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Multiplex PCR method to detect *Cyclospora*, *Cystoisospora*, and Microsporidia in stool samples

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Abstract

Cyclospora, *Cystoisospora*, and Microsporidia are eukaryotic enteropathogens that are difficult to detect in stool samples because they require special stains and microscopy. We developed a multiplex PCR reaction with 4 primer sets to amplify *Cyclospora cayetanensis*, *Cystoisospora belli*, *Enterocytozoon bieneusi*, and *Encephalitozoon intestinalis*. Detection of the amplicon is through specific probes coupled to Luminex beads. Sensitivity of the assay was evaluated using *Encephalitozoon intestinalis* spores and revealed detection of 10¹ spores spiked into stool. No cross reactivity was observed. We evaluated the assay on diarrheal specimens from Thailand, Tanzania, Indonesia, and the Netherlands that had been previously tested by microscopy and the assay yielded 87–100% sensitivity and 88–100% specificity. Microscopy negative/PCR positive samples had lower Luminex values suggesting they were true but lower burden infections. In summary this is a convenient single PCR reaction that can detect *Cyclospora*, *Cystoisospora*, and Microsporidia without the need for cumbersome microscopic analysis.

INTRODUCTION

Cystoisospora belli (previously known as *Isospora*), Microsporidia (*Encephalitozoon intestinalis* and *Enterocytozoon bieneusi*; reclassified as fungi from protozoa; Keeling et al. 2000), and *Cyclospora cayetanensis* are eukaryotic enteropathogens that are difficult to detect in stool samples because they require complex stains and skilled microscopists. The epidemiology of these infections is not well understood because of this diagnostic uncertainty, but they are often considered by clinicians in special cases of diarrhea, such as in the setting of persistent diarrhea, foodborne outbreaks, and in individuals with travel

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histories or immunocompromise. We have previously developed PCR based panels for several intestinal parasites, including *Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium* spp., *Ascaris lumbricoides*, hookworm, and *Strongyloides stercoralis* (Taniuchi et al. 2011), as well as a viral panel that includes rotavirus, adenovirus, astrovirus, sapovirus, and norovirus (Liu et al. 2011). In this work we augmented these panels to include the *Cyclospora*, *Cystoisospora*, and microsporidial targets. Of the microsporidial targets we chose to detect *Encephalitozoon intestinalis* and *Enterocytozoon bieneusi* since they are reported to be the major intestinal microsporidia (Didier and Weiss 2006). The assays use biotinylated primers followed by capture and detection via internal probes that are linked to beads. Amplicons are detected on the Luminex platform, which allows broad multiplexing capability and integration with other Luminex based assays. The result is a panel that provides sensitive and specific detection of *Cystoisospora*, Microsporidia, and *Cyclospora* that can be used either in isolation or in concert with other panels.

MATERIALS & METHODS

Control material

Positive control materials used in this study included purified *Encephalitozoon intestinalis* spores (Waterborne Inc., New Orleans, LA) and sequence-confirmed DNA extracted from clinical fecal specimens positive for *Cyclospora cayetanensis*, *Cystoisospora belli*, or *Enterocytozoon bieneusi*. We also synthesized an 825 bp sequence of the 5.8S/ITS region of the *Cystoisospora belli* (DNA2.0, Menlo Park, CA). Negative controls were water. Since *E. intestinalis* spores are the only organism available in purified form, we performed sensitivity testing by serially spiking *E. intestinalis* spores into 200mg aliquots of a single source stool sample from one healthy uninfected individual. For *Cystoisospora*, *E. bieneusi*, and *Cyclospora* we also generated plasmid DNA containing the amplicons (TA Cloning Kit, Invitrogen, Carlsbad, CA). Plasmids were transfected into *E. coli*, grown, and plasmid DNA extracted (QIAprep Miniprep Kit, Qiagen, Valencia, CA) and spiked into fecal DNA extracts from a healthy donor to determine analytical sensitivity.

Microscopy

Cyclospora and *Cystoisospora* were identified by microscopy after modified Ziehl-Neelsen acid fast stain or autofluorescence microscopy. Microsporidia were detected by optical white staining or modified Trichrome stain (Ryan-Blue), which required pinkish red spore walls with interior polar tube evidence for confirmation.

