes (DEG) analysis

The mapped reads were counted against the recently updated genome annotation file obtained from CryptoDB release 46 (https://cryptodb.org/common/downloads/release-46/CparvumIowaII/gff/data/CryptoDB-46_CparvumIowaII.gff). The featureCounts tool (Liao et al. 2013) was employed for preparing the gene counts table, which was then used as an input for the differential expression analysis using the R–based tool, DESeq2 (Love et al. 2014). In addition to the gene-based differential expression analysis, the transcript-based differential expression analysis was performed on the transcript abundance output obtained from the Salmon tool. The DESeq2 tool was then employed for the analysis using R version 4.0.2. The differential expression analysis output contained the list of genes with their log2fold change, associated p-values.

5. Results and general discussion

5.1. Preliminary tests on lectin magnetic separation (LMS)

The preliminary tests on the potential application of LMS for the concentration of the parasites prior to detection included the use of surrogate parasites *E. mitis* and *T. crassiceps*. The experiments showed contradictory results between the fluorescein-labelled lectins and those without fluorescein. Despite the high intensity of fluorescence observed for some lectins on parasite preparations dried to slides, for parasites in suspension and lectins coated onto paramagnetic beads appeared to bind poorly to the surfaces of the surrogate parasites (Figure 14). Different concentration of lectins and beads were tested to improve the binding efficiency, but no significant difference was observed. Therefore, no further tests were conducted on the use of LMS for concentration purposes.

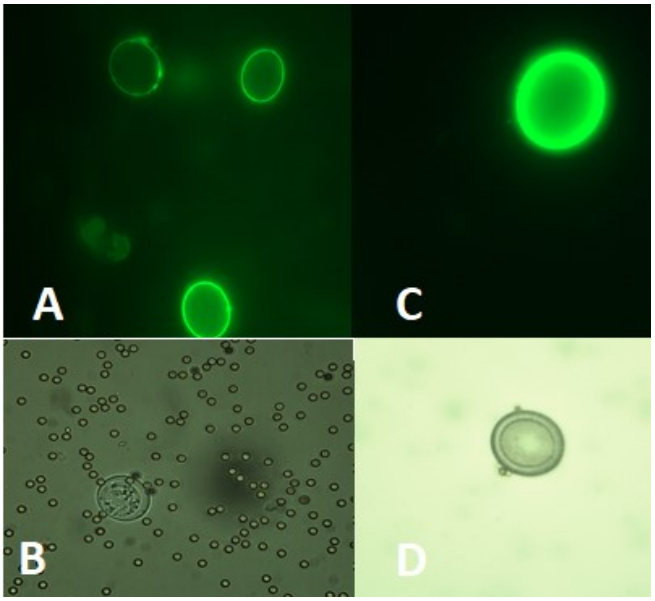


Figure 14. Lectin binding tests on the *Eimeria mitis* and *Taenia crassiceps*. *Eimeria* (A) and *Taenia* (C) stained with FITC labelled PHA-L, *Eimeria* (B) and *Taenia* (D) in a suspension containing streptavidin coated PHA-L.

5.2. Selection of DNA extraction kit

The preliminary results of DNA extraction kit comparison showed that the DNeasy PowerSoil kit outperformed the other kits. This was shown by the consistency of results across replicate samples and lower C_q values as compared to the other kits (Table 7).

Table 7. Preliminary comparison of commercially available DNA extraction kits using *Toxoplasma gondii* oocysts

Kit	Number of oocysts	Mean C_q	Remark
PowerSoil	10,000	20,1	
	1,000	23,4	
	500	25,6	
	100	28,3	
QiaAmp	10,000	18,9	
	1,000	21,8	2/3 positive
	500	25,0	
	100	25,6	only 2/6 positive
PowerFood	10,000	No C_q	
	1,000	24.4	only 1/3 positive
	500	Nd	
	100	Nd	
FastSpin	10,000	22,8	
	1,000	28,1	Only two replicates run due to shortage
	500	Nd	Not done due to kit shortage
	100	Nd	Not done due to kit shortage

The results of the preliminary experiments showed that the stages of the method prior to PCR detection are very important in order to obtain acceptable results. For satisfactory performance, a DNA isolation kit should result in inhibitor-free DNA, providing consistent results on independent replicates, and with the highest recovery of DNA as possible. The PowerSoil kit was later compared to another approach to DNA extraction based on UNEX buffer, which was shown to be effective for *C. cayetanensis* DNA extraction from stool samples (Qvarnstrom et al. 2018).

However, the results of our study demonstrated that DNeasy PowerSoil kit was superior for both *T. gondii, C. cayetanensis, and C. parvum* (Table 8). The details of the findings of this study are presented in paper I.

Table 8. 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*
	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*
	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*
	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	

*indicates significance

5.3. Development and optimization of methods for detection

A new protocol for *C. cayetanensis* detection (based on Paper II)

A new method that could detect *C. cayetanensis* from contaminated berries was developed and thoroughly evaluated for its application. The method showed acceptable performance characteristics according to the MIQE guideline. In addition to the *in-silico* specificity of the newly designed oligos, the method was found to be highly specific when tested *in vitro* against related parasites, including *T. gondii, C. parvum, C. canis,* and *E. mitis*. In addition to the qualitative assessments, the quantitative aspects of the method were evaluated including the limit of detection, linearity, efficiency, precision, and robustness.

The results of the experiment showed that the method could detect as few as 10 oocysts of *Cyclospora* per 30 g of berries. The LoD of the present method is lower than that of a previously reported PCR method that could detect 40 oocysts per 100 g of raspberries (Steele et al. 2003). A PCR method that could detect a single oocyst from basil wash has also been reported (Lalonde & Gajadhar 2008). However, the LoD of these methods are not strictly comparable because of the differences in the sample matrix and protocols for washing the berries. It should be noted that LoD could differ due to the sporulation status of the oocysts used in the experiments. This is because the number of gene copies would increase as the oocyst sporulates. In this study, none of the oocysts were sporulated.

The linearity, as shown by the coefficient of determination ($r^2 = 0.999$), of the new method was within the acceptable range, and the efficiency of the method was also close to the ideal performance (99 %). The linearity and efficiency of the method indicated that it could be applied for precise quantification.

The SD of C_q values from 12 replicate measurements of the same sample was 0.2 (95 % CI: 0.2, 0.3). The small SD of the C_q values indicates that the repeatability of the new method was quite high.

The robustness test that included intentional variations in the master mix, concentration of oligos, annealing temperature, and 'super mix' volume showed significant changes in the C_q values. The main factor that contributed to the variation was the change in the master mix, as indicated by the large regression coefficient (Figure 15). This demonstrates that the performance of a given method could be drastically affected by the choice of master mix. The added benefit of conducting such experiments during intra-laboratory validation phase is that it allows the identification of factors that need to be considered prior to investing time and resources in inter-laboratory comparison studies. Such inter-laboratory comparisons are a stated goal of PARABERRY and are planned for 2021.

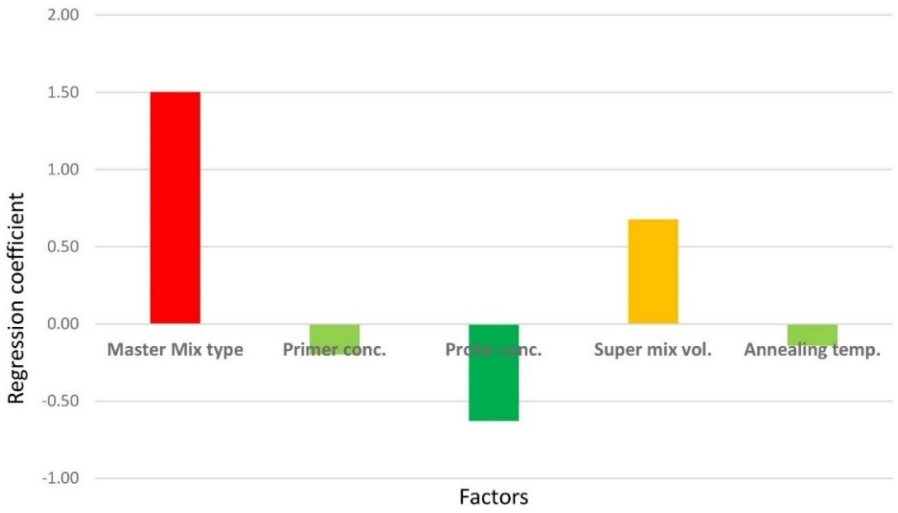


Figure 15. Screening for the main effects of factors that could affect the qPCR results.

A novel multiplex qPCR for detecting *E. multilocularis, T. gondii,* and *C. cayetanensis* (based on paper III)

Following the development of a new protocol for the detection of *C. cayetanensis*, a novel multiplex qPCR that could be used for simultaneous detection *of E. multilocularis, T. gondii,* and *C. cayetanensis* was developed to conduct the surveillance of berries.

The novel multiplex qPCR was thoroughly evaluated before it was used for the surveillance of berries. The evaluation protocol was essentially the same as for the *Cyclospora* method, as described above, apart from the inclusion of intermediate precision in the validation

protocol of the multiplex qPCR. The pooled SD of the C_q values from repeated measurements under varied conditions (analyst and day of analysis) showed no significant variation.

The method was found to be highly specific, both *in silico* and also *in vitro* when tested against available parasites. Moreover, the linearity and efficiency of the multiplex qPCR for each channel were well within the acceptable ranges as per the MIQE guidelines.

The comparative evaluation of the C_q values obtained by using the simplex protocol against its multiplex channel was not significantly different. This was especially true for both *T. gondii* and *E. multilocularis*, whereas the C_q values for the *C. cayetanensis* channel were consistently 1 C_q lower for the multiplex qPCR as compared to its simplex protocol.

5.4. Surveillance of berries (manuscript in preparation)

A total of 674 samples of imported berries, which included 266 blueberries, 228 raspberries, and 180 strawberries, were processed for laboratory analysis for parasite contamination. In addition, a total of 86 samples of berries (20 raspberries and 66 strawberries) produced in Norway were processed. The import countries were pragmatically prioritised based on the endemicity of the parasites under investigation. Countries such as Peru, Morocco, Chile, and Poland are among the priority import countries (Figure 16).

The samples were analysed for parasite contamination using multiplex qPCR for detection of *E. multilocularis, T. gondii, and C. cayetanensis*. The analyses of the DNA extracts for *Cryptosporidium spp.* and *G. duodenalis* is ongoing and therefore only the results of the multiplex qPCR are presented in this thesis.

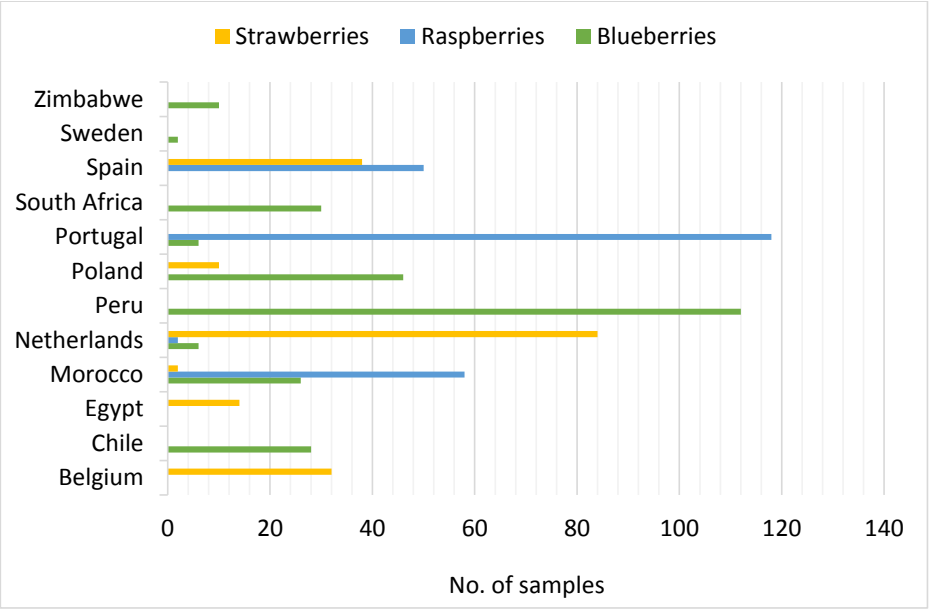


Figure 16. Frequency distribution of the sources of imported berries analysed for parasite contamination.

The multiplex qPCR analyses were completed for 80 % (608/760) of the total samples including 230 samples of blueberries, 214 samples of raspberries, and 164 samples of strawberries. The overall prevalence of parasite contamination of the berries using the multiplex qPCR was estimated to 3.8 % (23/608). The findings of the analyses showed that 1.7 % (4/230) of blueberry samples were contaminated with *T. gondii* and about 0.9 % (2/230) with *C. cayetanensis.* DNA of *T. gondii* was detected on 4.2 % (9/214) of the raspberries and 1 sample (0.5 %) was contaminated with *C. cayetanensis* DNA. Contamination was also detected on the strawberry samples, where 3 % (5/164) and 1.2 % (2/164) were contaminated with the DNA of *T. gondii* and *C. cayetanensis*, respectively. However, *Echinococcus multilocularis* was not detected in any of the berry samples. Figure 17 shows the distribution of country of origin for the berry samples found to be contaminated with *T. gondii* and or *C. cayetanensis*. The sources of berries contaminated with *Toxoplasma* include Chile, Poland, Norway, and Zimbabwe; whereas both *Toxoplasma* and *Cyclospora* contamination were detected on berries imported from Portugal, Morocco, Belgium, and the Netherlands.

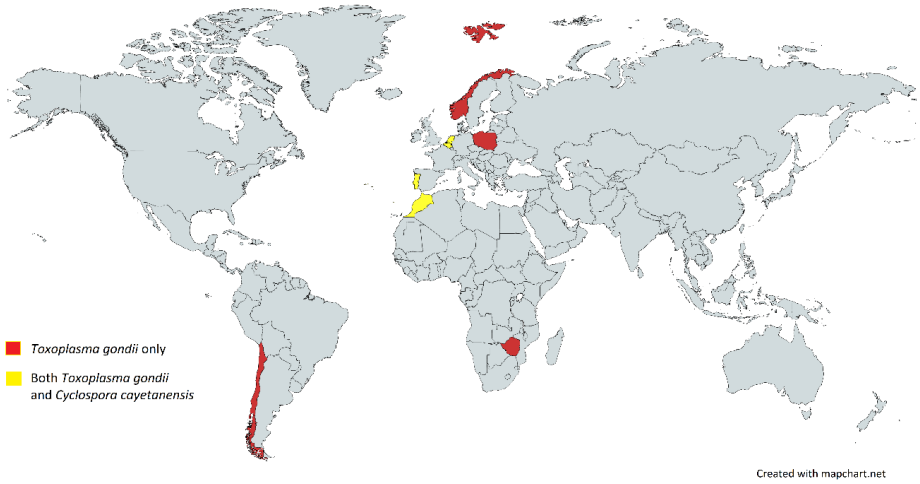


Figure 17. Countries of origin of berries contaminated with parasites

A prevalence of 3 % (18/608), for *T. gondii* is lower than the findings of 5 % (6/120) contamination of strawberries from Colombia (Pineda et al. 2020). The difference might be as a result of variation in the sample size and difference in the geographical origins of the berries. on the other hand, < 1% (0.82) of the samples were contaminated with *C. cayetanensis* which was quite similar to the findings from Colombia (Pineda et al. 2020).

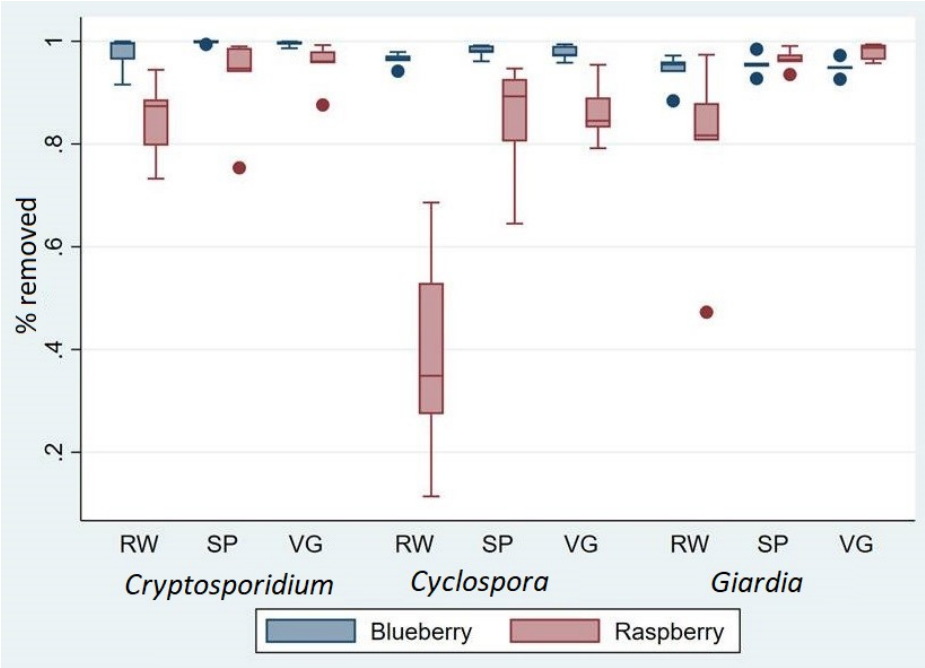
To the best of our knowledge, this is the first study to use molecular methods to investigate contamination of berries in the Norwegian market with parasites of public health relevance. The two surveys previously conducted in Norway used microscopy for screening the berries for parasite contamination (Johannessen et al. 2017; Robertson & Gjerde 2001). The sensitivity of the multiplex qPCR used in the present survey was estimated to 10 oocysts of *Toxoplasma* and *Cyclospora* per 30 g of berries. However, limitations pertaining to the amount of berries used for analysis and the multistep procedure in the detection of parasites means that there could be underestimation of the contamination.

5.5. Removal of parasites (manuscript in preparation)

The comparison of three washing techniques that included washing under running water (RW), using salad spinner (SP), and vinegar (VG) showed significant differences in terms of their removal efficiency of parasites from contaminated berries. The overall removal efficacy sorted in increasing order is RW < SP < VG. There were also some differences between berries and between parasites.

The quantile regression of the percent removal showed that the overall removal efficiency of washing berries under tap water (RW) was significantly lower than for the other two methods (Wald $\chi^2 = 7.9$, $p = 0.005$). The overall percentage removal of parasites was significantly lower on raspberries than on blueberries (Wald $\chi^2 = 15.4$, $p < 0.0001$), regardless of method. The RW was least effective at removing the parasite contamination, and this was especially so for *Cyclospora.* Although the combined data showed no statistically significant difference among the three parasites (Wald $\chi^2 = 1.4$, $p = 0.232$), separate analysis of the removal efficacy from raspberries showed that the percentage removal of *Cyclospora* was statistically significantly lower than for *Cryptosporidium* and *Giardia* (Wald $\chi^2 = 21.2$, $p < 0.0001$).

