Detection methods and prevalence of transmission stages of Toxoplasma gondii , Giardia duodenalis and Cryptosporidium spp. in fresh vegetables: a review

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Abstract

One of the ways of human parasitic infection is the accidental ingestion of vegetables contaminated with parasites, which represents a major human health hazard. This non-exhaustive review aims to evaluate studies carried out on five types of vegetables (lettuce, parsley, coriander, carrot and radish) since 2000, particularly the methods used for recovery, concentration, detection and identification of protozoan parasites as *Toxoplasma gondii, Giardia duodenalis* and *Cryptosporidium* spp., and the results of each work. Various studies have determined the presence of pathogenic parasites in fresh vegetables with different rates, this variation in rate depends particularly on the detection method used which is related to each parasite and each vegetable type.

The variation in parasitic prevalence in food could be due to different factors as the geographical location, the size of analyzed samples, and the methods used for parasite detection.

Key words: *Toxoplasma*. *Giardia*. *Cryptosporidium. Vegetables. Detection. Prevalence of contamination.*

Introduction

T. gondii, G. duodenalis and *Cryptosporidium* spp. are protozoan parasites that are considered as emerging food and waterborne pathogens according to Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2012). Sometimes, these parasites are present in the environment in high numbers in their transmission stages, which are oocysts for *T. gondii* and *Cryptosporidium* spp., and cysts for *G. duodenalis.* Therefore, these forms can contaminate water and soil and may be considered as vehicles of parasitic contamination and disease outbreak. However, these parasitic forms are not capable of multiplying as bacteria, which implies the need for sensitive methods for their detection in food matrices. The rates of parasitic contamination are different from one country to another and even between regions in the same country.

Vegetables and fruits are an important part of a balanced diet due to their nutritional value (Losio *et al.*, 2015). Nowadays there is a global increase in the tendency to eat raw or slightly cooked vegetables, which may increase the risk of food borne infections (FAO/WHO, 2012). Parasitic diseases are an important health problem that can, in some cases, occur by the transmission of parasites to humans through the ingestion of contaminated water or foodstuffs as vegetables. Infections as giardiasis and cryptosporidiosis are usually characterized by diarrhea, stomach pain, and vomiting. The presence of these pathogens in fresh vegetables, even in very low concentrations, poses a high risk to the consumer (Ali *et al.*, 2004). The extent of contamination depends on various factors that include among others, the use of untreated or contaminated water for irrigation, irregular hygiene practices during post-harvest handling as well as environmental pollution (Kumar *et al.*, 2014).

In the past, the risk of human infection due to parasites was limited to distinct geographic regions, because of parasites' adaptation to definitive hosts, specific intermediate hosts and environmental conditions. These barriers have been gradually removed due to the expansion of international commerce of foodstuffs, facilitated by refrigerated transport (El Said Said, 2012). Indeed, several parasitic infections cases were linked to imported foodstuffs, for example, horse meat imported from Canada and Brazil was implicated in particular cases of toxoplasmosis in individuals in France (Pomares *et al.*, 2011), since there are virulence differences in *Toxoplasma* strains which are associated to geographic localization, the exchange of fresh or chilled meat – but not frozen –may induce the spreading of such strains (Robertson *et al.*, 2014). On another hand, imported fresh produces to non-native regions or countries were incriminated in multiple foodborne outbreaks, for example, imported raspberries from Guatemala were responsible for cyclosporiasis outbreak in Canada and the United States (Ortega and Sanchez, 2010).

The lack or the absence of adequate methods for the detection of parasites in fresh vegetables can lead to the underestimation of foodborne parasitic outbreaks or even their non-detection. In fact, standardized methods exist only for the microscopic detection of *Cryptosporidium* spp. and *G. duodenalis* in leafy green vegetables and berry fruits after immunomagnetic separation (Method 18744) (ISO, 2016). Other harmful parasites as *T. gondii* require the establishment of reliable and practical methods for their detection.

Existing studies on the prevalence of parasites are heterogeneous due to numerous factors, such as i) sampling methods, ii) detection procedures with different recovery efficiencies and also variable detection limits, iii) geographic locations, iv) levels of development between countries, as well as v) seasonal changes within countries.

The aim of this review is to compare the findings of the multiple studies carried out in order to analyze leafy greens (lettuce (*Lactuca sativa*), parsley (*Petroselinum crispum*), coriander (*Coriandrum sativum*)) and root

vegetables (radish (*Raphanus raphanistrum* subsp. *sativus*), carrot (*Daucus carota* subsp. *sativus*)) for the presence of *T. gondii* and *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts; this includes the methods used and the results obtained in order to demonstrate prevalence rates of parasitic contamination.

Literature selection criteria

In this review, we have evaluated the different monitoring methods of *T. gondii*, *G. duodenalis* and *Cryptosporidium* spp. in some fresh leafy green vegetables as lettuce, parsley and coriander, and root vegetables as carrot and radish, and we have listed the different described procedures used for the detection of the three protozoan in fresh vegetables and the prevalence rates reported worldwide. For this purpose, we have performed a search of the literature databases since 2000 (e.g., PubMed, ScienceDirect, and Google Scholar), in which, the terms "detection of parasites in vegetables", "prevalence of parasites in vegetables", "vegetables and protozoan parasites", "*T. gondii* in vegetables", "*G. duodenalis* in vegetables", "*Cryptosporidium* in vegetables", and "foodborne parasites" were applied. The obtained literature included about 200 papers relevant to the detection of protozoan and helminths parasites in various fresh vegetables. We have selected the articles investigating the presence of at least one of the parasites of interest (*T. gondii*, *G. duodenalis* or *Cryptosporidium* spp.), in marketed fresh vegetables, even if the studies involved other parasites. As we have also taken into account the studies reported on the detection of the three protozoan parasites, in different vegetables types including at least one of the vegetables in focus. This selection led to keeping 48 papers as represented in Table 1.

1. Detection of parasitic transmission stages in fresh vegetables

1.1. sampling

For the detection of *T. gondii*, *G. duodenalis* and *Cryptosporidium* spp. in fresh vegetables, the commonly consumed fresh vegetables in each study area were collected randomly from retail markets, supermarkets, or street vendors. Then, they were transported in plastic or nylon sterile labeled bags to the laboratory for parasitological analysis. Different types of vegetables were analyzed, especially leafy ones, as lettuce, parsley, coriander, etc.

Parasites were investigated in portions of about 10g-1kg of each vegetable depending on the studies. Three distinct steps were followed: elution, concentration and detection. The methods used to detect parasites and their prevalence in fresh vegetables, according to different studies, are summarized in Table 1.

