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A cloth-based hybridization array system for rapid detection of the food- and waterborne protozoan parasites *Giardia duodenalis*, *Cryptosporidium* spp. and *Toxoplasma gondii*

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ABSTRACT

Protozoan parasites in food or water samples are generally detected using microscopy or PCR followed by Sanger sequencing. However, microscopy is subjective, requires a high degree of expertise and has limited sensitivity, while DNA sequencing requires expensive and specialized equipment and facilities. This study describes a cloth-based hybridization array system (CHAS) that is an alternative to Sanger sequencing to confirm PCR-positive samples. CHAS is an inexpensive, rapid and reliable method for the simultaneous detection of multiple protozoan parasite species based on the colorimetric detection of PCR amplicons on a polyester cloth. PCR primers and CHAS hybridization probes were developed to detect the protozoan parasites *Giardia duodenalis*, *Cryptosporidium* spp. and *Toxoplasma gondii*. In addition, CHAS probes were designed for the differentiation of *G. duodenalis* Assemblages A and B. In artificially contaminated fresh produce (lettuce, parsley) and water samples (river water, wastewater), this CHAS assay allowed for the successful detection of *G. duodenalis*, *Cryptosporidium* spp., and *T. gondii*. The present study demonstrates that the CHAS detection method is a simple and inexpensive alternative to DNA sequencing for the confirmation of PCR-positive results in laboratories testing for parasites in food or water samples. This assay may also be beneficial in developing countries, where DNA sequencing facilities may not be readily available.

1. Introduction

Giardia duodenalis, Cryptosporidium spp., and *Toxoplasma gondii* are amongst the most prevalent protozoan parasites implicated in food- and waterborne diseases (Devleesschauwer et al., 2017). All three parasites are wide-spread and cause varying degrees of symptom severity depending upon an individual's immune status. Environmentally resistant cysts (*Giardia duodenalis*) and oocysts (*Cryptosporidium* spp. and *T. gondii*), are shed in the faeces of definitive hosts, and contaminate the environment, including water sources and various foods, particularly fresh produce. However, cysts and oocysts are often present in very low concentrations in food and water samples, and simple enrichment is not possible, making detection very challenging. As a result, foodborne parasites, in particular, are amongst the most neglected pathogens affecting humans (Gajadhar, 2015).

Giardia duodenalis (syn. *G. lamblia*, *G. intestinalis*) is a flagellate protozoan parasite infecting the upper intestinal tract of humans and

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a wide range of other mammals worldwide. This species is further divided into eight assemblages (A to H) which, despite their morphological similarity, show considerable genetic heterogeneity and differ in their host ranges (Dixon, 2021; Ryan et al., 2019). The vast majority of human infections involve the zoonotic Assemblages A and B, and distinct sub-assemblages and subtypes within these assemblages have been recognized as infecting both humans and animals (Dixon, 2021). *Cryptosporidium* species are intracellular apicomplexan parasites infecting a wide range of animals worldwide, with *C. parvum* and *C. hominis* accounting for the majority of human infections (Xiao and Feng, 2017). The parasite, *Toxoplasma gondii*, represents the only species within the genus. These intracellular apicomplexan parasites are also infectious to humans and a very wide range of animals, with felids serving as the only definitive hosts (Djurković-Djaković et al., 2019).

Direct microscopic examination, staining, immunofluorescence microscopy and PCR are all commonly used to detect protozoan parasites on fresh produce and in water samples (Berrouch et al., 2020; Efstratiou et al., 2017b; Li et al., 2020). There are, however, limitations to the use of both microscopical methods and PCR. Specifically, microscopy is time consuming, requires a high level of expertise, has limited sensitivity, and often leads to ambiguous results, while PCR alone, without sequencing, may not be specific enough to differentiate amongst species and genotypes. Additionally, amplicon sequencing may not be readily available, leading to increased cost and time if the sequencing facility is far from a remote research location where the food and/or water testing is performed (Helmy et al., 2016). Lastly, sequencing results and differentiation amongst genotypes may be difficult to interpret accurately without specific expertise and background knowledge (Blais and Gauthier, 2007).

Newer molecular methods for the detection of food and waterborne parasites include quantitative PCR (qPCR), loop-mediated isothermal amplification (LAMP), fluorescence in situ hybridization (FISH) and aptasensors (Ahmed and Karanis, 2018; Berrouch et al., 2020; Chalmers et al., 2020; Efstratiou et al., 2017b; Hassan et al., 2020; Lalonde et al., 2021). While these methods are rapid and highly sensitive in the detection of parasites, some require access to specialized instruments that are not necessarily available to all researchers, especially in remote or developing regions.

