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Detection of *Cyclospora cayetanensis*, *Echinococcus multilocularis*, *Toxocara* spp. and microsporidia in fresh produce using molecular methods: – A review

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ABSTRACT

The current trend for a healthy lifestyle corresponds with a healthy diet, which is associated with regular and frequent consumption of raw fruit and vegetables. However, consumption of ready-to-eat (RTE) food without heat treatment or sufficient washing may pose a risk to consumers. Among the well-known protozoan parasites associated with RTE food and water are *Cryptosporidium* spp., *Giardia duodenalis* and *Toxoplasma gondii*. These belong among prioritized parasitic pathogens, as they are associated with numerous disease outbreaks in humans all around the world. Nevertheless, other parasitic agents such as *Cyclospora cayetanensis*, *Toxocara cati*, *Toxocara canis*, *Echinococcus multilocularis* and zoonotic microsporidia should not be neglected. Although these selected parasites belong to phylogenetically diverse groups, they have common characteristics associated with fresh produce and each of them poses a health risk to humans.

Ensuring healthy food is produced requires the standartization of laboratory methods for the detection of parasitic agents. This article reviews the molecular methods currently used in laboratories for detection of *Cyclospora cayetanensis*, *Toxocara cati*, *Toxocara canis*, *Echinococcus multilocularis* and zoonotic microsporidia in fresh produce.

Contents

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1. Introduction

On the farm-to-fork pathway there are many sources of possible contamination for ready-to-eat (RTE) food. RTE foods such as fresh vegetables, fruit, herbs and sprouts, which are usually eaten without cooking or heating, are quite susceptible to parasite contamination. Parasitic infections are often referred to as neglected diseases, although such infections may have serious consequences for human health. In the past few decades there has been an effort to reduce the global consumption of "modern diets" which are based on unhealthy fried meals and semi-finished products. These negatively influence human health and contribute to the growth in obesity worldwide. Nowadays diets are undergoing changes due to the inclusion of more fruit and vegetables in common dishes (Ramos et al., 2013). It is therefore logical that the number of outbreaks is also increasing. Identifying foodborne or waterborne outbreaks is difficult in cases involving retail purchases of produce over a large geographical area (Dawson, 2005). In many countries, the rising consumption of fresh smoothies and shakes in addition to the availability of various pre-prepared salad mixes for direct consumption without the need for any preparation contributes to the possibility of foodborne infection. It is clear that the consumption of raw, unwashed fresh products may be risky for humans in terms of the possibility of infection by certain pathogens (Tefera et al., 2018). Nowadays, waterborne parasites, together with foodborne parasites, are in the forefront of interest (European Food Safety Authority and European Centre for Disease Prevention and Control, 2018). Monitoring and risk assessment of known water-related zoonotic parasites such as *Cryptosporidium* spp., *Giardia duodenalis* and *Toxoplasma gondii* are important, but this could be expanded to include other less monitored and well-known parasites. Many articles discussing *Cryptosporidium* spp., *Giardia duodenalis* and *Toxoplasma gondii* detection have already been published. The aim of this review is to present the molecular methods of selected parasites, namely protozoa *Cyclospora cayetanensis*, helminths *Toxocara cati* and *Toxocara canis*, *Echinococcus multilocularis*, one of the smallest tapeworms, and zoonotic representatives of microsporidia (Plutzer and Karanis, 2016). It includes pre-treatment steps, DNA isolation and PCR assays used on fresh produce.

The parasites that we focus on in this study are excreted with feces into the environment as environmentally resistant stages (ERS): oocysts, cysts, eggs, spores. These ERS possess unique abilities in terms of environmental survival (weeks or months) and resistance to various temperatures and humidity. Furthermore, due to their small sizes (2–90 μm), low specific gravity, low infectious dose and resistance to routinely used chemical disinfections, there is substantial waterborne transmission (Thevenet et al., 2017; Ortega and Sanchez, 2010; Dumetre and Darde, 2003; Thevenet et al., 2005). Possible sources of infection include water (water for irrigation, water used for washing produce and other food processing), wastewater from human settlements, soil and soil environments including fecal waste from warm-blooded animals, manure and other fertilizers, animals and insects or handling by pickers and handlers (Berger et al., 2010; Dixon, 2016; Tefera et al., 2018; Geldreich, 1996; Dudlova et al., 2015). Nevertheless, all these sources are also common for many other pathogens, not only parasites. Fresh produce may be contaminated by ERS from a variety of parasites and use of the appropriate laboratory method is crucial for identification. Generally, examination of fecal samples is easier because of the high concentration of excreted parasites. Examination of food and water samples, however, requires more complex methods involving concentrating, pre-processing, isolation and detection steps. The concentration step is crucial but unfortunately varies depending on the type of matrices and parasitic species (Steele et al., 2003; Chandra et al., 2014). Studies show that the fluctuations in the prevalence of vegetables and fruit contamination are related to differences in plant morphology and structure, as well as in the way that these products are grown (Berrouch et al., 2020). For example, strawberries have an uneven surface with trichomes, which allow the ERS to adhere to the surface in contact with contaminated water or soil. In contrast, tomatoes have a smooth surface, which may tend to reduce the rate of ERS adhesion (Giangaspero et al., 2015). Rapid, specific and accurate detection of the agents of interest is necessary in order to monitor their occurrence in food and water, especially when an outbreak occurs. The use of molecular methods fulfils these requirements. Furthermore, the ability for PCR-based methods to simultaneously perform pathogen detection in a single reaction reduces operating costs. However, microscopic methods, although time consuming and not entirely species-specific, should not be ruled out. They are still used in low and middle-income countries for parasitic detection due to their low operating costs (Zarlenga and Trout, 2004).

For this publication The Web of Science® (WOS) Core Collection database (Thomson Reuters) and PubMed were used to retrieve publications related to the topic of molecular detection of selected parasitic agents and fresh produce. Only relevant articles published in English were considered.