The results show that simple washing of berries under the cold tap for 1 min could remove at least 80 % of the parasites, except for *C. cayetanensis* (11.4 - 68.6 %), which seems to be stickier than both *G. duodenalis* and *C. parvum* (Figure 18). The stickiness of *Cyclospora* had been found stronger than that of *Cryptosporidium* and *Giardia* probably due to their specific adhesins (Ortega & Sanchez 2010). The findings also showed that contaminant parasites were easier to remove from blueberries than raspberries. Approximately 97 %, 94 %, and 96 % of *Cryptosporidium o*ocysts, *Giardia* cysts, and *Cyclospora* oocysts were removed from blueberries by washing under running water. The percent removal was slightly lower for raspberries - at about 84 %, 79 %, and 39 % of *Cryptosporidium* oocysts, *Giardia* cysts, and *Cyclospora* oocysts, respectively (Table 9).



Key: RW- running water; SP- salad spinner; VG- vinegar

Figure 18. Box plot of the percentage removal of parasites by 3 different washing techniques.

The findings of significant differences between blueberries and raspberries in this study were not unexpected and corroborate reports from previous studies (Kniel et al. 2002). A relevant result of the study is that washing contaminated berries prior to consumption by the consumer removes a considerable proportion of parasite and thereby lowers the risk of ingesting of parasite transmission stages.

Table 9. Percentage removal of parasites from raspberries and blueberries after washing with three different washing techniques in 5 replicates.

Percent removal of parasites from raspberries

Washing alternative	*C. parvum*					Median	95 % CI	
Running water	94.45	87.37	88.69	73.26	79.69	87.37	73.25	94.44
Vinegar	87.61	99.24	95.71	98.06	96.09	96.09	87.61	99.24
Salad spinner	94.01	94.7	98.71	98.99	75.36	94.70	75.36	98.99
	G. duodenalis							
Running water	87.97	80.67	97.37	47.26	81.67	81.67	47.26	97.37
Vinegar	98.71	99.39	96.39	99.36	95.73	98.71	95.73	99.39
Salad spinner	96.43	95.92	97.44	99.08	93.52	96.43	93.51	99.08
	C. cayetanensis							
Running water	68.61	52.97	27.41	11.41	34.88	34.88	11.41	68.61
Vinegar	84.52	95.41	79.18	89.07	83.19	84.52	79.17	95.41
Salad spinner	92.67	89.28	80.48	94.69	64.51	89.28	64.51	94.68

Percent removal of parasites from blueberries

	C. parvum					Median	95 % CI	
Running water	96.44	91.57	99.86	99.97	99.55	99.55	91.57	99.97
Vinegar	99.95	99.81	99.94	99.33	98.64	99.81	98.63	99.95
Salad spinner	99.99	99.99	99.82	99.39	99.99	99.99	99.38	99.99
	G. duodenalis							
Running water	96.07	95.47	97.21	94.01	88.41	95.47	88.41	97.21
Vinegar	94.97	97.23	94.6	92.62	95.04	94.97	92.61	97.23
Salad spinner	95.64	95.04	95.64	98.46	92.75	95.64	92.74	98.45
	C. cayetanensis							
Running water	97.22	94.17	97.91	96.63	96.12	96.63	94.17	97.91
Vinegar	99.1	97.02	95.83	99.4	97.37	97.37	95.82	99.40
Salad spinner	99.18	96.11	98.13	99.18	97.73	98.13	96.11	99.18

5.6. Method development for viability testing (based on Paper IV)

As described in section 4.10, one focus of my PhD studies was to develop a RT-qPCR method that relies on the relative quantification of differentially expressed gene/s following oxidative stressor challenges. Methods based on RT-qPCR were considered most appropriate, both in terms of implementation feasibility and in terms of providing useful and relevant information.

In this study, a novel RT-qPCR method was developed and evaluated. This technique could be used for differentiating between viable, potentially infectious, oocysts of *C. parvum* and inactivated ones. Its value lies particularly in assessing the extent to which potential inactivation technologies are successful, rather than in determining whether parasites contaminating fresh produce are alive or not. This is because large number of parasites are required for the assessment and usually few parasites are detected as contaminants.

RNA quality assessment

The quality of RNA, as indicated by the RIN values, was assessed to qualify samples for submission to RNA-Seq. The quality of the RNA was significantly affected by the duration of bead beating as shown in Table 10. The RNA extracted from samples subjected to heat shock at 45°C for 20 min did not produce RIN values and hence was not submitted for RNA-Seq.

RNA-Seq analysis

The results of the RNA-Seq analysis showed that the samples subjected to oxidative stressor from the enzymatic reaction of xanthine oxidase and hypoxanthine, as well as those subjected to heat shock, were placed distinctly separate from one another and from the other three groups (Figure 19). Samples treated with MSB showed no difference from the control samples. The samples exposed to the oxidative stressor showed higher intensities of log2fold change in gene expression than those exposed to heat-shock. Therefore, the oxidative stressor challenge was selected for further evaluation in the RT-qPCR method development.

Table 10. The assessment of quality of RNA, using Agilent's Bioanalyzer, extracted from 10 million oocysts of *C. parvum* under different conditions and kits

Bead-beating condition	RIN	
	RNeasy Plus mini kit	PureLink RNA mini kit
4 m/s for 15 s (2 cycles)	9,2	9,2
4 m/s for 30 s (2 cycles)	9,0	8,5
4 m/s for 60 s (2 cycles)	NA	ND
4 m/s for 20 s (3 cycles)	9.5	ND
4 m/s for 20 s (3 cycles) [a]	NA	NA
6 m/s for 20 s (3 cycles)	NA	NA
6 m/s for 20 s (3 cycles) [b]	7	ND
6 m/s for 20 s (3 cycles) [a b]	NA	ND

[a] garnet beads used instead of the lysing matrix tube E
[b] extracted by using QIAcube automated system
ND- not done; NA- RIN not available

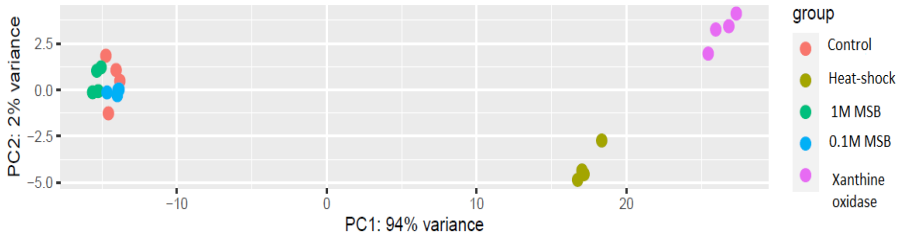


Figure 19. The principal component analysis of the RNA-Seq data after rlog transformation.

RT-qPCR method development and evaluation

Preliminary experiments investigating the six different target genes selected for the RT-qPCR method development showed that all were upregulated following oxidative stress. Among the six target genes evaluated, COWP7 and thioredoxin were selected for further evaluation. The COWP7 gene was selected because of its high log2fold change and the potential role of COWP genes in protecting the oocysts and surviving the environmental

stresses (Templeton et al. 2004). In addition, COWP7 was the only gene among the six genes evaluated that contained introns, making it a suitable candidate for RT-qPCR method. The thioredoxin gene was selected because, in addition to the high log fold change, it has a potential role in the oxidative stress response, as it is a well-known anti-oxidant enzyme that protect cells from cytotoxicity elicited by oxidative stress (Nishinaka et al. 2001).

The most commonly used method for gene expression induction prior to RNA extraction is heating the oocysts at 45°C for 20 min (Bajszár & Dekonenko 2010; Garcés-Sanchez et al. 2013; Travaillé et al. 2016). However, in our work, exposure of oocysts to 45°C for 20 min resulted in poor quality RNA, probably due to degradation, which could not be used for RNA-Seq. Although these samples were not suitable for RNA-Seq, the RT-qPCR results showed that gene expression could be induced by heat shock, but at about 8-fold lower quantities than the present approach of exposure to oxidative stress (Figure 20).

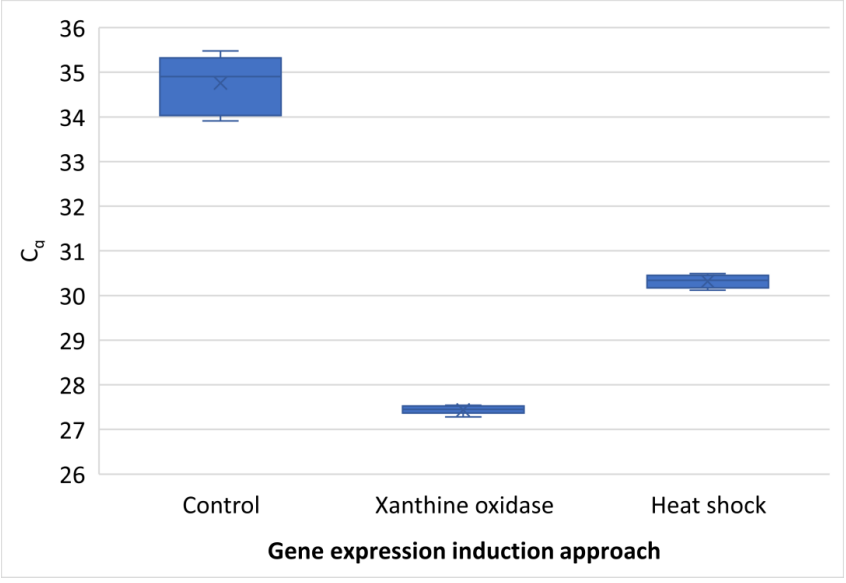


Figure 20. Comparison of gene expression induced by heat shock (45°C for 20 min) with that induced by oxidative stressor challenge catalysed by xanthine oxidase.

Evaluation of the RT-qPCR with samples that included mixtures of inactivated and viable oocysts in different proportions provided promising results, with the thioredoxin RT-qPCR results indicating that the gene expression level was proportionally related to the number of viable oocysts in the sample.

The novel RT-qPCR method was also evaluated for its use in testing the efficacy of some of the potential physical and chemical approaches used for inactivation of oocysts. The results of the study showed that not all oocysts were inactivated after storage in the freezer (-20°C) for 2 h but the oocysts were completely inactivated after 24 h and 48 h. However, chlorination did not inactivate the oocysts.

The novelty of the present RT-qPCR method lies in the application of oxidative stressor challenges to distinguish live from inactivated oocysts as the method exploits the relative quantification of genes expressed in response to oxidative stress. This differs significantly from the assumption used in various other efforts to develop viability assays that rely upon simple mRNA detection as indicative of viability; as we see here, and has been reported previously (Chambers 2005), mRNA can be detected in inactivated oocysts.

The evaluation of the novel RT-qPCR method on oocysts subjected to different inactivating conditions such as freezing, heating, and bleach exposure demonstrates that this approach may be of value in testing the efficacy of different inactivation/disinfection regimens. The use of 18s rRNA as normaliser and inclusion of untreated control samples along with test samples would enable the log-reduction effects from different treatments to be estimated. This is more in line with how the effects of inactivation technologies on bacterial foodborne pathogens are expressed.

6. Future work

Due to the relevance of factors such as globalisation, lack of awareness from agencies, and gaps in surveillance and control, it is anticipated that FBP remain an important public health concern in spite of the recent advances in research on FBP (Trevisan et al. 2019). The data obtained from the present thesis could be used for risk assessments. In line with the top 5 research priorities on FBP in Europe (Trevisan et al. 2019), i.e., methodological issues, and considering the results of the studies presented in this thesis, further research needs in the field are presented as follows.

Inter-laboratory validation of detection methods

The best way to evaluate whether a given analytical method is fit for intended purpose is to conduct an interlaboratory comparison study. This could be done for the novel multiplex qPCR developed for the detection of *E. multilocularis, T. gondii,* and *C. cayetanensis* on contaminated berries. The validation would include evaluation of the robustness of the qPCR when the same sample is analysed by different laboratories. It could also include testing the overall protocol including the washing and elution of parasite stages on berries, the extraction of DNA and the qPCR.

Optimisation of sample processing for surveillance of berries

The probability of detecting parasite contaminants depends on the number of parasites found on the berries' surfaces, which indirectly depends on the weight of berries used for analysis (although not true for point contamination). So far, there has been no standard method for sampling and analysis of berries for parasite contamination and the present study also did not consider it. The literature search shows a range of 30 g to 500 g of berries per sample, and different approaches to sample processing. On the other hand, efficacy of elution of parasite stages from the surface of berries affect the outcome of the qPCR analysis. That means that the technique for washing the berries prior to concentrating the pellet and extracting the DNA should be optimal. This might be affected by the type and volume of wash solution, and the duration and speed of shaking. Here we propose that it could be possible to standardise the sample processing for each berry type considering the most important factors that could affect the result of qPCR analysis.

Optimisation of the RNA extraction for viability RT-qPCR

Further optimization of the RNA extraction protocol for viability RT-qPCR presented here is highly recommended. Although effort was directed towards obtaining good quality RNA for the RNA-Seq, no further attempt was made to optimise the protocol for the RT-qPCR method due to time constraints. Optimisation should consider increasing the RNA yield as to improve the sensitivity of the RT-qPCR. On the other hand, it should also take in to account the quality of the RNA, as higher RNA yield should not be achieved at the expense of the quality required for the RT-qPCR protocol.

Validation of the RT-qPCR using animal models or cell culture

Before implementing the RT-qPCR as described here for viability testing, it would be relevant to verify and validate the method against the gold standard mice infectivity assay or the cell culture assay.

Testing the RT-qPCR approach for *Toxoplasma* and *Cyclospora*

Assuming the phylogenetic relatedness of coccidian parasites, it might be useful to test the homologues of some of the target genes that are upregulated in response to the oxidative stressor, for *Toxoplasma* and *Cyclospora*. However, this might not be straightforward and may require that many potential target genes are tested. Alternatively, the entire experimental protocol, described here for *Cryptosporidium,* could be followed for these related parasites. This includes induction of gene expression followed by RNA-Seq and target gene identification as described in the present project. This would, of course, require a large number of fresh oocysts that poses a practical challenge. Nevertheless, with proper planning such a project is feasible.

Application of the novel RT-qPCR

The novel RT-qPCR could be employed to evaluate the inactivation efficacy of different treatments that could be used by the fresh produce industry such as ozone, high pressure processing, and UV radiations. This is also a goal of PARABERRY, and such investigations are planned for 2021.

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Supplementary file

The RNA-Seq data have been submitted to the sequence read archive of NCBI and are available for public use with the accession number PRJNA669334 (http://www.ncbi.nlm.nih.gov/bioproject/669334).

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

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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

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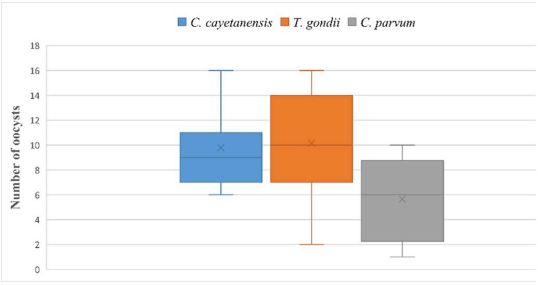

Fig. 1. Distribution of the oocyst counts from 10 independent spikes of *C. cayetanensis*, *T. gondii,* and *C. parvum*.

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.

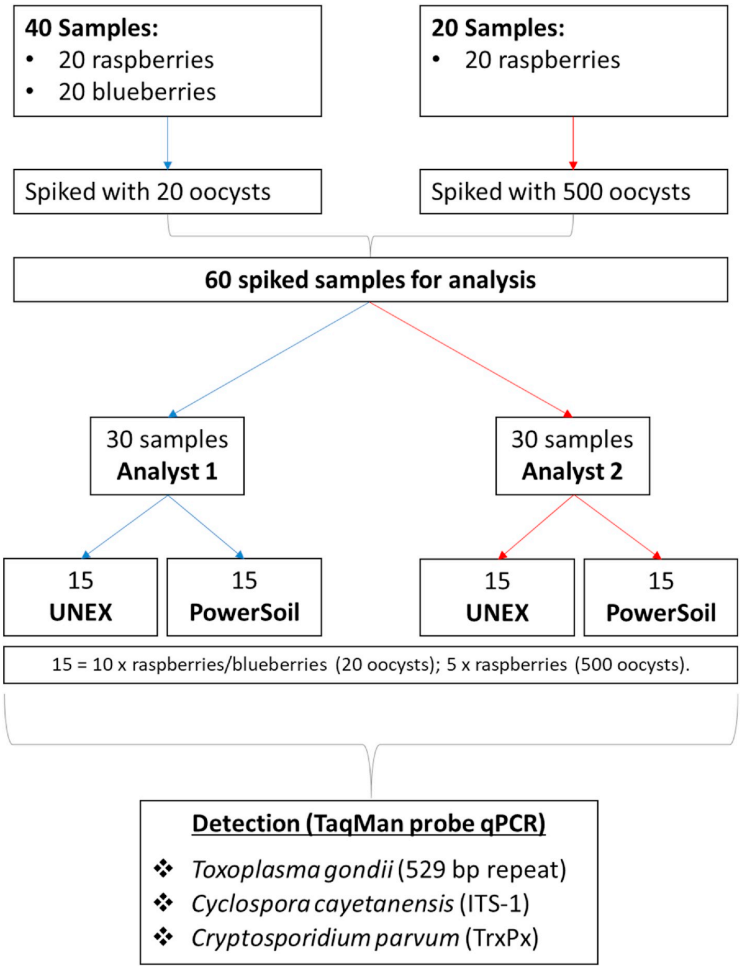

Fig. 2. The experimental design for the comparison of DNeasy PowerSoil kit with UNEX-based DNA extraction of *C. cayetanensis, T. gondii,* and *C. parvum* as contaminants of berries.

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.