To address these issues, a simple, inexpensive cloth-based hybridization array system (CHAS) was developed in the present study for the rapid detection and differentiation of common food- and waterborne protozoan parasites. CHAS is an alternative to amplicon sequencing and related downstream analyses, as it is based on a simple colorimetric assay to detect the presence of target DNA in a sample (Blais et al., 2012). Furthermore, CHAS probes can be designed for the simultaneous detection of multiple PCR amplicons, and can be used to detect, and differentiate amongst, different species or different genotypes within a species.

In this study, we describe the development of a CHAS method for the detection and differentiation of three important food- and waterborne parasites: *G. duodenalis*, *Cryptosporidium* spp., and *T. gondii*. To demonstrate the feasibility of using the CHAS to detect parasites in food and environmental samples, we tested the assay on artificially spiked fresh produce and water samples.

2. Materials and methods

2.1. Parasites

Giardia duodenalis (isolate H3, Assemblage B) cysts, *Giardia muris* (Roberts-Thomson isolate) cysts, and *C. parvum* (Iowa isolate) oocysts were obtained from Waterborne Inc. (New Orleans, LA, USA). While *G. duodenalis* Assemblage A cysts could not be purchased commercially, trophozoites from an Assemblage A cell culture (isolate WB) were kindly provided by Drs. André Buret and Thibault Allain (University of Calgary, Calgary, AB, Canada). *Toxoplasma gondii* (ME-49 strain) oocysts were generously provided by Drs. J. P. Dubey (USDA, Beltsville, MD, USA) and Eric Villegas (USEPA, Cincinnati, OH, USA).

Table 1
PCR primers used in this study.

Parasite	Gene	PCR	Primer Name	DNA Sequence (5′ to 3′)	Reference
Giardia spp.	16S	outer + nested	RH11F	CATCCGGTCGATCCTGCC	Appelbee et al. (2003)
			RH4R	AGTCGAACCCTGATTCTCCGCCAGG	(Appelbee et al., 2003)
Giardia duodenalis Assemblages A and B	tpi	outer + nested	AL3544m	CCCTTCATCGGTGGTAACTTC	Sulaiman et al. (2003)
			AL3546	CAAACCTTTTCCGCAAACC	Sulaiman et al. (2003)
Cryptosporidium spp.	COWP	outer	BCOWP·F	ACCGCTTCTCAACAACCATCTTGTCCT	Pedraza-Diaz et al. (2001)
			BCOWP.R	CGCACCTGTTCCCACTCAATGTAAACCC	Pedraza-Diaz et al. (2001)
		nested	Cr-COWPcry.15F	GTAGATAATGGAAGAGATTGTG	Pedraza-Diaz et al. (2001)
			Cr-COWPcry.9R	GGACTGAAATACAGGCATTATCTTG	Pedraza-Diaz et al. (2001)
Toxoplasma gondii	B1	outer	B1outF	GGAACTGCATCCGTTCATGAG	Di Guardo et al. (2011)
			B1outR	TCTTTAAAGCGTTCGTGGTC	Di Guardo et al. (2011)
		nested	B1intF	TGCATAGGTTGCAGTCACTG	Di Guardo et al. (2011)
			B1intR	GGCGACCAATCTGCGAATACACC	Di Guardo et al. (2011)
Internal amplification control (IAC)	–	nested	IAC-1	CATAATATCACTCGCGTCCGTTGAAGCTTA	Leggate (2006)
			IAC-2	GACGAAATCGTAAGCTTCAA	Leggate (2006)

2.2. Food and water samples

Romaine lettuce and parsley were purchased at a local supermarket in Ottawa, ON, Canada and transported in clean food grade plastic bags. Both lettuce and parsley are very commonly tested in surveillance studies on parasites in fresh produce (Berrouch et al., 2020). Furthermore, lettuce has also been widely used in method development studies as it is well suited to spiking and recovery experiments. Wastewater was collected at the Robert O. Pickard Environmental Centre sewage treatment plant in Ottawa, ON, Canada. River water was collected from the Ottawa River on Bate Island (45°24′33.8"N 75°45′14.4"W) in March 2019 where the ice on the river was breaking up. Water samples were collected in sterile plastic containers. All samples were stored at 4 °C prior to use. Twenty-five gram samples of lettuce and parsley, and 200 ml of river or wastewater, were spiked with 50 μl containing 6.25×10^4 cysts of *G. duodenalis* Assemblage B, or oocysts of *C. parvum* or *T. gondii*. Spiked food samples were added to 200 ml phosphate buffered saline (PBS) containing 0.01% Tween 80, and placed on an orbital shaker (DW 150 Waver, VWR, Mississauga, ON, Canada) for 15 min. Both the food rinses and the water samples were vacuum filtered using a custom-made 35 μm filter, and then concentrated by centrifugation at 2000 ×*g* for 15 min at 4 °C. To rupture the cysts or oocysts, the concentrated suspension was subjected to 5 cycles of 2 min freeze (in liquid nitrogen) followed by 2 min thaw at 95 °C in a water bath. Finally, DNA was extracted using the Qiagen DNeasy Blood and Tissue kit (Qiagen, Toronto, ON, Canada).