1.2. Elution

The elution step aims to extract parasites from vegetable samples. It is crucial since all subsequent steps depend on the efficiency of (oo)cyst recovery. Therefore, the use of adequate elution buffers and efficient separation (isolation) methods is required to maximize the (oo)cyst recovery as well as the accuracy of the results. Various elution methods were evaluated for the elution of protozoan parasites from vegetable matrices. Indeed, Cook *et al.*, (2006) evaluated different elution buffers (e.g., PBS [phosphate-buffered saline], pH 7.2; 0.1M tricine, pH 5.4; 1% lauryl sulfate; 1M Glycine, pH 5.5) for the detection of *C. parvum* in lettuce, as they tested various elution procedures (e.g., stomaching, pulsification, rolling, orbital shaking), coupled with immunofluorescence assay (IFA). They demonstrated that the extractants and physical extraction methods influenced the percentage of recovered oocysts. According to this study, Glycine buffer '1M Glycine', pH of 5.5 was the cheapest and effective buffer compared to the other ones, especially when it was coupled with stomaching for 1min (recovery efficiency "RE" of *C. parvum* oocysts from lettuce was 59.0±12.07%). However, using similar methods, the recovery of parasites from lettuce has varied as reported by Rzezutka *et al.*, (2010) (RE of *Cryptosporidium* was

19%) and Utaaker *et al.*, (2015) (RE were 53% for *Cryptosporidium* and 33% for *Giardia*), that could be related to the number of replicates tested. Noting that slight changes in the method could affect the recovery as illustrated in the finding of Amoros *et al.*, (2010) (RE were 24.5±3.5% for *Cryptosporidium* and 16.7±8.1% for *Giardia* in fresh produce) when reducing the time of stomaching to 30 seconds. Otherwise, Shields *et al.*, (2012) compared various buffers as deionized water, 1M Glycine, pH 5.5, a detachment solution, and 0.1% Alconox®, to recover *C. parvum* and *Cyclospora cayetanensis* from lettuce, herbs and raspberries; the RE of *C. parvum* from spinach and raspberries using deionized water were 38.4±10.1% and 34.9±6.2%, respectively, whereas there was no statistically significant difference between the deionized water and 1M glycine, pH 5.5. Furthermore, they revealed that 0.1% Alconox® allowed a significant improved recovery (72.6±6.6%) of *C. parvum* oocysts from basil. On another hand, elution buffers (EB) composed of Laureth 12, 1M Tris buffer; pH 7.4, EDTA and Antifoam A were also used (Robertson and Gjerde, 2000; Bethea, 2014) and evaluated when coupled with rotating and IFA, revealing a RE of 42% for *Cryptosporidium*, and 67% for *Giardia* in lettuce, Chinese leaves, and strawberries (Robertson and Gjerde, 2000). A recent study (Shapiro *et al.*, 2019) described a method for simultaneous detection of *C. parvum*, *G. duodenalis*, *T. gondii* and *C. cayetanensis*, that coupled leaf-washing with m-PCR (multiplex Polymerase Chain Reaction) allowing higher recoveries (46% for *Cryptosporidium*, 38% for *Giardia* and 43% for *T. gondii*) with 90% certainty of their detection when contamination levels ranged from 1 to 10 (oo)cysts/g of spinach, and permitting more consistent detection of parasites compared to stomaching processing. Furthermore, various studies employed physiological saline solution to detach parasites from vegetables (Daryani *et al.*, 2008; Abougrain *et al.*, 2010; Ezatpour *et al.*, 2013; Benti and Gemechu, 2014; Alhabbal, 2015; Gabre and Shakir, 2016), followed by microscopic examination (Table 1) since it is a simple and low coast approach, but the RE were not determined.

The difference in recovery efficiencies (Table 2) between the different elution buffers could be stemmed from their composition and interaction with the foodstuffs tested. For lettuce, 1M glycine with optimal pH allowed for instance no excess debris formation and did not influence *Cryptosporidium* oocysts recovery in IMS (Cook *et al.*, 2006), contrarily to EB that has a low molarity and a neutral pH that made the recovery suboptimal. This later could be overcome by increasing both molarity and pH (Cook *et al.*, 2006). While 0.1% Alconox® that contains both synthetic detergent surfactant agents and food additives with emulsifying and dispersing properties, allowed an efficient removal of oocysts from vegetable surfaces (Shields *et al.*, 2012), but when used with stomaching, produced an excessive foam that interferes with oocyst recovery (Lalonde and Gajadhar, 2016a).

1.3. Concentration

The concentration step is usually done by centrifugation (Maikai *et al.*, 2012; Benti and Gemechu, 2014; Avazpoor *et al.*, 2015; Ahmad *et al.*, 2016; Marchioro *et al.*, 2016), as it can then be followed by isolation methods as immunomagnetic separation (IMS) (Robertson and Gjerde, 2001a; Rzezutka *et al.*, 2010; Utaaker *et al.*, 2017). This latter is recommended in the standardized method 18744 for *Cryptosporidium* spp. and *G. duodenalis* detection in fresh leafy green vegetables and berry fruits (ISO, 2016), but it is time consuming and expensive, thus not suitable for industrials trying to establish parasitic control for foodstuffs. In this context of minimizing the cost and the duration of sample treatment, Utaaker *et al.*, (2015) reduced the number of IMS reagents and modified the buffering system, while others replaced the immunofluorescence method by molecular techniques.

1.4. Detection

Among the described methods, direct microscopic examination, acid-fast stains as modified Ziehl-Neelsen staining (used for *Cryptosporidium* detection) or Lugol's Iodine stain (used to detect *Giardia*) were commonly used (Table 1), because of their low cost. Whereas compared to other methods, they were suboptimal for the detection of parasites. Indeed, Ramirez-Martinez *et al.*, (2015) reported a recovery efficiency of 20±4.3% and a detection limit of 20 cysts of *Giardia*/g of lettuce, using light microscopy, while PCR targeting the β-giardin gene was 10-fold more sensitive. Another method is the IFA, which was used for the detection of parasites (oo)cysts in various vegetable matrices, it is in fact the method recommended by the 18744 method (ISO, 2016). The combination of immunofluorescence, size and morphology allowed to identify and recognize (oo)cysts from the other particles (Miller *et al.*, 2006; Keserue *et al.*, 2012). However, the auto-fluorescence of some particles present in foodstuffs matrices could interfere with labelled (oo)cysts making the IFA unsuitable for their analysis. Besides, this method was claimed to be time consuming, requiring an expensive technology and a microscopy expertise (Ahmed and Karanis, 2018). Since the IFA does not provide the genotype of detected parasites, it was coupled to PCR to improve the specificity as used by Rzezutka *et al.*, (2010) and Utaaker *et al.*, (2017). On the other hand, different studies have described molecular approaches that allow the detection of protozoan parasites in vegetables, these methods' efficiency is strongly correlated with the recovery of (oo)cysts from vegetables as well as the DNA extraction step. Technically this later could be performed by altering cycles of freezing/thawing, which is time consuming, or using commercial kits based on a mechanical disruption of (oo)cysts walls, that was proved to be more suitable for the DNA extraction of *T. gondii* (Lass *et al.*, 2012), *G. duodenalis* and *C. parvum* from complex matrices as vegetables (Berrouch et *al.*, data not shown). Throughout the described studies, Marchioro *et al.*, (2016) conclude that the detection limits (LOD) of *T. gondii* in lettuce were 10 and 100 oocysts per µl, using a PCR targeting the B1 gene and the 529 bp fragment, respectively. Taking into account that this difference was explained by the fact that the B1 gene consists of a highly conserved region of the genome of the parasite and is repeated 35 times while the 529 bp fragment is repeated 200–300 times in the genome and corresponds to a non-coding region (Marchioro *et al.*, 2016). In addition, Hohweyer *et al.*, (2016) evaluated the LOD of *T. gondii*, *G. duodenalis* and *C. parvum* in basil using qPCR, the results were 3 (oo)cysts/g for both *C. parvum* and *G. duodenalis*, and below 1 oocyst/g for *T. gondii.* Whereas Lass *et al.*, (2012) reported a LOD of 100 oocysts *T. gondii* in radish, by qPCR. Lalle *et al.*, (2018) have also assessed the LOD of this parasite in baby lettuce at 0.5 oocyst per g, using LAMP-Chromatographic Lateral-Flow Dipstick. Although this technique requires more time compared to qPCR, it requires cheaper equipment which makes it a valuable molecular test to be established in resource limited cases (Table 2).