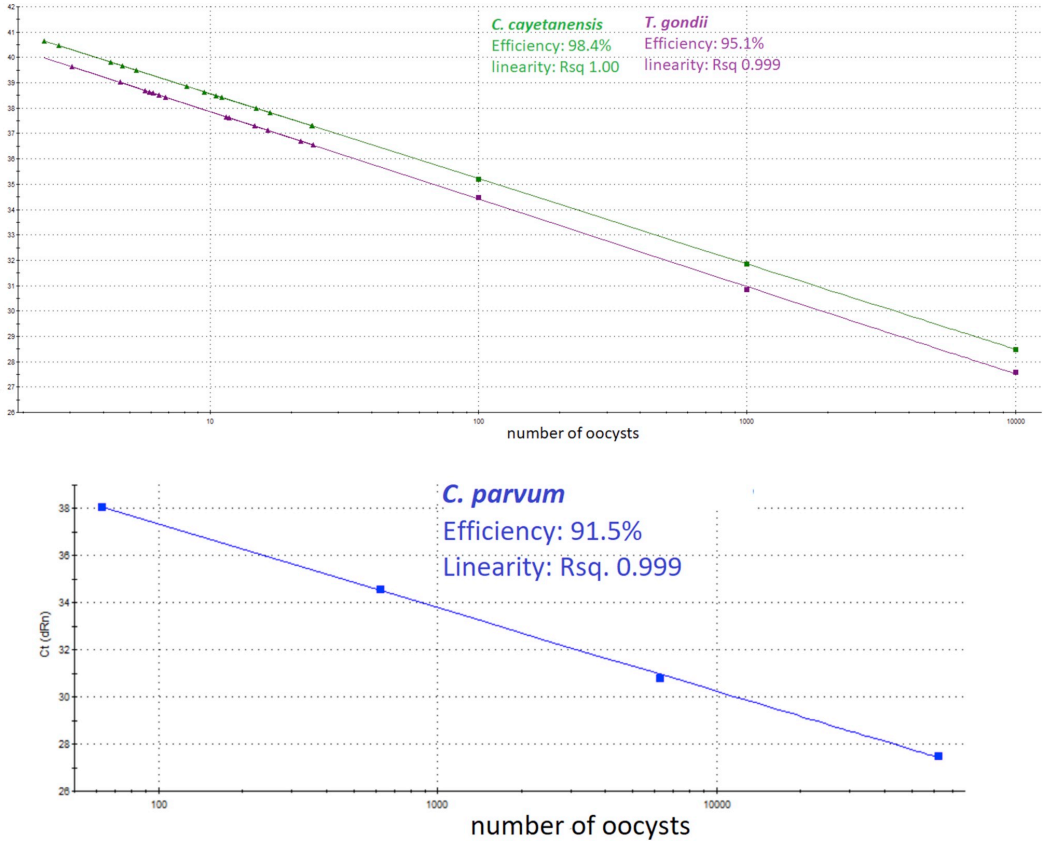

Fig. 3. The standard curves of the qPCR assays used for the detection of *C. cayetanensis, T. gondii, and C. parvum.*

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.

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Paper II

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METHODS
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A New Protocol for Molecular Detection of *Cyclospora cayetanensis* as Contaminants of Berry Fruits

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Cyclospora cayetanensis is a coccidian parasite that is associated with foodborne outbreaks of gastrointestinal illnesses. Raspberries have been implicated as a vehicle of infection in some of these outbreaks. Most of the molecular techniques used for the detection of parasites commonly use the 18s rRNA as a target gene, which is highly conserved. The conserved nature of the 18s rRNA gene among coccidia means that there is potential for cross-reactivity from primers intended to target this gene in *C. cayetanensis* with the same gene in related coccidia. This provides an additional challenge in developing a specific detection method. The aim of this study is to develop a new, more specific assay to detect *C. cayetanensis* in berry fruits. This new assay, targeting the internal transcribed spacer 1 (ITS-1) region, was tested on three different berry matrices: raspberries, blueberries, and strawberries. The new assay showed good efficiency (102%), linearity (r^2 = 0.999), repeatability (standard deviation of C_q 0.2 (95% CI: 0.2, 0.3) and specificity for *Cyclospora*, with no cross-reactivity with related coccidia (*Toxoplasma gondii, Eimeria mitis, Cystoisospora canis,* and *Cryptosporidium parvum*) when tested *in vitro*. The method development was initially conducted using *Cyclospora* DNA only. After it was confirmed to have an acceptable performance, the method was evaluated using the oocysts of *C. cayetanensis*. The method was also improved by incorporating an internal control as a duplex in order to monitor PCR inhibition due to sample matrix components. The duplex assay also showed a good efficiency (100%) and linearity (r^2 = 0.99).The results showed that the new assay has potential for standard use in food testing laboratories. Furthermore, results regarding important factors related to assay robustness are discussed.

Keywords: *Cyclospora cayetanensis*, berries, TaqMan probe, internal transcribed spacer-1, method development, duplex qPCR, contamination, detection

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INTRODUCTION

Cyclospora cayetanensis is a coccidian parasite that has been associated with extensive foodborne outbreaks of gastrointestinal disease. The symptoms of cyclosporiasis include watery diarrhoea, nausea, loss of appetite, cramping, bloating, increased gas, weight loss, fatigue, and, less commonly, vomiting and low-grade fever. There have been frequent outbreaks of cyclosporiasis in the United States, with hundreds of people affected every year.

According to Centers for Disease Control and Prevention (CDC), 1065 laboratory-confirmed cases of cyclosporiasis from 40 states were recorded in spring/summer outbreaks in 2017, 384 laboratory-confirmed cases in 2016, 546 in 2015, and 304 in 2014[1]. However, the number of cases showed a dramatic increase in 2018; as of 1 October, 2018, 2,299 laboratory-confirmed cases of cyclosporiasis had been reported from 33 states. Epidemiological investigations indicated that some of the cases were linked to prepackaged vegetable trays sold at a convenience store chain and salads sold at a fast-food chain (Casillas et al., 2018). Previous outbreaks of cyclosporiasis have been commonly associated with imported contaminated raspberries, cilantro, basil, mesclun lettuce, and snow peas (Chacin-Bonilla, 2017).

As the number of parasites contaminating fresh produce is often likely to be low and there are difficulties in obtaining clean sample eluates, detection methods based on light microscopy are probably hampered by low sensitivity. Given that the sensitivity of molecular techniques, such as polymerase chain reaction (PCR), is often considerably higher than that of microscopy techniques, there have been considerable efforts directed towards development and validation of new protocols for detecting *C. cayetanensis* (Shields et al., 2013; Murphy et al., 2017, 2018; Almeria et al., 2018).

Fresh produce may be contaminated by oocysts from a variety of coccidian parasites (e.g., *Eimeria* spp., *Cryptosporidium* spp., and *Toxoplasma gondii*), and although there are many factors that can influence the likelihood of fresh produce being contaminated with different coccidian parasites, the level of specificity of any detection method is crucial. Molecular detection methods often target conserved regions of the genome, e.g., 18s rRNA, that are found in multiple copies. On the one hand, the multi-copy features of these loci are beneficial for assay sensitivity. On the other hand, because these are conserved regions they are very similar across closely related species. The latter feature makes it challenging to develop primers and probes that are specific for the target parasite and do not amplify corresponding genes in related parasites.

The more closely related non-target species are to a target species, the more likely that primers or probes may bind. Thus, although *C. cayetanensis* seems to infect only humans, there are several other species of *Cyclospora* that infect other animal hosts (including cattle, primates, and reptiles) (Lainson, 2005).These species are not infectious to humans but, if contaminating fresh produce, may be amplified due to their sequence similarity to *C. cayetanensis*. Less closely related species, such as coccidia in the genera *Cystoisospora, Eimeria*, and *Toxoplasma*, or even *Cryptosporidium*, may also be amplified by primers intended for *C. cayetanensis*, particularly if the target gene is highly conserved.

In our laboratory, cross-reactivity with the DNA from *T. gondii* was observed using the primers and probe described in the method currently used by the FDA for detection of *C. cayetanensis* from fresh produce (Murphy et al., 2017), albeit that in our laboratory the probe was labelled differently and had a different quencher. In addition, we found that the same primers and probe with the unmodified PCR conditions (Verweij et al., 2003) also cross-reacted with DNA from *Eimeria mitis* and *T. gondii*.

Thus, although the modification used in our laboratory was not identical with regards to the labelling and quenching, omission of the internal quencher would not affect the probe's specificity because the internal quencher decreases background fluorescence, and hence increases the sensitivity and precision of the assay; this effect is significant for probes longer than 30 bp[2].

Furthermore, given the increasing modifications and advances in PCR technology, it is important that assays designed for diagnostic testing are sufficiently robust that the principal components can be applied successfully with similar specificity and sensitivity despite minor alterations in, for example, primer and probe concentrations, annealing temperature, etc.

The internal transcribed spacer region of the genome, due to its non-coding nature, has a high degree of inter-species variation. The ITS-1 region of *C. cayetanensis* was shown to have a variation within and between samples collected from different geographical locations (Olivier et al., 2001). Another study showed that the high variability in the ITS-1 region of *C. cayetanensis* was intragenomic (Riner et al., 2010). This implies that the sensitivity of a method targeting the ITS-1 gene might be lower than one targeting the 18s-rRNA because the number of ITS-1 copies that matches a set of primers and probes can vary between oocysts.

Another molecular method using primers targeting the ITS-2 region of the *C. cayetanensis* genome was developed by Lalonde and Gajadhar (2008). As this method is based on conventional PCR it is both more time consuming and potentially less specific than methods based on TaqMan probe qPCR.

A further challenge when working with environmental samples, such as berries, is the presence of inhibitors in the sample matrices. It is known that berry fruits contain PCR inhibitors such as polysaccharides (e.g., pectin) and polyphenols (Schrader et al., 2012). This has been observed in the method developed for the detection of *C. cayetanensis* from cilantro and fresh raspberries (Murphy et al., 2017), where it was reported that inhibition due to matrix factors could result in complete absence of amplification. Significant inhibition, but not leading to complete absence of amplification might also occur and could be monitored by including an internal control as a duplex assay (Murphy et al., 2017). Although the inhibition could be reduced by fourfold dilution of the template, it must be borne in mind that diluting the template might also result in false-negative results, particularly when the concentration of the target DNA is very low. This means that using a DNA isolation protocol that counteracts PCR inhibitors would be preferable to diluting the template. The protocol developed by Murphy et al. (2017) has been approved by the United States. Food and Drug Administration (FDA) and is currently used for regulatory purposes (Almeria et al., 2018).

In the present study, we aimed to develop and evaluate a new protocol for molecular detection of *C. cayetanensis* from berry fruits, with emphasis on specificity, sensitivity and

[1]https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/index.html

[2]https://eu.idtdna.com/pages/education/decoded/article/two-quenchers-are-better-than-one

assay robustness, using the ITS-1 region as a target. We also incorporated an internal control, in a duplex method, to enable monitoring of inhibition due to matrix components. Although determining whether berries are contaminated requires the optimisation of various steps, from sample choice through to detection, the focus of our work here was towards the final detection step.

MATERIALS AND METHODS

Sample Preparation

C. cayetanensis DNA

Purified DNA isolated from *C. cayetanensis* oocysts was kindly provided by Dr. Ynes Ortega, University of Georgia, United States. The DNA was isolated from positive faecal samples from Peru as previously described (Chandra et al., 2014). The DNA had a concentration of 32 ng/μl and this was serially diluted tenfold (3.2, 0.32, 0.032, and 0.0032 ng/μl) for the purpose of preparing the calibration curve. The concentration of the DNA was estimated from a spectrophotometric measurement of the purified qPCR product (NanoDrop ND-1000 Spectrophotometer, Saveen Werner AB).

Purified DNA of *C. cayetanensis* from four different sources (Guatemala, Malaysia, Israel, and unknown country of infection) were kindly provided by Dr. Jessica Beser, Public Health Agency of Sweden. These DNA isolates were used for testing the applicability of the new method for detecting isolates of *C. cayetanensis* from different geographic locations.

Oocysts of *C. cayetanensis*, *E. mitis, T. gondii, Cryptosporidium parvum,* and *Cystoisospora canis*

Unsporulated oocysts of *C. cayetanensis* in faeces were kindly provided by Dr. Kristin Elwin, Public Health Wales Health Protection Division, United Kingdom. The faecal sample was collected from a patient in Wales who had recently travelled to Mexico. The faecal sample containing the *Cyclospora* oocysts was washed twice with 0.5% SDS and the oocysts isolated using saturated salt flotation. The oocysts were suspended in distilled water and then stored in the refrigerator. These oocysts were used for evaluation of the performance characteristics of the developed method as applied on the berry matrix. Furthermore, the oocysts were sorted by fluorescence-activated cell sorting (BD FACSAria cell sorter), using their auto-fluorescence and size, into 96-well PCR plates at Ullevål Sykehus, Oslo, Norway.

Oocysts of *E. mitis* were isolated from chicken faeces, *C. canis* from canine faeces, and *C. 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. Oocysts of *T. gondii* from a previous project were also used; the details of the oocyst strain and origin are described elsewhere (Harito et al., 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).

Berry Matrices

Sample matrices were prepared from store-bought raspberries, blueberries, and strawberries as follows. About 30 g of berries was weighed into plastic boxes to which 200 ml of 0.1 or 1% Alconox™ (Alconox, Inc., NY, United States) 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 and strawberry samples were shaken at 600 rpm for 10 min.

The eluate was then transferred into four 50 ml tubes for concentration by centrifugation at 1,690 rcf for 10 min and the supernatant removed by vacuum suction, leaving 10 ml of the sediment. The pooled sediment was centrifuged at 3,803 rcf for 10 min with a deceleration break set to 6 (on a scale of 0–9) and about 1.5 ml of the sediment was further concentrated down to 250 μl by centrifugation at 13,000 rcf for 5 min.

Isolation of DNA

DNA was isolated from the oocysts of all five coccidian parasite species using the 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 rcf for 1 min at room temperature, and 500 μl of the supernatant used for the subsequent step in the protocol. The effects of background DNA and PCR inhibitors from the sample matrix were tested by spiking the berries with *Cyclospora* oocysts and this was subjected to DNA extraction as described above. The final elution volume was 50 μl. Samples were stored at −20°C until further analysis.

Real-Time PCR (qPCR) Assay

Primers and Probe Design

The primers and probe for *C. cayetanensis* were designed and tested using Geneious 11.1.4[3] to amplify a product of 141 bp from a target region of the ITS-1 region, based on a consensus of nine sequences retrieved from the GenBank (GenBank Accession No. AF301386, AF301389, AF302506, AF302529, AF302533, AF302546, AF302558, GU295381, and GU295248). The oligos used in the present study are presented in **Table 1**.

The primers and probe for detection of the internal control, Phocine herpesvirus-1(PhHV1), were as described previously (Niesters, 2002). Reverse-phase cartridge (RP1) purified primers and HPLC purified probes were purchased from Sigma-Aldrich.

qPCR Conditions

The PCR was performed in a 0.3 ml PCR plate without skirt (Multiply®, Sarstedt, Norway). The reaction conditions for the qPCR setup was a 20 μl reaction volume that included a

[3]https://www.geneious.com

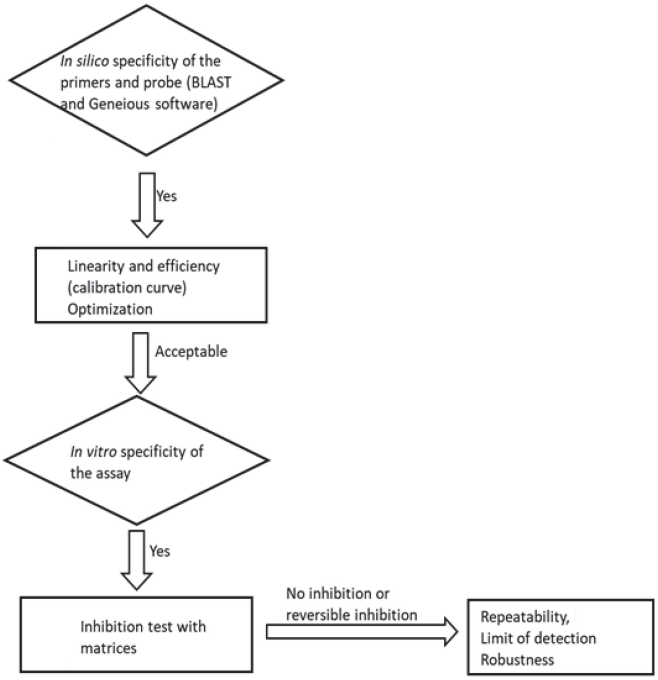

FIGURE 1 | A flowchart of the method evaluation steps followed in this study.

template volume of 2 μl, 10 μl of 2 × KiCqStart® Probe qPCR ReadyMix™, low ROX™ (Sigma-Aldrich, Norway), 0.5 μM of each primer and 0.1 μM of the probe. ROX was used as a reference dye against which the target fluorescence data were normalised.

Method Evaluation

The method was evaluated for its specificity, efficiency, linearity, inhibition, limit of detection (LoD), precision, and robustness. The planned steps for method evaluation are summarised in a flowchart (**Figure 1**).

Specificity

The specificity of the primers and probe were investigated *in silico* using BLAST searches against coccidia in general and separately for the *Cyclospora* genus. In order to increase the level of specificity, the "somewhat similar sequences (blastn)" algorithm was selected to allow cross-species comparison. The specificity was evaluated *in vitro* by running agarose gel electrophoresis (1.5%) of the qPCR product to confirm the amplicon size. In addition, DNA from related coccidia (*Toxoplasma, Eimeria, Cystoisospora,* and *Cryptosporidium*) were included in the qPCR run. Moreover, the qPCR product was sequenced by a commercial company (Eurofins Genomics, Germany GmbH) to confirm the specificity of the assay.

Efficiency and Linearity

The method was first evaluated for its efficiency and linearity for the range of concentrations used in this study (Section "Sample Preparation"). The calibration curve was prepared using tenfold serial dilutions of the pure *Cyclospora* DNA (64 ng, 6.4 ng, 0.64 ng, 64 pg, and 6.4 pg). The linearity of the method was assessed by obtaining the coefficient of determination, with $r^2 \geq 0.98$ considered acceptable.

Inhibition

Inhibition from the berry matrices was tested using a tenfold serial dilution of berry washes spiked with *Cyclospora* oocysts. To evaluate the applicability of the new method in outbreak investigations, where the samples may become old and deteriorated before reaching the laboratory, blueberries and raspberries kept in the fridge for 32 days were spiked with *Cyclospora* oocysts and processed for the qPCR detection.

Precision

The precision of each assay was estimated, under repeatability conditions, for three different berry matrices (raspberry, strawberry, and blueberry) containing approximately 0.16 and 3.2 ng of the target DNA and expressed as the standard deviation of C_q from 12 replicates of each.

Limit of Detection

The limit of detection was determined by dilution of the DNA and approximately 32, 12.8, and 6.4 pg of the DNA roughly estimated to be equivalent to 5, 2, and 1 oocyst based on the gene copy number, respectively, were tested. The qPCR was run with six replicates of the 32 pg and nine replicates of the 12.8 and 6.4 pg.

The LoD was also estimated using the flow-sorted *Cyclospora* oocysts (section "Oocysts of *C. cayetanensis, E. mitis, T. gondii, Cryptosporidium parvum,* and *Cystoisospora canis*"). The oocysts spiked into a tube containing the eluates of blueberry washes ready for DNA extraction. Accordingly, five replicates of the two oocysts, five oocysts, 10 oocysts, and 100 oocysts sorted by FACS were used. Based on the preliminary results from this experiment, 10 and 50 oocysts of *Cyclospora* (each in triplicate) were used for direct spiking on the berries before washing. In this experiment, about 20 μl of suspension containing the oocysts was used for the spiking and this was distributed to different individual berries. The spiked berries were left to dry at room temperature for 3 h and then stored in the refrigerator overnight. The berries were then subjected to washing as described in section "Berry Matrices". The eluates were spiked to obtain an approximation of the LoD of the qPCR. Spiking of berries was conducted to assess the LoD of the entire method (including washing, DNA extraction, and detection with qPCR).

