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Detection and Genotyping of *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, and *Cryptosporidium parvum* by Oligonucleotide Microarray

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Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* are the most frequently identified protozoan parasites causing waterborne disease outbreaks. The morbidity and mortality associated with these intestinal parasitic infections warrant the development of rapid and accurate detection and genotyping methods to aid public health efforts aimed at preventing and controlling outbreaks. In this study, we describe the development of an oligonucleotide microarray capable of detecting and discriminating between *E. histolytica*, *Entamoeba dispar*, *G. lamblia* assemblages A and B, and *C. parvum* types 1 and 2 in a single assay. Unique hybridization patterns for each selected protozoan were generated by amplifying six to eight diagnostic sequences/organism by multiplex PCR; fluorescent labeling of the amplicons via primer extension; and subsequent hybridization to a set of genus-, species-, and subtype-specific covalently immobilized oligonucleotide probes. The profile-based specificity of this methodology not only permitted for the unequivocal identification of the six targeted species and subtypes, but also demonstrated its potential in identifying related species such as *Cryptosporidium meleagridis* and *Cryptosporidium muris*. In addition, sensitivity assays demonstrated lower detection limits of five trophozoites of *G. lamblia*. Taken together, the specificity and sensitivity of the microarray-based approach suggest that this methodology may provide a promising tool to detect and genotype protozoa from clinical and environmental samples.

Entamoeba histolytica, *Giardia lamblia* (syn. *intestinalis* or *duodenalis*) and *Cryptosporidium parvum* are three of the most common intestinal protozoan parasites infecting humans worldwide (25). The disease manifestation of *E. histolytica* infections, invasive intestinal amoebiasis, causes up to 100,000 deaths per year globally (31). Even more staggering are the 600 million estimated intestinal infections caused by the diplomonad *G. lamblia* (giardiasis) and the apicomplexan *C. parvum* (cryptosporidiosis). Both are recognized as common causes of diarrheal disease worldwide, and although usually self-limiting in immunocompetent individuals, *G. lamblia* and *C. parvum* infections can become chronic and deadly in immunocompromised patients and malnourished children (3, 37). In addition to posing a continuing health threat to civilian populations, the disabling gastrointestinal disease caused by these organisms also presents a serious threat to the overall mission readiness of deployed military personnel as outbreaks of all three diseases usually occur through fecal-oral transmission and are most often associated with the consumption of untreated water or contaminated food (18).

The global distribution and increasingly frequent presence of *E. histolytica*, *G. lamblia*, and *C. parvum* in ambient and source waters suggest that rapid and accurate identification methods are important for public health efforts to prevent and control outbreaks. Traditionally, laboratory detection of these three parasites has mostly relied on the microscopic examination of stool samples and water concentrates (25, 41), which is laborious, insensitive, and requires professional training. The paramount limitation of this method is its inability to differentiate closely related species and heterogeneity within species, as it is often difficult to differentiate these pathogenic organisms from the cysts of nonpathogenic intestinal protozoa via microscopic examination. For example, the nonpathogenic protist *Entamoeba dispar* is morphologically indistinguishable from its sister species, pathogenic *E. histolytica* (11), but is responsible for approximately 10 times as many infections as *E. histolytica* that do not require treatment (15). In addition to genus-level identification, species differentiation and subtype differentiation also provide important epidemiological, surveillance, and host range information. *G. lamblia* isolates capable of causing infectious disease in humans are morphologically indistinguishable, yet can genetically be differentiated into two major groups, designated assemblages A and B (42). Similarly, morphologically indistinguishable *C. parvum* isolates can be differentiated into two distinct genotypes, 1 and 2: genotype 1 members exclusively infect humans and nonhuman primates, while genotype 2 members have a wider range of hosts, including humans and livestock (27). In this regard, antibody-based diagnostic methods are useful in that they can discriminate between different species or genotypes, but are far from optimal due to problems of nonspecific binding, variability among clinical isolates, and interference from sample debris (28).

Molecular methods, such as PCR, have aided in alleviating some of the sensitivity and specificity issues traditionally associated with the detection of protozoan pathogens. A number of PCR-based assays, including gene amplification with specific primers (17, 24, 33), multiplex PCR (12, 32), restriction fragment length polymorphism (3, 5, 10, 46), and real-time PCR (2, 4, 16, 23, 40), have been developed for the identification of

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protozoan infections. However, the shortcomings of PCR-based assays become apparent during practical applications. The generation of nonspecific DNA fragments from environmental and clinical samples poses a significant problem that often results in false-positive results. Conversely, the failure to amplify a single diagnostic sequence due to inhibitors in the sample or possible mutations in the primer binding region may result in false-negative results. Furthermore, although real-time PCR assays are sensitive enough to detect a single cell (4), the limited number of probes that can be applied in one reaction hinders its utility for confident multitarget detection and genotyping analyses.

Recently, oligonucleotide microarrays have been used successfully for the detection of bacterial and viral pathogens (7, 8, 13, 43, 44). The distinct advantage of this detection approach is that it combines powerful DNA amplification strategies with subsequent hybridization to oligonucleotide probes specific for multiple target sequences. This method allows for the simultaneous analysis of a larger number of genetic features in a single experiment (8). Thus, the amplification and hybridization approach produces a highly sensitive and specific platform with high-throughput capacity for pathogen detection and genotyping. Due to the increasing reliance upon genetic tests for identification and differentiation, the low concentration or number of organisms required to cause disease, and the often-found presence of multiple protozoan species in a single environmental or clinical sample, such methods would be ideally suited for the detection of waterborne protozoan parasites. In the present study, we demonstrate the first oligonucleotide microarray capable of simultaneously detecting and differentiating the primary waterborne protozoa pathogenic for humans, *E. histolytica*, *G. lamblia*, and *C. parvum*.

MATERIALS AND METHODS

Parasite isolates and DNA. Purified genomic DNA extracted from cultures of *E. histolytica* strain HM-1:IMSS clone 9, *E. dispar* SAW760 and *Entamoeba moshkovskii* strain Laredo were kindly provided by C. Graham Clark (London School of Hygiene and Tropical Medicine). Trophozoites of *G. lamblia* WB, GS-H7, and CM strains were generous gifts from Theodore E. Nash (National Institutes of Health, Bethesda, Md.). Genomic DNA and oocysts of *C. parvum* TU502, UG502, GCH1, *C. meleagridis*, and *C. muris* were obtained from Donna Akiyoshi (Tufts University, Medford, Mass.). Formalin-fixed oocysts of *C. parvum* Iowa isolates were purchased from Waterborne, Inc. (New Orleans, La.). Genomic DNA from *G. lamblia* trophozoites was isolated using the DNA STAT-60 kit (Tel-Test, Inc., Friendswood, Tex.) or simply released by suspension in distilled water. *C. parvum* DNA from purified oocysts was extracted following three cycles of freezing and thawing (35).