Currently, there is one standardized method 18744 (ISO, 2016) for the detection and enumeration of *Cryptosporidium* and *Giardia* in fresh leafy green vegetables and berry fruits. However, there are many parasites as *T. gondii* which are harmful for humans and can't be monitored using this method, since there are no monoclonal antibodies for *T. gondii* oocyst walls to perform IMS separation and IFA detection. Considering the finding of Dumètre and Dardé, (2005) where the monoclonal antibody mAbs 3G4 was described and incorporated in an IMS procedure against *T. gondii* : wall, Hohweyer *et al.*, (2016) tested the IMS Toxo method (using the monoclonal antibody mAb 3G4 coupled with Super paramagnetic beads (Activ Master Beads® #02650)) for two food matrices, basil and raspberries, and the mean recovery rates in basil were 0.2%

and 35% by microscopy and qPCR, respectively, while they were 2.0% by microscopy against 29% by qPCR, in raspberries.

2. Prevalence of *T. gondii* and *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts in vegetables:

As summarized in Table 1, fresh vegetables were found to be contaminated with protozoan parasites namely *T. gondii, G. duodenalis* and *Cryptosporidium* spp. with different rates.

2.1. Prevalence *of T. gondii* oocysts*:*

There have only been few studies on the prevalence of *T. gondii* in fresh produce that reported different rates of *T. gondii,* based on different elution methods and a variety of microscopic and molecular assays (Table 1). The contamination rates of fresh produce marketed in Canada (Lalonde and Gajadhar, 2016b) (lettuce 0%), United States of America (lettuce 2%, parsley 0%) and Peru (Bethea, 2014) (lettuce 3.6%, parsley 4.3%) were largely lower than those reported in Egypt (Ahmad *et al.*, 2016) (lettuce 41%, carrot 12.2% and parsley 5.7%) or Saudi-Arabia (Al-Megrm, 2010) (leafy vegetables 6.6%). The contamination of fresh produce with *T. gondii* could be affected by various factors (see section 3 below) as it could be underestimated since there is a loss of oocysts during the recovery procedures as demonstrated by Lass *et al.,* (2012) when comparing the results of qPCR performed with DNA extracted from *T. gondii* oocysts suspension in distilled water with the results of recovery from experimentally contaminated vegetables, in the case of vegetables samples, ten-fold loss of *T. gondii* oocysts was recorded. Furthermore, Escotte-Binet *et al.,* (2019) proved the improvement of *T. gondii* oocysts recovery when using mechanical procedure for the lysis of oocysts' walls instead of alternate freeze-thaw cycles, since *T. gondii* oocysts possess very robust cell walls, as they underlined the interference of many parameters with the efficiency of oocysts recovery as the composition of analyzed matrices. Consequently, pretreatment and detection procedures should be adapted depending on the matrices.

While many studies have been conducted on the presence of other protozoan parasites (e.g., *Giardia* and *Cryptosporidium*) that have been incriminated in numerous foodborne outbreaks worldwide, only few studies have been reported on *T. gondii* in vegetables due to the lack of standardized methods for the investigation of this parasite in food matrices and the under-recognition of this mode of transmission for *T. gondii*. Another factor that have contributed to the paucity of surveillance studies on fresh produce was the low number of reported toxoplasmosis foodborne outbreaks, indeed, there have been only two outbreaks of toxoplasmosis associated with the consumption of fresh produce (Ekman *et al.,* 2012) or juice (Morais *et al.,* 2016), both reported in Brazil. Robertson, (2016) concluded that the rarity of reported toxoplasmosis associated with the consumption of salad vegetables could be due to the many potential routes of infection for this parasite and the fact that infections are often asymptomatic.

There is no doubt that the prevalence of *T. gondii* genotypes in the environment remains unknown (Lass *et al.*, 2012), which is due, in part, to the low number of oocysts present in environmental samples. However, it is an important information for human health. Lass *et al.,* (2012) have investigated the presence of *T. gondii* in 216 fresh produce samples collected in Poland and determined two genotypes: SAG2 type I (in six samples) and SAG2 type II (in two samples), which is surprising as type II was found to be the most prevalent genotype in human disease in Europe (mainly in France) (Ajzenberg *et al.*, 2002).

2.2. Prevalence of *G. duodenalis*

Giardia duodenalis has been incriminated in numerous foodborne outbreaks. In the last one reported in El Salvador (Kasper *et al.*, 2012), the consumption of local food prepared without proper monitoring has been

suspected as a factor for this diarrheal illness. Besides, in 2016, Adam *et al.*, (2016) have analyzed all the giardiasis outbreaks reported to the Centers for Disease Control and Prevention for 1971–2011, the 242 outbreaks affecting ~41 000 persons, resulted in 15.7% from foodborne transmission. Otherwise, an expert elicitation study (Hald *et al.*, 2016) estimated the proportion of cases of giardiasis caused by the foodborne route, it indicated that the importance of the foodborne route of infection was quite similar across regions (e.g., 0.11 in African Region; 0.13 in Eastern Mediterranean Region and 0.12 in European Region). Recently, numerous studies worldwide investigated the occurrence of *Giardia duodenalis* in fresh produce, using different elution procedures and detection methods based on immunofluorescence (Robertson and Gjerde, 2001a; Amoros *et al.*, 2010; Utaaker *et al.*, 2017), fast-stains (e.g., Lugol's iodine staining) (Ahmad *et al.*, 2016; Amaechi *et al.*, 2016; Mohamed *et al.*, 2016; Alemu *et al.*, 2019) and molecular assays (Utaaker *et al.*, 2017). The contamination rates were variable, in leafy greens as lettuce; 61.5% was recorded in Spain (Amoros *et al.*, 2010), 2% in Norway (Robertson and Gjerde, 2001a), 2.7% in Egypt (Ahmad *et al.*, 2016), 0% in India (Utaaker *et al.*, 2015) and Southern Ethiopia (Alemu *et al.*, 2019), while in parsley 0% was reported in Norway (Robertson and Gjerde, 2001a), Iran (Shahnazi and Jafari-Sabet, 2010) and Turkey (Erdoğ rul and Şener, 2005), 25.5% in Egypt (Eraky *et al.*, 2014). Whereas in root vegetables as carrot, 14% was reported in India (Utaaker *et al.*, 2017), 4% in North Central Nigeria (Amaechi *et al.*, 2016) and 6.4% in Southern Ethiopia (Alemu *et al.*, 2019). The difference in contamination rates could be affected by multitude factors as detailed in section 3 bellow.