2. Identification of selected parasites

2.1. Cyclospora cayetanensis

Cyclospora cayetanensis is a human coccidia parasite that causes cyclosporiasis (Mansfield and Gajadhar, 2004). The disease occurs worldwide with certain seasonality. Itis classified as an emerging pathogen for humans. People with this disease suffer

from diarrhea and associated anorexia, and other symptoms of gastroenteritis. The disease is more severe in immunocompromised individuals The transmission of *C. cayetanensis* oocysts in the environment is associated with water and consumption of RTE food, as confirmed by the many cases of cyclosporiasis in the United States (Varma et al., 2003; Ortega et al., 1993; Casillas et al., 2019).

In animals, many species of *Cyclospora* have been identified. *C. cayetanensis* is the only species found affecting humans. At the end of the 1970s, *Cyclospora* was first identified as the causative agent of human disease and in 1993 Ortega et al. (1993) named it *Cyclospora cayetanensis*. Relman et al. (1996) performed phylogenetic analysis of the *18S rRNA* gene of this coccidium, which showed a close relationship with other coccidia of the genus *Eimeria*. Pieniazek and Herwaldt (1997) confirmed this analysis and clarified the similarity to mammalian *Eimeria*. Oocysts of *Eimeria* (*Eimeria papillata*) were used as a surrogate for those of *C. cayetanensis* in artificial contamination experiments to detect it in fresh produce (Lalonde and Gajadhar, 2016a). The earliest paper describing the detection of *C. cayetanensis* in fresh produce is from 1998, but only one or two publications a year on *C. cayetanensis* detection in fresh produce have been published since 2013. It is common for publications to describe the detection of *C. cayetanensis* along with that of other zoonotic parasites (Orlandi and Lampel, 2000; Dixon et al., 2013; Lalonde and Gajadhar, 2016a; Temesgen et al., 2019b; Pineda et al., 2020). The advantage for *C. cayetanensis* is the existence of a validated regulatory method (FDA Method, 2004) which is regularly updated. These updates are essential, as the original method involved the use of FTA filters, which are no longer available. Even so, only one study detailed the detection of *C. cayetanensis* in cranberries using the aforementioned filters (Orlandi and Lampel, 2000). In 2017, a validated new U.S. Food and Drug Administration method (FDA BAM) was developed for detection of *C. cayetanensis* in produce using real-time PCR. Improved sample preparation together with a real time PCR assay provides a rapid, robust, and less laborious procedure for detecting *C. cayetanensis* (Murphy et al., 2017).

The types of analyzed fresh products are summarized in Table 1, which includes the quantity and selected sample matrices. The studies that focus on herbs (basil) and berries (strawberries, raspberries) use maximum amounts of 25/30 g and 50/100 g, respectively, for the analysis. Leafy greens and other kinds of vegetables are analyzed less often. The largest sample volumes were used for analyzing the external parts of melons, cucumbers or tomatoes (1000 g; Giangaspero et al., 2015). In addition to the amount of sample, it is also important to consider pre-treatment washing and subsequent selection of the kit for DNA isolation. A third of the studies did not include pre-treatment. Pre-treatment usually consists of freeze-thaw cycling in liquid nitrogen or bead beating. One study describes only the freeze-thaw cycling for DNA "isolation" from *C. cayetanensis* oocysts (Jinneman et al., 1998). In a large number of studies QIAGEN kits were used for DNA isolation. Raspberries and strawberries are matrices often used for *C. cayetanensis* detection. In such matrices, PCR analysis is challenging due to the presence of many inhibitory substances and the low concentration of parasitic agents. When inhibition of qPCR is observed in the detection of *C. cayetanensis* in cilantro, Assurian et al. (2020b) recommended using various DNA clean-up commercial kits. Therefore, care must be taken in the washing of samples (Murphy et al., 2017).

As shown in Table 2, four detection targets are used for the detection of *C. cayetanensis* in fresh produce. The oldest and most commonly used target is the *18S rRNA* gene. To ensure specificity and to differentiate between *Cyclospora* and *Eimeria*, nested PCR combined with restriction fragment length polymorphism analysis (RFLP) was used (Dixon et al., 2013; Shapiro et al., 2019). The *18S rRNA* target was used in both nested PCR and real time PCR. When using real time PCR, the amplicon products are shorter and therefore primers are designed differently for nested PCR. The conserved nature of the *18S rRNA* gene among coccidia means that there is potential for cross reactivity with other related coccidian species. This provides a challenge in developing more specific detection methods based on other targets. Other *C. cayetanensis* specific primers includes internal transcribed spacer 2 (*ITS 2*), the *hsp70* gene and internal transcribed spacer 1 (*ITS 1*; Lalonde and Gajadhar, 2008, Shields et al., 2013, Temesgen et al., 2019b). The study on berries shows that the limit of detection for qPCR analysis focused on the ITS region can be 10 oocysts/30 g of berry fruits and approximately 32, 12.8, and 6.4 pg of DNA roughly estimated to be equivalent to 5, 2, and 1 oocyst based on gene copy number respectively. However, the high variability in the *ITS 1* region for further source observation and in epidemiological studies makes the design of primers and probes of all isolates demanding (Temesgen et al., 2019b).

2.2. Echinococcus multilocularis

One of the smallest tapeworms, *Echinococcus multilocularis*, is of interest in Central and Eastern Europe and is emerging in parts of North America and Asia (Bouwknegt et al., 2018; Szostakowska et al., 2014). *E. multilocularis* is responsible for a disease known as alveolar echinococcosis (AE). AE is caused by the larval stage of *E. multilocularis* which creates characteristic lesions similar to a tumor-like growth in the affected organ, predominantly the liver. The symptoms of human AE tend to be severe (e.g. weight loss, abdominal pain, general malaise and signs of hepatic failure), even potentially lethal. A major complication in diagnosis is that the cause of the disease may not be detected and the illness may last for years without proper treatment (Eckert and Deplazes, 2004). Infection in humans is caused by direct or indirect ingestion of *E. multilocularis* eggs excreted by a definitive host (fox and other canids) through its feces into the environment (soil, water). Infection among foxes is widely distributed throughout the northern hemisphere and is associated with the occurrence of intermediate hosts (rodents). The occurrence of infection is

Table 1
Cyclospora cayetanensis: sample, pre-treatment and molecular detection methods.