2.3. Incorporation of DIG-labelled dNTPs using PCR

The PCR primers (Sigma Aldrich, St. Louis, MO, USA) used in this study are shown in Table 1. To detect all *Giardia* species, we targeted a region of the 16S gene. To distinguish between *G. duodenalis* Assemblages A and B, we used one set of primers for the tpi gene but the PCR product was then applied to a CHAS cloth with two distinct probes, one that hybridizes to *G. duodenalis* Assemblage A amplicons, and the other that hybridizes to Assemblage B amplicons. The tpi primers only amplify *G. duodenalis*, and can be used to differentiate *G. duodenalis* from other *Giardia* species. To detect all *Cryptosporidium* species, we targeted the *Cryptosporidium* outer wall protein (COWP) gene. Because of the highly conserved genes in *Cryptosporidium* spp., we were unable to design primers or probes that could reliably distinguish amongst the different *Cryptosporidium* species. For *Toxoplasma gondii*, we used primers for the B1 gene since this is a multicopy gene that would boost sensitivity. An internal amplification control (IAC) was incorporated into the CHAS to exclude false negative PCR results due to inhibitors, as described previously (Blais et al., 2012).

Two rounds of PCR with different primer sets were performed for each gene locus to improve sensitivity, with the exception that the same primer sets were used in both PCR rounds for *Giardia* 16S and tpi genes. All PCR reactions were prepared to a total volume of 50 μl using the Qiagen Multiplex PCR PLUS kit (Qiagen, Toronto, ON, Canada). Each outer PCR reaction contained 5 μl of template DNA, 1× Multiplex Master Mix, 1× CoralLoad, 0.5× Q solution, 2% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA) and 200 nM of each outer PCR primer pair, except B1outF + B1outR which was used at 100 nM concentration (Table 1).

A nested PCR reaction was prepared with 1 μl of the product from the outer PCR, 1× Multiplex Master Mix, 1× CoralLoad, 0.5× Q solution, 2% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA), 200 nM of each primer pair (including IAC-1 + IAC-2), 10 nM of 3-amino-3-deoxydigoxigenin-9-dCTP (DIG-dCTP) (PerkinElmer, Waltham, MA, USA) and 10 nM of digoxigenin-11-dUTP (DIG-dUTP) (Roche Diagnostics, Mannheim, Germany). Because multiplex PCR reactions favor the amplification of the shortest fragments, B1intF and B1intR were used in a concentration of 100 nM throughout the experiments. Immunoenzymatic detection only occurs when anti-DIG-HRP (horse-radish peroxidase) conjugates bind to DIG.

Cycling conditions for both the outer and nested PCR were identical. Initial denaturation was performed at 95 °C for 5 min. This was followed by 35 cycles of denaturation, annealing and extension for 30 s at 95 °C, 90 s at 60 °C, and 60 s at 72 °C, respectively. Final extension was carried out at 72 °C for 10 min, followed by a 10 °C hold.

2.4. Cloth preparation

CHAS cloth preparation was performed as described previously (Blais and Gauthier, 2007), with modifications. A strip of polyester cloth, custom-printed with five adjacent 9 × 9 mm squares (Dupont, Wilmington, DE, USA), was saturated with 95% ethanol for 1 min. It was then placed on a bottle top filter (Sarstedt, Nümbrecht, Germany) and washed with deionized water seven times, using a vacuum to aspirate between each wash. The cloth was left to dry overnight in an open petri dish in the dark at room temperature (RT).

Capture probes (Sigma Aldrich, St. Louis, MO, USA) were designed to be complementary to an amplified region of the genes of

Table 2
DNA sequences of CHAS probes immobilized to cloth.

Parasite	Gene	Probe	DNA Sequence (5′ to 3′)	Reference
Giardia spp.	16S	Pan Giardia Probe 2	GCGGACGGCTCAGGACAACGGTTGCACCCCCCGCGGCGGTCCCTGC	This study
Giardia duodenalis Assemblage A	tpi	tpi A Probe 3	GTCCAAGATGCTCTGGAAGGAG	This study
Giardia duodenalis Assemblage B	tpi	tpi B Probe 3.3	ATTGGAGAATCAAAGAAGTTATGGGAGAACGTTGTA	This study
Cryptosporidium parvum	COWP	COWP Probe 3	CCTCCTGGATTCAATTTGTCAGGACAACAATGTATGGCACCAGAATCAG	This study
Toxoplasma gondii	B1	Toxo B1 Probe	CGAGCTCCCCTCTGCTGGCGAAAAGTGAAATTCATGAGTATCTGTGC	This study
Internal amplification control (IAC)	–	IAC Probe	CATAATATCACTCGCGTCCGTTGAAGCTTACGATTTCGTC	Leggate (2006)

interest. Table 2 lists the CHAS probes used in this study to detect the PCR amplicons, including the probe name, their sequences and the targeted gene. The probes were designed to have the same hybridization temperature. The lengths of the probes were adjusted to their GC content.