Robustness

The robustness of the assay was evaluated by 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). A screening experimental design that enables detection of the main effects was used for this experiment (**Table 2**). Nine replicates of *Cyclospora* DNA (approximately 1.28 ng) and a negative control were included per experimental setup (the six different combinations of the different factors).

Furthermore, considering the within species variation of ITS-1 copies, the newly developed method was tested on different isolates of *C. cayetanensis* DNA obtained from different sources. The sources included Guatemala, Malaysia, Israel, the United Kingdom, and one isolate for which the country of origin was not reported.

Duplexing With an Internal Control (PhHV-1)

In order to monitor success of DNA extraction as well as the PCR, the inclusion of PhHV-1 (EVAg Ref-SKU: 011V-00884) as an internal control was evaluated. The PhHV-1 sample (10 μl of the 1000-times diluted stock) was mixed with the *Cyclospora* oocysts samples for co-extraction of DNA. The specific details of the duplex assay are presented in **Table 1**.

The duplex assay was evaluated for the variation in the C_q value of the internal control by varying the concentration of *C. cayetanensis* DNA while keeping the concentration of PhHV-1 DNA constant. The DNA extracted from about 10^5 oocysts was serially diluted to get approximately 10^4, 10^3, 10^2, and 10 oocysts. The PCR was run in 20 μl volume with a 3.5 μl template, with 2 μl of templates added from the DNA extracted from the *C. cayetanensis* oocysts and 1.5 μl of PhHV-1 DNA was added to each well of the PCR plates.

Similarly, the effect of the internal control on the low concentration of *Cyclospora* DNA was also assessed by

TABLE 1 | The overview of setup for the Duplex assay.

	C. cayetanensis	**Phocid herpesvirus 1 (PhHV-1)**
Forward primer (5′ → 3′)	CylTS1_TT-F ATGTTTTAGCATGTGGTGTGGC	GGGCGAATCACAGATTGAATC
Reverse primer (5′ → 3′)	CylTS1_TT-R GCAGCAACAACAACTCCTCATC	GCGGTTCCAAACGTACCAA
Probe (5′ → 3′)	CylTS1_TT-P HEX-TACATACCCGTCCCAACCCTCGA-BHQ1	6FAM-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ1
Primers conc.	0.5 μM	0.2 μM
Probe conc.	0.15 μM	0.1 μM
Amplicon size	141 bp	89 bp
Thermal profile	95°C for 3 min 1 × 95°C for 15 s 45 × 60°C for 30 s 45 ×	

TABLE 2 | Experimental design for testing the robustness of the new assay.

Factor	**Combination**						**The new method**
	Test-1	**Test-2**	**Test-3**	**Test-4**	**Test-5**	**Test-6**	
Master mix type	−1	−1	−1	1	1	1	KicqStart
Primer conc.	1	1	−1	−1	−1	1	0.5 μm
Probe conc.	1	−1	1	−1	1	−1	100 nm
Super mix vol.	1	−1	−1	−1	1	1	18 μl
Annealing temp.	1	−1	1	−1	−1	1	60°C

Sign used	**Master mix type**	**Primer conc.**	**Probe conc.**	**Super mix vol.**	**Annealing temp.**
−1	KicqStart	0.4 μM	80 nM	17.1 μl	59°C
1	PerfeCTa Multiplex qPCR ToughMix	0.5 μM	100 nM	18.9 μl	61°C

simultaneously running the singlex and duplex assays on the same serially diluted templates.

Data Collection and Analysis

The fluorescence data were collected by Stratagene Mx3005P. The raw fluorescence intensity was evaluated against the recommended range of the instrument by using the multicomponent view. Each analysis was run in triplicate unless otherwise stated, and the mean C_q was used for calculations. The data obtained with the Mx3005P were then exported to an Excel sheet (Microsoft® Office Excel® 2010) for further statistical analysis by JMP® Pro version 14.1.0 software (SAS institute, Inc.). The SD of C_q was calculated and presented using its 95% confidence interval. The efficiency of the qPCR was calculated automatically by the MxPro™ qPCR software.

Quality Control

An unspiked berry control was included in the experimental setup to ensure that the amplifications are specific to the *Cyclospora*. Every qPCR run was performed in triplicate and no template control (NTC) was included in every run. The TaqMan probes were prepared in small volumes of working solution to reduce the potential for damage from repeated freeze-thawing. Furthermore, to ensure that the fluorescence obtained was only from amplification of the template, no amplification control (NAC) was run after repeated freeze-thaws of the probe.

RESULTS AND DISCUSSION

The results of experiment conducted to select an appropriate concentration of Alconox for the washing of blueberries indicated that there was no meaningful difference in C_q values for the two concentrations (data not shown).

Method Evaluation Using *C. cayetanensis* DNA

Specificity: *In silico* Test

The specificity test using BLAST search indicated that for both primers there was no hits for any other coccidia other than *C. cayetanensis*. The "blastn" search for the probe returned, in addition to *C. cayetanensis*, four hits for *Neospora caninum* complete genome (GenBank Accession No. LN714484.1, LN714488.1, FR823386.1, FR823385.1), and one hit for *Hammondia hammondi* phospholipid-translocating P-type ATPase (GenBank Accession No. XM_008885584.1). However, these alignments had higher E values (8), which indicates that the probability of the alignment to be just by chance is very high. Furthermore, the *Neospora* and *Hammondia* sequences were imported to the Geneious software to test the primers and probe for their potential to amplify and detect these unintended targets. The analyses showed no cross-reactivity with *H. hammondi* and *in silico* testing against the sequences of *N. caninum* identified no match with the set of primers and probe. However, it should be noted that there were many bases consecutively represented by "N" (which represents any of the four bases, A, G, T, or C of DNA) in this whole genome sequence of *N. caninum*.

Efficiency and Linearity

We found an ideal efficiency (102%) and linearity (r^2 = 0.999) for the tested concentration range at a threshold fluorescence of 0.02 (**Figure 2**).

Specificity: *In vitro* Test

As the *in silico* test cannot entirely replace the practical test of specificity in the laboratory, the assay was evaluated for cross-reactivity against four genera of related coccidian parasites (*Cryptosporidium*, *Eimeria*, *Cystoisospora*, and *Toxoplasma*). Such empirical testing for *Hammondia* and *Neospora* was not conducted due to the lack of availability of these parasites. However, the *in silico* testing provided confidence that cross-reactivity would be highly unlikely to occur. No cross-reactivity between the related coccidian parasites tested, and the primers and probe used in the present assay was detected. The sequencing result also confirmed that the qPCR product was indeed from the amplification of the intended target in the ITS-1 region of *C. cayetanensis* based on the BLAST search of the sequences obtained. The BLAST search result showed 100% identity with many (at least 100) of the sequences of *C. cayetanensis* ITS-1 found in the GenBank (AF302599.1, AF302508.1, GU295401-GU295404, GU295365 - GU295371, to mention a few) with 100% query cover.

Inhibition Test

It is well known that berry fruits contain PCR inhibitors such as polysaccharides (e.g., pectin) and polyphenols (Schrader et al., 2012). It is therefore important that every assay designed to detect parasite contamination of this fresh produce type should determine the magnitude of inhibition from these matrices. In the present method, we detected no inhibition from the berry matrix. The applicability of the new method for outbreak investigation, as assessed using old berries spiked with *Cyclospora* oocysts, gave positive results with no inhibition from the matrix. This was confirmed by including the two-fold and four-fold diluted templates in the duplex qPCR run. Here it is worth noting that the amount of debris after the final concentration was more than twice than that obtained with fresh berries. However, the volume of the debris did not affect the DNA isolation, and there was no requirement to divide the sample into multiple tubes. Nevertheless, for samples resulting in a much larger sediment than required (250 μl) it would be possible to divide the sample between two tubes, and to then combine it into one spin column during the DNA binding step to avoid the risk of dilution.

Limit of Detection

The LoD was shown to be approximately 6.4 pg, with a probability of about 77% (seven positives out of nine replicates).

Precision: Repeatability

The repeatability of the assay was demonstrated by the minimal difference between replicates of the run, as shown in **Table 3**. At a lower concentration of template (160 pg), the overall repeatability with the berry matrices showed a C_q with SD of 0.4 (95% CI: 0.3, 0.5). The SD of C_q for raspberry was 0.5 (95% CI: 0.3, 0.8) and showed the highest deviation. For blueberry and strawberry, the SD was 0.3 (95% CI: 0.2, 0.5).

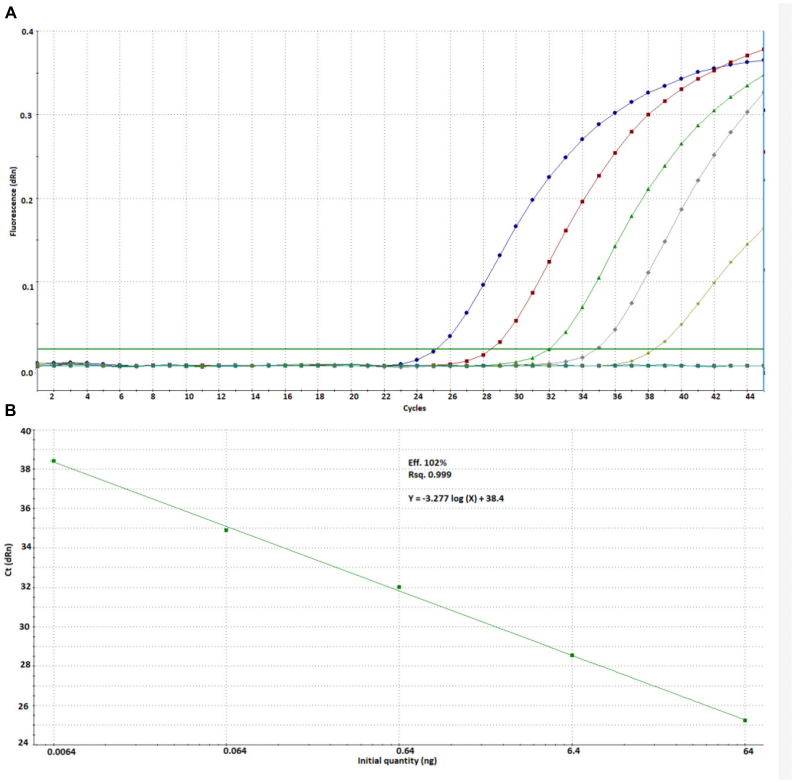

FIGURE 2 | Amplification plot **(A)** and calibration curve **(B)** prepared from *Cyclospora* DNA by using the new method.

TABLE 3 | Repeatability study of the new method for the three berry matrices spiked with *Cyclospora* DNA.

	Mean C_q ± SD	95% CI	
0.160 ng DNA			
Raspberry (*n* = 11)	35.07 ± 0.51	34.73 ± 0.36	35.41 ± 0.90
Strawberry (*n* = 12)	35.18 ± 0.30	35.00 ± 0.21	35.37 ± 0.50
Blueberry (*n* = 12)	34.74 ± 0.29	34.56 ± 0.20	34.92 ± 0.49
3.2 ng DNA			
Raspberry (*n* = 12)	30.71 ± 0.17	30.60 ± 0.12	30.82 ± 0.29
Strawberry (*n* = 12)	31.16 ± 0.22	31.02 ± 0.16	31.30 ± 0.37
Blueberry (*n* = 12)	30.98 ± 0.18	30.87 ± 0.13	31.09 ± 0.30

The repeatability was also evaluated for a higher concentration of template (3.2 ng). The results of the experiment showed a better precision, with an overall SD of 0.2 (95% CI: 0.2, 0.3) and the SDs for each matrix were similar.

Robustness Test

The findings of the experiments on robustness of the new method identified factors (as described in the sub-section on Robustness, in the section on Method Evaluation.) that could significantly affect the qPCR results. A visual summary of the experiment is presented by the Box–Whisker plot in **Figure 3**. The overall standard deviation of the C_q for the six combinations was 0.8 (95% CI: 0.7, 1.0). The coefficient of variation (CV) of the copy numbers was estimated to be 50% (95% CI: 48%, 54%). These figures might indicate that the new method was not sufficiently robust.

However, the experiment clearly indicated which factor(s) most contributed to the large CV. As shown in **Figure 4**, the master mix type, the concentration of probe, and the volume of "super mix" contributed most to the deviations obtained. Changing the master mix type and using excess "super mix" volume resulted in higher C_q (the large and positive coefficients indicate increase in the C_q). Nevertheless, maintaining the concentration of the probe as described in the protocol had a positive effect, indicated by the negative coefficient in the figure. There were no significant effects from either primer concentration or annealing temperature. The largest proportion of variation was due to the change in master mix type. In a separate run, in which all other assay conditions were kept

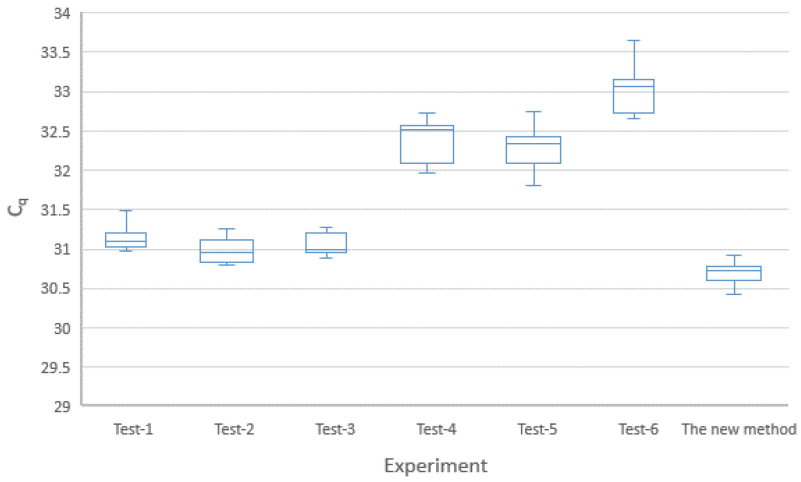

FIGURE 3 | Box-plot (with 95% CI) representation of the six different experiments on the combinations of the five factors included in the robustness test (see **Table 2**). KicqStart Master Mix: Tests 1, 2, 3; PerfeCTa master mix: Tests 4, 5, 6. Primer concentration of 0.4 μM: Tests 3, 4, 5; Primer concentration of 0.5 μM: Tests 1, 2, 6. Probe concentration of 80 nM: Tests 2, 4, 6; Probe concentration of 100 nm: Tests 1, 3, 5. Super mix volume of 17.1 μl: Tests 2, 3, 4; Super mix volume of 17.1 μl: Tests 1, 5, 6. Annealing temperature at 59°C: Tests 2, 4, 5; annealing temperature at 61°C: Tests 1, 3, 6.

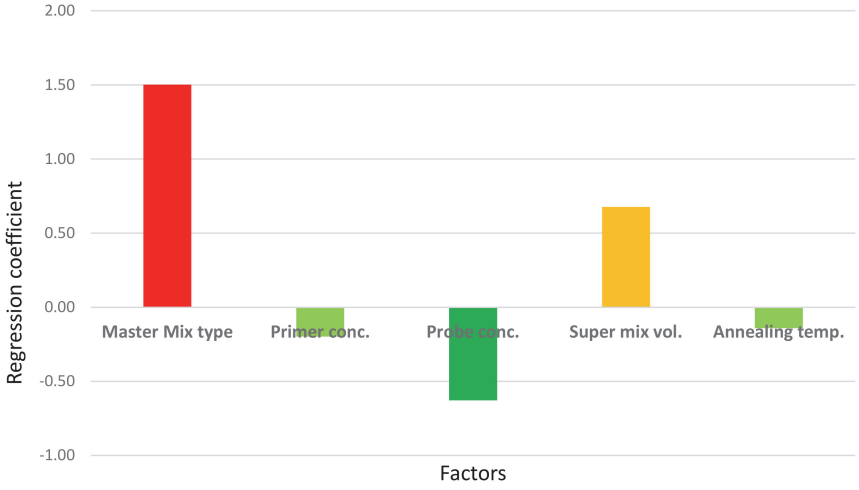

FIGURE 4 | Screening for the main effects of factors that could affect the qPCR results. The sign (+ or –) of the coefficients indicates the direction of the factor's effect on the C_q when changed from –1 to 1 (see **Table 2**).

constant except the master mix type, a difference of one cycle was noted (data not shown), which was also shown as the major contributor to the large CV in the robustness test results. Therefore, it should be noted that the performance of commercially available master mixes differ and should be considered during interpretation of qPCR results.

These results highlight the fact that in order for a new method to be applied in different laboratories, it should be sufficiently robust to be unaffected by small changes in the various factors that potentially impact on the performance of the assay. Conducting a robustness test is very helpful in predicting the outcome of inter-laboratory validation and enables necessary

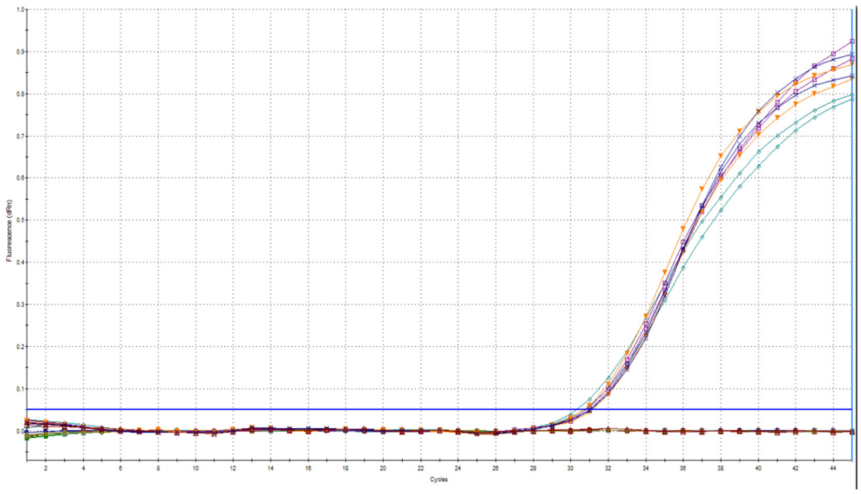

FIGURE 5 | Different concentrations of *C. cayetanensis* (10^5, 10^4, 10^3, 10^2, and 10 oocysts) did not affect the C_q value of PhHV-1.

adjustments to be made to a protocol before investing a great deal of time and resources on inter-laboratory comparisons.

Evaluation of the Method Based on Experiments With *C. cayetanensis* Oocysts

Considering the intergenomic and intragenomic variation of ITS-1 region, it might be beneficial to test different oocysts from various geographical locations. But one challenge is that there is a limited access to the oocysts of *C. cayetanensis* as humans are the only host. Oocysts of *C. cayetanensis* were obtained after completion of the development and evaluation of the method using the *C. cayetanensis* DNA.