Fecal specimens

One hundred twenty one fecal specimens were obtained from an ongoing study at Kilimanjaro Christian Medical Centre (KCMC), Moshi, Tanzania. Additionally, 91 specimens were obtained from the Leiden University Medical Center (LUMC) clinical microbiology laboratory and Department of Parasitology's collaborative epidemiological projects in multiple countries. Furthermore 24 stool samples were tested at the Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. All samples were tested by microscopy as above. Of note, the AFRIMS and LUMC samples were also tested by in-house or published real-time PCR assays (Verweij et al. 2003, Verweij et al. 2007, ten Hove et al. 2008). In order to establish statistical power, we sought to obtain at least 25 microscopy positive samples per organism with the exception of *E. intestinalis* for which positive samples were limited. All protocols were approved by the respective institutional review boards at UVA, KCMC, AFRIMS, and Leiden University.

DNA extraction

DNA was extracted using a modified QIAamp DNA Stool Mini Kit protocol (Qiagen Inc., Valencia, CA) for specimens from AFRIMS and KCMC. Briefly, 1.4ml of the lysis buffer ASL was added to the stool then the suspension was pretreated by bead beating with 0.15 mm garnet beads (MO-BIO Laboratories, Inc, Carlsbad, CA, US) for 2 min followed by incubating for 7 min at 95°C prior to extraction. DNA extraction for the specimens from LUMC was performed using regular QIAamp mini Kit spin columns with modifications which included a pretreatment with PVPP (polyvinylpyrrolidone) and boiling (Verweij et al. 2001). *E. intestinalis* positive controls were extracted using the QuickGene DNA tissue kit S on the QuickGene-810 platform (Fujifilm, Tokyo, Japan), with a modification to allow a larger input stool volume of 200 mg. Briefly, 1ml of the tissue lysis buffer MDT was added to the stool then the suspension was pretreated by bead beating with a tube of 0.15 mm garnet beads (MO-BIO Laboratories, Inc, Carlsbad, CA, US) for 2 min followed by boiling for 7 min prior to extraction. Other modifications included the addition of 100µl of EDT solution (Proteinase K) from the kit and a longer incubation time following the addition of EDT solution for 90 min. All DNA samples were stored at –80°C until use in PCR.

Multiplex PCR

Primer sequences for *C. cayetanensis* (Chu et al. 2004) and *E. intestinalis* (Verweij et al. 2007) have been described previously. Primers for *C. belli* and *E. bieneusi* were developed during this work. Target genes included the small subunit ribosomal RNA gene for *C. cayetanensis*, *E. bieneusi*, and *E. intestinalis* and 5.8s rRNA and ITS2 for *Cystoisospora belli* (Table 1). Briefly, PCR amplification reaction for the 4-plex panel was performed in 25 µl volume with 12.5 µl of iQ Multiplex Powermix (Bio-Rad, Hercules, CA) which contains dNTPs and 12mM MgCl₂, 0.33µM of each forward primer, and 0.33µM of each reverse primer. PCR cycling condition consisted of initial 3 min 95°C cycle followed by 40 cycles of 15sec at 95°C and 60sec at 58°C.

Internal probes were amine modified at the 5'-end and included 12 carbon spacers. All probes and primers were purchased from Integrated DNA Technologies, Inc (Coralville, Iowa). Probes were coupled to carboxylated Luminex beads with EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride). Hybridization of beads to the PCR products was performed at 50°C for the assay for 35 min using the Oligonucleotide Hybridization Protocol from Luminex Corporation (Luminex 2006). Detection of the amplicon was performed by the Bioplex 200 (Bio-Rad, Hercules, CA) or Luminex 100 (Luminex Corporation, Austin, TX) after addition of streptavidin PE as the reporter molecule. Negative control material (nuclease free water) and positive controls were included with each run. All discrepant samples were retested by assays in a real-time PCR format with the same primer/probe sequences or a previously published real-time PCR assay.

Luminex Data

Luminex data was reported as Median Fluorescence Intensity (MFI). We calculated a corrected MFI (cMFI) which normalizes to background fluorescence as follows: $cMFI = [MFI (sample) - MFI (background)] / MFI (background)$ in order to accommodate testing on the four sites' Luminex platforms given differences in softwares (Star Station v.2.0 in the Netherlands, Luminex 100 IS Software v.2.3 in Thailand, and Bioplex v.5.0 at UVA and Tanzania) and in high vs. low photomultiplier tube voltages.

Statistics

The cMFI values yielded values that were not normally distributed thus were compared using Mann-Whitney (SPSS Inc., Chicago, IL). Data shown as mean $\pm$ standard error of the mean (SEM) unless otherwise indicated. We calculated a 95% confidence intervals for sensitivity and specificity based on the sample size chosen for this study using the formula (confidence interval = $p \pm 1.96 \times [p(1-p)/n]^{1/2}$) where p=sensitivity or specificity and n=number of infected samples for sensitivity or uninfected samples for specificity determined by microscopy, the gold standard test (Banoo et al. 2006).