In parallel with the detection, the determination of genotypes is important to identify the source of contamination and the risk to human health. For *Giardia*, either humans or animals could be the source of contamination. The most frequent assemblages detected in humans are Assemblage A and B. The finding of Utaaker *et al.*, (2017) revealed the presence of both Assemblages A and D in fresh produce (cucumber and tomato) sold in Chandigarh, in India. Assemblage D, which was found contaminating cucumber, indicates most likely a contamination from canine feces, and probably does not represent a risk to human health. Whereas Assemblage A, which was found contaminating tomatoes, is infectious to both humans and a range of other animals, and thus it is not possible to narrow down the likely source of contamination.

2.3. Prevalence of *Cryptosporidium* spp.

Cryptosporidium spp. is considered as one of the most important foodborne parasites. It is responsible for several well-documented foodborne outbreaks worldwide (Chalmers *et al.*, 2019; Gharpure *et al.*, 2019; Robertson *et al.*, 2019) that were mainly associated with lettuce, pre-cut mixed salad leaves, apple cider, milk chicken salad and raw meat. The proportion of cryptosporidiosis outbreaks originated from food was higher in African Region (0.15) than other regions as European Region (0.10) or Western Pacific Region (0.10), as estimated by Hald *et al.*, (2016).

Fresh produce that is consumed after minimal preparation is most likely a vehicle for the transmission of *Cryptosporidium* infection (Amoros *et al.*, 2010). Therefore, several studies investigated the presence of *Cryptosporidium* in fresh produce based on molecular or immunofluorescent assays, as well as acid-fast stains (e.g., modified Ziehl-Neelsen staining) that are commonly used (Table 1). The contamination of leafy greens as lettuce was low in Canada (0%) (Lalonde and Gajadhar, 2016b) and India (0%) (Utaaker *et al.*, 2017), compared to that reported in African regions as Egypt (43.3%) (El Said Said, 2012) and (26.3%) (El Sherbini *et al.*, 2016). Similarly for root vegetables as that ranged from (0%) in India (Utaaker *et al.*, 2017) to (33.3%) in Seoul (Korea). The distribution of parasites in fresh produce could be affected by numerous factors (see section 3).

Unlike microscopic assays that are unable to identify the infectious *Cryptosporidium* species, molecular analyses allow the determination of species and genotypes that may provide information on the sources of contamination, particularly whether the parasites are likely to be from humans (*Cryptosporidium hominis*) or animals (generally C. parvum). Rzezutka *et al.*, (2010); Hong *et al.*, (2014); Lalonde and Gajadhar, (2016b) and Utaaker *et al.*, (2017) reported that DNA isolated from vegetable samples positive for *Cryptosporidium* belong to *C. parvum*, the source of contamination of fresh produce could thus be from either dirty hands or infected livestock or other animals.

3. Factors affecting the prevalence of parasitic contamination of vegetables:

The variation of the parasitic prevalence reported among the different studies could be related to multiple factors, these were: i) the processing procedures of analyzed vegetables, ii) the climatic conditions, iii) the geographic location, iv) the vegetable type, v) the quality of irrigation water and fertilizers, and vi) the source of vegetables.

3.1. Processing procedures

As detailed in section 1, there are various methods to analyze fresh vegetable samples for the presence of protozoan parasites, these methods showed variable sensitivity, efficiency and detection limits as demonstrated by several authors. Alongside these studies, Duedu *et al.*, (2014) evaluated the effect of three elution buffers namely tap water, PBS and saline (0.85%) on the recovery of *Cryptosporidium* and *Giardia*, saline was the most effective and recovered (52%) of parasites followed by PBS (34%) and tap water (14%). There was however some correlations between the methods used and the type of parasite recovered, *Cryptosporidium* was the most parasite recovered in saline whereas others as *S. stercoralis* recovered most from PBS. Concordantly, Al-Binali *et al.*, (2006) demonstrated that the use of Tris-buffer-saline for the extraction of *Cryptosporidium*, *Giardia* and other parasites, significantly increased the isolation rate (27.2%) of the parasites compared with the use of tap water (7.8%). Furthermore, Robertson and Gjerde, (2001b) evaluated factors affecting the recovery of both *Cryptosporidium* oocysts and *Giardia* cysts from vegetable produce (lettuce). These were: (i) sample weight, (ii) contribution of elution procedure to losses, (iii) IMS kit used, and (iv) strength of magnet during IMS. The evaluation indicated that the differences in sample weight (30g and 100g), rather than elution technique, IMS kit or magnet strength were responsible for the increase of (oo)cysts recovery. This later may be related to several factors, namely lighter samples required the use of smaller volumes of elution buffer which may reduce the potential for losses during manipulation and centrifugation. In addition, lighter samples required less space in the bag for washing that provided more contact space between elution buffer and samples. On another hand, Macarisin *et al.*, (2010) demonstrated that *Cryptosporidium* oocysts were capable of strongly adhering to spinach plants after contact with contaminated water and were also internalized within the leaves, that made this pathogen resist to washing. Hence samples processing using an effective elution method may result in preventing a better recovery of oocysts internalized in vegetable leaves.

Otherwise, Robertson and Gjerde, (2000) proved that both the washing procedure and the subsequent separation by IMS will have contributed to the improved recovery efficiencies of *Giardia* and *Cryptosporidium*. The IMS provides improvement by selecting the parasites from the other debris present in the concentrate. In addition, Lass *et al.*, (2012) underlined the contribution of the DNA extraction step to the improvement of *T. gondii* oocysts recovery, accordingly, the reported rates of *T gondii* in vegetables could be underestimated, because of the resistance of oocyst wall resulting in difficult *T. gondii* DNA extraction. Moreover, PCR inhibitors such as

humid acids or polysaccharides play a role in environmental samples. They may reduce the PCR sensitivity by up to 100–1,000 times.

3.2. Climatic conditions

The distribution of parasitic contamination could be influenced by seasonal changes. Indeed, Daryani *et al.*, (2008); Al-Megrm, (2010) and Fallah *et al.*, (2012) revealed that the rate of contamination was significantly higher in warm seasons than in cold ones, which may be related to the frequent use of untreated wastewater for irrigation of vegetables during spring and summer resulting in higher rate of parasitic contamination in these seasons. However, Charron *et al.*, (2004) correlated the presence of parasites to both drought and rainy periods since the increases in precipitation could intensify erosion leading to potential parasitic contamination of surface and groundwater, and decrease the effectiveness of water treatment. As the heavy rain following drought could lead to more severe runoff and risk of water contamination.