With the use of the DNA extracted from the oocysts, the method showed a very good efficiency (92%) and linearity (0.999) (**Supplementary Figure S1**).

Duplexing With an Internal Control (PhHV-1)

The results indicated that the internal control could be included in a duplex assay for the detection of *C. cayetanensis* from berries without affecting the performance of the protocol. The assay showed a good efficiency (100 and 99.5%) and linearity (r^2 = 0.99 and 1.00) for *C. cayetanensis* and PhHV-1, respectively (**Supplementary Figure S2**).

Furthermore, the test for the reliability of the internal control in a range of different concentrations of *C. cayetanensis* DNA showed that there was no significant variation in the C_q value (**Figure 5**). The standard deviation of the C_q for PhHV-1 was 0.2 (95% CI: 0.16, 0.49).

This indicated that PhHV-1 could be reliably used for monitoring inhibition due to matrix components. Adding the internal control to the sample before DNA extraction serves a dual role, monitoring both the presence of inhibition and the efficiency of the DNA extraction. This is best achieved by including a diluted template in the qPCR run. In cases where both the diluted and undiluted template results are negative for the internal control, failure of the DNA extraction is indicated. This is different from the approach used by Murphy et al. (2017) in which the internal control monitors the presence of inhibition only. However, it should be noted that a positive internal control might not be indicative of perfect DNA extraction because the efficiency of DNA extraction would not be the same for PhHV-1 as for the robust oocysts of *Cyclospora*. Nevertheless, this control would be useful to detect a major and inadvertent decline in the efficiency of the DNA extraction.

The internal control had no effect on detection of a low concentration of *Cyclospora* DNA (**Table 4**).

Here it is also worth mentioning that the C_q value of the target is dependent on the matrix used during DNA extraction. With the use of DNeasy PowerSoil kit for the DNA isolation, it was noted that the C_q value of the target parasites were higher when

TABLE 4 | The singlex and duplex qPCR results on the serially diluted oocysts of *Cyclospora* but with equivalent quantity of PhHV-1.

Estimated no. of oocysts	C_q value obtained	
	Singlex assay	Duplex assay
10^4 oocysts	28.3	27.7
10^3 oocysts	31.9	30.8
10^2 oocysts	34.7	33.6
10 oocysts	37.9	37.6
No template control	No C_q	No C_q

purified samples were used. But using background matrices, such as berry washes, during the DNA extraction apparently reduced the C_q values. This was true for the qPCR tests on *E. mitis*, *T. gondii*, *C. cayetanensis*, and PhHV-1 (data not shown). In this study, the berry matrix seemed to improve the efficiency of the DNA extraction rather than reducing it. It is assumed that this enhanced DNA extraction may be due to a carrier function of one or more components in the berry matrix, but this was not further explored.

The duplex assay was also tested for its specificity, as was done for the singlex assay, and the result showed no C_q value for the related coccidian parasites (*Eimeria, Cryptosporidium, Toxoplasma,* and *Cystoisospora*). In every qPCR run, the NTC was included to rule out non-specific amplification. Furthermore, gel electrophoresis of the qPCR product of the duplex assay confirmed that there was no unintended amplification (**Supplementary Figure S3**).

LoD of the Duplex Assay

The findings of the present study showed that five oocysts of *Cyclospora* as the LoD of the duplex assay (**Table 5**).

Other studies on the detection of *C. cayetanensis* from fresh produce have reported different detection limits. A PCR method that could detect 40 oocysts per 100 g of raspberries has been reported (Steele et al., 2003). Lalonde and Gajadhar (2008) reported a PCR method that could detect a single oocyst from basil wash. However, the LoD of these methods are not strictly comparable because of the differences in the sample matrix and protocols for washing the berries.

It should be noted that the LoD could differ due to the sporulation status of the oocysts used in the experiments. This is because the number of gene copies would increase as the oocyst sporulates. In this study none of the oocysts were sporulated. It is also worth noting that the aim of the study should be considered for comparison of the LoD of methods. For studies intending to assess the sensitivity of the PCR, using a synthetic positive control gene target (Murphy et al., 2017) would be appropriate. However, this should not be confused with the LoD of the whole method that includes all the steps of analysis; washing, concentration, extraction of DNA, and PCR.

The results from berries spiked with 10 and 50 oocysts of *Cyclospora* and subjected to washing and DNA isolation for the detection with the duplex qPCR showed that it is possible to detect 10 oocysts of *Cyclospora* from 30 g of raspberries and blueberries (**Table 6**).

Here we have shown that this protocol is able to detect as few as ten unsporulated *Cyclospora* oocysts from 30 g of berries. However, as the ploidy of a sporulated oocyst is presumably 4 times higher than that of an unsporulated oocyst, we might speculate that for sporulated oocysts, or for mixed populations of sporulated and unsporulated oocysts, the LoD could be even lower. Due to the lack of availability of sporulated oocysts we were unable to test this empirically.

To maximise the sensitivity of the method, use of larger volumes of template (e.g., 5 μl) is recommended, and also replicates of the sample (at least triplicate). The template volume depends on whether the template contains inhibitors and this, in turn, depends on the efficacy of the DNA isolation kit at removing potential inhibitors. Thus, the practicality of using a larger template volume should be assessed in the laboratory. In the present study, the use of 2, 3, and 5 μl of the template was tested and lower C_q values were obtained with the higher template volume, which indicates that there was no inhibition due to the matrix components.

LIMITATIONS

We assumed that the most appropriate DNA extraction protocol for *Cyclospora* oocysts would be similar to that for *T. gondii* oocysts, taking in to account their genetic and morphological similarities. In our laboratory, it has been shown that different commercially available DNA extraction kits have highly significant differences in their efficacy at extracting DNA from *T. gondii* oocysts (unpublished data). This might be due to differences in the lysis buffers in the kits or inhibition of qPCR due to chemicals used in the kits. Therefore, future standardisation of the present assay should include testing the efficiency of the DNA extraction protocol on oocysts of *C. cayetanensis*.

TABLE 5 | qPCR results of the eluates of blueberry washes spiked with 2, 5, 10, and 100 *Cyclospora* oocysts.

Independent replicates	qPCR result			
	2 oocysts	5 oocysts	10 oocysts	100 oocysts
1	No C_q	Pos.	No C_q	Pos.
2	No C_q	Pos.	Pos.	Pos.
3	No C_q	No C_q	Pos.	Pos.
4	No C_q	Pos.	Pos.	Pos.
5	No C_q	No C_q	Pos.	Nd

Key: Nd – not done; Pos. – positive.

TABLE 6 | The duplex qPCR results of the berries spiked with 10, and 50 oocysts of *Cyclospora*.

Types of berries	qPCR result		
	Negative control (0 oocyst)	10 oocysts	50 oocysts
Raspberry			
1	No C_q	Pos.	Pos.
2	No C_q	Pos.	Pos.
3	Nd	Pos.	Pos.
Blueberry			
1	No C_q	Pos.	Pos.
2	No C_q	Pos.	Pos.
3	Nd	No C_q	Pos.

Furthermore, the high variability of the ITS-1 region of *C. cayetanensis* genome might affect the PCR. It was noted that the probe has up to three mismatches with some of the sequences of *C. cayetanensis* that have been submitted to GenBank. Although high variability at a target gene might be advantageous for source tracking and some epidemiological studies, it might also mean that it is challenging to design a primer pair and probe that is appropriate for amplification of DNA from all *C. cayetanensis* isolates. Nevertheless, it is worth noting that the ITS-1 variation reported did not show geographic cluster, but the variation was between and within samples examined (Olivier et al., 2001). In this study, *Cyclospora* isolates from six different sources were successfully amplified with the newly developed duplex qPCR method; although it would obviously be preferable to use further isolates. Thus, these data suggest that this detection method could be a suitable alternative for use in the analyses of berry samples and other fresh produce for *Cyclospora* contamination. It should be noted that in implementing new laboratory protocols, sequencing is an option for providing confidence regarding positive results.

In the present study, we have attempted to investigate the robustness of the new method by manipulating factors such as the annealing temperature, super mix volume, master mix brands, the concentration of primers and probes. However, there are also factors that could affect the robustness of the assay that has not been considered in our study. These include factors such as the analyst performing the test and the qPCR instrument brand. Such factors would be most appropriate to address by inter-laboratory comparison studies.

CONCLUSION

In this study, a new assay targeting the ITS-1 target was developed for detection of *C. cayetanensis* as contaminants of berries and shown to be an effective approach that could be suitable alternative for food testing laboratories. The high specificity of the new detection method, as shown by both *in silico* and *in vitro* investigations, is a very important and relevant aspect. In addition to the specificity, the new protocol is robust (can tolerate minor changes in the annealing temperature, primers and probe concentration, and the reaction volume) and relatively simple, which makes it convenient for regular use in food testing laboratories. However, in order to standardise this method, further tests are warranted. This should include an inter-laboratory comparison for validation of the method's fitness for purpose. Furthermore, any changes in the method should be assessed accordingly.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and the **Supplementary Files**.

AUTHOR CONTRIBUTIONS

KT and LR conceived and supervised the study, wrote the grant proposal, obtained the funding, and contributed to the design of experiments. TT designed and performed the experiments, analysed the data, and drafted the manuscript. All authors revised the manuscript and read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.01939/full#supplementary-material

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Paper III

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

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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

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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′)	CyTIS1_TT-F ATGTTTTAGCATGTGGTGTGGC	Tox-9F AGGAGAGATATCAGGACTGTAG	EmMGB_F GTGCTGCTYATAAGAGTTTTTG
Reverse primer (5➔3′)	CyTIS1_TT-R GCAGCAACAACAACTGCTCATC	Tox-11R GCGTGGTCTC GTCTAGATCG	EmMGB_R CTATTAAGTGCTAAACAATACCATA
Probe (5′➔3′)	CyTIS1_TT-P HEX-TACATACCCGTCCCAACCCTCGA-MGBEQ	Tox-TP1 Cy5-CCGGCTTGGGTGCTTTTCCT-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).

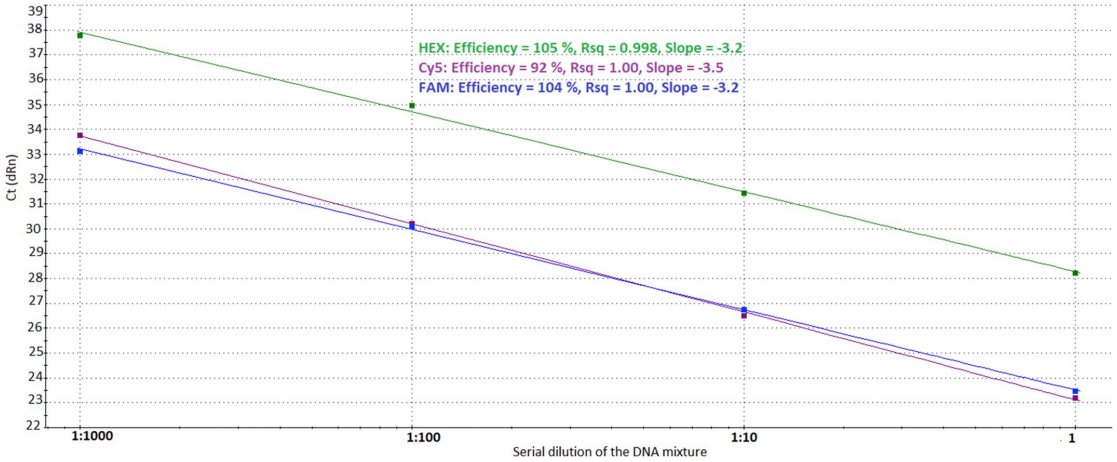

Fig. 1. Calibration curve prepared for the Triple qPCR using a mixture of the three parasites. Note: Fluorescent channel representations HEX for *Cyclospora*, Cy5 for *Toxoplasma,* and FAM for *Echinococcus*.

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.

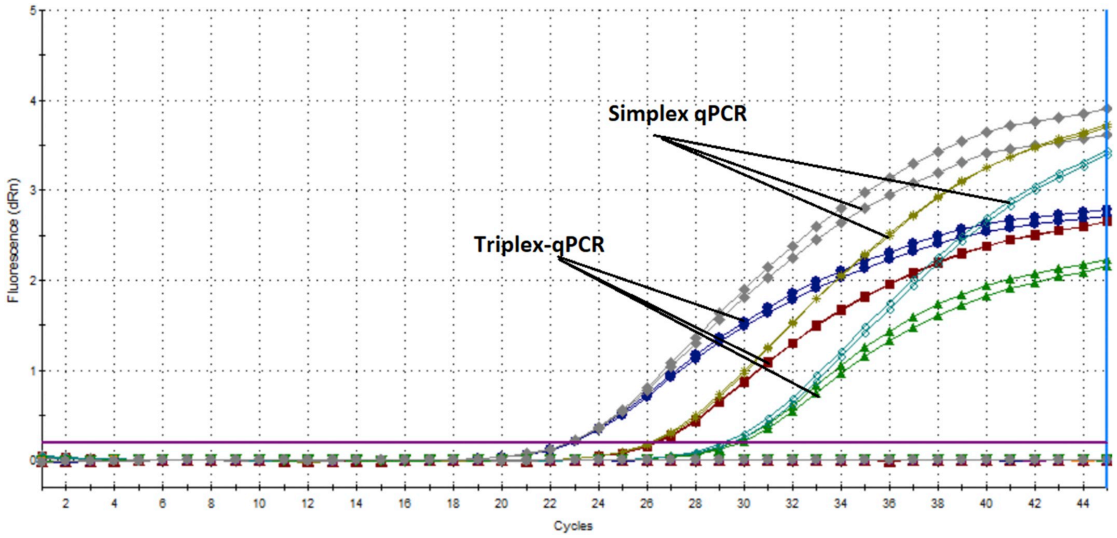

Fig. 2. Amplification plot of the triplex qPCR and simplex assay for *T. gondii* (Cy5 channel).

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

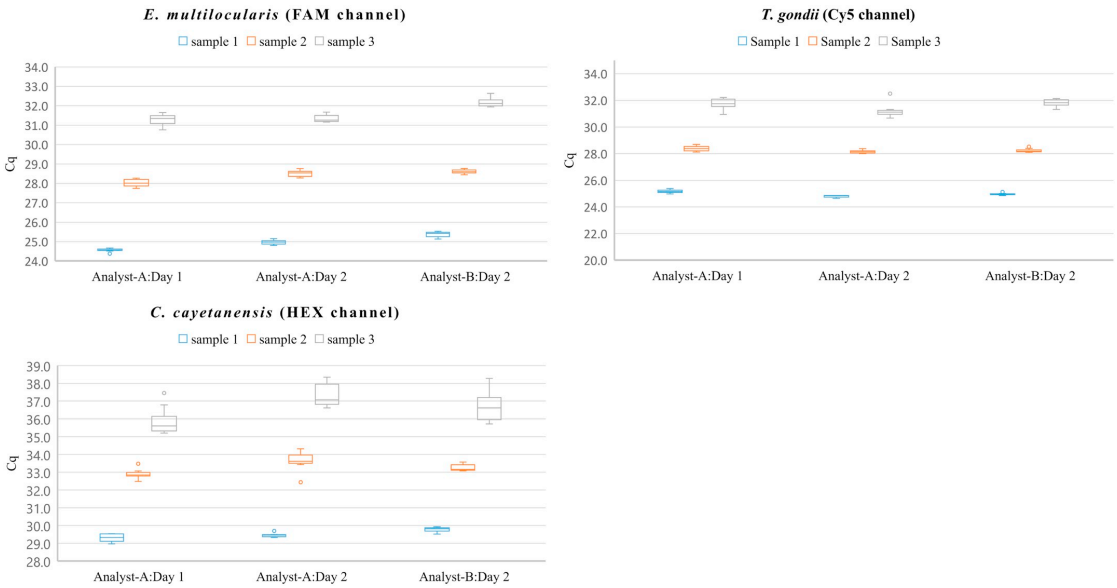

Fig. 3. Graphical representation of the results obtained from the assessment of intermediate precision.

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.

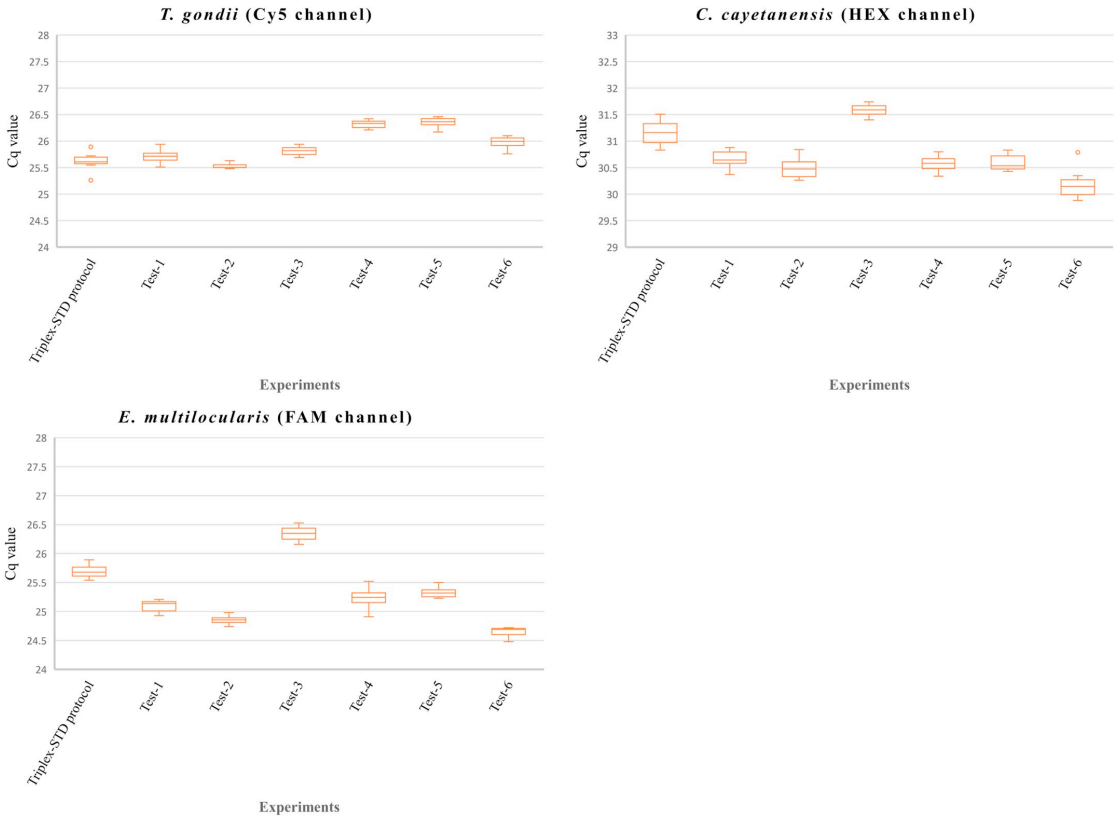

Fig. 4. Investigation of the robustness of the triplex qPCR by making deliberate changes in different factors of the qPCR as described in Table 2.