Origin/item	Spiking (No of oocysts) No of positive/tested	Amount (g)	Pre-treatment	DNA isolation	Detection method	Recovery or LOD (oocyst)	Reference
Raspberry wash sediment extract	YES (50)	250 g	6 × 2 min in N_2/98 °C	DNA released by F-T	nested PCR	25	Jinneman et al. (1998)
Raspberries	YES (N/A)	100 g	NO	6 mm FTA filters	nested PCR	10–30/100 g	Orlandi and Lampel (2000)
Basil leaves	YES (10^1–10^3)	30 g	8 × 1 min in N_2/98 °C	QIAamp DNA microkit or DNeasy blood and tissue kit	PCR	10 in 9/15; 1 in 2/15 sampl	Lalonde and Gajadhar (2008)
Raspberries, basil, mesclun lettuce	YES (10–4000)	100 g	NO	QIAamp DNA Stool Mini Kit	nested PCR	LOD: tens of unit[a]; Recovery: 5/50 g	Steele et al. (2003)
Washes from raspberry, basil, pesto	YES (50 or 500, 5000)	25 g basil; 50 g raspberries	NO	FastDNA SPIN Kit for soil, UltraClean™ Soil DNA Isolation Kit, QIAamp DNA Mini Stool Kit, UNEX method	nested PCR; qPCR	LOD: 1 copy of gene	Shields et al. (2013)
Leafy greens	NO (9/544)	25 g	5 × 2 min in N_2/90 °C	Qiagen DNeasy Blood and Tissue Kit	nested PCR	N/A	Dixon et al. (2013)
Basil	YES (100 or 1000)	25 g	NO	FastDNA Spin for Soil Kit	nested PCR	N/A	Chandra et al. (2014)
Cucumber, lettuce, fennel, celery, tomato, melon, chicory	NO (6/49)	100 g external leaves of vegetable[b]; 1000 g melon or tomato	NO	Nucleospin tissue/stool kit	qPCR	N/A[c]	Giangaspero et al. (2015)
Leafy greens (herbs, green onions), berry fruits	YES (5000 *E. pappilata*)	35 ± 0.5 g	8 × 1 min in N_2/98 °C	QIAamp DNA Mini Kit	qPCR	5 of *E. papillata*/g	Lalonde and Gajadhar (2016b)
Leafy greens[d]	NO (6/1171)	35 ± 0.5 g	8 × 1 min in N_2/98 °C	QIAamp DNA microkit/DNeasy blood and tissue kit	qPCR	Recovery: 3%–18%	Lalonde and Gajadhar (2016a)
Packaged salads[e]	NO (1.3%/64)	100 g	15 × 1 min N_2/65 °C	QiAmp Plant Mini Kit	qPCR	46–1580/g	Caradonna et al. (2017)
Perilla leaves, winter-grown cabbages, chives, sprouts, blueberries, cherry tomatoes	NO (5/44)	20 g perilla; 30 g sprouts; 50 g rest	NO	QIAquick stool mini kit	qPCR, nested	13–348/g	Sim et al. (2017)
cilantro, raspberries	YES (0, 5, 10, 200)	25 g cilantro; 50 g raspberries	BB	FastDNA SPIN Kit for Soil	nested PCR, qPCR	N/A	Murphy et al. (2017)
Cilantro, raspberries	YES-interlaboratory (0, 5, 10, 200)	25 g cilantro; 50 g raspberries	BB	FastDNA SPIN Kit for Soil	nested PCR, qPCR	LOD: 0.2/g	Murphy et al. (2018)
Carrot, cabbage, basil, parsley, Coleslaw with dressing	YES (5 or 10, 200); 141	25 g	BB	FastDNA SPIN Kit for Soil with a FastPrep-24 Instrument	qPCR	5/25 g	Almeria et al. (2018)
Strawberries, blueberries, raspberries	YES (10, 50)	30 g	2 × BB 4 m/s for 60 s	DNeasy PowerSoil Kit	qPCR/multiplex	LOD: 10/30 g	Temesgen et al. (2019a, 2019b)
Vegetables, fruits[f]	NO (2/1099) (0,2%)	25 g	NO	E.Z.N.A.R® Stool DNA Kit	nested PCR	N/A	Li et al. (2019)
Spinach	YES (10^1–10^4)	10 g	1 × 4 min N_2/100 °C	DNeasy Blood and Tissue Kit	nested multiple, qPCR, RFLP	1–10/g	Shapiro et al. (2019)
Berry fruit	YES (20,200)	30 g	BB	DNeasy PowerSoil kit/UNEX-based DNA extraction	qPCR	10/30 g	Temesgen et al. (2020)
Fresh and frozen berries[g]	YES (1,20)	50 g	BB	FastDNA SPIN Kit for Soil	qPCR	5/50 g	Assurian et al. (2020a)
Fresh berries, berry farm soil	YES ($0-10^3$)	50 g	NO	ZymoBIOMICS DNA Kit	nested PCR	1/g	Resendiz-Nava et al. (2020)

Table 1 *(continued)*

Origin/item	Spiking (No of oocysts) No of positive/tested	Amount (g)	Pre-treatment	DNA isolation	Detection method	Recovery or LOD (oocyst)	Reference
Cilantro	YES (5, 10, 200)	25 g	BB	FastDNA SPIN Kit for Soil with a FastPrep-24[h]	qPCR		Assurian et al. (2020b)
Strawberries	NO (1/120)	30 g	BB	DNeasy PowerSoil kit	multiplex PCR		Pineda et al. (2020)