PCR primers. The primers used in the multiplex PCRs to amplify fragments from different target genes are listed in Table 1. These primers were either derived from publications or designed by PCR primer design software: Primer3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and Oligos (http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm).

Microarray design and fabrication. Target sequences were downloaded from either GenBank, the TIGR parasite database (The Institute for Genomic Research, Rockville, Md.), or the *Giardia lamblia* Genome Project (Marine Biology Laboratory, Woods Hole, Mass.). Orthologous sequences between *E. histolytica* and *E. dispar* and polymorphic sequences within *G. lamblia* and *C. parvum* were aligned by using ClustalW (http://www.ebi.ac.uk/clustalw/). Oligonucleotide probes were designed with Array Designer 2.02 (Premier Biosoft, Palo Alto, Calif.) and the Oligonucleotide Properties Calculator (http://www.basic.nwu.edu/biotools/OligoCalc.html) by using the following criteria: (i) probe length range between 23 and 30 nucleotides (nt) and (ii) melting temperature range of 55 to 65°C. At least two oligonucleotide probes were designed for each target sequence to identify and differentiate species of *Entamoeba*, *G. lamblia* assemblages, and *C. parvum* genotypes. For example, probes ssrDNA and locus1-2.3 are common to both *E. histolytica* and *E. dispar* (genus-specific probes), whereas the remainder of the *Entamoeba* probes were species specific. For *G. lamblia*, both species-specific and assemblage-specific probes were selected from the gene coding for giardin and *gdh*, whereas the two *hsp70* probes were only species specific and the *tpi* and *c4* probes were assemblage specific. As the available *C. parvum* sequences with polymorphic characteristics were limited, probes from only three genes (*dhf*, *ptg*, and *p23*) were genotype specific, while the remaining probes were species specific. The sequences of all 92 probes and one additional internal positive control probe used in this study are presented in Table 2. Based on the design specificities, the expected hybridization patterns are shown in Fig. 2B (internal positive hybridization spots at left and right ends of each array not included). The probes were synthesized with a 5′ amino modifier and 12-carbon spacer (QIAGEN Operon, Alameda, Calif.) and were resuspended in a carbonate-bicarbonate buffer (100 mM, pH 9.0) at a final concentration of 50 μM. The probes were printed onto 3-aminopropyltriethoxysilane (silanization)-plus-1,4-phenylene diisothiocyanate (cross-linker)-modified glass slides for covalent probe immobilization (6), using a Virtek ChipWriter Pro contact printer at KamTek, Inc. (Gaithersburg, Md.). The printed slides were stored desiccated at room temperature.

Multiplex PCR and synthesis of Cy5-labeled targets. Multiplex PCR was conducted with the HotStarTaq Multiplex PCR kit according to the standard protocol (QIAGEN, Valencia, Calif.). The amplification reaction mixture (25 μl) consisted of 12.5 μl of 2× Master buffer, a mixture of 200 nM (each) forward and reverse primers, and 1 to 2 μl of template DNA. PCR was carried out in a Peltier Thermal Cycler PTC225 (MJ Research, Inc., Reno, Nev.) with an activation step at 95°C for 15 min; followed by 45 cycles of 94°C for 30 s, 54°C for 90 s, and 72°C for 90 s; and a final extension at 72°C for 10 min. PCR products (5 μl) were analyzed by electrophoresis with 2% agarose gels, and the remaining reaction volumes were purified with the DNA Clean & Concentrator-25 kit (Zymo Research, Orange, Calif.) and eluted in 35 μl of water or directly transferred to the following labeling step. Cy5-labeled ssDNA for microarray hybridization was synthesized from the multiplex PCR products by the primer extension method (20, 43). The labeling reaction was performed in a volume of 25 μl containing 2.5 U of Taq polymerase (QIAGEN); 1× PCR buffer with 2 mM $MgCl_2$; 200 nM (each) reverse primers; 200 nM dATP, dGTP, and dTTP; 40 nM dCTP; 40 nM Cy5-dCTP (Amersham Bioscience UK Ltd., Amersham, Buckinghamshire, United Kingdom); and 2.5 μl of unpurified double-stranded DNA (dsDNA) or 16 μl of purified dsDNA from the previous multiplex PCRs. The primer extension protocol included preliminary denaturing at 95°C for 1 min followed by 35 cycles at 95°C for 20 s, 52°C for 20 s, and 72°C for 2 min, with a final extension step at 72°C for 10 min. The Cy5-labeled ssDNA products were then purified and dried.

Microarray hybridization and processing. Oligonucleotide-printed slides were blocked with a 3% bovine serum albumin–casein solution (pH 7.4) for 15 min at room temperature, rinsed with distilled water, air dried, and placed in a MAUI hybridization dual chamber lid (BioMicro, Salt Lake City, Utah) immediately prior to hybridization. The fluorescently labeled ssDNA was resuspended in 20 μl of 1× hybridization buffer (5× Denhardt's solution, 4× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.2% sodium dodecyl sulfate) containing 0.1 μM Cy5-labeled internal positive control probe (Table 2). The target hybridization sample was denatured at 95°C for 5 min, chilled on ice for 2 min, and applied to the microarray. Hybridization was performed on MAUI Hybridization System (BioMicro) for 1 h at 58°C. After hybridization, the slides were washed once with 4× SSC plus 0.2% sodium dodecyl sulfate at 58°C for 5 min and twice with 1× SSC at room temperature for 1 min. Slides were dried under a nitrogen stream and subsequently scanned with a GSI Lumonics ScanArray Lite confocal laser-scanning system (Perkin-Elmer, Torrance, Calif.). Unless otherwise noted, the microarray images were captured at laser power 80/PMT gain 80.

RESULTS

Target selection and multiplex PCR. In addition to conserved genes, such as rRNA and *hsp*, which have been widely used as diagnostic markers, many genus- and species-specific genes were selected as amplification targets so as to avoid potential coamplification and cross-hybridization issues. The selected targets and their respective primer sets are shown in Table 1. Each of the *Entamoeba* targets was selected from previously reported variable regions: extrachromosomal rRNA,