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.

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Paper IV

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Manuscript Draft

Manuscript Number: WR58928

Title: A novel comparative reverse transcription quantitative PCR method for assessing the viability of Cryptosporidium oocysts: a potential tool for inactivation efficacy trials

Article Type: Research Paper

Keywords: Viability; Cryptosporidium; RT-qPCR; RNA-Seq; Oocysts; Gene expression

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Abstract: Considerable effort has been directed towards developing laboratory methods for detecting low numbers of protozoan parasites contaminating environmental matrices, particularly water. However, it would be even more useful to have a method that could differentiate between inactivated and viable parasites. The aim of the present study was to develop a novel comparative RT-qPCR method for viability assessment of Cryptosporidium parvum oocysts. Although RT-qPCR has been previously explored as a tool for viability assessment of Cryptosporidium oocysts, the novelty of the approach described here lies in the use of RNA sequencing (RNA-Seq) analysis for the identification of inducible target genes and exploiting the 18s rRNA gene as a normaliser in the relative quantification of gene expression among samples exposed to an oxidative stressor.
To identify the most suitable targets for viability assessment, Cryptosporidium parvum oocysts were subjected to different stressors before total RNA extraction. Bioinformatics analysis of the RNA-Seq data identified several differentially expressed genes in the samples exposed to heat shock and oxidative stress due to xanthine oxidase catalysed reactions. Target genes were then pragmatically selected for developing RT-qPCR method using xanthine oxidase catalysed reaction as the induction approach. The targets included in the experiment included thioredoxin, Cryptosporidium oocyst wall protein 7 (COWP7), heat-shock protein 70 (HSP70), malate dehydrogenase, prohibitin, and UDP-glucose 6-dehydrogenase (UGDH). The RT-qPCR was evaluated on oocysts exposed to the oxidative stressor. Preliminary experiments investigating the six different target genes selected for the RT-qPCR method development showed that all were upregulated following oxidative stress. Thioredoxin and COWP7 were selected for further evaluation of the RT-qPCR on a mixture of viable and inactivated oocysts, as well as on oocysts subjected to various inactivation treatments such as freezing and chlorination. Evaluation of the RT-qPCR with samples that included mixtures of inactivated and viable oocysts in different proportions provided promising results, with the gene expression level proportionally related to the number of viable oocysts in the sample.

The results of the study show that this approach could be used for testing the efficacy of different inactivation treatments. The RT-qPCR method offers a quick and cost-effective alternative to viability assessment compared with mouse infectivity and cell culture assays. However, before use as a standard method, this approach should be validated against the gold standard bioassay (mouse infectivity test).

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Cover Letter, For Editor only

Date: October 20, 2020

To Water Research

Elsevier

Manuscript submission: A novel comparative reverse transcription quantitative PCR method for assessing the viability of *Cryptosporidium* oocysts: a potential tool for inactivation efficacy trials

Dear editor-in-chief,

We hereby submit our manuscript entitled "**A novel comparative reverse transcription quantitative PCR method for assessing the viability of *Cryptosporidium* oocysts: a potential tool for inactivation efficacy trials"** for consideration for publication in *Water Research.*

The manuscript is a research paper aimed at developing and evaluating a novel RT-qPCR method for viability assessment of *Cryptosporidium* oocysts. The present method explored oxidative stress challenges to induce gene expression and exploited the RNA-Seq data analysis to select the appropriate target genes. The novel RT-qPCR method was extensively evaluated for its application in the assessment of efficacy of inactivation treatments.

In our opinion, given the importance of *Cryptosporidium* as a waterborne pathogen, with numerous waterborne outbreaks recorded, this work could be of interest to your readers.

We hereby confirm that the manuscript has not been published nor submitted for peer-review in any other journal, and that all authors approve the submitted version.

We look forward to receiving editorial and reviewer comments in due course.

Best regards,

*Highlights (for review)

Highlights

- Water industry lacks good tools for assessing viability of *Cryptosporidium* oocysts
- Xanthine oxidase identified as promising induction agent for gene expression
- RNA-seq used to assess differentially expressed genes for viability assessment
- RT-qPCR method at two genes developed for viability testing
- Method successfully used for testing the efficacy of different inactivation treatments

A novel comparative reverse transcription quantitative PCR method for assessing the viability of *Cryptosporidium* oocysts: a potential tool for inactivation efficacy trials

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Abstract

Considerable effort has been directed towards developing laboratory methods for detecting low numbers of protozoan parasites contaminating environmental matrices, particularly water. However, it would be even more useful to have a method that could differentiate between inactivated and viable parasites. The aim of the present study was to develop a novel comparative RT-qPCR method for viability assessment of *Cryptosporidium parvum* oocysts. Although RT-qPCR has been previously explored as a tool for viability assessment of *Cryptosporidium* oocysts, the novelty of the approach described here lies in the use of RNA sequencing (RNA-Seq) analysis for the identification of inducible target genes and exploiting the 18s rRNA gene as a normaliser in the relative quantification of gene expression among samples exposed to an oxidative stressor.

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Keywords: Viability; Cryptosporidium; RT-qPCR; RNA-Seq; Oocysts; Gene expression

61

62 ## 1. Introduction

63 *Cryptosporidium* is a coccidian parasite responsible for cryptosporidiosis, a gastrointestinal
64 disease manifested as watery diarrhoea, nausea, vomiting, fatigue, and other signs and
65 symptoms. Humans acquire the infection through ingestion of the sporulated oocysts, each of
66 which contain four sporozoites that invade the cells of intestinal epithelium. Transmission is
67 often due to ingestion of drinking water or recreational water (incidental swallowing of water in
68 lakes, rivers and swimming pools) that has been contaminated with infectious oocysts.
69 Outbreaks of cryptosporidiosis attributed to swimming pool or drinking water contamination
70 have been frequently recorded. It is considered as the leading cause of outbreaks of diarrhoea
71 linked to water contamination in the U.S (Gharpure et al. 2019). This is partly due to the highly
72 robust and environmentally resistant oocysts that are well suited for transmission via
73 contaminated environmental matrices such as water and food. Infection via contaminated food
74 or direct host-to-host transmission is also possible. Some species of *Cryptosporidium*, such as
75 *Cryptosporidium parvum*, are zoonotic, with a wide range of potential hosts, although young
76 ruminants are predominantly associated with human infections.

77 Although *Cryptosporidium* infections can be asymptomatic or cause self-limited disease, it may
78 also cause severe diarrhoea that lasts for 2 or more weeks and the symptoms are worse in
79 immunocompromised individuals. The watery diarrhoea typical for cryptosporidiosis, can
80 sometimes be profuse and prolonged and result in dehydration and wasting. This may cause

81 critical illness (Bouzid et al. 2013) and, due to the lack of effective treatment, mortality rates
82 may be high, especially for immunocompromised patients.

83 Considerable effort has been directed towards developing methods for detecting
84 contamination of water and other environmental matrices with *C. parvum* oocysts. However, it
85 would also be useful to have an appropriate method for determining whether oocysts are
86 infectious or not, particularly for investigating the efficacy of different inactivation treatments.
87 Due to the complex nature of parasites, it has been challenging to develop sensitive *in vitro*
88 methods for assessing their viability. The current gold standard method for viability testing of
89 parasites is *in vivo* animal bioassay. Alternative *in vitro* techniques that have been explored
90 include experimental infections in cell cultures, in vitro excystation, sporulation, vital dye
91 inclusion/exclusion, propidium monoazide PCR (PMA-PCR), nucleic acid sequence-based
92 amplification (NASBA), reverse transcription quantitative PCR (RT-qPCR), and fluorescence *in*
93 *situ* hybridisation (Rousseau et al. 2018).

94 Although there are several published protocols for viability assessment of *C. parvum* based on
95 RT-qPCR, there is no consensus on the best approach and there are different rationales for
96 selecting an appropriate genetic marker. Heat-shock protein 70 (HSP70) has been commonly
97 used for viability assessment of *C. parvum* and often the protocol includes heat induction (45°C
98 for 20 min) for improving the sensitivity of the method (Bajszár & Dekonenko 2010; Garcés-
99 Sanchez et al. 2013; Travaillé et al. 2016). In contrast, another study argued that the ideal
100 viability marker should not be altered by an external stressor and hence suggested the use of
101 CP2 over HSP70 (Lee et al. 2008). Others have suggested using the ratio of mRNA to DNA for

the assessment of viability following heat-induction (Garcés-Sanchez et al. 2013). Collectively, these results indicate that RT-qPCR based methods could be a rapid, sensitive, and reliable estimate of the viability of parasites if the right approach to selecting the target genes, experimental setup, and interpretation of the results is followed. A potentially useful approach would be to induce gene expression by exposure to stressors and compare the relative quantity of expression of relevant genes in test and non-exposed control samples (normalized against reference genes to correct for artificial differences between the samples). This approach would avoid the problem of over-estimation of viability, due to detection of residual RNA in inactivated oocysts, as has been previously reported (Chambers 2005; Widmer et al. 1999).

The present study aimed at using this approach to develop a novel comparative RT-qPCR method for the viability assessment of *C. parvum* oocysts, with identification of inducible target genes by RNA-Seq analysis and then using the 18s rRNA gene as a normaliser in the relative quantification of the gene expression among samples exposed to an oxidative stressor and an untreated control.

2. Materials and methods

2.1. Parasites

Cryptosporidium parvum IOWA strain oocysts were purchased from Bunch Grass Farm (Deary, USA) and used within three months after passage. The oocysts had been stored in PBS supplied with antibiotics (1000 IU penicillin, 1000 µg streptomycin), wrapped in cold gel pack, and shipped overnight by courier. An additional isolate of *C. parvum* oocysts was purified from calf faeces that had been submitted to the Parasitology lab, Norwegian University of Life Sciences

for diagnostic examination. The comparison of glycoprotein 60 (GP60) gene fragments from this isolate with the IOWA strain showed about 87% identity.

2.2. Experimental setup

In order to achieve the objective of the study, the experimental setup was designed to include four main sections including: i) different induction approaches to stimulate upregulation of a range of genes; ii) identification of these genes using RNA-Seq and differentially expressed genes (DEG) analysis; iii) selection of appropriate targets and designing RT-qPCR protocols for their relative quantification; iv) use of these protocols to determine viability of oocysts following exposure to different chemical and physical potential inactivation treatments. The experimental setup is summarised in Fig. 1.

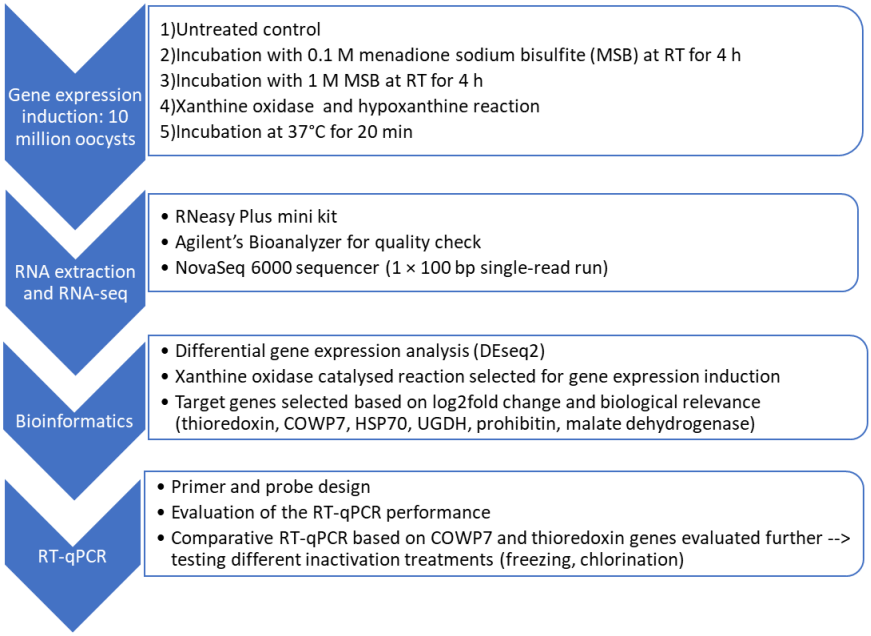


Fig. 1. Flow chart indicating the 4 stages of the RT-qPCR method development

2.2.1. Identification of inducible target genes using RNA-Seq and DEG analysis

2.2.1.1. Induction of upregulation of gene expression

Five different gene-expression induction approaches were explored using, in brief: a) two concentrations of menadione sodium bisulfite (MSB); b) an enzymatic reaction composed of xanthine oxidase and hypoxanthine, and c) heat shock at two different temperatures; see Table 1 for details. All chemicals used were purchased from Sigma Aldrich, Norway. Each of the 5 induction groups contained four independent replicates of approximately 10 million *C. parvum*

143 oocysts. Oocysts were pre-washed twice in water before being subject to the different
144 treatments. A control group was stored refrigerated prior to RNA extraction.

145 Table 1. Description of the gene expression induction approaches explored for RNA-Seq analysis

Treatment	**Brief description**
1M MSB	200 µL of oocysts were mixed with 500 µL of 1 M MSB, vortexed, then held at room temperature for 4 h. The suspension was then washed 3 times with water before total RNA extraction
0.1M MSB	200 µL of oocysts were mixed with 500 µL of 0.1 M MSB, vortexed, then held at room temperature for 4 h. The suspension was then washed 3 times with water before total RNA extraction
Xanthine oxidase and hypoxanthine	200 µL of oocysts were vortexed and 50 µL of xanthine oxidase (20 U/mL) added to the suspension. The suspension was brought up to 500 µL with PBS (pH 8.5) and incubated at 37°C for 10 min. Thereafter, 500 µL of 0.5 mM hypoxanthine was added to the mixture, briefly vortexed and further incubated at 37°C for 30 min with the lids open. The sample was then washed 3 times with water before total RNA extraction.
Heat shock	200 µL of oocysts were incubated at 37°C for 20 min. The sample was then washed 3 times with water before total RNA extraction.
Heat shock	200 µL of oocysts were incubated at 45°C for 20 min. The sample was

	then washed 3 times with water before total RNA extraction.
Control	Control samples of oocysts were washed five times with water before total RNA extraction.

2.2.1.2. RNA extraction and quality assessment for RNA-Seq

The RNA extraction protocol for this study was based on RNeasy plus mini kit (Qiagen, Norway) with slight modifications to the lysis approach. Briefly, oocyst lysis was performed in a lysing matrix E tube (MP Biomedicals, France) to which 600 µL of the RLT plus buffer was added, and the tube was subjected to 2 cycles of bead-beating at 4 m/s for 25 s with a 3-minute pause on ice. The lysate was centrifuged at 12,000 rcf for 2 min and then added to the gDNA eliminator spin column before elution into 55 µL of nuclease-free water and storage at -20°C.

RNA quality was assessed using an Agilent 2100 Bioanalyzer and the RNA 6000 Nano kit was used for sample preparation. The RNA integrity number (RIN) produced by the Bioanalyzer provides an indication of the RNA quality; RIN ranges from 1-10, where 10 indicates intact RNA. The RNA extracted from samples subjected to heat shock at 45°C for 20 min did not produce RIN values and hence was not submitted for RNA-Seq.

2.2.1.3. RNA sequencing (RNA-Seq)

The RNA samples were sequenced at a national technology core facility (Norwegian Sequencing Centre, Ullevål Hospital, Oslo). The RNA library preparation was performed using Truseq stranded RNA prep kit and sequenced in the NovaSeq SP flow cell with the NovaSeq 6000

sequencer. After indexing all samples were sequenced in the same flow cell using single-read sequencing of 100 bp and a sequencing depth of ca 20 million reads per sample.

2.2.2. RT-qPCR method development

The gene expression induction approach selected for the RT-qPCR method development was based on the oxidative stressor challenge as shown in Fig. 2.

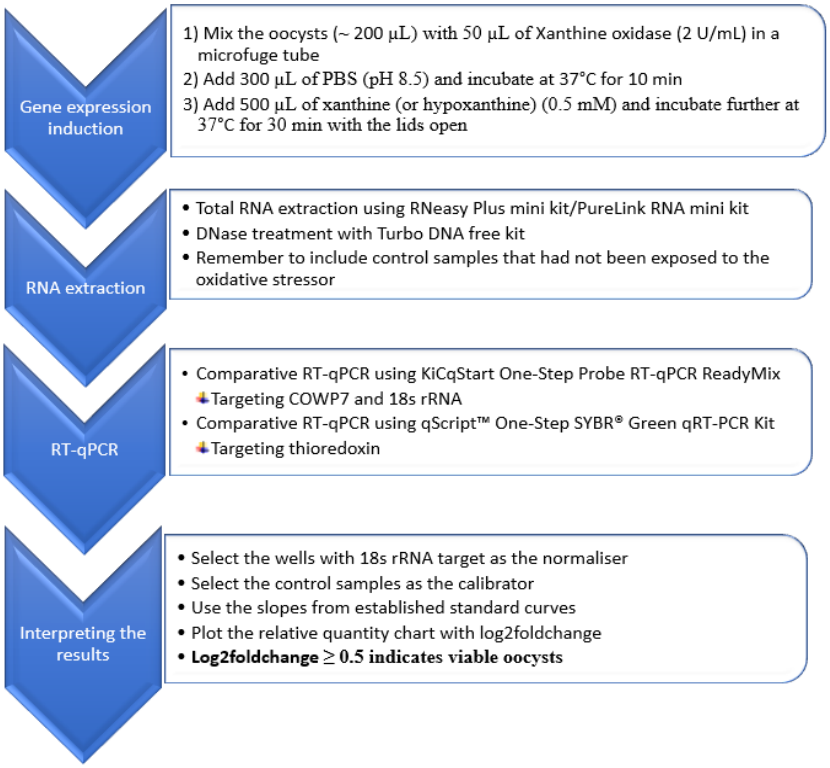


Fig. 2. Flow chart representing the summary of the novel comparative RT-qPCR method for the assessment of viability of *Cryptosporidium* oocysts

2.2.2.1. Target genes and primers

Based on the differentially expressed genes (DEG) analysis (see section 2.3.2), six different target genes were selected for further testing using RT-qPCR. These genes included COWP7, type 3 malate dehydrogenase, thioredoxin, prohibitin, heat shock protein 70 (HSP70), and UDP-glucose 6-dehydrogenase (UGDH). In order to evaluate the targets identified by the DEG analysis by RT-qPCR, appropriate primers were designed in Geneious Prime, with intron-spanning sites included where possible (Table 2). In addition to the differentially expressed target genes, 18s rRNA was selected as the reference gene to normalise variation between samples.

