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Research Paper

Optimized Molecular Detection of *Cryptosporidium* Within the Water-Soil-Plant-Food Nexus: Advancing Surveillance in Agricultural Systems

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ABSTRACT

Cryptosporidium, a protozoan parasite causing severe diarrheal illness in humans and animals, poses detection challenges due to low parasite concentrations, inhibitors, and inefficient DNA extraction. This study optimized DNA extraction and detection of Cryptosporidium in environmental samples and evaluated their practical use in agriculture. After evaluating 11 DNA extraction methods from spiked phosphate-buffered saline (PBS) samples, three methods for molecular detection of *Cryptosporidium* in water, soil, and fresh produce were selected and further tested using real-time PCR. A total of 188 artificially contaminated samples were prepared, consisting of distilled water (n = 36), environmental water (n = 44), soil (n = 36), and fresh produce (lettuce and spinach; n = 72). Each sample was inoculated with serial dilutions of 12,500 to 5 *Cryptosporidium* oocysts and tested using real-time PCR and droplet digital PCR (ddPCR) to evaluate detection sensitivity. Results demonstrated that extraction performance varied by matrix, with two spin-column kits excelling for water and another for soil and produce. DNA from as few as five oocysts was occasionally detectable, with ddPCR being less prone to be affected by PCR inhibitors than real-time PCR. These methods were then applied to detect Cryptosporidium in 210 environmental samples (water, soil, produce) from South African small-scale farms. None of the samples tested positive with real-time PCR, while ddPCR detected *Cryptosporidium* in 13.6% of water, 23.3% of soil, and 34.7% of fresh produce samples. Surface water showed the highest contamination at 28.6%. Soil amended with both fertilizer and manure had a 45% contamination rate. Among vegetables, roots were most affected (46.7%), followed by fruiting (40%) and leafy greens (30.15%). These findings highlight the health risks of Cryptosporidium in food systems and the need for improved detection methods to enhance surveillance and inform future outbreak prevention strategies.

Cryptosporidium is a waterborne and foodborne protozoan parasite that significantly impacts both human and veterinary health, causing gastrointestinal illnesses ranging from self-limiting diarrhea to severe, chronic conditions, particularly in immunocompromised individuals (Ryan & Hijjawi, 2015; Khan et al., 2019; Li et al., 2020). The parasite is transmitted through the ingestion of oocysts from contaminated water and food, often originating from feces of infected humans, animals, or birds (Daraei et al., 2021). Due to its low infectious dose, even a single oocyst can cause infection, highlighting the importance of accurate detection and prevention (Messner & Berger, 2016; Innes et al., 2020). Moreover, *Cryptosporidium* is highly persistent in the environment, where it can remain viable for two years in aquatic environments and soil (Nasser, 2022; Golomazou et al., 2024). Despite advances in molecular detection, there is still a gap in standardized methods for monitoring *Cryptosporidium* in environmental settings, which poses challenges in mitigating public health risks, particularly through food and water sources (Moss et al., 2014; Mthethwa et al., 2022).

The increasing global demand for fresh produce, coupled with agricultural practices such as manure application and the use of irrigation water from potentially contaminated sources, exacerbates the risk of *Cryptosporidium* contamination in food systems (Li et al., 2020; Razakandrainibe et al., 2020). In low- and middle-income countries like South Africa, where access to safe water remains limited, contamination from environmental sources, including surface water and soil, poses significant risks to public health, especially in rural and *peri*-urban communities reliant on small-scale farming (The World Bank Group. 2024, 2024; Kruger et al., 2022). In South Africa, small-scale farming is not

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solely defined by land size, as small farms can also achieve commercial outputs with high-value crops (Carelsen et al., 2021). Instead, a small-scale farm is suggested to be characterized as an agricultural operation too small to attract essential support services for significant productivity improvements, typically generating an annual income of R500,000 or less (Carelsen et al., 2021). These farms rely on family labor for subsistence and commercial purposes, contributing to rural food security while facing challenges such as limited land access, technology, education, and financial constraints (Carelsen et al., 2021). Despite the substantial evidence of *Cryptosporidium* infections in vulnerable populations in Southern Africa, little is known about its presence in fresh produce or the broader environmental context, such as irrigation water or soil (Omolabi et al., 2022; Trelis et al., 2022).

This study aimed to address this gap by developing a molecular-based workflow for detecting *Cryptosporidium* in the water-soil-plant nexus of small-scale farms in South Africa. The research evaluated various DNA extraction methods using real-time PCR and ddPCR to optimize detection across water, soil, and fresh produce matrices, while also assessing *Cryptosporidium* prevalence and its potential waterborne and foodborne transmission risks.