TABLE 1. Protozoan gene-targeted primers used in this study

Target[a]	Name	Sequence (5′→3′)	Length (nt)	T_m (°C)[b]	GC (%)	PCR product size (nt)	Source or reference
Entamoeba							
Locus1-2	R1	CTGGTTAGTATCTTCGCCTGT	21	50.6	48		
	R2	CTTACACCCCCATTAACAAT	20	49.6	40	402/495	48
Locus5-6	R5A	CTAAAGCCCCCTTCTTCTATAATT	24	57.5	38		
	R6A	CTCAGTCGGTAGAGCATGGT	20	49.6	55	485/510	48
ITS	18SP1	AGGTGAACCTGCGGAAGGATCATTA	25	62.6	48		
	28SP2	TCATTCGCCATTACTTAAGAAATCATTGTT	30	65.2	30	433/423	34
sod-act	SAF2	GAGCTGCTTACTTAGAACATTGGTGG	26	59.1	46		14
	SAR2	CCAGATCCATTATCTACAACAAGTGC	26	57.0	42	497	This study
prrdx	prrdxF	GTCAAGAGAAAGAATGTTGTAAAGA	25	51.6	32		39
	prrdxR1	TTGATTTCTTTCAATTGTCCTGCA	24	56.3	33	274/304	This study
cpl	EntaCP1-F	GCAGCACTTGAAGGAAGATTATT	23	54.3	39		
	EntaCP1-R	CCATCAACAACACCATATCCAA	22	52.2	41	441	This study
G. lamblia							
hsp	ABB97F	AGGGCTCCGGCATAACTTTCC	21	60.5	57		
	ABB220R	GTATCTGTGACCCGTCCGAG	20	51.5	60	163	32
tpiA	TPIAF	CGAGACAAGTGTTGAGATGC	20	47.7	50		
	TPIAR	GGTCAAGAGCTTACAACACG	20	48.5	50	476	3
tpiB	TPIBF	GTTGCTCCCTCCTTTGTGCA	20	54.1	55		
	TPIBR2	AGGCAATTACAACGTTCTCCCA	22	56.3	46	384	This study
giardin	GIA40F	CCGACGACCTCACCCGCAG	19	58.5	74		5
	GIA773R	GAGAGGCCGCCCTGGATC	18	56.3	72	749	This study
gdh	GDHF	CCGCTTCCACCCCTCTGTCAA	21	59.6	62		26
	GDHR	CCTTGCACATCTCCTCCAGGAA	21	57.0	55	389	This study
c4-orf	C4-F	AGCTCATCTTCGTCCTCTA	19	46.4	48		47
	C4-R	CAATCTTGTTTGCATACGA	19	46.2	37	445	
C. parvum							
cowp1	CpR1-F2	AAAGAAGCACCTCCTGTTTCAG	22	53.5	46		21
	CpR1-R2	GCAGCTGCTAATCTTCTTAGTGC	23	54.6	48	485	
SSUrRNA	CpB-DIAGF	AAGCTCGTAGTTGGATTTCTG	21	50.8	43		30
	CpB-DIAGR	TAAGGTGCTGAAGGAGTAAGG	21	50.9	48	435	
dhf	DHFR1	TTGTTGTGGCAGCTTCTGTTTTGA	24	57.7	42		30
	DHFR4	AAAGTTATCCTTTAAAGCATCCCT	24	56.8	33	359	This study
ptg	PolyTF1	TCCCAGTTCAAACTCACAAGAGTA	24	52.9	42		30
	PolyTR1	GGAGGAATAATACCACCATCTTCA	24	55.2	42	496	This study
RAPD	SB012F	CTCCGTTCGATGATGCAGATG	21	51.2	52		45
	SB012R	CGGCCCCTGTAGAAATAAGTCA	22	57.0	50	433	
TRAP-C2	TRAP-C2F	CATATTCCCTGTCCCTTGAG	20	50.4	50		38
	TRAP-C2R	TGGACAACCCAAATGCAGAC	20	51.9	50	369	
hsp	CPHSPT2F	TCCTCTGCCGTACAGGATCTCTTA	24	57.2	50		
	CPHSPT2R	TGCTGCTCTTACCAGTACTCTTATCA	26	55.5	42	346	22
p23	P23-45	ATTATTTTTACGTTCCTTCCACTTG	25	57.0	32		
	P23-569	AACCTTAATAAAAAACACTCTATTG	25	51.9	24	537	37

[a] Abbreviations of target genes or gene products: ITS, intergenic sequence between rDNA; *sod-act*, intergenic region between superoxide dismutase and actin 3 genes; *prrdx*, perosiredoxin; *cp1*, cysteine protease 1; *hsp*, heat shock protein; *gdh*, glutamate dehydrogenase; *tpi*, triose phosphate isomerase; *cowp*, *Cryptosporidium* oocyst wall protein; ssu, small subunit; *dhf*, dihydrofolate reductase-thymidylate synthase; *ptg*, polythreonine-rich glycoprotein; TRAP-C2, thrombospondin-related anonymous protein 2.

[b] Basic melting temperature (T_m) was calculated with Oligos software downloaded from (http://www.biocenter.helsinki.fi/bi/bare-1_html/oligos.htm).

the intergenic region between the superoxide dismutase (*sod*) and actin genes, and the cysteine protease gene (*cp1*). Each pair of primers was adopted or designed to be able to amplify homologous genes from different species (*E. histolytica* and *E. dispar*) and genotypes (*G. lamblia* and *C. parvum*). Only the two sets of *tpi* primers were *Giardia* assemblage A and B specific. Individual PCRs with each primer set were conducted to confirm primer specificity and verify expected amplicon sizes (data not shown). Once confirmed, all genus-specific primers were pooled for *Entamoeba*-, *Giardia*-, and *Cryptosporidium*-specific multiplex PCRs (Fig. 1). Differences in the electrophoretic profiles between *E. histolytica* (lane 1) and *E. dispar* (lane 2) are due to homologous gene-length size variation as shown in Table 1. A 749-bp fragment of the *giardin* gene appeared to be missing from *G. lamblia* GS-H7 (lane 4), suggesting that this gene was not efficiently amplified in the multiplex PCR. *C. parvum* type 1 (lane 5) and type 2 (lane 6) isolates showed nearly identical electrophoretic profiles. In addition to the expected amplified products, there were also a few unexpected bands on the gel due to nonspecific amplification.

Genus-level hybridization specificity. Genomic DNA from each of the six protozoan species displayed in Fig. 1 was subjected to multiplex PCR amplification, primer extension fluorescent labeling, and hybridization to the protozoan microarrays. The unique hybridization pattern for each of the six representative protozoan isolates is shown in Fig. 2A. Importantly, the target DNA from each genus did not cross-hybridize with extra-genus probes, indicating the genus specificity of each protozoan subarray.