LOD = limit of detection.
BB = bead beating.
[a] 40 oocysts/100 g raspberries, 10 oocysts/100 g basil, 1000 oocysts/100 g mesclun lettuce.
[b] Lettuce, fennel, celery or the external part of cucumber.
[c] The number of DNA copies per µl was calculated by correlating the Ct mean value, with the number of oocysts calculated assuming that an oocyst contains 15 copies of rDNA, depending on the stage of sporulation.
[d] Spinach, spring mix, leaf lettuce, romaine, kale, arugula, chard, collards, dandelion greens, rapini.
[e] Mixed salad (curly and escarole lettuce, red radish, rocket salad and carrots).
[f] Lettuce, coriander, celery, baby bok choy, leaf lettuce, water spinach, crown daisy, fennel plant, endive, spinach, schizonepeta, cabbage, leaf mustard, Chinese chive, chive and the stripped epidermis of cucumber, watermelon, potato, bean, green chili.
[g] Blackberries, strawberries, blueberries and mixed berries.
[h] Five DNA cleanup commercial kits: QIAquick® Purification kit, One step™ PCR inhibitor removal, Nucleospin® Genomic DNA clean up, DNA IQ™ system and DNeasy® Power Plant® Pro kit.

also influenced by climatic conditions (Miterpakova et al., 2006)Since *E. multilocularis* eggs are sensitive to desiccation and high temperatures, positive canids were more often found in areas with higher humidity (Veit et al., 1995).However, eggs can remain infectious in environmental conditions with residual humidity for up to one year (Otero-Abad and Torgerson, 2013; Veit et al., 1995). To date there is little information about the risk of *E. multilocularis* infection from fresh products. With increasing numbers of foxes (with territories near to human settlements) and the popularity of RTE food consumption, there is a possible risk of AE (Bastien et al., 2018).

Microscopic identification of ERS is difficult because the eggs of all *Echinococcus* and *Taenia* species are morphologically indistinguishable. Due to the microscopic size of the eggs concentration, often by flotation is recommended. However, the diagnostic sensitivity of double flotation-based protocols in the detection of taeniid eggs is only about 50% (Liccioli et al., 2012). After DNA isolation, Taenid eggs can be differentiated to the species level with PCR-based assays. This workflow is recommended (Trachsel et al., 2007)and standardized (Dinkel et al., 2011) for fecal samples. It is necessary to specify the laboratory concentration method for matrices such as fruit or vegetables. All the articles dealing with PCR detection of *E. multilocularis* in fresh produce have been published in the last five years (Table 3). Many types of fruit and vegetables have been analyzed for the presence of *E. multilocularis*. The sample volume ranged from 30 to 1800 g. This extremely high amount occurred in only one study, in which 1–40 heads of lettuce were examined (Federer et al., 2016). Malkamaki et al. (2019a) used a sample volume of 250 g of bilberries which proved to be the maximum volume suitable for further processing (sieving) of the sample. Eggs are concentrated using centrifugation or sieving, or more commonly by flotation. After the concentration step and before DNA isolation, a pre-treatment can be included. Pre-treatment can comprise of freez/thaw cycles (cycling of −70 and 30 °C) or bead beating. Although various isolation kits were used there is no apparent preferred commercial kit for DNA isolation of *E. multilocularis eggs*.

Originally, nested PCR was used as a detection method, but in recent studies the real time PCR targeting *nad1* or *12S rRNA* genes is preferred. Studies dealing with the detection of *E. multilocularis* in berries using specific PCR or broadly specific PCR analyses can target other foodborne zoonotic parasites such as *Toxoplasma gondii* and *C. cayetanensis* (Temesgen et al., 2019a) or other *Cestoda* (Malkamaki et al., 2019a) as well. Almost all publications listed in Table 3 first evaluate the detection method or the entire process (e.g. determination of the limit of detection, recovery, rinsing method, wash solution, DNA extraction kit etc.) by using artificial contamination of RTE foods (Frey et al., 2019). After evaluation of the whole isolation and detection procedure, detection of *E. multilocularis* in real samples follows. Non-zoonotic tapeworms can be used for validation. *Taenia pisiformis* eggs were used for spiking romaine lettuce and strawberries (Frey et al., 2019) and *Taenia laticollis* eggs for bilberries and lingonberries (Malkamaki et al., 2019b). The study using *T. laticollis* eggs also tested how many eggs can adhere to the surface of the berries in fresh or frozen conditions, and the authors described interesting data demonstrating that the freezing of fresh vegetables has a negative effect on the recovery of *E. multilocularis* eggs from the matrix (Malkamaki et al., 2019b).

Lass et al. (2015) carried out the first study dealing with PCR detection of *E. multilocularis* eggs in real samples (Table 4). A total of 103 samples of berries, mushrooms, vegetables (carrots, beets, celery, radishes, lettuce) and herbs (parsley) were analyzed. The samples originated from Polish forests, kitchen gardens and plantations. Analyzed samples were positive in 23.3% of cases, which is an unusually high number. However, it should be noted that the explored area is part of the region with the highest rate of human AE cases in Poland. In contrast, no positive samples (strawberries) were found in Colombia, a country that is outside of the geographical distribution of *E. multilocularis* (Pineda et al., 2020). One year later Federer et al. (2016) focused on vegetables (lettuce) and other fruits intended for animal feed (primates in a zoo). The samples originated from greenhouses or fields in the Basel region in northern Switzerland and from various wholesalers throughout Europe. *E. multilocularis* was not present in the samples but other taenid DNA was detected in a large number of samples, leading to the conclusion that samples of fruit and vegetables from area with infected canids could be a source of contamination.

Table 2
Summary of PCR oligonucleotides used for the specific detection of *Cyclospora cayetanensis* in fresh produce.