The climate could also affect (oo)cysts lifetime. In fact, Amahmid *et al.*, (1999) reported that after contamination induced by irrigation with sewage, three days were sufficient for the apparent disappearance of *Giardia* cysts. This decrease in the burden of *Giardia* cysts can be related to high temperatures and intense solar radiation, which would favor the desiccation of cysts. Indeed, factors as solar radiation, temperature, humidity and rainfall directly affect the persistence of microorganisms (Larkin *et al.*, 1978). Concordantly, Lélu *et al.*, (2012) proved that drought affect the survival of *T. gondii* oocysts as the proportion of oocysts surviving in soil after 100 days was 7,4% in dry conditions (281 mm of precipitation per year) and 43,7% in damp conditions (3648 mm of precipitation per year).

3.3. Geographic location

The geographic location reflects, in part, the levels of development and application of hygiene practices in regions/countries. For instance, Utaaker *et al.*, (2017) investigated the presence of parasites in vegetables sold in Chandigarh which is generally regarded as one of the cleanest cities in India, vegetables were contaminated by protozoan parasites, with 6% contaminated with *Cryptosporidium* oocysts and 5% with *Giardia* cysts. Studies from some other low- or middle-income countries where hygiene, sanitation and water quality may be sub-optimal (Dixon, 2016) have indicated similar or higher levels of contamination (El Said Said, 2012; Maikai *et al.*, 2013). In more developed countries, the rate of contaminated vegetables with these parasites tends to be lower (Robertson and Gjerde, 2001a; Rzezutka *et al.*, 2010; Lalonde and Gajadhar, 2016b).

3.4. Vegetables types

The structure of vegetables was largely involved in the level of parasitic contamination. El Said Said, (2012); Duedu *et al.*, (2014); Istifanus and Panda, (2018) reported that leafy greens as lettuce, cabbage and rocket were more susceptible for contamination compared to other vegetables with smooth surfaces, due to their curly leaves that may increase the attachment of parasites as *Cryptosporidium* and *Giardia*. In addition, Amahmid *et al.*, (1999) found that leafy greens as coriander and mint were relatively more contaminated with *Giardia* compared to carrots and radish, which could be related to their dense foliage offering a large contamination surface. Contrarily to Sleman Ali *et al.*, (2018) who showed that contamination rates of *Cryptosporidium* were not significantly related to vegetable type, which is similar to the results of Maikai *et al.*, (2013) and Rahman *et al.*, (2014) who found no difference between contamination rates of *Cryptosporidium* in different types of vegetables.

3.5. Quality of irrigation water and fertilizers

The quality of irrigation water was usually incriminated in the presence of waterborne parasites in fresh produce. Indeed, several studies found that the use of raw water in irrigation leads to contamination of vegetables with *G. duodenalis* (Amahmid *et al.*, 1999; Daryani *et al.*, 2008; Abougrain *et al.*, 2010; Amoros *et al.*, 2010; Shahnazi and Jafari-Sabet, 2010), and *Cryptosporidium* (Sleman Ali *et al.*, 2018), or both parasites (Amoros *et al.*, 2010). Another factor contributing to the contamination of crops with *Cryptosporidium* is the fertilization using manure from cattle and sheep, containing viable oocysts of *Cryptosporidium* (Moore *et al.*, 2007; Rzezutka *et al.*, 2010; Sleman Ali *et al.*, 2018), since oocysts can remain viable in manure for several months (Bukhari, 1995), and in soil for several weeks especially at low temperature (Nasser *et al.*, 2007).

3.6. Vegetables source

In the reviewed studies, some have tested vegetables samples from different sources to investigate the effect of this parameter on parasitic contamination. Duedu *et al.*, (2014) revealed a very low prevalence of parasites in vegetables obtained within the supermarkets and this was about ten times more among vegetables obtained from the open-aired markets. Moreover, Alemu *et al.*, (2019) found that vegetables directly supplied by farmers to vendors were 3.5 times more likely to be contaminated with parasites as compared to vegetables supplied by large scale vendors. Noting that large scale vendors in the study area used appropriate vehicles for the distribution of vegetables, while small scale vendors were supplied directly by farmers *via* back of animals or human labor, which exposed vegetables to contamination. Furthermore, Lass *et al.*, (2012) proved that the cultivation of vegetables in farms where the contact with feline feces is more probable increase the risk of contamination with *T. gondii.* Contrarily, Hassan *et al.*, (2012); Omowaye and Audu, (2012) showed no difference between the contamination rates found at the various markets, this could be explained, in part, by the various sources from which vegetables are brought to these markets.

Conclusion

T. gondii, *G. duodenalis* and *Cryptosporidium* spp. are considered as pathogens in human and are frequently present in the environment leading to the contamination of fresh vegetables worldwide. These matrices are potentially contaminated by protozoan and could represent a health risk for populations. Existing studies are heterogeneous supporting the need for further investigation of standardized methods

In this review, we underline the importance of the determination of (oo) cysts recovery efficiency and detection limits (LOD) to validate the methods for monitoring protozoan parasites. These standardized methods will enable us to compare the efficiency and sensitivity between methods as well as the prevalence of resistance forms between the different regions.

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Table 1: Methods used for monitoring *T. gondii*, *G. duodenalis* and *Cryptosporidium* spp. in fresh vegetables (lettuce, parsley, coriander, radish, and carrot), and the prevalence of these parasites according to different studies

Methods			Results % of positive samples			Vegetable samples			References
Elution	Concentration	Detection	*T. gondii*	*G. duodenalis*	*Crypto* spp.	Type	Number of analyzed samples	Sample size	
-1st elution: In zipper bags with 2-3 volumes of 0.1% liquinox 0.01 M phosphate-buffer saline (PBS; pH 7.4); with shaking for 15min; -2nd elution: in 0.01M PBS; pH 7.4, with shaking for 15min;	- Cf (5000 x *g*; 20min) of each washing solution; - Cf (1000 x *g*; 20min) of the pellets; - IMS.	Real time PCR *C. parvum* SWI2/SNF2 ATPase, Rad16 ortholog gene	X	X	33.3% *C. parvum*	Carrot	3	50g	(Hong *et al.*, 2014)
- With a glycine buffer (1 M, pH 5.5) using a stomacher;	- Cf (2000 x *g*; 15 min); - The sediment was subjected to a flotation using sucrose solution (sp.g: 1.26).	Real time PCR 18S rDNA	0.0%	X	0.0%	Lettuce	220	35g	(Lalonde and Gajadhar, 2016b)
- With distilled water/0.01% Tween 20, with shaking at 100rpm for 2h; - Vegetables were removed, and solutions of 1M $CaCl_2$ and 1M $NaHCO_3$ were added with shaking, then the pH was adjusted to 10 using 1M NaOH; - The mixture was left over night (without shaking); - The supernatant was discarded, and the pellet suspended in 10% NH_3O_3S solution;	- Cf (1700 x*g*; 10min; 4°C); - The pellet was suspended in 0.01% Tween 80 and the volume was adjusted with distilled water; - Cf (2500 x *g*; 10min; 4°C).	Real time PCR B1 gene Nested PCR-RFLP SAG 2 For genotyping positive samples determined by qPCR	18.0% 19.5% 5.0% All: 6 samples SAG2 type II 2 samples SAG 2 type I	X X X	X X X	Lettuce Carrot Radish	50 46 60	1 lettuce 500g 20 radishes	(Lass *et al.*, 2012)
-In stomacher bags with an	Cf (3000 rpm;	Nested PCR SAG 2	3.6%[b]	X	X	Lettuce	111	25g	(Bethea,