The 'Pan Giardia Probe 2' detects all *Giardia* species 16S amplicons, whereas 'tpi A Probe 3' and 'tpi B Probe 3.3' were designed to detect amplicons from *G. duodenalis* Assemblage A and B, respectively. Furthermore, 'COWP Probe 3' hybridizes to *Cryptosporidium* spp. COWP amplicons, and 'Toxo B1 Probe' hybridizes to *T. gondii* amplicons. The internal amplification control (IAC) consists of two complementary overlapping primers which, during successful PCR, form a "primer-dimer"-like structure. The overlapping region is underlined in Table 2. The 'IAC Probe' was designed to capture the entire "primer-dimer"-like PCR product created. One microliter of each 100 μM capture probe was mixed with 6 μL of high salt buffer (10 mM $MgCl_2$, 150 mM NaCl, 100 mM Tris-HCl, 70% (*v*/v) H_2O). The mix was denatured at 100 °C for 10 min using a thermocycler, then snap-chilled on ice for 5 min. Three microliters of 100% ethanol was added to the solution on ice, making a final concentration of the probes of 10 μM.

Each denatured capture probe was spotted onto a separate printed square on the cloth and incubated at 35 °C in an uncovered petri dish for 30 min. Capture probes were covalently linked to the polyester cloth through exposure to 120 mJ/cm^2 of UV light (254 nm) for 60 s, using a CL-1000 Ultraviolet Crosslinker (UVP, Upland, CA, USA). To prevent non-specific binding, 2.5 mL of hybridization solution (5× saline-sodium citrate, 1% (*w/v*) skim milk powder, 0.1% sarkosyl, 0.02% sodium dodecyl sulfate) was added to the cloth and incubated with a cover for 1 h at 35 °C. The cloth was then washed five times with PBS containing 0.05% Tween 20 (PBST), and vacuum filtered on a bottle top filter between each wash.

2.5. Hybridization and colorimetric detection

The DIG-labelled PCR product was denatured at 100 °C for 10 min, then snap-chilled on ice for 5 min. Forty microliters of DIG-labelled PCR products were added to 1.5 ml of hybridization solution containing 2 M urea. The mix was applied to the entire CHAS cloth with affixed probes in a petri dish (Fig. 1), and the dish was then covered with a lid and incubated at RT for 30 min. After incubation, the cloth was removed from the petri dish, placed on the bottle top filter and washed five times with PBST, with vacuum filtering between each wash. For immunoenzymatic detection, the cloth was saturated with 1.0 ml PBST containing 0.5% (*w/v*) skim milk powder and 1:2000 dilution of anti-digoxigenin antibody, conjugated with horse-radish peroxidase (HRP) (Sigma Aldrich, St. Louis, MO, USA). The cloth was then incubated at RT for 10 min and washed five times with PBST, with vacuum filtering between the washes. For colorimetric detection, the cloth was covered with 1.0 ml 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). Positive samples showed a colour reaction within 15 min at RT. To prevent fading of the reaction, cloths were stored in covered petri dishes in the dark. Digital images of the reaction on the cloths were taken using a smart phone.

3. Results

3.1. Nested multiplex PCR is capable of simultaneously amplifying Giardia spp., Cryptosporidium spp. and Toxoplasma gondii

The multiplex PCR was initially set up with equal primer concentrations per amplicon. However, some primers produced more amplicons than others. The most abundant PCR amplicon was seen for the shortest gene, *Toxoplasma* B1. Therefore, we tested both the outer and nested multiplex PCR with varying concentrations of *Toxoplasma* B1 primers, ranging from 25 nM to 200 nM, while keeping the concentrations of all other primers constant at 200 nM. Our results indicated that a *Toxoplasma* B1 primer concentration of 100 nM led to the most homogeneous amplification of all amplicons (Fig. 2). Therefore, these primer concentrations were used for all subsequent experiments, as described in the Materials and methods.