Material and methods

The experimental design of this study involved four phases, with Phase 1 serving as a baseline to evaluate DNA extraction methods for isolating *C. parvum* DNA. In Phase 1a, eleven extraction methods were assessed using real-time PCR on standard oocysts (Waterborne Inc., New Orleans, USA) from phosphate-buffered saline (PBS), spiked with *C. parvum* at concentrations ranging from 1,250,000 to 1.25 oocysts. This established a foundation for Phase 1b, where the three most effective methods (Methods 9, 10, and 11; Table 1) were selected for extraction from water, soil, and spinach spiked with heat-inactivated oocysts at the same concentration range, followed by real-time PCR detection. Phase 2 focused on the optimization of the droplet digital PCR (ddPCR) (Bio-Rad, California, USA) assay for *C. parvum* detection using reference DNA. Phase 3 aimed to determine the lowest detectable number of *C. parvum* oocyst that could be extracted from artificial contamination trials using the most effective extraction methods. Distilled (1L) and environmental (200 mL) water, soil, and fresh produce (lettuce and spinach) were tested with real-time PCR and ddPCR across oocyst concentrations of 12,500–5 oocysts. Phase 4 applied the optimized methods to determine the presence of *Cryptosporidium* spp. in water, soil, and fresh produce from smallholder farms in South Africa.

Phase 1: Comparison of DNA extraction methods for DNA from inactivated oocysts in different samples. Heat-treated, inactivated *C. parvum* oocysts (1×10^7 in 8 ml PBS) derived from the Iowa isolate were purchased (Waterborne Inc., New Orleans, LA, USA), and a ten-fold dilution series (1.25–1,250,000 oocysts/ml) was prepared as working solutions in PBS (Oxoid, Hampshire, UK) for evaluating DNA extraction methods.

Phase 1a: DNA extraction from PBS using real-time PCR. Eleven DNA extraction protocols with slight pretreatment and/or lysis step modifications to lyse oocyst cell walls were tested to select possible optimal extraction methods for each of the matrices, including three DNeasy PowerSoil Pro Kits, a modified Invisorb Spin Tissue Mini Kit, a Genesig easy DNA/RNA extraction kit, and six modified DNeasy Blood and Tissue Kits. (Table 1).

Phase 1b: DNA extraction from soil and spinach using real-time PCR. Three DNA extraction methods, selected from Phase 1a (methods 9, 10, and 11), were tested on soil and spinach samples spiked with oocysts covering a clinically relevant range (1,250,000, 125,000, 12,500, 1,250, 125, 12.5, 1.25 oocysts per 100 µl).

Soil: Fourteen 0.25 g commercial potting soil samples (7 per extraction method) were spiked with oocyst dilutions and incubated overnight at 4 °C before DNA extraction using methods 9 and 10 (Table 1).

Fresh produce (spinach): Twenty-one 30 g "washed ready-to-eat" spinach samples, purchased from a commercial retailer, (7 per extraction method) were weighed and spiked with oocyst dilutions by spotting on the leaves. After two hours of drying at room temperature, the samples were placed in labeled, sterile polyethylene stomacher bags (Seward Ltd., West Sussex, UK), sealed, and incubated overnight at 4 °C (Razakandrainibe et al., 2020). Nonspiked leaves served as a control for natural contamination. Spiked samples were homogenized for 30 s at 250 rpm in 200 ml PBS (Oxoid) with 0.01% Tween-80 in a 1:4 wt-to-volume ratio (Dixon et al., 2013). The homogenate, in 50 −ml falcon tubes, was centrifuged at 2,500*g* for 10 min, the supernatant was discarded, and the step was repeated until all the biomass was centrifuged. The pellet was subsequently resuspended in 1 ml distilled water, centrifuged at 10,000 rpm for three minutes, and the process was repeated before resuspending the pellet in 300 µl PBS for DNA extraction.

Detection of *Cryptosporidium* with real-time PCR. The presence of *Cryptosporidium* was determined using the Genesig Standard Kit for *Cryptosporidium* (PrimerDesign™) and PrecisionPLUS qRT-PCR Mastermix (PrimerDesign™). The PCR reaction mixture was prepared according to the manufacturer's instructions to a final volume of 20 µl, and a positive control of *Cryptosporidium* DNA and a negative control provided were also included in the qPCR assay. PCR conditions were as follows: 95 °C for two min, followed by 50 cycles of 95 °C for 10 s, 60 °C for 60 s. The Bio-Rad CFX Connect system was used for data analysis, with the cycle quantification (Cq) value used to quantify the target DNA.

Phase 2: Optimization of droplet-digital PCR analysis. To determine the detection limit of ddPCR, positive *C. parvum* DNA (2×10^5 copy number/µl) from the Genesig kit was diluted serially (0.02–2×10^5 copies/µl). Reaction mixtures each contained 13 µl of Bio-Rad supermix for probes (no dUTP) (Bio-Rad), 1.5 µl *Cryptosporidium* primers/probes included in the Genesig Standard Kit, 4.5 µl nuclease-free water, and 7 µl of template DNA to make a final reaction volume of 26 µl. A no-template control (NTC) was also included. The QX200 Droplet Digital PCR system (Bio-Rad) was used for amplification and droplet partitioning. Data analysis was performed using QuantaSoft software version 1.2 (Bio-Rad). PCR amplification was conducted on a T100 thermal cycler (Bio-Rad), using the conditions: 95 °C for 2 min, followed by 50 cycles of denaturation at 95 °C for 10 s and annealing at 57 °C for 60 s, and a final extension at 72 °C for 10 min. The threshold line was based on the negative and positive droplet separation. The synthetic DNA was further serially diluted using a 1:10 dilution factor to determine the detection limit of the ddPCR protocol.