***Entamoeba* subarray.** Hybridization of *E. histolytica* strain HM-1:IMSS clone 9 and *E. dispar* SAW760 amplified products to the protozoan microarray (*Entameoba* subarray) resulted in

TABLE 2. Oligonucleotide probe set used in this study

Name	Sequence[a]	Position[b]	Length (nt)	T_m (°C)	GC (%)	Coordinate(s)[c]
Entamoeba						
Ehlocus1-2.3	CTGGTTAGTATCTTCGCCTGTCACG	1	25	59.3	52	A3, C3
EssrDNA.1	AATGAATTGCGATAAGTGATAGGAAC	171	26	55.0	35	B3, D3
E. histolytica						
Ehlocus5-6.1	TCTTTGAGACTTATTTCTACTTTATTTCTT	37	30	53.7	23	A1
Ehlocus5-6.2	TGTACGTCTTTAACTTTAAAAACAA	415	25	54.7	24	B1
Ehlocus1-2.1	ATATTCTTATCACTTCCTACTACTCTTATT	116	30	53.6	27	A2
Ehlocus1-2.2	TACTACTCTT*(6)CTTACTATACCT*(6)CTTACTAC	331	30	54.5	33	B2
EhlTS.1	AATCTACAAAGAAAATAATAATAAGTAAGA	54	30	50.4	17	A4
EhlTS.2	AATATCAATAGACAGACCAGACCAATA	368	27	54.8	33	B4
EhlTS.3	GCAAGTACAACAGAGAAGAAGTAGC	304	25	53.1	44	A5
EhSA.1	ACTTTTCCCCTCAATTATTTCGTTTT	76	26	55.1	31	A6
EhSA.2	AAGACAGGTATTTAAAGATCATAATAAACT	401	30	53.5	23	B6
Ehprrdx.1	GAAAGAATGTTGTAAAGAATGTTGTTGTCC	9	30	61.6	33	A7
Ehprrdx.2	AACAGAAATGATTGGATATAGTGAACTT	224	28	54.8	29	B7
Ehcp1.1	TATGGTTCAATGTACTAGGGAAGATG	73	26	55.1	39	A8
Ehcp1.2	AGTTATACAAGAGTGGAGCATATACAG	342	27	55.4	37	B8
E. dispar						
Edlocus5-6.1	CTATATTCTTTTTATGTACTTCCCTTA	33	27	54.3	26	C1
Edlocus5-6.2	TTCCTTTTATACAAATACTCTCATG	356	25	51.4	28	D1
Edlocus1-2.1	GTCACTTATACTATTAACTTTATCTATTCC	102	30	52.2	27	C2
Edlocus1-2.2	CCTACTATACCTACTACTCTTACTACTCCT	428	30	54.1	40	D2
EdITS.1	AATCTACAAAGAAAATAATAA*AAGTAAGA	54	29	55.9	17	C4
EdITS.2	TTAACCAGATATCTATAAGTGAGTTAATA	356	29	53.7	24	D4
EdITS.3	TTAGTAGAAGTGAGAAGTAGCTAGTG	303	26	50.1	39	C5
EdSA.1	GTTTTTGTTAAGTTTTTGGTATTACTG	110	27	55.5	26	C6
EdSA.2	GAGCCAGGTATTTAAAGATCATATCAA	401	27	58.2	33	D6
Edprrdx.1	GAGAAAGAATGTTGTAAAGAGTATTGTTGT	6	30	58.2	30	C7
Edprrdx.2	AACAGAAATGATTGGATATAGTGAAGTT	194	28	56.8	29	D7
Edcp1.1	GTACTAGAGATAATGGAAACAATGG	84	25	52.2	36	C8
Edcp1.2	GTGGTGCATACAGTGATACTAAATG	353	25	51.8	40	D8
G. lamblia						
Glhsp.1	AATTGATTAGTATTAGTAGGATGCCT	46	26	52.6	31	E1, G1
Glhsp.2	CTTGGCGTTCCCGAAGTCTGTC	113	22	59.8	59	F1, H1
girAB.1	TCAAGCTCAGCAACATGAACCAGC	36	24	58.8	50	E5, G5
girAB.2	ATGGAGAACGAGATCGAGGTCCG	84	23	60.4	57	F5, H5
gdhAB.1	GAGCAGATCCTGAAGAACTCCCT	49	23	57.0	52	E8, G8
gdhAB.2	GACAACGAGGTCATGCGCTT	134	20	54.1	55	F8, H8
Assemblage A						
tpiA.1	ATGGGTTTGAAGCATGTGATAGTAG	29	25	55.8	40	E2
tpiA.2	TATGACGATGATCGACATTCTTACG	505	25	55.7	40	F2
girA.1	ATGTACCTAACGATCAAGGAGGAGA	246	25	57.3	44	E3
girA.2	AGAACGCAGAAGGAAGAAGATGTA	442	25	56.9	40	F3
girA.3	ATCGCACACCTCGACAGGCTCAT	150	23	60.1	57	E4
girA.4	CCACGACAGAAGCGCTCACAAACA	561	24	60.0	54	F4
gdhA.1	AATCTTTCGATTCTCAAGTTCCT	20	23	57.6	35	E6
gdhA.2	GGTACCTGTACGGACAGTACAAG	246	23	57.8	52	F6
gdhA.3	TTGACCCAAAGGGCAAGTCCGA	113	22	61.8	55	E7
c4A.1	GCCATGATGAAAGGGAGTGCTGTA	37	24	58.9	50	E9
c4A.2	TGGACCCCACTGTTCAAAAGAAGA	158	24	58.5	46	F9
c4A.3	CATCCAAAGATATGAATGAGCTTCC	218	25	57.4	40	E10
c4A.4	CAGATGGAATCAAGCTTCTTAGCG	362	24	58.4	46	F10
Assemblage B						
tpiB.1	CTGAGCCATGTAATAATAGGACACT	145	25	54.9	40	G2
tpiB.2	GCCAATAACACTATGGAGGTGAATA	289	25	55.4	40	H2
girB.1	ATGTACCTGACGATCAAGGAGGAGA	246	25	57.5	48	G3
girB.2	AGAACGCCGAGAGGAAGAAGATGTA	442	25	60.6	48	H3
girB.3	ATCGCGCACCTCGACAGACTCAT	150	23	60.4	57	G4
girB.4	CCACGACAGAGGCCCTCACAAACA	561	24	61.9	58	H4
gdhB.1	AACCTCTCGATCCTTAAGTTCCT	20	23	55.0	44	G6
gdhB.2	GTTATCTGTTTGGACAGTATAAGCG	246	25	54.5	40	H6
gdhB.3	TCGATCCTAAGGGCAAGTCGGA	113	22	60.2	55	G7
c4B.1	GGCTATGACGAAAGAAGATGCTGTA	36	25	57.8	44	G9