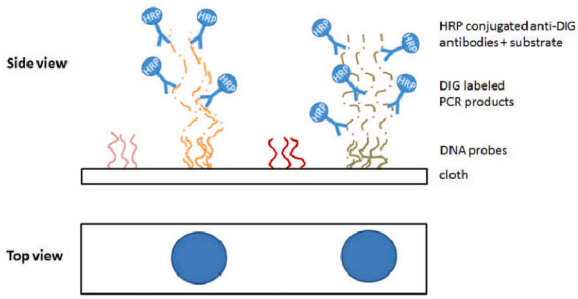

Fig. 1. Cloth-based hybridization array system (CHAS) setup. DIG, digoxigenin; HRP, horse-radish peroxidase.

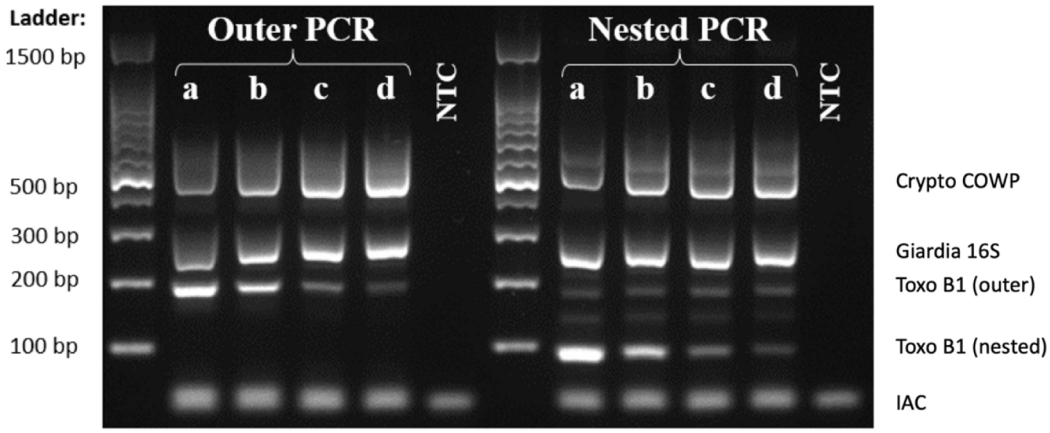

Fig. 2. Nested Multiplex PCR. Images of 2% agarose gels with various concentrations of B1 outer and B1 nested primers (band at 97 bp) while the concentrations of *Cryptosporidium* COWP, *Giardia* 16S, and *Giardia* tpi primers remained constant at 200 nM each. Primers are described in Table 1. B1 outer and nested primer concentrations were 200 nM (a), 100 nM (b), 50 nM (c), and 25 nM (d), respectively. Outer PCR: Crypto COWP (769 bp), *Giardia* 16S (292 bp), Toxo B1 (155 bp), IAC (50 bp). Nested PCR: Crypto COWP (553 bp), *Giardia* 16S (292 bp), Toxo B1 (97 bp), IAC (50 bp). Ladder: GelPilot 100 bp plus ladder (Qiagen, Toronto, ON, Canada). NTC = no template control.

3.2. *CHAS is specific for detection of Giardia spp., Cryptosporidium spp. and Toxoplasma gondii*

CHAS specificity was determined using parasite DNA amplicons individually and multiplexed. Amplification of nested PCR products leads to incorporation of DIG-dUTP/DIG-dCTP that can then be detected in the subsequent immunoenzymatic reaction.

To verify that there was no cross-reaction amongst PCR amplicons on the CHAS cloth, we applied the DIG-labelled PCR amplicons both individually and combined on cloths that were spotted with all probes used in this study (Fig. 3). In the absence of parasite amplicons, only the IAC showed a colorimetric reaction on the cloth. A positive IAC reaction verified that the DIG-incorporation and PCR amplification were successful and, therefore, excluded false negative results (Fig. 3b). When a mixture containing IAC and *G. duodenalis* amplicons was applied to the cloth, coloured spots only appeared in squares with the IAC probe and the *Giardia* spp. 16S probe (Fig. 3c). Similarly, coloured spots only appeared in squares with the IAC probe and the *Cryptosporidium* spp. COWP probe when a mixture containing IAC and *C. parvum* amplicons was applied to the cloth (Fig. 3d). Coloured spots only appeared in squares with the IAC probe and the *T. gondii* B1 probe when a mixture containing IAC and *T. gondii* amplicons were applied to the cloth (Fig. 3e).