Type of PCR	Target	Sequence	Amplicon size (bp)	Reference
Nested[a]	*18S rRNA*	1st: CYCIFE: 5'-TACCCAATGAAAACAGTTT-3'; CYC2RB: 5'-CAGGAGAAGCCAAGGTAGG-3′ 2nd: CYCF3E: 5'-CCTTCCGCGCTTCGCTGCGT-3'; CYC4RB: 5'-CGTCTTCAAACCCCCTACTG-3'	294	Jinneman et al. (1998)
		1st: CYCIFE + CYC2RB 2nd: CYCF3E + CYC4RB		Dixon et al. (2013)
nested		1st: F1E: 5′-TACCCAATGAAAACAGTTT-3′; R2B: 5′-CAGGAGAAGCCAAGGTAGG-3′ 2nd.: F3E: 5′-CCTTCCGCGCTTCGCTGCGT-3′; R4B: 5′-CGTCTTCAAACCCCCTACTG-3′	636 294	Orlandi and Lampel (2000)
		1st: F1E + R2B 2nd: F3E + R4B		Sim et al. (2017)
		1st: F1E + R2B 2nd: CC719: 5′-GTAGCCTTCCGCGCTTCG-3′; CRP999: 5′-CGTCTTCAAACCCCCTACTGTCG-3′	636 298	Shields et al. (2013)
		1st: F1E + R2B 2nd: CC719 + CRP999	636	Murphy et al. (2017, 2018)
		1st: CYCIFE + CYC2RB 2nd: CC719 + CRP999	630 298	Resendiz-Nava et al. (2020)
		1st: CYCF IE: 5'-GGAATTCCTACCCAATGAAAACAGTTT-3'; CYCR2B: 5'-CGGGATCCAGGAGAAGCCAAGGTAGG-3' 2nd: CYCF3E: 5'-GGAATTCCTTCCGCGCTTCGCTGCGT-3'; CYCR4B: 5'-CGGGATCCCGTCTTCAAACCCCCTACTG-3'	101	Steele et al. (2003)
		1st: ExCycF: 5′-AATGTAAAACCCTTCCAGAGTAAC-3′; ExCycR: 5′-GCAATAATCTATCCCCATCACG-3′ 2nd: NesCycF: 5′-AATTCCAGCTCCAATAGTGTAT-3′; NesCycR: 5′-CAGGAGAAGCCAAGGTAGGCRTTT-3	N/A	Li et al. (2019)
		1st: ExCycF: 5′-AATGTAAAACCCTTCCAGAGTAAC-3′; ExCycR: 5′-GCAATAATCTATCCCCATCACG-3′ 2nd: NesCycF: 5′-AATTCCAGCTCCAATAGTGTAT-3′; NesCycR: 5′-CAGGAGAAGCCAAGGTAGGCRTTT-3′	500 294	Chandra et al. (2014)
		HMPr46: 5′- TCGTGATGGGGATAGATTA-3′; HMPr43: 5′- GCTCTATTTACGCAACTTTC-3′; HMPro61 FAM: 5′-CTGGTCAGTCCAATGAGTTCACA-3′	200	Shapiro et al. (2019)
nested multiplex-[b,c]		1st: m18SeF: 5′-CGGGTAACGGGGAATTAGGG-3′; m18SeR: 5′-TCAGCCTTGCGACCATACTC-3′ 2nd: m18ScycF: 5′-TCGTGGTCATCCGGCCTT-3′; m18ScycR: 5′-TCGTCTTCAAACCCCCTACTG-3′	751–779 359	Shapiro et al. (2019)
qPCR MCA		Cyclo: 5′-AGTGACGAGAAATAACAATACAGG-3′; Cyclo: 5′-CCTGCTTGAAACACTCTATTTTTC-3′	315	Lalonde and Gajadhar (2016a, 2016b)
qPCR		HMPr46: 5′-TCGTGATGGGGATAGATTA-3′; HMPr43: 5′-GCTCTATTTACGCAACTTTC-3′; HMPro61: 5′-CTGGTCAGTCCAATGAGTTCACA-3′	200	Shields et al. (2013)
		Cyclo250F: 5'-TAGTAACCGAACGGATCGCATT-3'; Cyclo350RN: 5'-AATGCCACGGTAGGCCAATA-3'; [d]dd.IAC: 5'-CTAACCTTCGTGATGAGCAATCG-3'; [d]dd-IAC: 5'-GATCAGCTACGTGAGGTCCTAC-3'	101	Murphy et al. (2017, 2018))
		Cyclo250F + Cyclo350RN; dd.IAC: + dd-IAC		Almeria et al. (2018)
		Cyclo250F + Cyclo350RN; dd.IAC: + dd-IAC		Assurian et al. (2020a, 2020b)
SNP		F1E-R2B: 5′-CAGGAGAAGCCAAGGTAGG-3′; CC719: 5′-GTAGCCTTCCGCGCTTCG-3′	298	Orlandi et al. (2003)
qPCR	*hsp70*	HMPr36: 5′-GGGTAAGCCACTTATTGA-3′; HMPr40: 5′-GCCTCCTTAACTTCTTTG-3′; HMPro53: 5′-CCTTCATCTTCACCAGCACCA-3′	132	Shields et al. (2013)
conventional	*ITS-2*	CCITS2: 5′-GCAGTCACAGGAGGCATATATCC-3′; CCITS2: 5′-ATGAGAGACCTCACAGCCAAAC-3′	116	Lalonde and Gajadhar (2008)
		CCITS2 + CCITS2		Giangaspero et al. (2015)
qPCR MCA		CCITS2 + CCITS2		Caradonna et al. (2017)
qPCR		CCITS2 + CCITS2; Probe-HEX: 5′-CGACGAACAGCCACGCACGCACTTG-3′		Sim et al. (2017)
qPCR	*ITS-1*	CyITS1_TT: 5 -ATGTTTTAGCATGTGGTGTGGC-3′; CyITS1_TT: 5′-GCAGCAACAACAACTCCTCATC-3′; probe CyITS1_TT-P: HEX-TACATACCCGTCCCAACCCTCGA-MGBEQ; +IAC Phocine herpesvirus-1(PhHV1)	141	Temesgen et al. (2019a, 2019b, 2020); Pineda et al. (2020)

MCA = melting curve analysis.
SNP = single nucleotide polymorphism.
[a] After nested PCR RFPL from *MnlI* restriction enzyme was used to differentiate *Cyclospora* and *Eimeria* organisms.
[b] After nested PCR RFPL from *BsaBI* restriction enzyme was used to differentiate *Cyclospora*, *Eimeria*, *Cryptosporidium*, *Giardia* organisms.
[c] Primers m18SeF and m18SeR not specific only to *C. Cayetanensis.*
[d] Internal amplification control artificially designed, showing no homology to sequences in GenBank.