elution buffer (Laureth 12, 1M Tris buffer, pH 7.4; EDTA; Antifoam A), by rocking for 15min on each side.	20min).		2.0%[a] 4.3%[b] 0.0%[a]	X X X	X X X	 Parsley	100 47 100	25g 25g 25g	2014)
-In plastic bags with 1% Tween 80 by hand shaking for 1min; -Filtration using cellulose ester membrane (0.3µm pore size) under pressure.	Cf (600 x *g*; 15min).	PCR B1 gene, a 529 bp repeat fragment	1.6% 20.0%	X X	X X	Lettuce Parsley	62 5	50g 50g	(Marchioro *et al.*, 2016)
- With 1M Glycine, pH 5.5, in a filtered stomacher bag for 30sec	- Cf (2500 x *g*; 10min); - IMS.	IFA	X	61.5%	76.9%	Lettuce	19	50g	(Amoros *et al.*, 2010)
- In an elution buffer containing salts, detergent and antifoam A; - Washing, twice, in a rotating drum, for 1-5min	- Cf (1000 x *g*; 10min); - IMS.	IFA	X X X	2.0% 0.0% 17.0%	4.0% 0.0% 0.0%	Lettuce Parsley Radish	125 7 6	100g 10-80g 50g	(Robertson and Gjerde, 2001a)
-Stomaching in stomacher bags with 1M Glycine for 1min.	- Cf (2500 x *g*; 10min; 15°C); - IMS	IFA Nested PCR-RFLP (for positive samples determined by IFA) (18S rRNA, COWP, Lib13)	X	X	0.0%	Lettuce	24	30g	(Rzezutka *et al.*, 2010)
- In stomacher bags, with Glycine buffer for 4min, by hand manipulation;	- Cf (15500 rcf; 10min); - IMS.	IFA PCR (for positive samples determined by IFA) (*Giardia* 18SrRNA) (*Crypto* 18SrRNA, COWP)	X X X X	0.0% 14.3% 4.0% 0.0%	0.0% 7.1% 0.0% 1.1%	Lettuce Coriander Carrot Radish	284	30g	(Utaaker *et al.*, 2017)
- With physiological saline solution (0.85% NaCl); - The washing solution was left 10h to sediment, the supernatant was discarded	Cf (2164 x *g*; 15min).	ME: Lugol's iodine staining	X	4.0%	X	Lettuce	27	100g	(Abougrain *et al.*, 2010)
- With a solution of (TBS: 20mM Tris base, 0.5mM Sodium chloride, 0.2% Tween 20), using a mechanical shaker at 150rpm for 30min; - The washing solution was left	Cf (1500 rpm; 5min).	ME: Lugol's iodine and ZN staining	41.0% 5.7% 12.2%	2.7% 0.0% 7.3%	0.0% 3.4% 2.4%	Lettuce Parsley Carrot	73 88 41	100g 100g 100g	(Ahmad *et al.*, 2016)

1h to sediment, the supernatant was discarded									
-With a solution of (TBS: 20mM Tris base, 0.5mM Sodium chloride, 0.2% Tween 20), using a mechanical shaker at 150rpm for 30min; - The washing solution was filtered through gauze and left 45min to sediment, the supernatant was discarded	Cf (1500 rpm; 5min).	ME: 10% formal-saline	X	3.1%	6.3%	Vegetables including lettuce and radish	180	100g	(Al-Binali *et al.*, 2006)
- With physiological saline by shaking; - The washing solution was left overnight to sediment; the supernatant was discarded;	Cf (3000 rpm; 5min).	ME: -direct smear - Lugol's iodine and modified ZN staining	X X	0.0% 6.4%	8.7% 11.3%	Lettuce Carrot	23 62	100g 100g	(Alemu *et al.*, 2019)
- With physiological saline solution (0.95% NaCl) by vigorous shaking; - The washing solution was left 12h to sediment; the supernatant was discarded;	Cf (2000 rpm; 15min).	ME: direct smear and Lugol's iodine staining	X X X X	21.4% 10.0% 16.2% 8.0%	X X X X	Lettuce Coriander Parsley Radish	28 20 37 25	200g	(Alhabbal, 2015)
- With physiological saline solution (0.95% NaCl); - The washing solution was left overnight to sediment; the supernatant was discarded;	Cf (2000 x *g*; 20min).	ME: Lugol's iodine staining	6.6%	31.6%	X	Vegetables including lettuce and radish	470	250g	(Al-Megrm, 2010)
- With normal saline; - The washing solution was filtered through gauze; and the filtrate was left 10h to sediment; the supernatant was discarded;	Cf (5000 rpm; 5min).	ME: Lugol's iodine staining	X X	4.0% 4.0%	X X	Lettuce Carrot	25 25	Not mentioned	(Amaechi *et al.*, 2016)
- With physiological saline solution (0.95% NaCl; - The washing solution was left 10h to sediment; the supernatant was discarded;	- Cf (2164 x *g*; 15min); - (Bailenger, 1962) concentration method	ME	X X X	44.4%[c] 33.3%[c] 44.4%[c]	X X X	Coriander Carrot Radish	9 9 9	1kg 1kg 1kg	(Amahmid *et al.*, 1999)

- Samples were put in containers with water/ 0.2% dish washing liquid for 6h, and were stirred regularly every half hour; - The solution was left 16h to sediment, the supernatant was discarded; - The residue was filtered through a sieve (150µm pore size);	Cf (1000 rpm; 5min) twice.	ME: Lugol's iodine and ZN staining	X	7.5%	40.0%	Lettuce	80	500g	(Avazpoor *et al.*, 2015)
- With physiological saline solution (0.85% NaCl); - The washing solution was left 24h to sediment; the supernatant was discarded;	Cf (2000 rpm; 5min).	ME: Lugol's iodine staining	X	19.4%	X	Lettuce	72	100g	(Benti and Gemechu, 2014)
-Using sterile peptonated water 0.1%;	Cf (900 x *g*; 15 min).	ME: ZN and Weber staining	X	X	2.0%	Vegetables including lettuce and parsley	250	Not mentioned	(Calvo *et al.*, 2004)
- With physiological saline solution (0.95% NaCl); - The washing solution was left 24h to sediment, the supernatant was discarded;	Cf (2000 x *g*; 5min).	ME: Lugol's iodine staining	X X	Im.Veg 7.0% N. Veg 9.0%	X X	Vegetables including (lettuce, parsley, coriander, radish)	Im. Veg 96 N.Veg 45	250g 250g	(Daryani *et al.*, 2008)
-Washing using three elution buffers (tap water, saline 0.85%, PBS), with vigorous shaking (manually) for 3 min;	Cf (3000 x *g*; 15min).	ME: - direct smear - Lugol's iodine and modified ZN staining	X	6.0%	17.0%	Vegetables including carrot and lettuce	168	100-150g	(Duedu *et al.*, 2014)
- Pre- washing with tap water, for 6-7 min; - Washing with a solution of (PBS; pH 7.4, 0.01% Tween 80); - The washing solution was filtered through gauze;	Cf (2000 x *g*; 30min).	ME: -direct smear - Lugol's iodine and modified ZN staining	X X	15.0% 5.0%	43.3% 33.3%	Lettuce Parsley	60 60	200g 200g	(El Said Said, 2012)
- With three solutions	- Cf (2000 x *g*; 30	ME: modified ZN	X	X	26.3%	Lettuce	80	150g	(El Sherbini *et*