RESULTS

Selection of PCR reactions

In developing the Luminex assays, we found that biotinylating the primer located closest to the probe region yielded higher fluorescence for each amplicon (data not shown). Luminex-PCR reactions were all initially performed in singleplex reactions and then multiplexed. For the *E. bieneusi*, *E. intestinalis*, *Cyclospora*, and *Cystoisospora* assays we attempted several published and in-house designs in singleplex to obtain this multiplex reaction (Table 1).

Sensitivity and Specificity of the assays

E. intestinalis is the only pathogen which is available in a purified preparation and thus usable for spiking studies. Using dilutions of spores spiked into a common stool sample and the commonly-used fluorescence cutoff of 2.5, our assay was able to detect 10^1 spores of *E. intestinalis* per 200mg of stool (Table 2). We spiked plasmid with organism specific sequence inserts into stool DNA to determine analytical sensitivities for *Cyclospora*, *Cystoisospora*, and *E. bieneusi*. The limit of detection was 10^2 plasmid copies for *Cystoisospora* and *E. bieneusi* and 10^3 plasmid copies for *Cyclospora* (Table 2). Known positive specimens for *Giardia*, *Cryptosporidium*, *E. histolytica*, *Ascaris*, *Strongyloides*, *Necator*, *Ancylostoma*, *Trichuris*, enteroaggregative *E. coli*, enteroinvasive *E. coli*, enteropathogenic *E. coli*, enterotoxigenic *E. coli*, enterohemorrhagic *E. coli*, rotavirus, adenovirus, astrovirus, sapovirus, and norovirus were tested and none of them yielded detection above background with any of the assays; specificity was further evaluated by sequencing positive bands of the singleplex amplicons during early development and 100% identity was confirmed (data not shown).

Use on clinical specimens

The results of microscopy, real-time PCR, and the Luminex assay for the detection of *E. bieneusi*, *E. intestinalis*, *Cyclospora*, and *Cystoisospora* are summarized in Table 3. We were able to obtain over 30 specimens that were previously microscopically positive for each parasite except *E. intestinalis*, which is rare. Performance of the multiplex Luminex assay versus microscopy revealed 87–100% sensitivity and 88–100% specificity (Table 3). Available discrepant samples were evaluated by singleplex real-time PCR, which further supported the PCR-Luminex results in 10 of 20 specimens (see Table 3 footnotes for details). For *Cystoisospora* and *E. bieneusi*, the PCR Luminex assay detected about 50% more infections than microscopy. These microscopy–/PCR+ yielded lower cMFI values than their microscopy+/PCR+ counterparts, suggesting they were true positive lower level infections. Sequence data was available for 1 discrepant *Cyclospora* sample and revealed a single polymorphism in the Luminex probe region. Microscopy for microsporidia cannot discriminate species, and we observed 2 microscopy+/PCR– samples. On the side of caution we have attributed these to be *E. bieneusi* PCR– specimens but in fairness these could be different microsporidia species and not represent *E. bieneusi* PCR failure.

DISCUSSION

This multiplex PCR protocol provides a sensitive and specific assay for *Cyclospora*, *Cystoisospora*, and Microsporidia, eukaryotic enteropathogens that are difficult to detect with conventional methods. With the lack of skilled microscopists the need for molecular assays for such pathogens will increase and can be used to better define the epidemiology of these infections.

Several PCR based assays are emerging for enteropathogens and assembly of different panels is possible. In this context this multiplex assay for these pathogens can fill an important niche, for example if no other pathogen is implicated in a case of diarrhea. We developed the assay for detection on a Luminex platform however conversion to other platforms such as array or multichannel real-time PCR is certainly possible. Our assay is also modular, such that one could use only one or two of the reactions if desired. For instance, one could add our *Cryptosporidium* assay (Taniuchi et al. 2011) to create a 5-plex assay if one wanted to screen for these organisms without the need for special stains (we observed no diminution in signal when *Cryptosporidium* was added to the 4-plex panel on 36 samples, data not shown). Finally, the assay is robust, with field testing for this work having been successfully performed on-site in diverse sites including Thailand, Tanzania, Virginia, and the Netherlands. In summary we present a molecular assay validated on a large number of specimens for important eukaryotic enteropathogens that can be used in isolation or in the context of additional enteropathogen screening algorithms.

