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Simultaneous Detection of Four Human Pathogenic Microsporidian Species from Clinical Samples by Oligonucleotide Microarray

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Microsporidian species have been rapidly emerging as human enteric pathogens in immunocompromised and immunocompetent individuals in recent years. Routine diagnostic techniques for microsporidia in clinical laboratories are laborious and insensitive and tend to underestimate their presence. In most instances, they are unable to differentiate species of spores due to their small sizes and similar morphologies. In this study, we report the development of another protozoan oligonucleotide microarray assay for the simultaneous detection and identification to the species level of four major microsporidian species: *Enterocytozoon bieneusi*, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis*. The 18S small-subunit rRNA gene was chosen as the amplification target, labeled with fluorescence dye, and hybridized to a series of species-specific oligonucleotide probes immobilized on a microchip. The specificity and sensitivity of the microarray were clearly demonstrated by the unique hybridization profiles exhibited by each species of microsporidian tested and its ability to detect as few as 10 spores. In order to assess the applicability of this microarray in a clinical setting, we conducted microarray assays of 20 fecal samples from AIDS patients. Twelve of these samples were positive for the presence of microsporidia and could be confidently identified; 11 of them were positive for more than one species. Our results suggested that this microarray-based approach represents an attractive diagnostic tool for high-throughput detection and identification of microsporidian species in clinical and epidemiological investigations.

Enterocytozoon bieneusi, *Encephalitozoon cuniculi*, *Encephalitozoon hellem*, and *Encephalitozoon intestinalis* are four major members of the phylum *Microsporidia*. They are obligate intracellular protozoan parasites that infect both invertebrates and vertebrates (14, 31). Recent studies have revealed that as many as 40% of AIDS patients suffering from chronic diarrhea had infections of microsporidia (9, 20), which represent the most common enteric pathogens detected in these patients. Therefore, microsporidiosis was listed in the differential diagnosis of human immunodeficiency virus-related symptomatic diseases (26). Human pathogenic microsporidia have routinely been reported in clinical specimens, and their presence in environmental reservoirs such as surface water has also been detected recently (8, 12). Due to the fact that humans, animals, and birds shed infectious spores via body fluids and feces, it was suggested that surface water could be a potential transmission route for microsporidian outbreaks (18).

The global spread of microsporidia, as seen in the increasing frequency of infection rates in AIDS patients and through recent environmental surveillance studies, illustrates the need for rapid, sensitive, and accurate identification methods. However, there are inherent difficulties in many of the current diagnostic procedures due principally to the small size of the spores (<2 μm in diameter) and the numerous matrices that can harbor them. Routine diagnosis of microsporidia in clinical laboratories has relied mostly on special staining and microscopic techniques (14, 15). However, these are often laborious, time consuming, and insensitive and microsporidial infections are often missed or misdiagnosed. Another limitation is the inability to differentiate related species in a specimen. Although immunofluorescent staining techniques have been developed for species differentiation, they are far from optimal due to problems of limited availability of antibodies, nonspecific binding, and interference from the sample matrix (1, 23). The fluorescence in situ hybridization technique has been reported to detect microsporidian species in environmental and clinical samples (3, 17, 30) and has shown great sensitivity and specificity, but its performance requires expertise and a long hybridization time, and it has only been occasionally used in practice.

The recent development of PCR-based detection methods has greatly enhanced the sensitivity and specificity for identifying microsporidia (13–15, 19, 25), and they are slowly being introduced in clinical laboratories. Such methods have taken advantage of small regions of microheterogeneity in the small-subunit (SSU) rRNA sequence as the amplification target for microsporidial detection and differentiation. However, drawbacks to these PCR methods have become apparent during their practice. Most PCR assays have been using conventional methods to extract microsporidian DNA from clinical and environmental samples, which require multiple steps and harsh conditions to destroy spore walls (14). Differentiation of species requires designing a number of species-specific primers for multiple PCRs and even needs restriction enzyme digestion or Southern blotting following specific PCR, which is laborious and reagent consuming. Although the TaqMan real-time PCR approach is very sensitive and specific (18, 38), the limited

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number of hybridization probes that can be used in one reaction hampers its application in the simultaneous identification and differentiation of multiple species in one sample.