Continued on following page

TABLE 2—*Continued*

Name	Sequence[a]	Position[b]	Length (nt)	T_m (°C)	GC (%)	Coordinate(s)[c]
c4B.2	AGCCGGATGCTCATTGTTGCG	68	21	60.8	57	H9
c4B.3	TATCTGAGAAGGACATGAAGGACCT	215	25	55.7	44	G10
c4B.4	ATCCAGCTTCTCAGTGCTTCTCTG	370	24	56.0	50	H10
C. parvum						
dhf.1	GGATAGGAATTAACGGACAATTACC	45	25	58.3	40	I1
dhf.2	TCAATAGAGAATCTTATGAATGATGACT	281	28	54.8	29	J1
dhf.3	AATGTGACTCGAATAAGAAGAATGC	120	25	54.7	36	K1
dhf.4	AAGCCGATCCTAATGTTGTTGTATT	240	25	55.8	36	L1
ptg.2	GAGTGATGATTCCAGGTTCTTTAGG	415	25	55.9	44	J4
Cphsp.1	TGCCGTACAGGATCTCTTATTATTG	6	25	55.2	40	K4
cowp.1	TCTCCTGCTTCAACTGTATGTCCTA	133	25	57.7	44	I6
cowp.2	TGTGTTAGAAGAAGCCAATATGACT	391	25	54.9	36	J6
rDNA.1	TTTACTTTGAGAAAATTAGAGTGCTTAAA	106	29	54.3	24	K6
rDNA.2	TAACAGTCAGAGGTGAAATTCTTAGAT	249	27	55.2	33	L6
RAPD.1	CATACAGGTGAGGAAGTTGATCTAT	75	25	52.7	40	I7
RAPD.2	ATCGCAATAATCCTTGTAACTTGTG	372	25	56.5	36	J7
TRAPC2.1	CCTTGAGTTGTACTGTCTCTGAATG	14	25	56.1	44	K7
TRAPC2.2	GGAGGAAAGACCTTCAGATTGTTTA	324	25	55.4	40	L7
Type 1						
dhfI.1	CTGTTTTGAGCAGTGGGATAGGAAT	16	25	58.8	44	I2
dhfI.2	CAAAGATAACTAATAATAAATGTGACTCG	89	29	57.2	28	J2
ptgI.1	TTAGATCCAGTTAGTTTGATTCCATTC	161	27	57.3	33	I3
ptgI.2	ATCCAATCTCAGATGAGATCATGAAT	214	26	55.3	35	J3
Cphsp.2	TGGTATCTTGAATGTATCTGCTGTT	297	25	53.5	36	L4
p23I.1	CCAATTAGCAACCAAGCCCAACAAA	188	25	64.4	44	I5
p23I.2	TACACTC*(3)GAGTACTTAACATGGGTT	427	25	53.5	40	J5
Type 2						
dhfII.1	CTGTTTTGAGTAGAGGAATAGGAAT	16	25	53.6	36	K2
dhfII.2	CAAAGATAACTAGTAATAATTGTGACTCG	89	29	56.5	31	L2
ptgII.1	TTGGATCCAGTCAGTTTGATTCCGTT	161	26	61.8	42	K3
ptgII.2	ATCCAATATCAGATGAGATAATGAAT	214	26	53.4	27	L3
ptg.1	TTATCGGTATCATATCTTGCTGCTAA	117	26	55.0	35	I4
p23II.1	CCAATCAGCAACCAAGCTCAACAAA	188	25	61.4	44	K5
p23II.2	CTCAGAGAGTACTTAACATGGGTT	431	24	51.0	42	L5
Internal positive control						
fliC.1	AACGGTTAGCAATCGCCTGACCTGCGGCGTCAT CCTTCGCGCTGTTAATACGCAAGCCAGAAGAC AGACG		70	80.6	56	

[a] Mismatched nucleotides between homologous target sequences are underlined, and deleted nucleotides are designated by an asterisk (with the number in parentheses).
[b] The first nucleotide of the probe corresponding to the position within an amplicon starting from the 5′ end.
[c] The position of each probe on the microarray is represented by coordinate number corresponding to Fig. 2B.

the generation of two distinct profiles (Fig. 2C). As expected, the locus1-2.3 and ssrDNA probes, which are common for both *Entamoeba* species and served as *Entamoeba*-specific markers, showed strong signals in both the *E. histolytica* and *E. dispar* hybridizations. The *prrdx* and *cp1* genes, which are unique to the genus *Entamoeba*, were the only gene coding sequences used to differentiate the two species tested. However, the *E. histolytica prrdx* probes cross-hybridized with *E. dispar* target DNA and vice versa, albeit with lower average hybridization signal intensities when compared to the isogenic probe/target hybridizations. This result is not surprising based on the number and location of nucleotide mismatches. A single mismatch between Ehprrdx.2 and Edprrdx.2 and two mismatches between Ehprrdx.1 and Edprrdx.1 were all located at the 3′ ends of the probes (Table 2). *E. dispar* target DNA also cross-hybridized with the *E. histolytica* locus1-2.1 and locus1-2.2 probes, although there are more than 10 nt differences in these regions between the two species. Again, the cross-hybridization signals were of lower average signal intensities when compared to the isogenic probe/target hybridizations. False-negative results were observed for the Edlocus5-6.1 and Edlocus5-6.2 probes when tested with *E. dispar* target DNA, despite the presence of a 510-bp locus 5-6 amplicon (Fig. 1, lane 2) and appropriately designed probes. However, the few instances of cross-hybridization and false-negative results within the *Entamoeba* subarray did not interfere with the interpretation of array results: the observed hybridization patterns clearly permitted the differentiation of both species.

Another apparently noninvasive but prevalent *Entamoeba* species, *E. moshkovskii*, was tested on the microarray as it is morphologically indistinguishable from *E. histolytica* and *E. dispar* (1). When genomic DNA from *E. moshkovskii* strain Laredo was amplified by the same set of multiplex primers and hybridized to the *Entamoeba* subarray, only two probes,

Ehlocus1-2.3 and EssrDNA.1, which are common to both *E. histolytica* and *E. dispar*, showed positive hybridization signals (data not shown).

***G. lamblia* subarray.** The hybridization of *G. lamblia* WB (assemblage A isolate) and *G. lamblia* GS-H7 (assemblage B isolate) amplified targets to the *Giardia* subarray resulted in two distinct profiles (Fig. 2C). Probes targeting the *hsp70*, *giardin*, and *gdh* genes were used as species-specific markers and were positive for both assemblage members. All other probes were shown to be specific for either assemblage A (isolate WB, top two rows) or assemblage B (isolate GS-H7, bottom two rows). The profile results indicated a single false-positive result (girB.1) and false-negative result (tpiA.1) for the assemblage A representative and two false-negative results (girB.3 and girAB.2) for the assemblage B representative. A confirmatory profile generated by the hybridization of another assemblage B isolate, *G. lamblia* CM, was identical to that of *G. lamblia* GS-H7 (data not shown). Despite the observed absence of the 749-bp *giardin* amplicon from the *G. lamblia* GS-H7 multiplex PCR (Fig. 1, lane 4), six of the eight probes targeting the *giardin* amplicon were hybridization positive, thus highlighting microarray-based detection sensitivity.