(separately), tap water, saline (0.85%) and PBS, with manual shaking for 3min; - The eluent was filtered through gauze.	min); -The residue was concentrated by sucrose flotation (specific gravity (sp.g):1.18).	staining	X X	X X	20.5% 20.0%	Parsley Carrot	88 20	150g 150g	*al.*, 2016)
- Soaking for 15mn in physiological saline; - Washing with normal saline by vigorous shaking for 15min; -The washing solution was left 10h to sediment; - washing solution was filtered through a sieve (452µm) to remove debris;	- Cf (2000 rpm; 15min); - The supernatant was concentrated by zinc sulfate flotation.	ME: - direct smear; - Lugol's iodine and modified ZN staining	X X	34.0% 25.5%	X X	Lettuce Parsley	101 102	200g 200g	(Eraky *et al.*, 2014)
- With physiological saline solution (0.95% NaCl; - The washing solution was left 10h to sediment; the supernatant was discarded;	- Cf(2164 x *g* ;15min); - Concentration following (Bailenger, 1962)	ME	X X	20.0% 0.0%	X X	Lettuce Parsley	15 20	100g 100g	(Erdoğ rul and Şener, 2005)
- With physiological saline solution (0.95% NaCl), by vigorous shaking; - The washing solution was left 12h to sediment, the supernatant was discarded;	Cf (2000 rpm; 15min).	ME: direct smear and Lugol's iodine staining	X	0.0%	X	Radish	55	250g	(Ezatpour *et al.*, 2013)
- With physiological saline solution (0.95% NaCl); - The washing solution was left overnight to sediment, the supernatant was discarded;	Cf (2000 x *g*; 15min).	ME: direct smear and Lugol's iodine staining	X	8.2%	X	Vegetables including coriander, parsley and radish	304	250g	(Fallah *et al.*, 2012)
- With physiological saline solution (0.9% NaCl), by vigorous shaking; - The washing solution was left overnight to sediment; the supernatant was discarded;	- Cf (2000 rpm; 15min); - Sucrose flotation (sp.g: 1.21) at 1500 x *g* for 5min;	ME	X X	X X	Un. Veg 2.9% Pre. Veg 1.8%	Vegetables including lettuce and carrot	Un. Veg 453 Pre. Veg 448	250g 250g	(Fallah *et al.*, 2016)
- With physiological saline solution (0.85% NaCl);	Cf (2164 x *g*; 15min).	ME: Lugol's iodine staining	X	0.0%	X	Lettuce	50	100g	(Gabre and Shakir, 2016)

- The washing solution was left 10h to sediment, the supernatant was discarded;			X	8.0%	X	Carrot	50	100g	
- With normal saline, by vigorous shaking;	Cf (2000 rpm; 15min).	ME: direct smear and Lugol's iodine staining	X X X X X	0.0% 10.0% 8.3% 20.0% 25.0%	X X X X X	Lettuce Parsley Coriander Carrot Radish	5 10 12 10 4	Not mentioned	(Hassan *et al.*, 2012)
- With physiological saline solution (0.85% NaCl), by shaking for 20min -The washing solution was left overnight to sediment; the supernatant was discarded;	Cf (2000 rcf; 15min).	ME: Lugol's iodine staining	X X	23.3% 0.0%	X X	Lettuce Parsley	30 42	250g 250g	(Ismail, 2016)
- With distilled water.	Flotation and formol-ether concentration	ME	X	0.0% 0.0%	X	Lettuce Carrot	82 187	Not mentioned	(Istifanus and Panda, 2018)
-With distilled water, in stomacher bags, using stomacher for 2min at 260 rpm.	-Cf (1200 x *g*; 10min). -Concentration following (Bailenger, 1962)	ME: modified ZN staining	X X	X X	33.3% 55.5%	Coriander Lettuce	9 9	10g 10g	(Le Quynh Chau *et al.*, 2014)
-With physiological saline solution (0.95% NaCl); - The wash was left for 10h to sediment; the supernatant was discarded;	- Cf (1500 rpm; 5min); - Flotation using sucrose solution (sp.g 1.21).	ME: modified ZN staining	X X	X X	48.0% 24.0%	Lettuce Carrot	25 25	250g 250g	(Maikai *et al.*, 2013)
- With 10% formal saline;	-Cf (3000 rpm; 5min).	ME: direct smear and Lugol's iodine staining	X X X	9.1% 2.0% 4.2%	X X X	Lettuce Carrot Radish	11 50 24	Not mentioned	(Mohamed *et al.*, 2016)
- With a solution of 1% sodium dodecyl sulfate (SDS) / 0.1% Tween 80, for 10min;	Cf (3000 rpm; 10min).	ME: fixation with formaldehyde 4% for 10min; Lugol's iodine staining	X X X	8.3% 6.25% 0.0%	X X X	Parsley Coriander Radish	12 16 9	200g 200g 200g	(Nazemi *et al.*, 2012) Using the standard method proposed by FDA
Method 1: - Washing with	Method 1: Cf (2000	ME: Lugol's				Vegetables			

physiological saline solution (0.95% NaCl); - The washing solution was left overnight to sediment; the supernatant was discarded; Method 2: - Washing with sterilized water; - The washing water was left 8h to sediment at room temperature, the supernatant was discarded;	x *g*; 20min). Method 2: Cf (3000 rpm; 5min).	iodine staining	X	11.0%	X	including (lettuce, parsley, coriander, radish)	270	250g	(Olyaei and Hajivandi, 2013)
- With distilled water; - The washing solution was filtered through a double layered sieve;	- Cf (2500 rpm;1 min); - Cysts were concentrated using (Umeche, 1991) technique.	ME	X	2.6%	X	Vegetables including lettuce	761	100g	(Omowaye and Audu, 2012)
- With physiological saline solution (0.98% NaCl) by shaking at 150 rpm for 30min; -The washing solution was left 45min to sediment, the supernatant was discarded;	- Cf (1500 rpm; 5min); - The pellet was subjected to a sucrose flotation (sp.g 1.18).	ME: modified ZN staining	X X	X X	40.0% 31.4%	Lettuce Carrot	20 35	250g 250g	(Rahman *et al.*, 2014)
- With a solution of 1% SDS, 0.1% Tween 80, with stirring for 10min; - The washing solution was left 30min to sediment; the supernatant was discarded;	Cf (270 x *g*; 15min).	ME: modified ZN staining	X	X	6.7%	Coriander	90	200g	(Ranjbar-Bahadori *et al.*, 2013)
- With physiological saline solution (0.95% NaCl); - The washing solution was left overnight to sediment; the supernatant was discarded;	Cf (3000 rpm; 5min).	ME: Lugol's iodine staining	X	13.3%	X	Vegetables including lettuce, parsley and radish	135	500g	(Saki *et al.*, 2013)
- With physiological saline solution (0.95% NaCl); - The washing solution was left overnight to sediment; the supernatant was discarded;	Cf (2000 x *g*; 20min).	ME: Lugol's iodine staining	X	16.3%	X	Vegetables including parsley	72	400-500g	(Saida and Nooraldeen, 2014)