Oligonucleotide microarrays have been successfully applied in the diagnosis of many pathogenic microbial organisms in recent years (5, 6, 22, 28, 35, 36). Hybridization of amplified target DNA on multiple specific oligonucleotide probes immobilized on a microchip produces a highly sensitive, specific, and high-throughput platform for pathogen detection and differentiation. In a previous study, we demonstrated for the first time that an oligonucleotide array can be used to detect and genotype multiple waterborne pathogenic protozoan simultaneously (37). In this study, we developed another parasitic protozoan microarray which contains species-specific oligonucleotide probes derived from the 18S SSU rRNA genes of four microsporidian species (*E. bieneusi*, *E. cuniculi*, *E. hellem*, and *E. intestinalis*). Also, we incorporated the extract-free, filter-based template preparation technique (27) to efficiently remove PCR interference factors and rapidly amplify target genes from clinical samples. The assay was carried out by amplification of 1,300-bp SSU rRNA fragments with universal primers, followed by fluorescent labeling of single-stranded DNA with primer extension and lastly hybridization to the immobilized probes. In this manner, the array was able to simultaneously detect and differentiate four microsporidian species pathogenic in humans and was also successfully applied to clinical samples.

MATERIALS AND METHODS

Parasite isolates and DNA. *E. bieneusi*, *E. cuniculi*, *E. hellem*, and *E. intestinalis* spores were provided by G. S. Visvesvara (Centers for Disease Control and Prevention, Atlanta, GA). Sources included tissue culture-enriched isolates, urine, fecal specimens, and mucosal aspirates. All isolates were stored at 4°C. The identities of all isolates were verified by PCR using previously defined species-specific primer pairs as follows: *E. bieneusi*, EBIEF1 (5′-GAAACTTGT CCACTCCTTACG-3′) and EBIER1 (5′-CAATGCACCACTCCTGCCATT-3′) (10); *E. cuniculi*, ECUNF1 (5′-ATGAGAAGTGATGTGTGTGCG −3′) and ECUNR1 (5′-TGCCATGCACTCACAGGCATC-3′) (32, 33); *E. hellem*, EHELF1 (5′-TGAGAAGTAAGATGTTTAGCA-3′) and EHELR1 (5′-GTAA AAACACTCTCACACTCA-3′) (32, 33); *E. intestinalis*, SINTF1 (5′-TTTCGA GTGTAAAGGAGTCGA-3′) and SINTR (5′-CCGTCCTCGTTCTCCTGCCC G-3′) (11). Archived clinical fecal samples were collected from human immunodeficiency virus-infected patients presenting with chronic diarrhea caused by suspected but unverified microsporidian infection and were provided by Julie Ribes at the University of Kentucky. All samples had been preserved in 10% formalin for 12 to 18 months prior to analysis. DNA templates were prepared by the FTA filter method as previously reported (27). Briefly, sample aliquots (20 μl) were applied to FTA filters (Whatman, Inc., Waltham, MA) and the filters were dried on a 56°C heating block. Six-millimeter disks were punched out by using an individual hole punch and placed in a 1.5-ml microcentrifuge tube. FTA disks were washed twice with 0.5 ml of FTA purification buffer (Life Technologies, Gaithersburg, MD) for 2 min and twice with 0.5 ml of TE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) for 2 min and again dried on a 56°C heating block. The washed disks were used directly as the templates in PCR amplification.