***C. parvum* subarray.** Distinct profiles were also generated when the amplicons from representative *C. parvum* type 1 and type 2 strains, TU502 and GCH1, respectively, were hybridized to the *C. parvum* subarray (Fig. 2C). The observed type 1 hybridization profile revealed unexpected false-positive signals arising from three probes: dhfII.2, ptg1, and (low positive) p23II.1. The observed type 2 hybridization profile matched the expected profile (Fig. 2B). Confirmatory profiles generated by the hybridization of two other *C. parvum* isolates, UG502 (type 1) and Iowa (type 2), to the *C. parvum* subarray were identical to the presented TU502 (type 1) and GCH1 (type 2) profiles, respectively (data not shown). Although the *C. parvum* subarray contained the smallest number of genotype-specific probes, the probes designed to target three genes, *dhf*, *ptg*, and *p23*, were clearly enough to ably discriminate between the two major *C. parvum* genotypes.

Although the probes and primers used for *C. parvum* subarray-based detection were specifically designed to differentiate the two major human infectious genotypes, we sought to potentially expand the utility of the current assay by testing two nonhuman-pathogenic *Cryptosporidium* species, *C. meleagridis* (primarily a bird pathogen but which can infect humans) and *C. muris* (a rodent pathogen). The electrophoretic profiles demonstrated in Fig. 3A suggested that the *C. parvum* multiplex primers amplified *C. meleagridis* DNA (lane 3) almost as efficiently as *C. parvum* DNA (lanes 1 and 2), but only appeared to amplify a single product from the *C. muris* isolate (lane 4). Application of the *C. meleagridis* amplicons to the *C. parvum* subarray resulted in hybridization-positive signals from 22 of the possible 28 probes, including the *dhf*, *hsp*, *cowp*, rRNA, randomly amplified polymorphic DNA (RAPD; one of two), and TRAP-C2 probes that were common to both cryptosporidial genotypes (Fig. 3B). Hybridization of the *C. muris* amplified material to the *C. parvum* subarray revealed two cryptosporidial targets were amplified: *hsp70* and rRNA (Fig. 3C). The results suggested that (i) as expected, the *hsp70* and rRNA sequences are the most conserved sequences among *Cryptosporidum* species; (ii) based solely on the number of hybridization-positive probes (and hence, primary sequence conservation), *C. meleagridis* appears to be more closely related to *C. parvum* than is *C. muris* (a result consistent with *Cryptosporidium* gene phylogenies) (38, 46); and (iii) although specifically designed for the detection of *C. parvum*, this subarray may also be used for the detection of other *Cryptosporidium* spp. once characteristic profiles using known templates have been established.

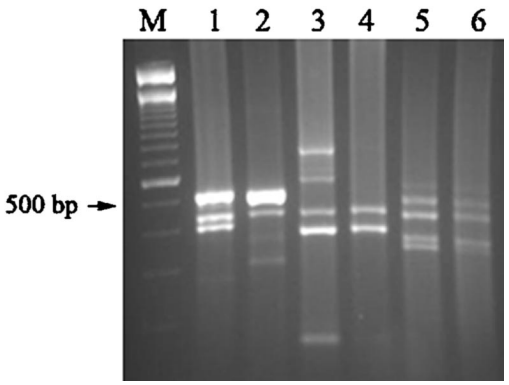

FIG. 1. Amplification of protozoan target genes by multiplex PCR. A combination of seven *Entamoeba*-specific primer pairs were used to amplify the genomic DNA of *E. histolytica* HM-1:IMSS (lane 1) and *E. dispar* SAW760 (lane 2). Similarly, six pairs of *G. lamblia* primers were used to amplify *G. lamblia* WB (lane 3) and *G. lamblia* GS-H7 (lane 4), and eight pairs of *C. parvum* primers were used to amplify the genomic DNA of *C. parvum* TU502 (lane 5) and *C. parvum* GCH1 (lane 6).

Sensitivity of protozoan microarray. One of the main advantages of microarray-based protozoan pathogen detection was assay sensitivity. As shown in Table 3, the combination of multiplex PCR, primer extension, and microarray hybridization resulted in the detection of as few as five *G. lamblia* WB and GS-H7 trophozoites. A comparison with individual PCR amplicon visualization via gel electrophoresis revealed that microarray-based detection sensitivity was either as sensitive or more sensitive in every instance, except for the detection of the GS-H7 *giardin* target amplicon.

As protozoan coinfections are not uncommon, we also sought to test whether the simultaneous amplification of two protozoan species would result in lower detection sensitivity for the less-abundant protozoan in the mixture. Two 10:1 *G. lamblia* mixtures (500:50 WB/GS-H7 and 50:5 WB/GS-H7 trophozoite ratios) were amplified and hybridized to the *Giardia* subarray. In both cases, all of the probes within the *Giardia* subarray showed hybridization-positive signals revealing *Giardia* assemblage A and B profiles (data not shown). These results indicated that the microarray was able to simultaneously detect members of both assemblages and that the detection of both coinfectants (at a 50:5 ratio) did not adversely effect the lower detection threshold of 5 trophozoites seen when detecting a single species (Table 3).

DISCUSSION

This study represents the first report documenting the use of oligonucleotide microarrays for the detection and genotyping of multiple protozoan parasites. The developed experimental protocol combining multiplex PCR, primer extension-based