Phase 3: Determination of the lowest number of oocysts detectable with real-time PCR and ddPCR using the best DNA extraction methods per sample matrix. Serial dilutions (5–12,500 oocysts) were tested in five replicates with real-time PCR and ddPCR. All spiked and negative control samples underwent DNA extraction and were analyzed using nanophotometer, real-time PCR, and ddPCR to determine the lowest detectable oocyst amount. In this study, positive results were defined as detection in at least three out of five replicates.

Water: For distilled water, seven 1L containers were spiked with 1 ml oocyst dilutions and filtered through a 0.45 µm membrane (Sartorius, Johannesburg, SA). The membranes were placed in Falcon tubes with 9 mL of 0.1% BPW (3M, Minnesota, USA), vortexed, and centrifuged for 10 min at maximum speed. After discarding the supernatant, the pellet was resuspended in 1 ml distilled water, centrifuged again at 10,000 rpm for 3 min, and then resuspended in PBS for DNA extraction using the modified DNeasy Blood and Tissue Kit. For environmental water, eight 1L irrigation water samples from two farms were collected and transported at 4 °C. The samples were split

Table 1
Summary of eleven different DNA extraction protocols tested for artificially spiked water with *Cryptosporidium*

		DNA Extractions										
		Method 1	Method 2	Method 3	Method 4	Method 5	Method 6	Method 7	Method 8	Method 9	Method 10	Method 11
Pretreatment		Freeze-thaw cycle	Freeze-thaw cycle		Freeze-thaw cycle							
Extraction procedure	Commercial name of kit	PowerLyzer isolation kit	Invisorb Spin Tissue Mini kit	DNeasy Blood and Tissue kit	PowerLyzer DA isolation kit	DNeasy Blood and Tissue kit	DNeasy Blood and Tissue kit	DNeasy Blood and Tissue kit	DNeasy Blood and Tissue kit	DNeasy Blood and Tissue kit	PowerLyzer DA isolation kit	Genesig easy DNA/RNA extraction kit
	Type of kit	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Spin column-based DNA extraction	Magnetic bead-based DNA extraction
	Starting step of kit	Step 2	Protocol 2	Step 1	Step 5	Step 1	Step 1	Step 1	Step 1	Step 3	Step 4	
	Lysis buffer	Buffer from kit	Buffer from kit	Buffer from kit	Buffer from kit	Buffer from kit	Buffer from kit	Buffer from kit	Buffer from kit	Modified lysis buffer with Proteinase K	Modified lysis buffer with Proteinase K	Buffer from kit
	Duration of incubation (if modified)	Manufacturers' instruction	Modified incubation step: 2 hr @ 52 °C	Modified incubation step: 3 hr @ 52 °C	Modified incubation step: 1 hr @ 65 °C	Modified incubation step: 90 min @ 56 °C	Modified incubation step: 5 hr @ 70 °C	Modified incubation step: 3 hr @ 70 °C	Modified incubation step: 5 hr @ 52 °C	Incubation step: 2 hr @ 55–56 °C	Incubation step: 2 hr @ 55–56 °C	Manufacturers' instruction
	Homogenizer system (company)	Mechanical TissueLyzer II (Qiagen)			Mechanical: TissueLyzer II (Qiagen)						Mechanical: TissueLyzer II (Qiagen)	
Molecular analysis (Genesig *Crypto-sporidium* standard kit)		Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)	Real-time PCR ddPCR (optimized using ddPCR supermix)
References				Modified from (Hagos & Molestina, 2022)	Modified from (Farhadkhani et al., 2022)	Modified from (Woolsey et al., 2019)	Modified from (Hagos & Molestina, 2022)	Modified from (Hagos & Molestina, 2022)	Modified from (Hagos & Molestina, 2022)	Modified from (Mthethwa et al., 2022)	Modified from (Mthethwa et al., 2022)	