180 Table 2. Oligos employed to evaluate the validity of selected target genes in the development of novel RT-qPCR method to assess

181 the viability of *Cryptosporidium* oocysts

Target gene	**Forward primer (5' → 3')**	**Reverse primer (5' → 3')**	**Probe**	**Product size**	**Ref.**
COWP7	CTATGGGATTCAATTTCGAAGTTCC	CCCAATACAAAATCTGCTACTTCCA	ATGGAATATCATCATCCCCTCA GCAA	97	cgd4_500
Thioredoxin	GAAAAGCTGAACCTCGCATTCG	CGTCCCGTGGTCAATGCAATAA	NA	134	cgd7_4080
Prohibitin	CCTTTTAGGTGCAATCGGAACA	CATGGGAGGAAGAAGTGGGTAC	NA	141	cgd7_4240
MDH	TCCTCTAGATGCGATGGTTTACTAC	CCACCTACAACAATGGCTGATACA	NA	162	cgd7_470
UGDH	CCTCCAACATTATCAGCTTTTTGAG	TGCATTTTAGAGTGAACCGCTT	NA	141	cgd8_920
HSP70	AGCCCGTATGAGTACAGAAGACT	GCCTGTGCCAAGAACCCTAAGA	NA	168	cgd4_3270
18s rRNA	JF1: AAGCTCGTAGTTggatTTCTG JF2: AAGCTCGTAGTTaatcTTCTG	JR: TAAGGTGCTGAAGGAGTAAGG	JT2: TCAGATACCGTCGTAGTCT	434	Elwin et al., 2020; UKWIR, 2020)

182

2.2.2.2. RNA extraction for the RT-qPCR method

The PureLink RNA mini kit (Thermo Fisher Scientific, Norway) was employed for total RNA extraction in the RT-qPCR method development. This kit was used here for pragmatic reasons; it was available in the lab and had demonstrated no difference compared with the RNeasy plus mini kit (Supplementary file 1). Briefly, the sample containing approximately 2 million oocysts of *C. parvum* (0.3 mL) was mixed with 0.6 mL of the lysis buffer containing 40 mM dithiothreitol in a lysing matrix E tube and subjected to bead-beating (two cycles of 4 m/s for 25 s with 3 min pause on ice in between the cycles). The lysate was then centrifuged at 12,000 × g for 2 minutes and the supernatant transferred to a new collection tube and purified according to the kit's instruction. RNA was eluted in 50 µL RNase free water before DNase treatment using Turbo DNA free kit (Thermo Fisher Scientific, Norway) and stored at -20°C until RT-qPCR analysis.

2.2.2.3. Reverse transcription quantitative PCR (RT-qPCR)

One-Step SYBR green RT-qPCR

RT-qPCR was performed in a total volume of 20 µL, which included 10 µL of One-Step SYBR Green master mix, 0.4 µL of the qScript RT-mix, 0.5 µM of each primer, and 2 µL of RNA template. The reaction assembly was performed according to the instructions provided with the master mix (Quantabio, Norway). The thermal profile of the reaction was as follows: cDNA synthesis at 50°C for 10 min, initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 15 s, and annealing/extension at 60°C for 1 min. Additionally, a melt-curve analysis step was included, a gradual increase of temperature from 65°C to 95°C with fluorescence data collected every 0.1°C.

One-Step probe RT-qPCR

In addition to the SYBR Green RT-qPCR, a probe-based One-step RT-qPCR was tested for the COWP7 target. The reaction was performed in a total volume of 20 µL that contained 10 µL of KiCqStart® One-Step Probe RT-qPCR ReadyMix, 0.5 µM of each primer, 0.25 µM probe, and 2 µL of the RNA template. The 18s rRNA target was detected and quantified using 0.6 µM of the forward primer (1:1 combination of JF1 and JF2) and reverse primer (JR) and 80 nM of the probe (JT2). The thermal profile of the reaction included cDNA synthesis at 50°C for 10 min, initial denaturation at 95°C for 3 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 30 s.

Standard curves were prepared to evaluate the linearity and efficiency of each primer pair.

An additional test was conducted to compare xanthine and hypoxanthine as the substrate in the oxidative stress induction.

2.2.3. Evaluation of the RT-qPCR method

The novel RT-qPCR method was evaluated for its applicability in the differentiation of viable and inactivated oocysts using different inactivation treatments. The main steps of the RT-qPCR are summarized in the flow chart presented in Fig. 2.

2.2.3.1. Inactivation of *Cryptosporidium* oocysts

In order to assess the applicability of the RT-qPCR method, physical and chemical inactivation approaches, which had been previously evaluated using mice bioassay (Fayer, R. & Nerad, T. 1996; Travaillé et al. 2016), were used to assess whether the method developed here could be

used to evaluate inactivation efficacy. Accordingly, *Cryptosporidium* oocysts were inactivated by heating at 80°C (on heat block) for 3 minutes and incubated at room temperature for 3 h prior to RNA extraction. Other inactivation tests included heating at 60°C for 2 min, freezing at -20°C for 2 h, 24 h, and 48 h. In addition to thermal inactivation, chemical treatment was assessed by subjecting oocysts to 4 mg/L and 0.2 mg/L free chlorine concentrations for 30 min.

2.2.3.2. Discriminating between viable and inactivated oocysts using the new RT-qPCR method

The new RT-qPCR method was evaluated for its ability to discriminate between different proportions of viable and inactivated oocysts by analysing samples containing different proportions of viable and inactivated oocysts. In brief, the oocyst mixtures (containing viable/inactivated oocysts in the following proportions: 0/100, 1/99, 10/90, 100/0 %) were exposed to an inactivation regime as described in section 2.2.3.1. Then the oocysts were exposed to xanthine oxidase catalysed oxidative stressor before RNA extraction. RT-qPCR targeting thioredoxin, COWP7, and 18s rRNA was used to determine relative quantity of thioredoxin and COWP7 genes from exposure to the oxidative stressor. Controls that contained the same proportion of viable/inactivated oocysts, but had not been exposed to the oxidative stressor, were included in each run.

2.3. Statistical analysis

2.3.1. Data pre-processing and mapping

Raw sequence data (fastq files) from the RNAseq were trimmed for adapter sequences and low quality reads (having phred score < 33) by using trimommatic 0.39 (http://www.usadellab.org/cms/?page=trimmomatic) and assessed for quality by using FASTQC tool. The trimmed reads were mapped to the reference genome of *C. parvum* IOWA II obtained from CryptoDB release 46 (https://cryptodb.org/common/downloads/release-46/CparvumIowaII/fasta/data/CryptoDB-46_CparvumIowaII_Genome.fasta) using STAR version 2.5 (Dobin et al. 2013). In addition, the Salmon tool (Patro et al. 2017) was applied for quasi-mapping of the trimmed reads against the annotated transcript of *C. parvum* IOWA II available from the CryptoDB release 46 (https://cryptodb.org/common/downloads/release-46/CparvumIowaII/fasta/data/CryptoDB-46_CparvumIowaII_AnnotatedTranscripts.fasta).

2.3.2. Differential gene expression analysis

The mapped reads were counted against the recently updated genome annotation file obtained from CryptoDB release 46 (https://cryptodb.org/common/downloads/release-46/CparvumIowaII/gff/data/CryptoDB-46_CparvumIowaII.gff). Gene counts table was prepared using featureCounts (Liao et al. 2013), which was then used as an input for the differential expression analysis with the R–based tool, DESeq2 (Love et al. 2014).

In addition to the gene-based differential expression analysis, a transcript-based differential expression analysis was performed on the transcript abundance output obtained from the Salmon tool and further analysed with DESeq2 in R. The differential expression analysis output

contained the list of genes with their log2fold change and associated p-values. The results of the DEG analysis were visualised using principal component analysis (PCA) plots and MA-plots.

3. Results

3.1. RNA-Seq analysis

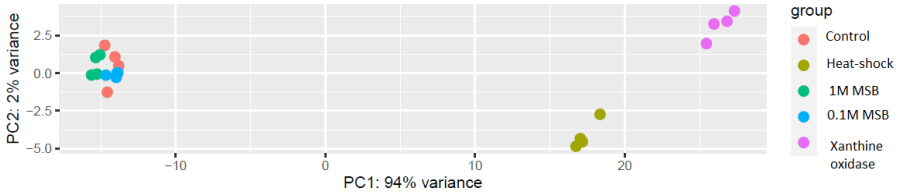
The sequencing reads were of high quality as indicated by the average Phred score of 36. The RNA-Seq data have been submitted to the sequence read archive of NCBI and are available for public use with the accession number PRJNA669334 (http://www.ncbi.nlm.nih.gov/bioproject/669334). The overall alignment rate of the reads against the reference genome was > 96 % whereas the alignment rate of the reads against the reference transcriptome was > 89 % (Table 3).

Table 3. The overall alignment rate of trimmed reads against the reference genome and transcriptome of *Cryptosporidium parvum*

Sample	**Raw reads**	**Trimmed reads**	**Overall alignment rate (%)**	
			STAR	**Salmon**
control 1	27878598	26620351	97.96	93.20
control 2	23459177	23075085	97.77	94.90
control 3	17486930	16539962	98.19	94.40
control 4	13061000	12752419	98.40	94.70
Xanth_ox_1	23377041	22510385	96.45	89.30
Xanth_ox_2	19825148	19397343	96.46	89.50

Xanth_ox_3	15911371	15380705	96.40	90.10
Xanth_ox_4	18693182	18319921	97.64	92.30
1M_MSB_1	20636906	19628468	98.75	94.50
1M_MSB_2	24400998	24019388	99.01	95.60
1M_MSB_3	19455385	18888633	98.78	94.70
1M_MSB_4	29212927	28039553	99.03	95.90
0.1M_MSB_1	17954612	16999783	98.68	95.10
0.1M_MSB_2	25264410	24818569	98.70	95.40
0.1M_MSB_3	20265589	19943975	98.61	95.50
0.1M_MSB_4	17021326	16172993	98.42	95.00
Heat_shock_1	25417560	24546200	98.32	92.70
Heat_shock_2	26218711	25825395	98.5	93.70
Heat_shock_3	23328379	22719854	98.42	93.50
Heat_shock_4	21647093	21266741	98.76	94.30

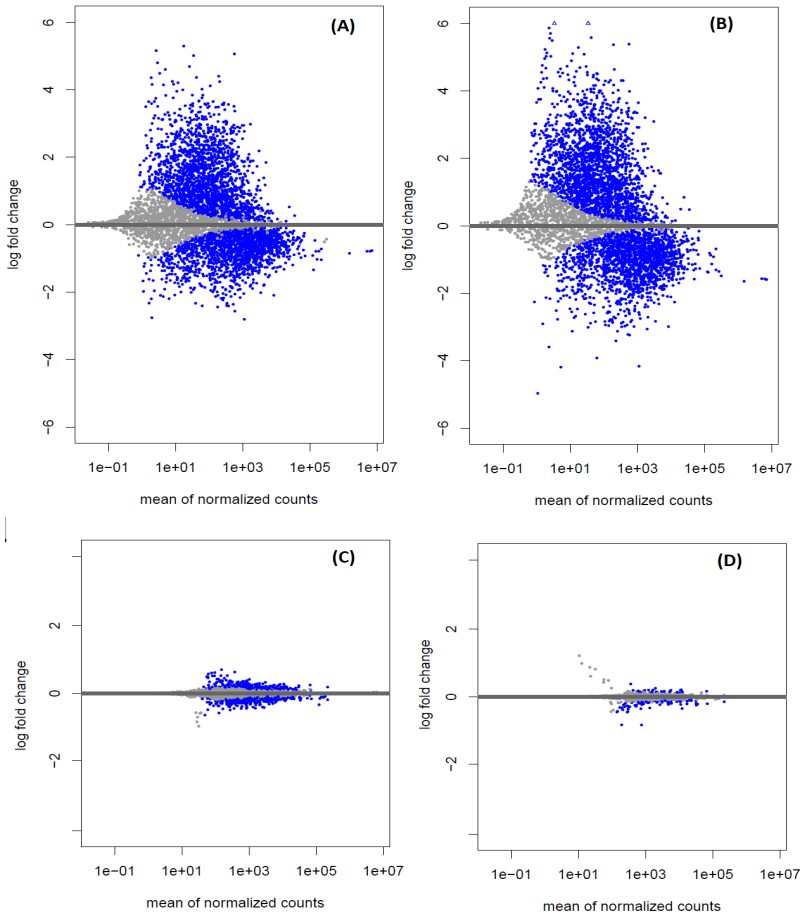
The differential expression analysis of the RNA-Seq data revealed that, as shown by PCA, two groups of samples were clearly separated from the rest (Fig. 3). The PCA plot shows that the samples subjected to oxidative stressor from the enzymatic reaction of xanthine oxidase and hypoxanthine, as well as those subjected to heat shock, were placed distinctly separate from one another and from the other three groups. The samples treated with MSB showed no difference from the control samples.



280

281 Fig. 3. PCA of the RNA-Seq data after rlog transformation.

282 The MA plot (Fig. 4) for the RNA-Seq data also showed differences in the gene expression
283 between the sample groups, indicating that MSB-treated samples and untreated control
284 samples had similar gene expression levels, as shown by the distribution of genes around the
285 horizontal axis. In contrast, samples subjected to heat shock or to the xanthine oxidase
286 catalysed reaction had clearly visible differences in gene expression levels compared with the
287 untreated control.



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289 Fig. 4. The MA-plot representing gene expression differences following (A) heat shock
290 treatment (37 °C for 20 min), (B) xanthine oxidase and hypoxanthine reaction, (C) 0.1M MSB
291 treatment, (D) 1M MSB treatment compared to the untreated control group.

292 The DEG analysis showed that many genes were differentially expressed in response to the
293 oxidative stress (supplementary file 2). In order to develop an RT-qPCR method, six target genes

294 were selected based on their log2fold change and general biological relevance in the oxidative
295 stress response of cells (Table 4).

296 Table 4. List of potential target genes selected for the assessment of the viability of
297 *Cryptosporidium* oocysts using the novel comparative RT-qPCR method

Gene ID*	**Gene name**	**Log2foldchange**
cgd4_500	COWP7	5.3
cgd8_920	UDP-glucose 6-dehydrogenase	4.8
cgd7_4080	Thioredoxin	4.7
cgd7_4240	Prohibitin	4
cgd7_470	Type 3 Malate dehydrogenase	4
cgd4_3270	HSP70	2.6

298 * according to https://cryptodb.org/cryptodb/app

299

3.2. RT-qPCR method development

The performance of each primer pair (i.e., efficiency and linearity) was well within the acceptable ranges. The standard curve prepared to assess the efficiency and linearity of the RT-qPCR based on the COWP7 gene is presented as an example below (Fig. 5).

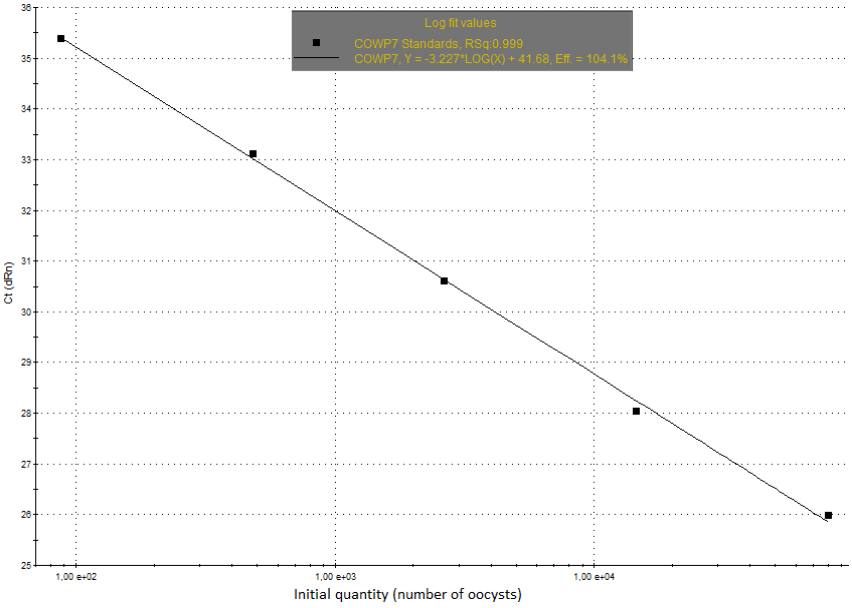


Fig. 5. The standard curve prepared by using 5-fold serial dilution of RNA extracted from 2 million oocysts of *C. parvum*.

Preliminary experiments investigating the six different target genes selected for the RT-qPCR method development showed that all were upregulated following oxidative stress, as indicated by consistently lower C_q values compared with those of untreated controls (Table 5). This confirmed the results obtained from the DEG analysis of the RNA-Seq data.

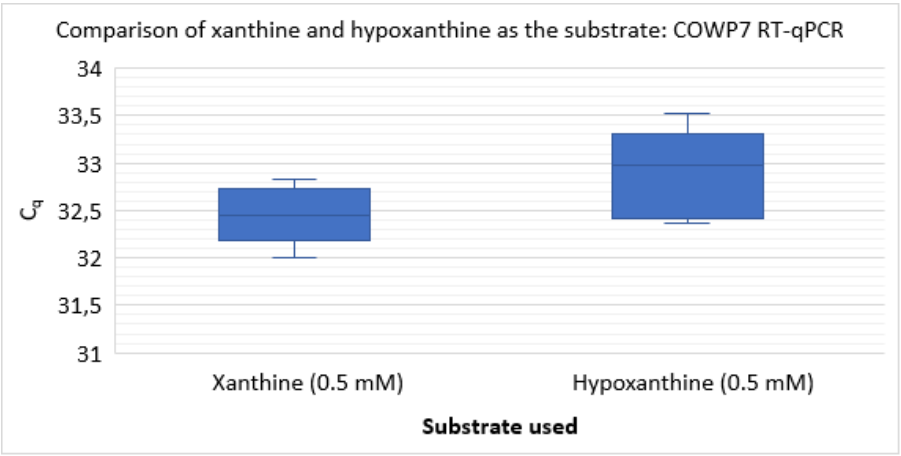
311 Table 5. Comparative quantitative analysis of selected target genes using 18s rRNA as the
312 reference gene (normaliser).

Target gene	Test samples (C_q) [a]				Control samples (C_q)			Log2FC
	T1	T2	T3	Mean	C1	C2	Mean	
COWP7	28.7	28.9	28.3	28.6	34.6	35.1	34.8	6.34
Thioredoxin	23.4	23.5	23.2	23.3	27.3	27.3	27.3	4.08
UGDH	25.1	25.2	25.1	25.1	29.6	29.4	29.5	4.49
MDH	22.7	22.6	22.6	22.6	26.5	26.7	26.6	4.11
HSP70	24.6	24.0	24.5	24.3	28.9	28.6	28.7	4.54
Prohibitin	26.0	25.8	25.9	25.9	30.0	30.1	30.0	4.28

313 [a] Samples subjected to oxidative stressor challenge using xanthine oxidase and xanthine
314 reaction.