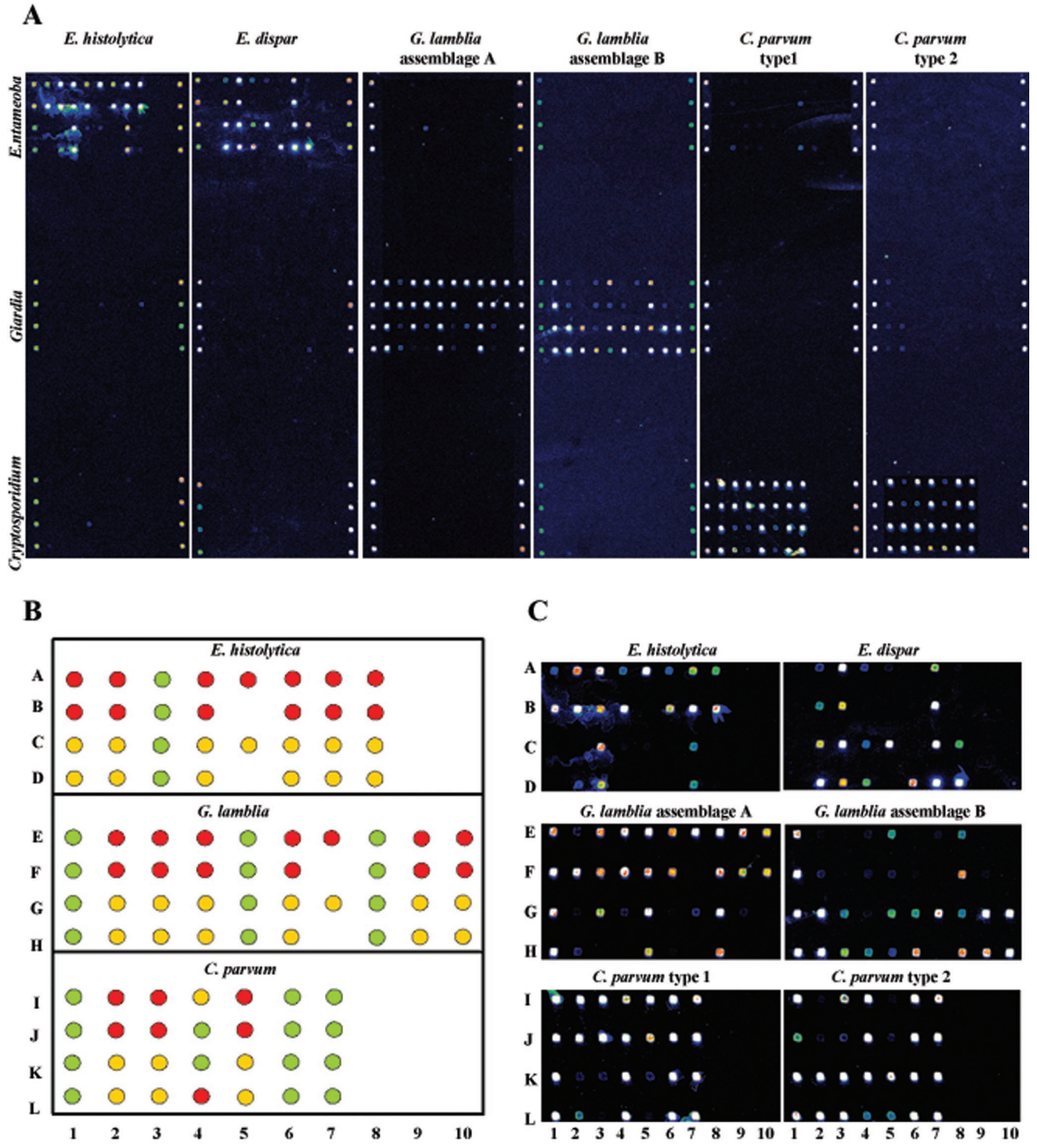

FIG. 2. Genus-, species-, and subtype-level microarray specificity. (A) Identical protozoan microarrays were individually hybridized with the labeled multiplex PCR products from the six protozoan strains shown in Fig. 1. The portions of the array dedicated to a particular genus are indicated on the left, whereas the original hybridization sample source is indicated above each array. The hybridization spots at the left and right ends of each array represent internal positive controls. (B) Microarray template showing the coordinates and identity of each probe (see Table 2) and the expected subarray hybridization pattern for each species, assemblage, or genotype. In each template, red spots represent *E. histolytica*, *G. lamblia* assemblage A, or *C. parvum* type 1-specific probes; yellow spots represent *E. dispar*, *G. lamblia* assemblage B, or *C. parvum* type 2-specific probes; and green spots represent probes common for both *E. histolytica* and *E. dispar*, *G. lamblia* assemblages A and B, or *C. parvum* types 1 and 2. (C) Observed subarray hybridization patterns.

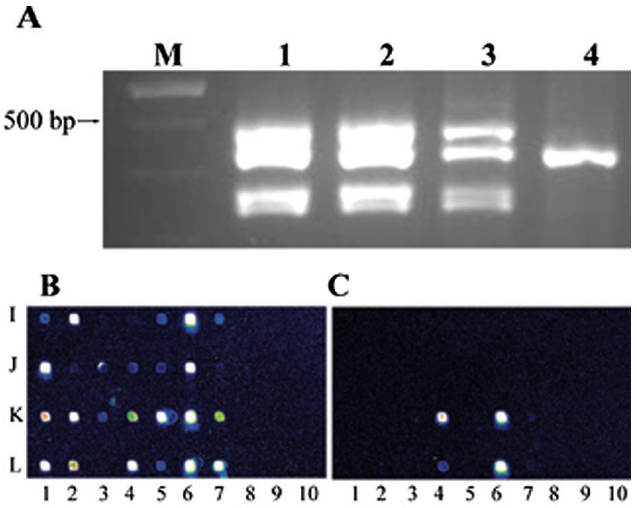

FIG. 3. Electrophoretic and microarray hybridization profiles of three species of *Cryptosporidium*. (A) Comparative electrophoretic profiles of *C. parvum* TU502 (lane 1), *C. parvum* GCH1 (lane 2), *C. meleagridis* (lane 3), and *C. muris* (lane 4) multiplex PCR amplicons. (B and C) Hybridization pattern of *C. meleagridis* (B) and *C. muris* (C) according to the *Cryptosporidium* subarray.

labeling, and microarray hybridization was shown to be a successful strategy for obtaining genus-level specificity capable of unequivocally detecting and differentiating members of the genera *Entamoeba*, *Giardia*, and *Cryptosporidium*. Furthermore, the hybridization of diagnostic targets to species- and genotype-specific probes generated unique profiles enabling the identification and differentiation of *E. dispar* from *E. histolytica*, *G. lamblia* assemblages A and B, and *C. parvum* genotypes 1 and 2 by using a single assay. In addition to distinguishing between the targeted principal genotypes, this assay may also have utility in detecting other related isolates, such as *C. meleagridis* and *C. muris*, and differentiating innocuous species (*E. moshkovskii*) from pathogenic species (*E. histolytica*).

The current accepted methods for the environmental detection of *Cryptosporidium* and *Giardia* spp. are labor and resource intensive. For example, Environmental Protection Agency method 1623 requires a series of filtration, immunomagnetic separation, and immunofluorescence assays for detection and determination of pathogen concentrations followed by vital dye staining and microscopy confirmatory assays. Although effective, the observed limits in sensitivity, specificity, and reproducibility have warranted the development of alternate detection strategies. Molecular detection methods, primarily PCR based, have become increasingly common for the identification of viral and bacterial pathogens and appear to be especially attractive for the detection of protozoan parasites that are difficult to culture, morphologically similar, and capable of causing disease in low infectious doses. Thus, the sensitivity and specificity afforded by molecular detection methods such as PCR, restriction fragment length polymorphism, and real-time PCR have resulted in the development of rapid approaches for the detection and genotyping of protozoa in clinical and environmental samples (4, 16, 40).