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Table 1

Primers and probes used in the PCR-Luminex assays.

Organism	Target	GenBank Accession	Reference	Name	Sequence (5'→3')
<i>Cyclospora cayentanensis</i>	18S rRNA	AF111183	Chu et al. 2004 this paper	Cc280F	GTAGCCTTCGCGCTTCG
				Cc280R*	CGTCTTCAAAACCCCTACTGTCTG
				Cc280P	GCAATTTGCCAAGGATGTTTT
<i>Cystoisospora belli</i>	5.8s rRNA & ITS2 region	AF443614	this paper this paper this paper	Ib213F	GGATATTCCCTGCAGCATGT
				Ib213R*	CGGGACACAACTCAACACTG
				Ib213P	GTCACAGCGCGCTTTACGC
<i>Encephalitozoon intestinalis</i>	18S rRNA	U09929	Verweij et al. 2007 this paper	Eimt214F	CACCAAGTTGATTCTGCCTGAC
				Eimt214R*	CTAGTTAGGCCATTACCCCTAAGTACCA
				Eimt214P	CGAGCCCAAGTAAAGTTGTAGG
<i>Enterocytozoon bienersi</i>	ssrRNA	AF024657	this paper this paper this paper	Eb202F	CCAGGGTCAAATCATTCGTT
				Eb202R*	TATTGTATTGCGCTTGCTGC
				Eb202P	GATGCCCTTAGATATCCTGG

* biotinylated primer in the PCR-Luminex assay

Table 2

Analytical Sensitivity of Serially Spiked Samples.

Organism	Quantity spiked in stool or stool extract	cMFI
<i>E. intestinalis</i> spores	10 ⁵ organisms	56.85 ± 8.06
	10 ⁴	43.51 ± 6.68
	10 ³	4.89 ± 1.39
	10 ²	4.29 ± 1.17
	10 ¹	2.61 ± 1.33
	10 ⁰	1.81 ± 1.88
<i>E. bienersi</i>	10 ⁸ plasmid copies	6.26 ± 2.29
	10 ⁶	7.42 ± 1.89
	10 ⁴	6.94 ± 0.54
	10 ²	4.29 ± 0.28
	10 ⁰	0.02 ± 0.01
<i>C. cayetanensis</i>	10 ⁹ plasmid copies	52.48 ± 1.30
	10 ⁷	49.04 ± 2.39
	10 ⁵	53.47 ± 1.02
	10 ³	25.66 ± 2.65
	10 ⁰	-0.23 ± 0.04
<i>Cystoisospora belli</i>	10 ⁸ plasmid copies	16.84 ± 4.30
	10 ⁶	18.83 ± 0.67
	10 ⁴	13.82 ± 2.45
	10 ²	5.51 ± 2.52
	10 ⁰	-0.12 ± 0.11

* cutoff cMFI = 2.50. All samples performed in triplicate.

Table 3

Performance of multiplex PCR-Luminex vs. conventional microscopy.

Organism	microscopy+	microscopy–	Sensitivity (95% CI)	Specificity (95% CI)
<i>Cyclospora</i> PCR/Luminex+ ^a	41	0	0.87 (0.76–0.97)	1.00
<i>Cyclospora</i> PCR/Luminex–	6	189		
<i>Cystoisospora</i> PCR/Luminex+ ^a	26 (cMFI 48.5±1.3) ^b	14 ^c (cMFI 16.2±1.3) ^b	0.93 (0.84–1.00)	0.93 (0.90–0.96)
<i>Cystoisospora</i> PCR/Luminex–	2 ^c	194		
<i>E. intestinalis</i> PCR/Luminex+ ^a	1	2	1.00	0.99 (0.98–1.00)
<i>E. intestinalis</i> PCR/Luminex–	0	233		
<i>E. bienersi</i> PCR/Luminex+ ^a	40 (cMFI 21.8±0.5) ^b	23 ^c (cMFI 8.2±0.4) ^b	0.95 (0.88–1.00)	0.88 (0.88–0.93)
<i>E. bienersi</i> PCR/Luminex–	2 ^c	171		

^aLuminex positive was defined as cMFI ≥ 2.5 for all pathogens.^bP<0.01 comparing cMFI in microscopy+ vs. microscopy– specimens using the Mann Whitney test.^cTwenty of the *Cystoisospora* and *E. bienersi* discrepant samples were available and upon retesting by singleplex real-time PCR 10 (50%) yielded the Luminex-PCR result.