2.3. Microsporidia

Microsporidia are obligate intracellular pathogens which form durable spores that survive in the environment and infect animals, humans or invertebrates (Wittner, 1999)There are more than 1200 species of microsporidia, however only four are known to be pathogenic to humans (*Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Ecephalitozoon cuniculi, Encephalitozoon hellem*) and can cause microsporidiosis. The spores are shed with feces or urine in the environment (soil, water etc.). Infection

Table 3
Echinococcus multilocularis: sample, pre-treatment and molecular detection methods.

Origin/item	Amount (g)	Pre-treatment	DNA isolation	Detection method	Spiking (egg amount)/No of positive/tested	Recovery (No. of eggs)	LOD	Reference
Fruit, vegetable, mushrooms[a]	300–500 g fruits/mushrooms, 500 g vegetables, one head of lettuce, two bunches dill or chives	3× F-T[b]	Sherlock AX Kit AntyInhibitor Kit	nested PCR	YES (10^1–10^3) 24/103	100	N/A	Lass et al. (2015)
Lettuce, fruits and other vegetables	at least 40 heads lettuce, 1400 g vegetables and fruits.	NO[b]	Chelex-100 + Qiamp DNA mini kit	conventional PCR	NO 0/141[c]	N/A	N/A	Federer et al. (2016)
Fruit, vegetable, mushrooms[a]	300–500 g fruits/mushrooms, 500 g of vegetables, one head of lettuce, two bunches of dill or chives	3× F-T[b]	Sherlock AX Kit AntyInhibitor Kit	nested PCR	NO 7/104	100	N/A	Lass et al. (2017)
Leafy greens, berries (romaine lettuce and strawberries)	35 g romaine lettuce, 55 g strawberry samples	BB 8× F-T	FastDNA™ SPIN Kit for soil (include BB), QIAamp® DNA Stool Mini Kit (include F-T)	qPCR	YES[d]	5/sample	N/A	Frey et al. (2019)
Bilberries	250 g	NO[e]	Tissue and Hair Extraction Kit + OneStep™ PCR Inhibitor Removal Kit	qPCR	YES (0, 1, 5, 50) 0/42	sensitivity 100% with 3 eggs	4.37×10^{-5} ng/μl	Malkamaki et al. (2019a)
Bilberries, lingonberry	26–300 g				YES (5, 10, 50, 100, 500)			Malkamaki et al. (2019b)
Berries (raspberries or blueberries)	30 g	BB	DNeasy PowerSoil Kit +2× BB 4 m/s for 60 s	Multiplex qPCR	YES (5, 10	5	5 eggs/30 g assay could detect 1 egg of *EM*	Temesgen et al. (2019b)
Strawberries	30 g	BB	DNeasy PowerSoil Kit +2× BB 4 m/s for 60 s	Multiplex qPCR	NO 0/120	2 eggs/sample	N/A	Pineda et al. (2020)
Lettuce	300 g, 900–1800 g (totally 158 kg ~1413 lettuce heads)	NO[b,f]	Chelex-100 + Qiamp DNA mini kit		YES (2, 4, 10, 20, 40, 120); 2/157		N/A	Guggisberg et al. (2020)

F-T = cycles of freezing at −70 °C and thawing at 30 °C.
BB = bead beating.
EM = *Echinococcus multilocularis*.
[a] Berry, mushroom, carrot, parsley, beet, celery, radish, lettuce, dill.
[b] Before DNA isolation, the sample was concentrated by flotation using $ZnCl_2$ solution, usage of sieves.
[c] Detected *Echinococcus granulosus* in 2 samples.
[d] Spiked by *Taenia pisiformis*; lettuce: 500, 100, 50, 10, 5; strawberries 100, 50, 10, 5.
[e] Before DNA isolation, the sample was concentrated by usage of sieves.
[f] Before DNA isolation, the sample was concentrated by centrifugation.

becomes symptomatic predominantly in immunocompromised individuals, mostly as gastrointestinal illness in the form of mild or severe diarrhea that can become chronic. Infection of the respiratory, reproductive, muscle, excretory, and nervous systems has also been reported (Mathis et al., 2005). Human pathogenic microsporidia have been detected in drinking water, wastewater, groundwater and irrigation water (Izquierdo et al., 2011; Thurston-Enriquez et al., 2002; Ghosh and Weiss, 2009). These findings indicate that waterborne route of transmission is possible and poses a risk of direct infection or the contamination of fresh produce (Jedrzejewski et al., 2007; Thurston-Enriquez et al., 2002).

Microsporidia occurrence on fresh vegetables is rarely monitored. There are only two studies dealing with the detection of microsporidia on fresh produce using molecular methods (Table 5). Although the detection of microsporidia in water by PCR was described in 1998 (Dowd et al., 1998), all other studies dealing with fresh produce are from the last two years. These articles do not focus on the method of detection but on the testing of real samples. Javanmard et al. (2018) and Li et al. (2019) focused on sample processing and PCR method detection. Use of species-specific pair primers or general primer sets for the amplification of all *Encephalitozoon* spp. for genotyping in environmental samples (water, fresh produce) is beneficial and leads, for example, to

Table 4
Summary of PCR oligonucleotides used for detection of *Echinococcus multilocularis* in fresh produce.