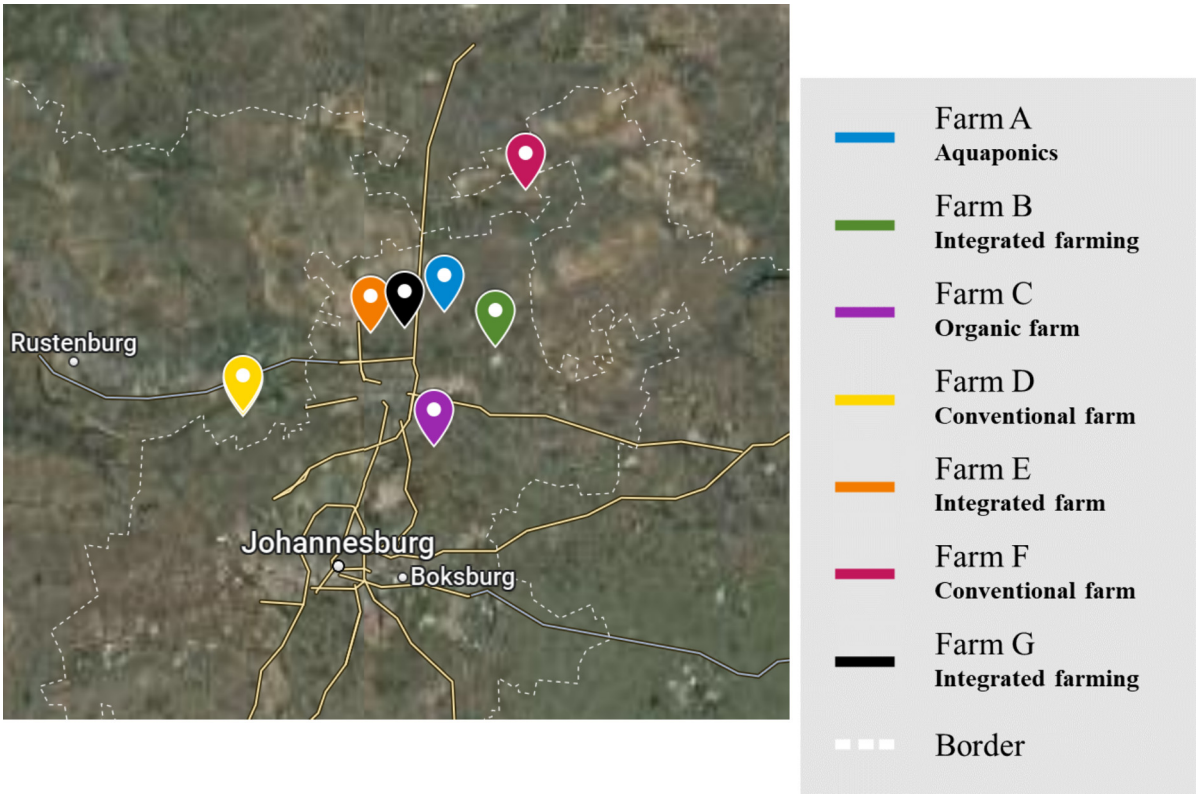

Figure 1. Map of South Africa showing the selected sampling sites for detection of *Cryptosporidium* in small-scale fresh produce production.

Table 2
Description of seven small-scale farm sites including farming practice, crop type sampled, and water source

Sampling Area	Province	Farming practice	Crop type sampled	Farm water source (irrigation method)
Farm A	Gauteng	Integrated Farming (Animal Husbandry) Aquaponics	Kale and spinach Lettuce	Borehole water Aquaculture system
Farm B	Gauteng	Integrated Farming (Animal husbandry)	Kale, spinach and rape	Borehole water
Farm C	Gauteng	Organic Farming	Lettuce, radishes and rocket	Borehole water
Farm D	North West	Conventional Farming	Baby carrots, leeks, spring onions and rocket	River water
Farm E	Limpopo	Integrated Farming (Animal husbandry)	Tomatoes, green peppers and spring onion	Dam water
Farm F	Gauteng	Conventional Farming	Spinach, green peppers and onions	Municipal water
Farm G	Gauteng	Integrated Farming (Animal husbandry)	Peas	River water

into 200 mL portions, spiked with oocysts, and processed as described for the distilled water samples.

Soil: Commercial potting soil (0.25 g) was spiked with 100 µl oocyst dilutions and incubated overnight at 4 °C. DNA was extracted using the DNeasy PowerSoil Pro Kit.

Fresh produce: Seventy-two samples of "ready-to-eat" spinach and lettuce (30 g each) were weighed in replicates of five and 100 µl spike by spotting on the leaves, and sample processing and DNA were extracted as described in Phase 1b.

Phase 4: Application of protocol for *Cryptosporidium* detection in South African smallholder farms. The optimized protocols from Phases 1–3 were applied for detecting *Cryptosporidium* in water, soil, and fresh produce samples from seven smallholder farms across three provinces in South Africa.

Site selection: Seven farms were selected from Gauteng (Farm A, B, C, F, G), North West (Farm D), and Limpopo (Farm E) (Fig. 1), focusing on diverse farming practices. The different farming practices, produce sampled, and irrigation water sources of each of the farms were summarized in Table 2. The farms were selected with guidance from the Department of Land Reform and Rural Agriculture Development (DALRRD). The connections between the different sample types, namely water sources, fresh produce, and soil amendments, are depicted in Figure 2, highlighting the flow of potential contamination across the water-soil-food nexus.

Sample collection and processing. A total number of 210 samples were collected from seven selected small-scale farms in the Gauteng, North West, and Limpopo Provinces. Seven sampling trips were conducted on different days. Fresh produce was placed in sterile paper bags, and soil samples were collected in clean, sealed containers. Disposable gloves were worn and changed between each sample, while equipment was sterilized using 70% ethanol to minimize the risk of cross-contamination. All samples were transported on ice to the laboratory and stored at 4 °C until further processing.