315 Among the six target genes evaluated, COWP7 and thioredoxin were selected for further
316 evaluation. The intron-spanning primer pair designed for the detection of COWP7 showed no
317 amplification from genomic DNA in the absence of DNase treatment, confirming the reliability
318 of comparative quantitation of the transcript among the different groups of samples (data not
319 shown). For thioredoxin, the DNase treatment of the RNA sample with Turbo DNA-free kit was
320 effective, as no amplification was detected in the absence of the reverse transcriptase enzyme.

The comparison of xanthine and hypoxanthine as a substrate for the xanthine oxidase catalysed reaction showed no significant difference. However, samples treated with xanthine had consistently lower C_q values when tested on COWP7 and thioredoxin (Fig. 6). Therefore, xanthine was employed for the rest of the RT-qPCR evaluation.



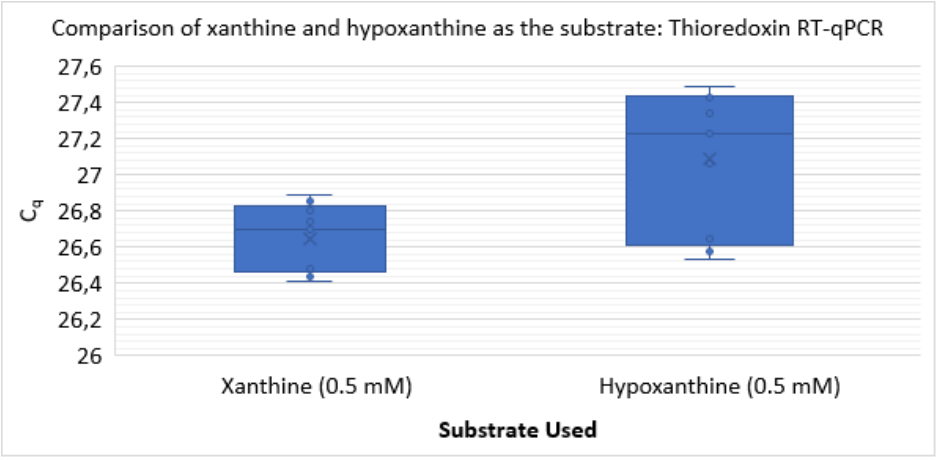


Fig. 6. Comparison of xanthine and hypoxanthine as the substrate in the oxidative stress challenge as indicated by RT-qPCR results for COWP7 (top) and thioredoxin (bottom) tests.

Comparison of the xanthine oxidase catalysed gene expression induction approach with that using heat shock (45°C for 20 min) indicated better results were achieved with the present approach, i.e., xanthine oxidase catalysed oxidative stressor challenge. Although the heat shock resulted in induction of gene expression, it was considerably lower than from exposure to the oxidative stress. The difference was about 3 C_q, which amounts to approximately 8.5-fold (Fig. 7).

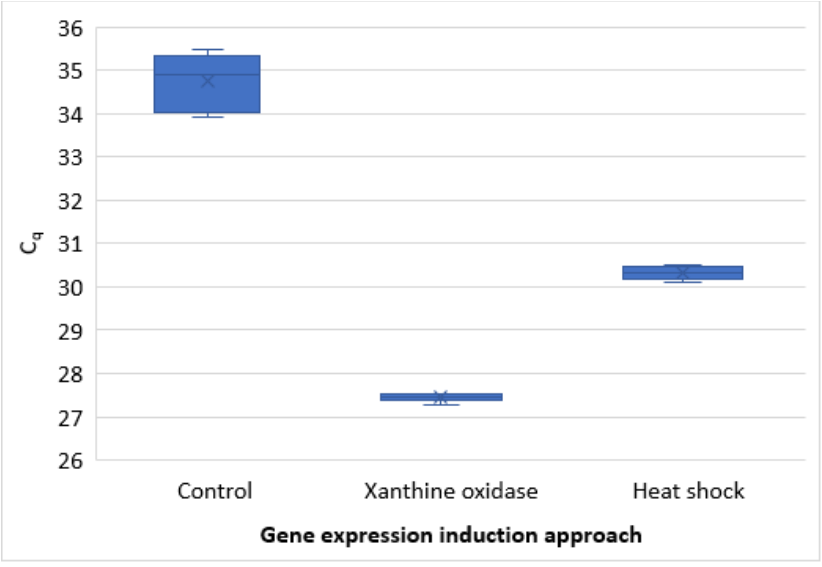


Fig. 7. Comparison of gene expression induced by heat shock (45°C for 20 min) with that induced by oxidative stressor challenge catalysed by xanthine oxidase.

339

340 ### 3.3. Evaluation of the RT-qPCR method

341 The thioredoxin RT-qPCR results indicated that the level of gene expression was proportionally
342 related to the number of viable oocysts in the sample (Table 6). Induction of the oxidative
343 stress response resulted in significantly more transcripts than its respective control that had not
344 been exposed to the oxidative stressor.

345 Table 6. Evaluation of the RT-qPCR targeting thioredoxin by using mixtures of viable and
346 inactivated oocysts of *C. parvum*

Sample preparation			Induction treatment group		
	Viable (%)	Inactivated (%)	Control C_q value	Oxidative stress C_q value	ΔC_q
Sample 1	100	0	29.9	25.6	4.3
	100	0	30.0	25.8	4.2
Sample 2	10	90	34.9	29.6	5.3
	10	90	33.6	28.6	5
Sample 3	1	99	No C_q	32.0	NA
	1	99	36.4	31.5	4.9
Sample 4	0	100	No C_q	No Ct	NA
	0	100	35.9	36.0	-0.1

The novel RT-qPCR protocol was further tested on a different isolate of *Cryptosporidium parvum* that had been purified from a sample delivered for diagnosis at the Parasitology lab, Norwegian University of Life Sciences. The results with this isolate indicated that the protocol was applicable to other *Cryptosporidium* isolates in addition to the IOWA strain used for the development of the method. The results of the comparative RT-qPCR showed that the sample subjected to oxidative stress had log2foldchange of 3 to 3.9 (data not shown).

The novel RT-qPCR method was also evaluated for its use in testing the efficacy of some of the physical and chemical means of inactivation of the oocysts. The results of the experiments showed that oocysts were completely inactivated following freezing (-20°C) for 24 h and 48 h. However, oocysts frozen for only 2 h were not inactivated as indicated by the oocysts' response to the oxidative stressor challenge, with mean C_q (± SD) of 28.5 ± 0.1 which is very similar to control oocysts that had not been exposed to freezing temperature with mean C_q (± SD) of 28.6 ± 0.3 (Table 7).

Table 7. The effect of extreme temperature on the viability of *Cryptosporidium* oocysts

Treatment condition		C_q value COWP7		C_q value Thioredoxin	
		Control[a]	Test[b]	Control[a]	Test[b]
-20°C for 2 h	Replicate 1	34.1	28.4	26.9	24.6
	Replicate 2	34.3	28.5	26.7	23.6
	Replicate 3	33.7	28.4	26.9	23.9
	Mean C_q ± SD	34 ± 0.3	28.5 ± 0.1	26.8 ± 0.1	24.0 ± 0.5

-20°C for 24 h	Replicate 1	33.7	35.3	30.2	30.2
	Replicate 2	33.9	34.7	30.4	30.0
	Replicate 3	33.6	33.7	30.2	29.1
	Mean C_q ± SD	33.7 ± 0.2	34.6 ± 0.8	30.2 ± 0.1	29.7 ± 0.6
-20°C for 48 h	Replicate 1	36.6	37.8	30.7	30.8
	Replicate 2	37.9	37.6	30.7	32.8
	Replicate 3	35.1	36.4	29.9	31.2
	Mean C_q ± SD	36.5 ± 1.4	37.3 ± 0.7	30.4 ± 0.5	31.6 ± 1.1
60°C for 2 min	Replicate 1	37.8	36.7	31.8	30.8
	Replicate 2	37.7	35.1	32.9	31.0
	Replicate 3	No C_q	35.7	31.7	30.8
	Mean C_q ± SD	37.8 ± 0.1	35.8 ± 0.8	32.1 ± 0.7	30.9 ± 0.1
80°C for 3 min	Replicate 1	No C_q	No C_q	No C_q	No Cq
	Replicate 2	No C_q	No C_q	35.9	36.0
	Mean C_q ± SD	NA	NA	NA	NA
Control[c]	Replicate 1	34.6	28.7	27.3	23.4
	Replicate 2	35.1	28.9	27.3	23.5
	Replicate 3	ND	28.3	ND	23.2
	Mean C_q ± SD	34.9 ± 0.4	28.6 ± 0.3	27.3	23.4 ± 0.2

361 [a]- Not subjected to the oxidative stressor; [b] - Subjected to the oxidative stressor; [c] - Not exposed
362 to the low/high temperature; ND- Not done; NA- Not applicable

363 Furthermore, oocysts that had been heated at 60°C for 2 minutes showed some, but
364 incomplete, inactivation, compared with the control oocysts with mean C_q lower than that of
365 their corresponding control (not exposed to the oxidative stressor) but markedly higher than
366 control oocysts not exposed to the elevated temperature (Table 7). This means that following
367 exposure to this temperature inactivation, some oocysts were still able to respond to the
368 oxidative stressor challenge. However, oocysts exposed to 80°C for 3 min seem to be
369 completely inactivated as the RT-qPCR results showed no amplification for the COWP7 target.
370 However, some traces could be detected from the thioredoxin target (Table 7).

371 Chemical treatment of oocysts with 0.2 and 4 mg/L free chlorine for 30 min at room
372 temperature did not affect the viability of the oocysts (Fig. 8). The comparative RT-qPCR results
373 show the difference between oocysts treated with bleach and those stored at -20°C for 24 h.

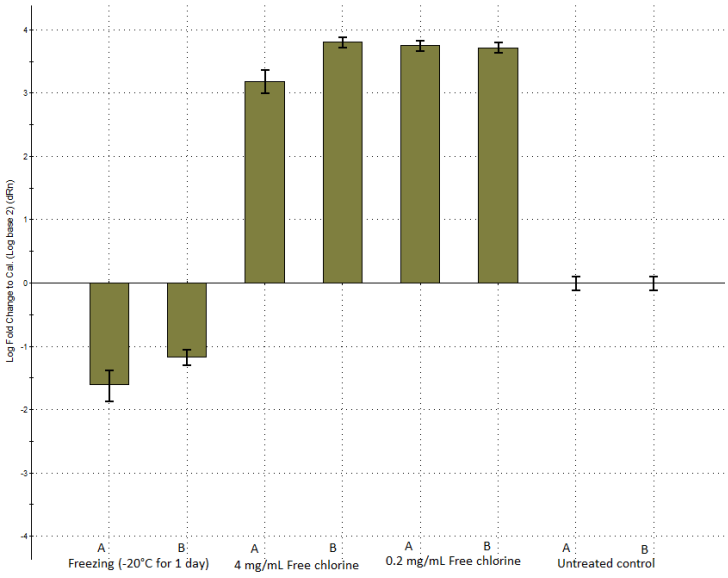


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Fig. 8. Relative quantity chart for Thioredoxin RT-qPCR test on samples frozen at -20°C for 24 h, treated with 4 mg/L free chlorine, and 0.2 mg/L free chlorine for 30 min (18s rRNA was used as the reference gene).

4. Discussion

In this study, we have developed and evaluated a novel RT-qPCR method that could be used for differentiating viable, potentially infectious, oocysts of *C. parvum* from inactivated ones. The method development approach was guided by the biological assumption that viable oocysts would respond actively to stressors. Rigorous testing by RNA-Seq analysis, and with complementary results obtained with the novel RT-qPCR, indicated this to be the case.

The most commonly used method for gene expression induction prior to RNA extraction is heating the oocysts at 45°C for 20 min (Bajszár & Dekonenko 2010; Garcés-Sanchez et al. 2013; Travaillé et al. 2016). However, in our work, exposure of oocysts to 45°C for 20 min resulted in poor quality RNA, probably due to degradation, which could not be used for RNA-Seq. Although these samples were not suitable for RNA-Seq, the RT-qPCR results showed that gene expression could be induced by heat shock, but about 8-fold lower than the present approach of exposure to oxidative stress. Exposure of *Cryptosporidium* oocysts to oxidative stress, resulting from reactions catalysed by xanthine oxidase, produced higher folds of changes in gene expression than the heat shock approach.

The reactions catalysed by xanthine oxidase as shown in Fig. 9 were further explored to compare xanthine and hypoxanthine as the substrate. It has been shown that uric acid is also a

pro-oxidant and could induce the formation of other radicals (Aziz and Jamil, 2020). Therefore, we assumed that the use of xanthine as substrate might result in greater stimulation of gene expression. The results of the comparative experiment showed consistently lower C_q values for xanthine than hypoxanthine, although the difference was relatively small. Nevertheless, for the experiments that involved xanthine as substrate, the precision of the quantification was superior.

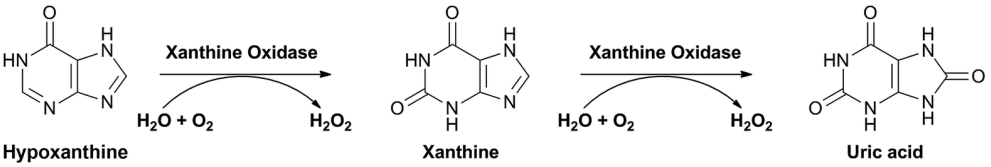

Fig. 9. Xanthine oxidase catalyzed reactions (source: (Rodrigues et al. 2015))

The present study was supported by the RNA-Seq analysis which revealed several DEGs in response to the oxidative stressor challenge. To the best of our knowledge, RNA-Seq has not previously been exploited for the purpose of developing methods for viability assessment of *Cryptosporidium* oocysts.

Among the six target genes evaluated, COWP7 and thioredoxin were selected for further evaluation. This was due to the high log fold change for each gene. Also, COWP7 was selected because of the putative role of COWP genes in protecting the oocysts and surviving environmental stresses (Templeton et al. 2004). In addition, COWP7 was the only gene, among the six genes examined that contained introns, making it a suitable candidate for RT-qPCR method. An additional reason for selecting thioredoxin was because of its potential role in the

oxidative stress response, as it is a well-known anti-oxidant enzyme that protects cells from cytotoxicity elicited by oxidative stress (Nishinaka et al. 2001).

Evaluation of the RT-qPCR method using oocysts exposed to various inactivating treatments indicated that this method may have value as a method of assessing the efficacy of different treatments at inactivating *Cryptosporidium* oocysts. The use of 18s rRNA as normaliser allows the estimation of log-reduction of different treatments. Inclusion of untreated control samples along with test samples would enable estimation of log-reductions in oocyst viability due to selected inactivation treatments.

The inactivation efficacy of freezing at -20°C was time dependent, with longer storage duration giving more effective the inactivation; although oocyst viability was not affected by freezing for 2 h, complete inactivation was seen after 24 h. These findings corroborate the results reported by Fayer and Nerad (1996), in which the mouse infectivity assay was employed to test the effect of low temperatures on the viability of oocysts. According to the results of that study, mice fed with the oocysts stored at -20°C for 1 h, 3 h, 5 h, and 8 h were infected, as indicated by the histological examination of the colon, ileum, and cecum of the mice showing the parasite's developmental stages (Fayer & Nerad 1996).

According to the novel RT-qPCR method presented here, chemical treatment of oocysts with 0.2 and 4 mg/L free chlorine for 30 min at room temperature did not affect the viability of the oocysts. It has long been known that *Cryptosporidium* oocysts are incredibly resistant to chlorination (Chauret et al. 2001; Korich et al. 1990), so although the results are not surprising

they demonstrate and confirm that the novel RT-qPCR method could be a useful method for determining the inactivation efficiency of different chemical treatments.

Complete inactivation of the oocysts after heating at 80°C for 3 min agrees with the findings of Travaillé et al., (2016), in which mice fed with oocysts heated at 80°C for 2 min were not infected. In addition, Travaillé et al., (2016) reported that mRNA could not be detected when mRNA extraction was performed after incubation of the sample at room temperature for 1 h. However, in contrast, our results were different, as mRNA was detected even after incubation of the heat-treated oocysts at room temperature for 3 h. This could be due to differences in the stability of mRNA as it has been shown that HSP70 mRNA is more stable than β-tubulin mRNA (Chambers 2005). Indeed, in the present study, it was shown that thioredoxin was more likely to be detected than COWP7.

The novelty of the present RT-qPCR method lies in the application of oxidative stressor challenges to distinguish live from inactivated oocysts, as the method exploits the relative quantification of genes expressed in response to oxidative stress. This differs significantly from the assumption used in various other efforts to develop viability assays that simple mRNA detection is indicative of viability. As we see here, and has been reported previously, mRNA can be detected in inactivated oocysts. The evaluation of the novel RT-qPCR method on oocysts subjected to different inactivating conditions such as freezing, heating, and bleach exposure demonstrates that this approach may be of value in testing the efficacy of different inactivation/disinfection regimens.

However, the present study is not without limitations and further validation should be performed, including comparison with the gold standard method (i.e., mouse infectivity bioassay). As we were unable to perform bioassays, the in-house evaluation of the novel RT-qPCR method was designed to include the parameters of inactivation treatments that had been assessed by bioassay in previous published work (Fayer & Nerad 1996; Travaillé et al. 2016).

Another limitation of the novel RT-qPCR method is that the sensitivity of the method is poor. This could be due to the mild lysis protocol used during the RNA extraction. This means that the method is not suitable for assessing the viability of oocysts detected in water samples, or other environmental matrices, as only low numbers of contaminating oocysts are usually detected. However, further optimisation may improve the sensitivity. The lysis approach used in the RNA extraction protocol was the same as that used for the RNA-Seq analysis, which is specifically intended to result in high-quality RNA. However, such high-quality RNA might not be necessary for RT-qPCR. Therefore, optimisation of the RNA extraction protocol to increase RNA yield, and thus the sensitivity of the RT-qPCR, may be a pertinent approach. However, it is important that the RNA yield is not achieved at the expense of the quality required for the RT-qPCR protocol.

5. Conclusions

The findings of the present study resulted in the following conclusions.

- The present study showed that RNA-seq and DEG analysis are indispensable tools in the identification of targets when developing RT-qPCR based methods for viability testing.

- Oxidative stressors are suitable for inducing mRNA expression of selected genes in *Cryptosporidium* oocysts.
- Evaluation of the novel RT-qPCR method showed that it could be used for testing the efficacy of different inactivation treatments on cryptosporidium oocysts.
- The present RT-qPCR method offers a reliable, rapid and cost-effective alternative to other approaches for assessing viability of *C. parvum* oocysts.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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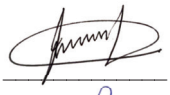
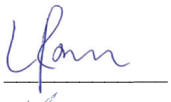
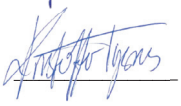
1.	Tamirat Temesgen		October 20, 2020
2.	Lucy Robertson		20 October 2020
3.	Kristoffer Tysnes		20 October 2020

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