Type of PCR	Target gene	Sequence	Amplicon size (bp)	Note	Reference
nested	*12S rRNA*	1st: p60for: 5′-TTAAGATATATGTGGTACAGGATTAGATACCC-3′ p375rev: 5′AACCGAGGGTGACGGGCGGTGTGTACC-3′	373	final PCR products sequenced	Lass et al. (2015, 2017)
		2nd: Em.nest/for: 5′-GTGAGTGATTCTTGTTAGGGGAAGA-3′ Em. nest/rev: 5′-ACAATACCATATTACAACAATATTCCTATC-3′	204		
qPCR (triplex)		EmMGB_F: 5´-GTGCTGCTYATAAGAGTTTTTG-3′; EmMGB_R: 5´-CTATTAAGTCCTAAACAATACCATA-3′; EmMGB_P FAM-ACAACAATATTCCTATCAATGT-MGBEQ	77	other targets: *TG* and *CC*	Temesgen et al. (2019b), Pineda et al. (2020)
conventional (triplex)[a]	*nad1*	Cest 1: 5′- TGC TGA TTT GTT AAA GTT AGT GAT C-3′; Cest2: 5′-CAT AAA TCA ATG GAA ACA ACA ACA AG-3′	395	PCR products sequenced	Federer et al. (2016), Guggisberg et al. (2020)
qPCR-MCA		Cest 1 + Cest2		analytical limit 1 egg set by *TaP*; PCR products sequenced	Frey et al. (2019)
		EmNAD1_88: 5´-TTGTGTGCTGGTTGGGGTAG -3; R: 5´-AACAAGCTTCAAACCTAACAGACC -3′	88		Malkamaki et al. (2019a)
	–[b]	5′-TCACAGTTTCGTAAGGGTCCAAAT-3′; 5′-CCAACTAACAACAACACCCC-3′	149		Malkamaki et al. (2019b)

TG = *Toxoplasma gondii*, TaP = *Taenia pisiformis*, *CC* = *Cyclospora cayetanensis*, MCA = melting curve analysis.
[a] Other targets: *Echinococcus* spp. + other cestoda.
[b] PCR specific for *Taenia laticollis*.

Table 5
Encephalitozoon intestinalis, *Encephalitozoon cuniculi*, *Encephalitozoon bieneusi*, *Encephalitozoon hellem* and *Toxocara* spp.: sample, pre-treatment and molecular detection methods.

Origin/item	Amount (g)	Pre-treatment	DNA isolation	Detection method	Spiking; No. of positive/tested	Recovery	Reference
Vegetable[a] (*Encephalitozoon* spp. *E. bieneusi*)	250 g	BB	YTA DNA extraction kit for stool	nested PCR	NO; 5/12 (41.7%); 1/12 (8.33%)	N/A	Javanmard et al. (2018)
Vegetables[b], fruits[b] (*E. bieneusi*)	25 g	NO[c]	E.Z.N.A.R® Stool DNA Kit	conventional PCR	NO; 3.5%; 38/1099	N/A	Li et al. (2019)
Lettuce (*Toxocara* spp.)	300 g[d]	sieving	Qiamp DNA mini kit	conventional PCR	YES; (4, 20); 4/157	2 eggs/sample	Guggisberg et al. (2020)

BB = bead beating.
N/A = not available.
[a] Lettuce, coriander, celery, baby bok choy, leaf lettuce, water spinach, crown daisy, fennel plant, endive, spinach, schizonepeta, cabbage, leaf mustard, Chinese chive, chive, cucumber, watermelon, potato, bean, green chili.
[b] Before DNA isolation the sample was concentrated by centrifugation.
[c] Before DNA isolation the sample was concentrated by FDA 2017.
[d] For spiking experiments the amount was 300 g, for real samples the amount was 900–1800 g (in total 158 kg ~1413 lettuce heads).

Table 6
Summary of PCR oligonucleotides used for detection of microsporidia (*Encephalitozoon intestinalis, Encephalitozoon cuniculi, Enterocytozoon bieneusi, Encephalitozoon hellem*) and *Toxocara* spp. in fresh produce.

Type of PCR	Target gene	Sequence	Amplicon size (bp)	Note	Reference
Nested	*18S rRNA* (*Encephalitozoon* spp., *E. bieneusi*)	1st: PMicF: 5′- GGTTGATTCTGCCTGACG -3′; PMicR: 5′ — CTTGCGAGC(G/A)TACTATCC -3′	779	Positive samples sequenced.	Javanmard et al. (2018)
		2nd: EnbF: 5′- GGTAATTTGGTCTCTGTGTG -3′; EnbR: 5′-CTACACTCCCTATCCGTTC -3′	440		
		EncepF: 5′- AGTACGATGATTTGDTTG-3′; EncepR: 5′-ACATAAGTCCAAGARCACA -3′	530		
conventional	*ITS* (*E. bieneusi*)	AL4038: 5′-AGGGATGAAGAGCTTCGGCTCTG-3′; AL4040: 5′-AATATCCCTAATACAGGATCACT-3′	392	Positive samples sequenced.	Li et al. (2019)
conventional	nad2 (*Toxocara* spp.)	F: 5′-GGAGTTGTTTAAGTTGGATGG-3′; R: 5′ -AGAACTCCGCCTTATCAAGACGAC-3′	227		Guggisberg et al. (2020)

the tracing of sources of infection or to the discovery of new genotypes. Javanmard et al. (2018) aimed to evaluate the presence of zoonotic microsporidia in treated wastewater and vegetable farms (vegetable samples) irrigated with treated wastewater over the course of a year. Among detected microsporidia from examinated samples of vegetables and wastewater and samples from animals and humans originated from the same region were revealed a close phylogenetic relationship. From 1099 samples of

vegetables and fruits, Li et al. (2019) identified eight previously known genotypes and four new genotypes of *E. bieneusi*, which were named CHV1, CHV2, CHV3,CHV4 For microsporidia detection on fruit and vegetables also non-molecular methods can be used. Fluorescence in situ hybridization (FISH) assays use fluorescent probes that bind only to the parts of the DNA sequence with a high degree of sequence complementarity. The microscopy is used for assessing the DNA bounded fluorescent probe. This approach is widely used for example in human sample analysis and can be adapted for fresh produce samples (Graczyk et al., 2007; Jedrzejewski et al., 2007). A total of 80 samples of fresh produce originating from commercial grocery stores, supermarkets, street vendors and markets in Poland were tested by multiplex FISH assay, revealing human-virulent spores (*E. intestinalis*, *E. bieneusi* and *E. cuniculi*; Jedrzejewski et al., 2007; Table 6).