Water: A total of 22 samples of 100L of water (i.e. river, borehole, canal, or holding dam) was collected from the water source and/or irrigation water source on the farms. Each sample was filtered on-site through a kidney dialysis ultrafilter (Isigidi Medical Supplies (PTY) LTD, Centurion, SA) (FDA 2021) using a peristaltic pump. The system was fitted with flexible silicone tubing (8 mm inner diameter, 16 mm outer diameter) and was sterilized before use. To minimize cross-contamination between samples, the tubing was either disinfected or replaced between each sampling event. Each sample was

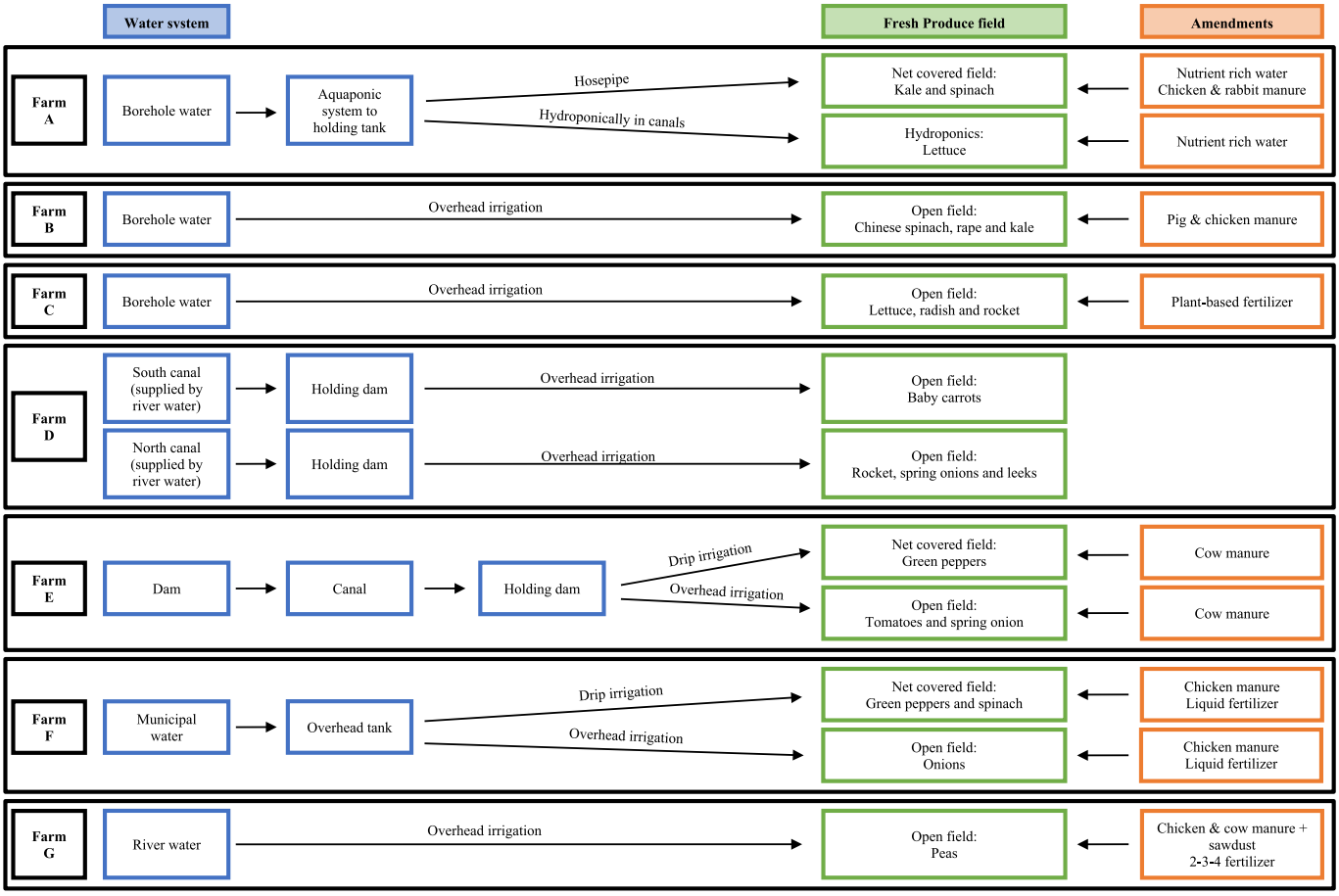

Figure 2. Description of water-soil-plant relationship on each of the selected farms.

Table 3
Performances of chosen DNA extraction protocol determined with real-time PCR

Sample type	Extraction protocol	Proportion of positive samples based on Cq values from real-time PCR						
		1,250,000 oocyst/µl	125,000 oocyst/µl	12,500 oocyst/µl	1,250 oocyst/µl	125 oocyst/µl	12.5 oocyst/µl	1.25 oocyst/µl
PBS	DNeasy Blood and Tissue kit	Cq 40.23	Cq 45.91	Cq 44.04	Cq 46.25	–	–	–
	PowerLyzer DA isolation kit	Cq 35.81	–	–	–	–	–	–
	Genesig easy DNA/RNA extraction kit	–	–	–	–	–	–	–
Spinach	DNeasy Blood and Tissue kit	–	–	–	–	–	–	–
	PowerLyzer DA isolation kit	Cq 37.15	Cq 35.13	–	–	–	–	–
	Genesig easy DNA/RNA extraction kit	–	Cq 46.24	–	–	–	–	–
Soil	PowerLyzer DA isolation kit		Cq 35.18	–	–	–	–	–
	Genesig easy DNA/RNA extraction kit	–	–	–	–	–	–	–

– Undetected.

transported in a cooler box at 4 °C until analysis within 24 h in the laboratory. The ultrafilter was backflushed with a 2.5 L solution containing sterile water supplemented with 0.5% Tween 80 (Ottesen & Kocurek, 2022). After filtering the filtrate through a 0.45 µm nitrocellulose membrane, the water samples were filtered and processed as previously described.