The CHAS assay also enabled the detection of all three parasite species simultaneously (Fig. 3f). Cloths were treated with either a

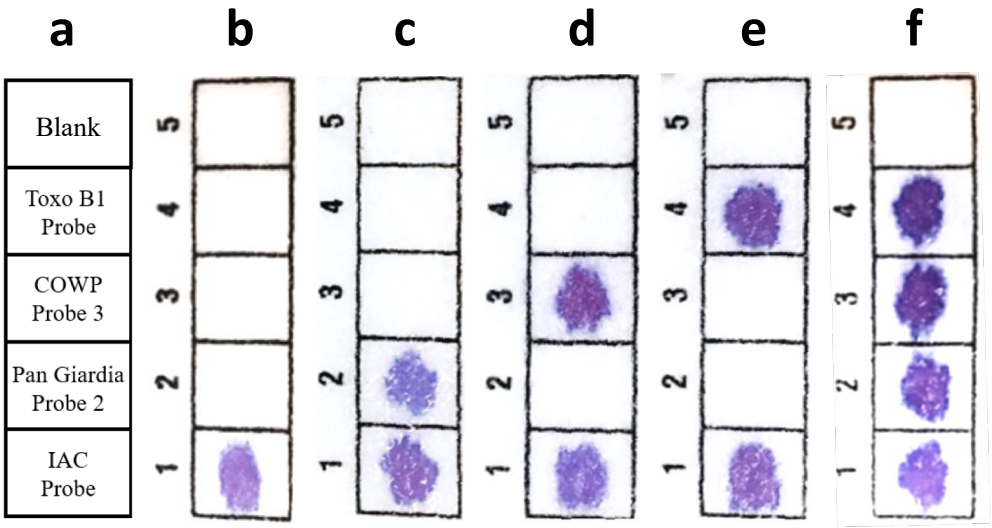

Fig. 3. PCR amplicon detection of the tested protozoan parasites on CHAS cloth. Photographs of polyester cloths showing results of the CHAS reaction. (a) Layout of CHAS cloth showing the arrangement of probes. Spot 5 was left blank as a negative control. (b-f) Results of CHAS reaction when different combinations of PCR amplicons were applied to the cloth. (b) IAC only; (c) IAC and *Giardia* spp. 16S; (d) IAC and *C. parvum* COWP; (e) IAC and *Toxoplasma gondii* B1; (f) IAC, *Giardia* spp. 16S, *Cryptosporidium* spp. COWP, and *Toxoplasma gondii* B1.

pool of individual amplicons for each parasite or with multiplex PCR reactions containing the same amplicons, both yielding the same correct pattern of results. When IAC, *G. duodenalis* 16S, *C. parvum* COWP, and *T. gondii* B1 amplicons were applied to the cloth, coloured spots developed in squares containing the IAC probe, the *Giardia* spp. 16S probe, the *Cryptosporidium* spp. COWP probe, and the *Toxoplasma gondii* B1 probe. Since square 5 was left as a blank, no colour developed. Thus, we showed that the target amplicons did not cross-react, nor bind non-specifically, to any of the other probes that were used in this study.

To determine if the CHAS assay was specific enough to detect and differentiate genotypes of the same species, a mixture of amplicons containing IAC, *Giardia* spp. 16S, and either *G. duodenalis* Assemblage A tpi or *G. duodenalis* Assemblage B tpi amplicons were applied to the cloths (Fig. 4). Tpi primers were designed to amplify only tpi from *G. duodenalis*, and not from *G. muris*. In Fig. 4c, a mixture of IAC and *G. muris* 16S amplicons was applied to the cloth. Only squares 1 and 2, containing the IAC probe and the *Giardia* spp. 16S probe, respectively, showed coloured spots. Because the *G. muris* tpi gene differs from that of *G. duodenalis*, no reaction occurred with probes designed to detect *G. duodenalis* Assemblage A or B tpi (Fig. 4c). As shown in Fig. 4d, the cloth strip incubated with IAC, *G. duodenalis* 16S, and *G. duodenalis* Assemblage A tpi amplicons, showed coloured spots in squares 1–3. No colour developed in square 4 (Fig. 4d), which had the Assemblage B tpi probe 3.3 bound to it. This indicated that Assemblage B tpi probe 3.3, designed to detect *Giardia* Assemblage B, did not cross-react with Assemblage A. Similarly, the cloth strip shown in Fig. 4e, which was incubated with IAC, *G. duodenalis* 16S, and *G. duodenalis* Assemblage B tpi amplicons, showed coloured spots in squares 1, 2, and 4, but not in square 3. Thus, Assemblage A tpi probe 3 was specific to Assemblage A tpi and did not cross react with Assemblage B tpi amplicons.

3.3. Spiking of fresh produce and water samples

Both fresh produce and water are important sources of infection with protozoan parasites (Efstratiou et al., 2017a; Gajadhar, 2015). However, testing for parasites in food and water samples is often complicated due to the presence of PCR inhibitors and non-target amplification. To confirm that our nested multiplex PCR and CHAS method worked with both food and water samples, we spiked samples of romaine lettuce and parsley, as well as river water and wastewater with equal numbers of all three parasite species in question. Nested multiplex PCR and CHAS was performed as described in the Materials and methods.