2.4. Toxocara cati and Toxocara canis

Another parasites distributed worldwide that are an examples of neglected zoonotic parasites are *Toxocara cati* and *Toxocara canis*, ascarid roundworms for which the hosts are felids and canids, respectively, that excrete eggs in feces into the environment. Over time the eggs develop into infective L3 larvae that are able to stay infective for months or even years, depending on the environmental conditions (Azam et al., 2012). The disease caused by *T. cati* and *T. canis* is called toxocariasis. The risk of infection for humans is high because cats usually bury their excrement in loose soil: thus vegetable beds are one of their most sought-after places. The prevalence of *T. canis* and *T. cati* infection in dogs and cats, respectively, ranges around 5% in Germany, the Netherlands and Australia. Even higher prevalence, from 1% to 45% in adult dogs and 3.2%–91% in cats, was observed in countries such as Portugal, Nigeria, India and China (Rostami et al., 2019). The estimated number of cats in Europe is over 106 million, and 87.5 million for dogs (FEDIAF, 2020; Ma et al., 2018). According to an extensive meta-analysis involving 109 studies, the estimated global prevalence of *Toxocara* eggs in public places is around 21%, depending on geographical longitude, latitude and relative humidity (Fakhri et al., 2018). Human toxocariasis can be symptomatic or asymptomatic depending on the anatomic site of the migrating parasite. *T. cati* and `T. canis` can migrate from the intestine by the circulatory system to throughout the body (liver, lungs, brain, eyes, muscles, CNS) and can encyst in these tissues. Clinical toxocariasis in humans often escapes attention due to its non-specific clinical presentation of the disease: visceral larva migrans, ocular larva migrans, neurotoxocariasis and covert toxocariasis (Rostami et al., 2019; Ma et al., 2018).

Over the last 20 years, several studies based on microscopic examination, discussed the detection of *Toxocara* spp. on vegetables. The results revealed a relatively high concentration of *Toxocara* spp. eggs on raw vegetables from local markets in northern Iran, Ethiopia, Poland, Libya, Turkey and Tunisia (Rostami et al., 2016; Tefera et al., 2014; Klapec and Borecka, 2012; Abougrain et al., 2010; Kozan et al., 2005; M'Rad et al., 2020). Only one molecular-based study focused on vegetables (lettuce). In their study, Guggisberg et al. (2020) purchased samples from farmer's markets and supermarkets in Zürich. The weight of a single analyzed sample varied from 300 g up to 1800 g of lettuce. In total, approximately 158 kg of lettuce heads was analyzed. The parasitic agents assessed were *E. multilocularis*, *T. gondii*, *T. cati* and *T.canis*. The authors improved the method for concentrating pathogens using a complex sieving system which simultaneously concentrated helminth and protozoa parasites. Such a complex approach has the potential for widespread use as a recovery and isolation method.

3. Discussion

Fruit and vegetables are principal components of a healthy diet and have been in high demand in recent years, although eating fresh, minimally processed food is not without risk. Since 1995 until now, major outbreaks of *C. cayetanensis* infection from fresh produce occurred in USA, Canada and Mexico, linked to the fresh produce originated from endemic countries (Hadjilouka and Tsaltas, 2020). From approximately 1100 globally reported outbreaks where etiological agent was identified, 4.5% were caused by parasites (Ramos et al., 2013). Many outbreaks of foodborne parasites on fresh produce are associated with products grown in countries with poor hygiene standards, water sanitation and sanitation in general (Lalonde and Gajadhar, 2016a). In spite of this, foodborne parasites are also an issue in Europe, a developed region. The FAO/WHO (2014) Risk Management recommended the following control measures to minimize the risk of contamination in the pre-harvest production phase: using properly treated water, restricting of animal access to produce, monitoring the health and hygiene of workers and providing good farm sanitation. Post-harvest recommendations include the use of treated water for washing and processing produce and washing of hands and all equipment. The basic prevention measures to reduce infectious parasites are treating domestic dogs and cats as well as stray carnivores with anthelmintics, thoroughly washing raw vegetables and fruit, and using adequate heat treatment on animal tissues (Ma et al., 2018). However, a key prevention measure for every household is to secure kitchen gardens from access by dogs, cats and wild carnivores (Poulle et al., 2017). Attention should be focused on ensuring safe fruit and vegetables and good water quality, as well as the possibility of inactivation of parasites prior to human consumption.

4. Conclusion

Molecular detection of parasitic agents in food is essentially in its infancy, in contrast to their detection in common matrices such as feces. There is no recommended procedure, and information on sample processing and detection methods (ISO standards) are spread throughout the literature. In an increasingly globalized world, involving movement of people, animals, food, the study and detection of many foodborne parasites is relevant and should be considered at the global level. At present, we must assume there is unlimited spread of parasites that were previously only considered a local issue. Furthermore, it is necessary to look at

foodborne parasites from a global perspective, taking into account the complex transport routes of food and globalization (Robertson et al., 2013). A summary of sample processing and detection methods seems to be the first step in harmonizing diagnostics and enabling us to compare results worldwide and map global trends regarding foodborne parasites.

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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