Soil: A total of 90 samples were simultaneously collected from five replicate points during harvest from the fresh produce production fields. Soil samples (approximately 15 g), comprised of three subsamples each, were collected at each of the five replicate fresh produce sampling points in the field in sterile fecal sample containers. Each sample was transported in a cooler box at 4 °C until analysis within 24 h in the laboratory. Soil (0.25 g) was weighed and processed as previously described.

Fresh produce: Based on availability, at least three different types of fresh produce samples were collected at harvest from each respective farm (n = 98), except Farm G, which only had peas available. For each type of fresh produce, five samples were collected, consisting of three composite samples. These samples were obtained using a 70% ethanol-sterilized knife, employing systematic random sampling across rows in the farm field. The samples were then placed in labeled paper bags for transportation in a cooler box at 4 °C until analysis within 24 h in the laboratory. Fresh produce (50 g) was weighed and processed as previously described.

Results

Phase 1: Determination of the best DNA extraction method from inactivated oocysts for each sample type. The Qubit HighSensitivity measurements showed very low detectable nucleic acid concentration for genomic DNA extracted from spiked *C. parvum* oocysts using all methods. Despite the methods tested in this study displaying varying low detectable DNA concentrations, *Cryptosporidium* was detected from some of the spiked samples (Table 3). In water samples, the DNeasy Blood and Tissue kit was the best extraction method, extracting DNA from as low as 1,250 oocysts (Cq 46.25) (Table 3). For soil and spinach samples, the PowerLyzer DA isolation kit was chosen as the best extraction method since it showed better positive results compared to the other extraction methods tested (Table 3). Therefore, the DNeasy Blood and Tissue kit method was chosen as the best-performing method for further experiments in water samples, and PowerLyzer DNA isolation kit for soil and fresh produce samples.

Phase 2: Optimization of ddPCR for the detection of *Cryptosporidium* based on positive reference DNA. The ddPCR assay was successfully optimized for detecting and quantifying *C. parvum* using the *Cryptosporidium* mix primers/probes from the PrimerDesign™ Genesig Standard Kit for *Cryptosporidium* (Crypto) Genomes. The assay had a clear separation of positive and negative droplets and no evidence of inhibitors or nonspecific binding. Diluting the sample at a 1:10 dilution factor revealed a decrease in droplet concentration (Supplementary Fig. S1). Based on the obtained results (Table 4), the limit of detection for the ddPCR assay targeting *C. parvum* was 0.14 copies in a 26 µl reaction. Further dilutions led to "no-call" or undetectable copies.

Table 4
Limit of detection for the droplet digital PCR assay using reference DNA

Dilution	Copies detected in ddPCR (26 µl reaction volume)	Detection rate (X detected/Y (2) replicates)
10^{-1}	1,000,000	2/2
10^{-2}	4,365.5	2/2
10^{-3}	393	2/2
10^{-4}	44.3	2/2
10^{-5}	4.2	2/2
10^{-6}	0.77	2/2
10^{-7}	0.14	2/2
10^{-8}	0	0/2

Phase 3: Determination of a lower detection limit (LOD) of the proposed extraction protocol for *C. parvum* oocysts. Variations in detection limits depending on the oocyst load, sample type, and detection method were observed (Table 5). Overall, ddPCR showed a higher sensitivity for each of the samples compared to real-time PCR. No natural occurrence of *Cryptosporidium* oocyst contamination was detected in any of the spiked samples, as all nonspiked leaves were negative. Additionally, it is worth noting that instances of positivity were observed even when the criterion of three out of five positive replicates was not met. For example, oocysts were detected at five oocysts for 1L of water and spinach with real-time PCR, and five oocysts for soil and spinach with ddPCR (Table 5).

Phase 4: Occurrence of *Cryptosporidium* in water, soil, and fresh produce across different regions. The frequencies of *Cryptosporidium* based on farming production practices are displayed in Table 6. For this scoping study, certain trends were observed between the type of farming practices, water source type, soil amendment, and fresh produce type. However, the number of samples was not enough for a comprehensive statistical analysis. Given the limited sample sizes, the ranking of contamination rates provides preliminary insights rather than statistically definitive conclusions, and further studies with larger sample sizes are required to confirm potential trends with greater statistical confidence.

Type of farming: The analysis indicates that organic farming had the highest number of positive samples (10/32, 31.3%), followed by conventional farming (23/80, 28.75%) and integrated farming systems (25/98, 25.5%).

Water source type: *Cryptosporidium* was not detected using real-time PCR, however, out of 22 processed water samples, three samples (13.6%) tested positive for *Cryptosporidium* oocysts using ddPCR. The farms utilized different water sources: three farms used borehole water, three used surface water, and one relied on municipal supply. Contamination was detected only at the water sources of Farm C (borehole water) in 50% (1/2) of the samples and Farm E (surface water from a dam) in 100% (2/2) of the samples; *Cryptosporidium* was not detected at the irrigation points (Table 6). The groundwater samples showed a lower percentage of positive samples (1/6, 16.7%) compared to surface water used for irrigation purposes (2/7, 28.6%). Notably, none of the municipal water samples tested positive for *Cryptosporidium* (Table 6).