Fig. 5 shows representative results of the various artificially contaminated sample types after amplification using nested multiplex PCR and CHAS detection with all three protozoan parasites. Unlike, PCR followed by sequencing, the detection and differentiation of parasites using this multiplex PCR and CHAS method could be accomplished in a single day. The cost per sample using the CHAS method is approximately CAD $4, with high sensitivity due to the use of multiplex PCR, and high specificity due to the lack of cross-reaction with off-target amplicons.

4. Discussion

The aim of this study was to develop a novel method for simple and rapid detection of *Giardia* spp., *Cryptosporidium* spp., and *Toxoplasma gondii*, which are important protozoan parasites commonly linked to water- and foodborne diseases (Devleesschauwer

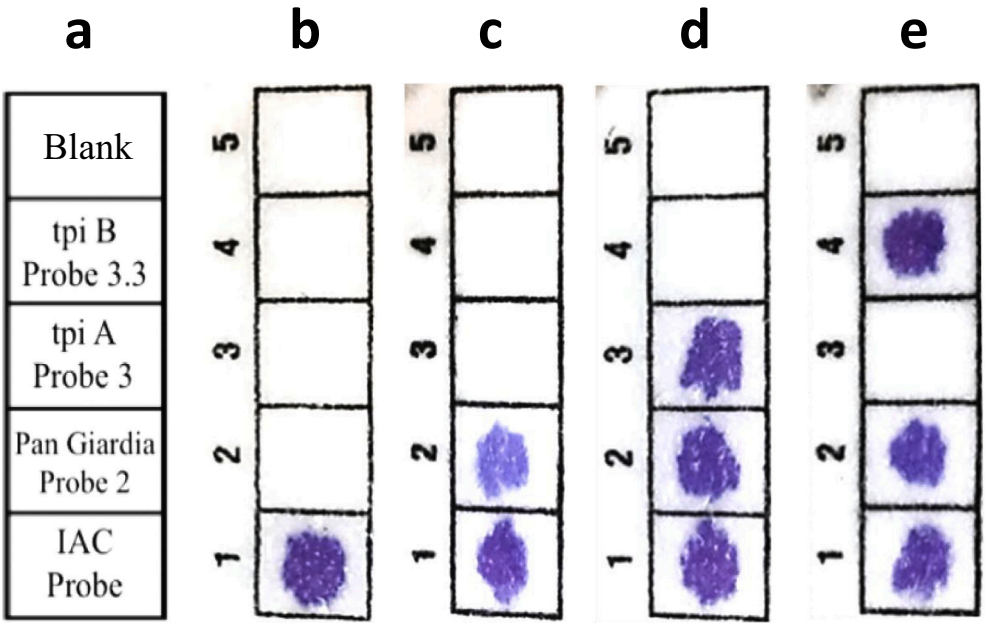

Fig. 4. *Giardia duodenalis* genotype detection on CHAS cloth. (a) Layout of CHAS cloth showing the arrangement of probes. (b-e) CHAS results after addition of different combinations of PCR amplicons. (b) IAC only; (c) IAC and *G. muris* 16S; (d) IAC, *G. duodenalis* 16S, and *G. duodenalis* Assemblage A tpi; (e) IAC, *G. duodenalis* 16S, and *G. duodenalis* Assemblage B tpi.

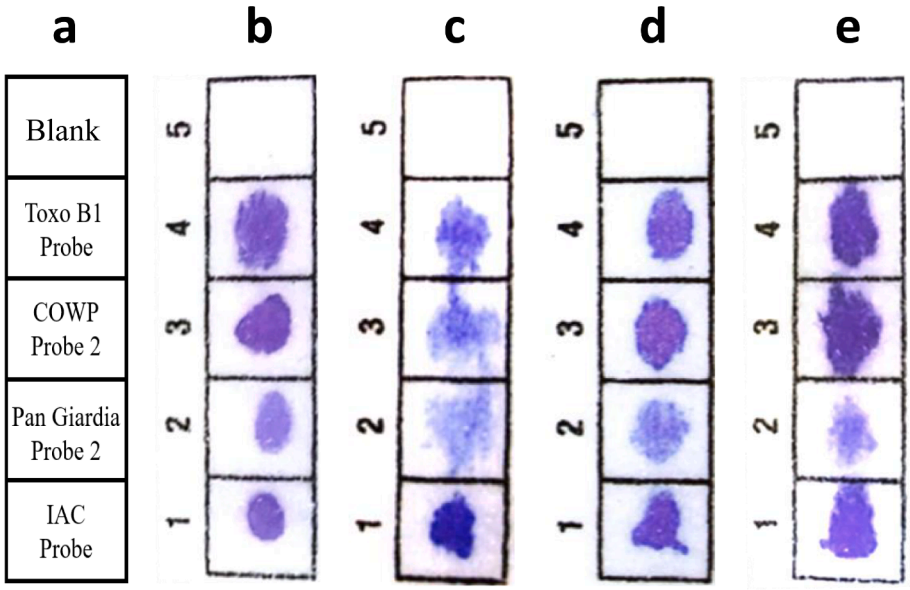

Fig. 5. Parasite detection in spiked food and water samples. (a) Layout of CHAS cloth showing the arrangement of probes; (b) romaine lettuce; (c) parsley; (d) wastewater; (e) river water.