- Pre-washing with tap water; - Disinfection with calcium hypochlorite solution 200ppm for 30min; - Rinsing in an automated vegetable-fruit washer; - Washing with a detergent solution containing 1% (w/v) SDS and 0.1% (v/v) Tween 80; - Sonication for 10min.	Cf (1500 x *g*; 15min).	ME	X X X X	0.0% 0.0% 0.0% 0.0%	X X X X	Lettuce Parsley Coriander Radish	23 21 24 29	200g 200g 200g 200g	(Shahnazi and Jafari-Sabet, 2010)
Washing with distilled water.	-Cf (250 rpm; 1min). -Zinc sulfate flotation.	ME	X	0%	X	Carrot	10	Not mentioned	(Simon-Oke *et al.*, 2014)
-Samples were kept eight hours in water containing anionic detergent (washing solution 1); -Washing by high pressure water for three times (washing solution 2); -The two washing solutions were mixed and left 14h to sediment; - The supernatant was discarded.	Cf (1500 rpm; 5min).	ME: direct smear and Lugol's iodine staining	X X X X	25.0% 10.0% 5.0% 0.0%	X X X X	Lettuce Coriander Parsley Radish	20 20 20 20	500g 500g 500g 500g	(Siyadatpanah *et al.*, 2013)
- With physiological normal saline (0.95% NaCl) by mechanical shaking; - The top layer was discarded, and the residue was filtered through gauze.	Cf (2000 rpm; 15min).	ME: ZN staining	X X	X X	6.25% 6.25%	Parsley Radish	80 80	400-500g 400-500g	(Sleman Ali *et al.*, 2018)
- With normal saline; - The washing solution was left overnight to sediment; the supernatant was discarded;	Cf (3000 rpm; 5min).	ME: modified ZN staining	X	7.5%	18,2%	Vegetables including lettuce and carrot	360	200g	(Tefera *et al.*, 2014)

Cf: Centrifugation; FDA: Food and Drug Administration of United States of America; IFA: Immunofluorescence assay; IMS: Immunomagnetic separation; Im.V: Imported vegetables; ME: Microscopic examination; N.V: Native vegetables; PCR: Polymerase chain reaction;Pre.V: Prewashed vegetables; Un.V : Unwashed vegetables ; X : Not done; ZN: Ziehl-Neelsen; [a]: samples from USA; [b]: samples from Peru; [c]:Crops of vegetables irrigated with raw wastewater

Table 2: Recovery efficiencies and detection limits of published methods for the detection of *Cryptosporidium*, *G. duodenalis* and *T. gondii*, in leafy greens and root vegetables

Processing method	Detection method	Matrices (grams)	Parasites [a]	Inocula size (Oo)cysts/sample	Recovery %	Detection limit (oo)cysts/g	References
Washing in rotating drum and sonication (Laureth 12, 1M Tris buffer; pH 7.4, EDTA and Antifoam A)	IFA [c]	Lettuce (100), Chinese leaves (100), and strawberries (100)	*Cryptosporidium*, *Giardia*	50-100	*Cryptosporidium* 42.0%, *Giardia* 67.0%	NR [b]	(Robertson and Gjerde, 2000)
Stomaching (1M Glycine, pH 5.5)	IFA	Lettuce (30) Raspberries (60)	*C. parvum*	100	59.0±12.0% (lettuce), 41.0±13.0% (raspberries)	NR	(Cook *et al.*, 2006)
Stomaching (1M Glycine, pH 5.5)	IFA	Fresh produce (50)	*Cryptosporidium* *Giardia*	NR	*Cryptosporidium* 24.5±3.5%, *Giardia* 16.7±8.1%	NR	(Amoros *et al.*, 2010)
Stomaching	IFA / Nested	Lettuce (30)	*Cryptosporidium*	100	19.0%	NR	(Rzezutka *et*

(1M Glycine)	PCR-RFLP [d]						*al.*, 2010)
Washing (0.01% Tween 20)	qPCR	Radish (20 radishes)	*T. gondii*	Ten-fold serial dilutions (10-10^4)	NR	<100 oocysts	(Lass *et al.*, 2012)
Washing (0.1% Alconox)	Fluorescent microscopy	Basil (25)	*C. parvum*	1123	72.6±6.6%	NR	(Shields *et al.*, 2012)
NR	IFA	Lettuce (25)	*G duodenalis*	Two-fold serial dilutions (10-300)	68.4%	1.4	(Ganz *et al.*, 2015)
Washing (buffer containing 0.01% Tween 80)	PCR/ microscopy	Lettuce (50)	*G. duodenalis*	NR	20±4.3%	20	(Ramirez-Martinez *et al.*, 2015)
Stomaching (1M Glycine, pH 5.5)	IFA	Lettuce (30)	*Cryptosporidium* *Giardia*	NR	*Cryptosporidium* 53.0%, *Giardia* 33.0%	NR	(Utaaker *et al.*, 2015)

Washing, mechanical agitation (1M Glycine pH 5.5)	qPCR	Basil (30) Raspberries (30)	*C. parvum*, *G. duodenalis*, *T. gondii*	5-10^4	*Cryptosporidium* 11.0%, *Giardia* 2.0%, *T. gondii* 35.0% (basil), *Cryptosporidium* 14.0%, *Giardia* 21.0%, *T. gondii* 2.5% (raspberries)	*Cryptosporidium* and *Giardia* 3, *T. gondii* <1 (all): <1	(Hohweyer *et al.*, 2016)
Washing (1% Tween)	PCR	Lettuce (50)	*T. gondii*	Ten-fold serial dilutions (10-10^4)	NR	10 oocysts/µl	(Marchioro *et al.*, 2016)
Stomaching (1M Glycine pH 5.5),	LAMP [e] / chromatographic lateral-flow dipstick	Baby lettuce (50)	*T. gondii*	Two-fold serial dilutions (25-100)	NR	0.5	(Lalle *et al.*, 2018)
Washing (0.1% Tween 80)	multiplex PCR	Spinach (10)	*C. parvum*, *G. duodenalis*, *T. gondii*	Ten-fold serial dilutions (10-10^3)	*C. parvum* 46.0%, *G. duodenalis* 38.0%, *T. gondii* 43.0%	*C. parvum* 3.5, *G. duodenalis* 4.5, *T. gondii* 1.0	(Shapiro *et al.*, 2019)

[a] Most studies targeted detection of one or two of the protozoan parasites, while two publications described methods for detection of the three parasites, [b] Not reported, [c] Immunomagnetic separation – immunofluorescence assay, [d] Polymerase chain reaction-restriction fragment length polymorphism, [e] Loop mediated isothermal amplification.