Soil amendment: *Cryptosporidium* was not detected using real-time PCR; however, out of 90 processed soil samples, 21 (23.3%) tested positive for *Cryptosporidium* oocysts using ddPCR, which included six

Table 5
Performances of the chosen DNA extraction protocol were determined with real-time PCR and ddPCR (1L water, lettuce, spinach and soil done in replicates of five and environmental water done in triplicate)

Sample type	Extraction protocol	Molecular detection protocol	Proportion of positive samples at each concentration (%)							Overall proportion of positive samples
			12,500 oocysts	1,250 oocysts	500 oocysts	125 oocysts	50 oocysts	12.5 oocyst	5 oocysts	
1 L Water	DNeasy Blood and Tissue kit	Real-time PCR	80	80	20	0	20	20	40	37.1
		ddPCR	100	80	60	0	40	40	0	45.7
Environmental spiked water: Farm 1		Real-time PCR					33.33	33.33		9.5
		ddPCR	100	66.66	33.33	33.33			33.33	38.1
Environmental spiked water: Farm 2		Real-time PCR	66.66			33.33				14.3
		ddPCR	100	100					33.33	33.3
Lettuce	PowerLyzer DA isolation kit	Real-time PCR	100	100	60	20	0	0	0	40
		ddPCR	100	80	40	40	0	0	40	42.9
Spinach		Real-time PCR	100	100	20	40	20	20	0	42.9
		ddPCR	100	80	60	60	20	0	20	45.7
Soil		Real-time PCR	100	100	80	0	0	0	0	40
		ddPCR	100	100	80	80	80	20	40	60

Table 6
Detection of *Cryptosporidium* in water, soil, and fresh produce samples from small-scale farms according to the different production practices used

	Variable	"Positive" samples (n)/Total samples	Percentage of total "Positive" samples
Type of farming	Integrated (Aquaponics and Animal husbandry)	25/98	25.5%
	Conventional	23/80	28.75%
	Organic	10/32	31.3%
Total samples (farming type)		58/210	27.62%
Water source type	Ground water	1/6	16.7%
	Surface water	2/7	28.6%
	Municipal water	0/2	0%
Total samples (water source)		3/15	20%
Soil amendment	Combination of commercial fertilizer and manure	9/20	45%
	Manure	6/35	17.1%
	Organic fertilizer	4/15	26.7%
	No amendment	2/20	10%
Total samples (soil amendment)		21/90	23.3%
Fresh produce type	Leafy Green vegetable	19/63	30.15%
	Root vegetable	7/15	46.7%
	Fruiting vegetable	8/20	40%
Total samples (fresh produce)		34/98	34.7%

of the seven farms tested. Soil treated with a combination of commercial fertilizer and manure exhibited the highest number of positive samples (9/20, 45%), while soil amended solely with manure had 17.1% (6/35) of samples testing positive. This may suggest that the application of manure could potentially introduce or elevate the presence of pathogens such as *Cryptosporidium*. Furthermore, soil enriched with plant-based organic fertilizers derived from composting exhibited a 26.7% positivity rate, with 4 out of 15 samples testing positive. In contrast, untreated soil demonstrated the lowest positivity rate, with only 2 out of 15 samples (10%) testing positive. Overall, Farm G had the highest number of samples that tested positive for *Cryptosporidium* (3/5, 60%), followed by Farm F (6/15, 40%), with both farms using a combination of manure (chicken and cow, and chicken, respectively) and chemical fertilizer. Farm E showed a lower number of samples testing positive (5/15, 33.3%), followed by Farm C (4/15, 26.7%), Farm D (2/20, 10%), and Farm B (1/15, 6.7%). No soil samples tested positive on Farm A.

Fresh produce type: *Cryptosporidium* was not detected by real-time PCR, but of 98 processed fresh produce samples, 34 (34.7%) tested positive for *Cryptosporidium* oocysts using ddPCR, across all seven farms. Fourteen fresh produce types were examined, with 13 types testing positive (Table 6). The highest number of positive samples was observed in radish (5/5, 100%), followed by tomato (4/5, 80%), peas (3/5, 60%), rocket (5/10, 50%), baby carrots (2/5, 40%), onions (2/5, 40%), spring onion (4/10, 40%), kale (3/10, 30%), spinach (4/15, 26,67%), lettuce (2/8, 25%), leeks (1/5, 20%), and green pepper (1/10, 10%). No contamination was detected in rape samples. Produce types were categorized as leafy green vegetables (kale, spinach, rape, lettuce, rocket, spring onion, leeks), fruiting vegetables (tomato, green pepper, peas), and root vegetables (radish, baby carrots, onion). The percentage of samples positive for *Cryptosporidium* varied: root vegetables were highest at 46.7% (7/15), followed by fruiting vegetables at 40% (8/20), and leafy green vegetables at 30.15% (19/63) (Table 6).