However, as most PCR-based detection methods are reliant upon amplicon size analyses or generic dsDNA intercalating dye fluorescence, subsequent sequencing or hybridization assays are necessary to confirm the identity of the amplified target. In comparison to a single PCR-based detection method, the combined amplification and microarray hybridization strategy employed in this study to detect and genotype protozoa had the following advantages. (i) Assay sensitivity was achieved not only by amplification of target sequences, but also by DNA-DNA hybridization, which was visualized by fluorescent labeling. Our data demonstrated that as few as five trophozoites of *G. lamblia* could be accurately detected by this method, whereas the use of PCR analysis alone at identical concentrations generated false-negative results (Table 3). (ii) Pathogen identification was not reliant upon amplicon size analyses or nonspecific fluorescent dye intercalation, but rather two sequential hybridization events—primer hybridization for target generation and target/probe hybridization. The combination of primer and probe specificity enhanced assay specificity as genetic variants were unambiguously identified and genotyped despite the existence of nonspecific multiplex PCR products that tend to confound electrophoretic analyses. (iii) Multiplex PCR amplification and microarray hybridization allowed for the simultaneous detection of multiple genetic markers. The redundancy of species- and genotype-specific targets and probes not only increased the confidence in the results but also reduced the vulnerability to spontaneous mutations that may occur in circulating clinical or environmental isolates. This experimental redundancy was found to be necessary for accurate and reliable data interpretation. (iv) Assay throughput was increased as the microarray format enabled the simultaneous analysis of multiple organisms with a large number of genetic markers in one experiment.

The sensitivity and specificity afforded by amplification and hybridization schemes has been highlighted by a number of

TABLE 3. Microarray versus PCR sensitivity with *G. lamblia* trophozoites

Gene (no. of trophozoites)	Detection by[a]:			
	PCR		Microarray (no. positive/no. of probes)	
	WB	GS-H7	WB	GS-H7
hsp				
200	+	+	2/2	2/2
5	+	−	2/2	2/2
tpi				
200	+	+	1/2	2/2
5	−	+	1/2	2/2
giardin				
200	+	+	6/6	6/6
5	?	?	6/6	0/6
gdh				
200	+	+	5/5	5/5
5	−	−	5/5	4/5
c4				
200	+	+	4/4	4/4
5	−	−	4/4	4/4

[a] Detection of each gene by PCR is designated as positive (+), ambiguous (?), or negative (−). Detection of each gene by microarray is represented by the number of positive spots out of the number of probes per gene.

microarray-based pathogen detection studies that have utilized a single highly conserved gene as the amplification and hybridization target (9, 13, 19, 29, 36). As demonstrated in these studies, the amplification of a single conserved target sequence with a pair of specific or degenerate primers is often more efficient and convenient than multiplex or random amplification, but there are two salient caveats. (i) Often the selected gene, such as 16S rRNA, is so highly conserved that sequence variability among species and strains is small or nonexistent, making it difficult to confidently differentiate between closely related species or subtypes. (ii) The reliance upon a primer pair to amplify a single target region provides little margin for error. A mutation in the primer binding site could potentially reduce or prevent target amplification and subsequently produce false-negative results. The potential pitfalls associated with the use of a single target led us to select multiple diagnostic sequences, both conserved and highly variable, as amplification and hybridization targets. Highly conserved target genes, such as rRNA and *hsp70*, were selected for general identification of the genus or species. Identification down to the species, assemblage, or genotype level was accomplished by targeting highly variable genes or genes that were unique to *Entamoeba* (*cp1*), *Giardia* (the gene encoding giardin and *c4*), and *Cryptosporidium* (*cowp*, *ptg*, *RAPD*, the gene encoding TRAP-C2, and *p23*). As demonstrated in this study, the use of multiple genetic markers, both conserved and variable, increased the certainty of detection and discrimination.

The use of short probes also aided in the differentiation of species as 20- to 30-mer oligonucleotides are ideal for distinguishing closely related species and monitoring intraspecies genetic variability. For example, Straub et al. recently reported genotyping *C. parvum* with an *hsp70* single-nucleotide polymorphism (SNP) microarray using short probes (36). In this study, we also chose to target the *Cryptosporidium hsp70* gene and designed short probes based on primary sequence from a *C. parvum* genotype 1 human isolate (accession no. AF221535). As hybridization to the *C. parvum* subarray with *C. parvum* genotype 1 (TU502) and genotype 2 (GCH1) demonstrated, genotype 2 strain GCH1 target hybridization to probe Cphsp.2 generated a markedly lower fluorescent signal (14-fold less) than that observed with the genotype 1 strain TU502 (Fig. 2C, *C. parvum* subarray, coordinate L-4). A comparison of the Cphsp.2 probe sequence to *C. parvum* genotype 2 *hsp70* genes in GenBank (accession no. U71181) revealed an SNP at position 1404 of *hsp70*. This SNP finding turned out to be the same as described by Straub et al. when using the *hsp70* SNP microarray (36). Thus, in addition to only using the presence or absence of fluorescent signals to determine genotype, in certain instances, the variations in hybridization signal intensities due to SNPs were also useful in differentiating closely related strains.

Although they did not hinder the interpretation of results, each of the subarrays generated a few false-positive and false-negative hybridization results. We suspect that these erroneous results were due to the amount of available sequence information, the probe location within the target, and/or probe design. First, in comparison to the amount of primary sequence information available for viral and bacterial enteric pathogens, the amount of sequence data available for protozoan enteric pathogens is limited. Thus, limited or incorrect sequence information in public databases may have resulted in the design of PCR primers and oligonucleotide probes that were not representative of the targeted genes. For example, the numbers of *E. dispar* sequences deposited in GenBank are limited, and often there was only a single sequence available for each gene target. This limitation was clearly highlighted by the number of false-positive and false-negative results obtained within the *E. dispar* SAW760 hybridization profile. The lack of sequence redundancy and sampling may have resulted in a potentially high design error rate for both primers and probes. Secondly, the positions of oligonucleotide probes (as they relate to the target sequence) and chosen method of labeling may have hindered results. Usually, two probes were designed for each target, with one probe located near the 5′ end and the other near 3′ end of the amplicon. In most cases, false-negative or low-intensity hybridization signals were found associated with probes located on the 5′ end of the target amplicon (such as Ehlocus5-6.1, EhITS.1, EhSA.1, and tpiA.1). As the amplicons were labeled by primer extension incorporation of Cy5-dCTP from the 3′ end, it is possible that premature termination of polymerization and dye incorporation resulted in fewer labeled full-length amplicons, thus decreasing the number of labeled targets for hybridization to the 5′-localized probes. In addition, as the probes were covalently immobilized via a 5′ reactive amine group, hybridization to the 5′ target terminus may have been hindered by increased steric and spatial constraints at the probe-target interface.

In summary, we have developed a microarray for the parallel detection and genotyping of *E. histolytica*, *G. lamblia*, and *C. parvum*. The amplification and hybridization of multiple diagnostic regions to short genus-, species-, and subtype-specific probes allowed for the unequivocal detection and discrimination of *E. histolytica*, *E. dispar*, *G. lamblia* assemblages A and B, and *C. parvum* genotypes 1 and 2 in a single assay. Thus, this method may aid in confidently expediting the detection of these three major waterborne parasites while simultaneously providing valuable epidemiological information. The relatively rapid and accurate nature of this platform has great potential for use as a diagnostic tool, and efforts are currently under way to further test the utility of this microarray on environmental water and clinical human fecal samples.

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