et al., 2017). Primers described in this study can be used individually or in a multiplex PCR. When using a multiplex PCR approach, it is common to see a shift in amplicon size migration compared to singleplex PCR (Deng et al., 2000). Shorter amplicons tend to outcompete longer amplicons and are, therefore, overrepresented (Sint et al., 2012). Furthermore, *Toxoplasma* B1 is a multicopy gene. Gradual reduction of the primers for the shortest amplicon, *Toxoplasma* B1, was done until a homogeneous amplification of all amplicons was achieved (Fig. 2).

We were able to demonstrate in this study that CHAS could detect, and differentiate amongst, *G. duodenalis, Cryptosporidium* spp., and *T. gondii*, both in suspension and in artificially contaminated fresh produce and water samples. The CHAS probes did not cross-react with non-target amplicons. Furthermore, the tpi probes (tpiA and tpiB) are specific to *G. duodenalis* Assemblage A and B, respectively. The probes were designed to anneal to a tpi gene region where Assemblage A and B differ by at least eight nucleotides. Upon designing the CHAS assay, the triosephosphate isomerase (tpi) gene was selected for detection of *G. duodenalis* since this gene is conserved within the assemblages but differs amongst them. The tpi gene is present in all *Giardia* species, but has high genetic heterogeneity. Primers and probes designed for this study were selected so as to be able to distinguish *G. duodenalis* Assemblages A and B, and not cross react with other *Giardia* species, such as *G. muris*.

An IAC was incorporated into each step of the CHAS process to ensure there was no inhibition in the amplification of target genes due to PCR inhibitors. PCR inhibitors are ubiquitous in a variety of samples (Hoorfar et al., 2003). The IAC is made up of a primer pair that anneals to one another, producing a "primer-dimer"-like structure. This structure can be detected by hybridizing with its specified oligonucleotide probe. If the target genes were not amplified properly due the presence of PCR inhibitors, the IAC would also fail to amplify. Additionally, the IAC acts as a positive control in the CHAS process. A colour reaction should always develop where the IAC probe is bound. This prevents the reporting of false-negative results due to failed PCR amplification, unsuccessful immobilization of the probes to the cloth, or an issue with the reagents used.

Variation in colour development on the cloth may be influenced by multiple parameters. An important factor that determines the strength of the colour on the cloth is the amount of DIG-labelled dNTPs that is incorporated into the amplicon. Immunoenzymatic detection only occurs when anti-DIG-HRP conjugates bind to DIG (Blais et al., 2012). If an insufficient amount of DIG is present, fewer anti-DIG-HRP conjugates are bound, therefore converting less TMB to a blue colour. A mix of DIG-dUTP and DIG-dCTP ensured that sufficient DIG-labelled dNTPs would be incorporated into amplicons, regardless of GC content.

Unlike many of the newer molecular methods available for the detection of food and waterborne parasites, the CHAS assay does not require specialized equipment. Moreover, it does not require access to a computer for data analysis, and photographs can be used for archiving results. Another major benefit of the CHAS is that it is a simple visual test. However, it relies on a robust PCR with internal amplification controls. Because of its specificity, CHAS could replace DNA sequencing, which requires down-stream analyses, expensive equipment and highly trained personnel. CHAS has the potential to be an inexpensive, rapid, and reliable method for detection and characterization of microorganisms in remote areas or developing countries, where sequencing facilities may not be readily available (Helmy et al., 2016). To further verify the results, the nested multiplex PCR amplicons may still be analyzed with Sanger sequencing.

This study demonstrated the feasibility of using CHAS for the detection and differentiation of parasites in food and water samples, and suggests that this novel method may be a useful tool for testing laboratories, especially those lacking the resources to perform DNA

sequencing. Follow-up studies are planned to determine the sensitivity of the CHAS method in the detection of parasites in a wider variety of food commodities and water samples by spiking with different concentrations of cysts and oocysts. As with any detection methods, the low concentrations of parasites in naturally-contaminated food and water, limit their detection due to the inherent loss of cysts and oocysts during washing and concentration.

Authors' statement

BRD, NC, and SJR designed the experiments. SJR, SZ, RB, and HM collected and analyzed the data. SJR and BRD wrote the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors would like to thank Dr. Burton Blais and Martine Gauthier, Canadian Food Inspection Agency, for providing CHAS cloths and expert advice. 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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