Discussion

This study is the first to optimize a molecular workflow for the detection of *Cryptosporidium* within the water-soil-plant nexus, by comparing the effectiveness of real-time PCR and ddPCR. A real-time PCR assay kit was successfully adapted for ddPCR, enabling occasional detection of *Cryptosporidium* from as few as five oocysts per sample. Both methodologies demonstrated high detection sensitivity. This is important as even low concentrations of oocysts can cause infections (Zacharia et al., 2019). Molecular techniques present a viable alternative to microscopy for the analysis of various sample types for *Cryptosporidium*. These methods are capable of detecting low concentrations of *Cryptosporidium* oocysts while requiring less hands-on time (Adeyemo et al., 2018; de Souza et al., 2019; Fradette et al., 2022). However, molecular methods face challenges, such as inefficient DNA extraction due to the tough oocyst cell wall and environmental inhibitors (Adamska et al., 2012; Mthethwa et al., 2022). Previous literature has emphasized the need for more rigorous methods to disrupt and recover DNA for analysis using molecular techniques (Adamska et al., 2012). To address this, we tested various DNA extraction methods, including freeze-thaw cycles, which degraded DNA and hindered amplification. Similar to Mthethwa et al., 2022, the current study found that using a lysis buffer with proteinase K digestion, was most effective for breaking down the oocyst cell wall, and improving DNA extraction from water, soil, and fresh produce samples.

The current study also examined the impact of plant matrices on oocyst recovery, as variations in food matrices and surface morphology can impact oocyst adherence and, consequently, their removal efficiency (Chandra et al., 2014; Razakandrainibe et al., 2020). Utaaker et al. (2015) demonstrated that *Cryptosporidium* recovery from spinach leaves was 9%, which is lower than the 53% recovery rate previously reported for iceberg lettuce (Utaaker et al., 2015; Razakandrainibe et al., 2020). Contrary to these findings, both vegetables yielded similar detection rates (>40%) using real-time PCR and ddPCR, with ddPCR showing slightly better sensitivity in the current study. Environmental water, soil, and fresh produce samples pose significant challenges for DNA extraction due to inhibitors and low oocyst concentrations (Mthethwa et al., 2022).

On the farm level, 27.62% of samples tested positive for *Cryptosporidium*, with varying detection levels linked to farming practices and water sources. Similar to the findings of Daraei et al., 2021, surface water had higher contamination than groundwater which is likely

attributable to the protective qualities of the (sub)soil (Chique et al., 2020). Although a limited sample size was tested, preliminary results indicated that manure-amended soils showed higher *Cryptosporidium* prevalence, supporting the link between livestock waste and contamination. The findings align with those of other studies, which indicate that manure is a significant pathway for *Cryptosporidium* transmission (Vermeulen et al., 2017). At Farm E, water, soil, and fresh produce samples all tested positive for *Cryptosporidium*. A recent study that included this farm also found *Cryptosporidium* contamination in sheep, goats, and cattle (Seanego, 2024). These findings highlight the interconnectedness of animal health, environmental contamination, and food safety, with manure from infected livestock playing a significant role in pathogen spread.

The implications of these findings are particularly important when considering the consumption of unwashed, raw vegetables, a key route for the transmission of parasitic diseases (Gad et al., 2020). Contamination can occur at various stages from farm to fork, including through soil, manure, irrigation wash water, equipment, or handling by people (Lalonde & Gajadhar, 2016). Detection rates of pathogenic parasites in fresh vegetables are influenced by the methodologies employed for the detection and the specific type of vegetable being analyzed (Berrouch et al., 2020). In this study, root vegetables had the highest contamination rates (31.4%), followed by fruiting vegetables (29.3%) and leafy green vegetables (29.1%), aligning with findings from Rahman et al. (2014) in Bangladesh and Bafghi et al. (2020) in Iran. In contrast, Maikai et al. (2013) found the highest contamination in leafy vegetables (37.3%), followed by fruiting vegetables (32%) and root vegetables (24%) in Nigeria.

Conclusion

This study developed an optimized protocol for detecting *Cryptosporidium* in water, soil, and fresh produce using real-time PCR and ddPCR, with the latter demonstrating superior sensitivity and resistance to inhibitors. These methods address challenges in detecting low concentrations of *Cryptosporidium* in diverse matrices, offering robust tools for environmental monitoring and pathogen surveillance. The contamination observed in the field studies on South African small-scale farms underscores the public health risks that *Cryptosporidium* can pose, as well as the interconnectedness of agricultural practices, water quality, and food safety. This work fills an important gap in the literature by providing a comprehensive understanding of *Cryptosporidium*'s distribution and transmission in diverse agricultural systems, which is critical for improving food safety and public health surveillance. Mitigating these risks requires integrated approaches involving public health professionals, policymakers, and stakeholders. Key actions include improved water quality management, food safety training for farmers, and public awareness campaigns targeting vulnerable populations, such as children and immunocompromised individuals. Continued surveillance and further research into health impacts, seasonal variations, and species identification are essential to refine mitigation strategies. This study demonstrates that ddPCR offers a reliable alternative to traditional methods for *Cryptosporidium* detection, with significant implications for improving public health and food safety in agricultural systems.

CRediT authorship contribution statement

Robyn Marijn Schipper: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Loandi Richter-Mouton:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Lise Korsten:** Writing – review & editing, Supervision, Resources, Funding acquisition.

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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Appendix A. Supplementary material

Supplementary material to this article can be found online at https://doi.org/10.1016/j.jfp.2025.100568.

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