PCR conditions. Primary PCR amplifications used primers MICRO-F (5′CA CCAGGTTGATTCTGCCTGA-3′) and MICRO-R (5′-TAATGATCCTGCTA ATGGTTCTCCAAC-3′) (32, 33) to amplify 1,300-bp SSU rRNA fragments from all four species tested. The PCRs were conducted with the HotStarTaq Multiplex PCR kit (QIAGEN, Valencia, CA) and with the prepared FTA disks as the templates in a 200-μl volume consisting of 100 μl Master buffer and a mixture of 200 nM (each) forward and reverse primers. PCR was carried out in a DNA Engine TETRAD2 (MJ Research, Inc., Reno, NV) with an activation step of 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. Species-specific PCR analysis was carried out in a 25-μl reaction mixture containing 1 μl of each of the primary PCR products, 2.5 U *Taq* polymerase (QIAGEN), 1× PCR buffer, 2 mM $MgCl_2$, 200 nM species-specific primer pairs, and 200 mM each dATP, dCTP, dGTP, and dTTP. The PCR program consisted of 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min, with a final extension step of 72°C for 7 min.

Oligonucleotide probe design and array fabrication. SSU rRNA gene sequences of four microsporidian species (accession no. L16868, L17072, L19070, and SIU09929) were downloaded from GenBank and aligned by using ClustalW (http://www.ebi.ac.uk/clustalw). Twenty-five-mer oligonucleotide probes with a melting temperature range of 54°C to 61°C were designed with the Oligonucleotide Properties Calculator (http://www.basic.nwu.edu/biotools/OligoCalc.html). Six groups consisting of a total of 33 oligonucleotide probes were selected based on sequence specificity and are shown in Table 1: four microsporidian probes recognized by all four species, three genus-specific probes common to three *Encephalitozoon* species, eight *E. bieneusi*-specific probes, and six species-specific probes for each *Encephalitozoon* species. The probes were synthesized with a 5′ amino modifier and a 12-carbon spacer (QIAGEN Operon, Alameda, CA) and were suspended 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 (4) using an OmniGrid Accent Microarrayer (Genemachines, San Carlos, CA). The expected hybridization pattern is shown in Fig. 1A.

Target DNA labeling, microarray hybridization, and processing. The primer extension method (34) was used to label 1,300-bp PCR products previously amplified from spores or clinical stool samples. A degenerate reverse primer, MspR3 (5′-TTTMMAACGGCCATGCACCAC-3′), with a sequence shared by all microsporidian species, was designed to synthesize an approximately 900-bp negative single-stranded DNA fragment. The labeling reaction was performed in a volume of 25 μl containing 1× PCR buffer with 2 mM $MgCl_2$; 200 nM MspR3 primer; 200 nM dATP, dGTP, and dTTP; 40 nM dCTP; 40 nM Cy5-dCTP (Amersham Bioscience UK Ltd., Amersham, Buckinghamshire, United Kingdom); 2.5 μl primary PCR product; and 2.5 U *Taq* polymerase (QIAGEN). The primer extension reaction consisted of 95°C for 1 min, followed by 35 cycles of 95°C for 20 s, 52°C for 20 s, and 72°C for 2 min, with a final extension step of 72°C for 10 min. The Cy5-labeled single-stranded DNA products were then purified with a QIAGEN PCR purification kit (QIAGEN) and 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], 0.2% sodium dodecyl sulfate) containing 0.1 μM Cy5-labeled internal positive control probe (37). Microarray hybridization was performed in a MAUI hybridization dual-chamber lid (BioMicro, Salt Lake City, UT) 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 (37). Hybridized slides were scanned with a GSI Lumonics ScanArray Lite confocal laser-scanning system and analyzed with ScanArray software (Perkin-Elmer, Torrance, CA). The microarray images were captured at a laser power of 100 and a PMT gain of 80.

RESULTS

Oligonucleotide probe design. Primers binding to two conserved regions of the SSU rRNA sequence were used to amplify 1,300-bp fragments from four microsporidian species. Use of an amplicon of this size, however, would result in a low hybridization efficiency. To decrease the target DNA size through nested amplification and to design species-specific oligonucleotide probes, multiple sequence alignment analyses were used to identify five conserved regions from each of four representative SSU rRNA genes. One region (MspR3), which is approximately 900 bp downstream from primer MicroF, was selected as the reverse primer for primer extension labeling. The other four (Msp1 to Msp4) were chosen as family-specific probes that could be cross-hybridized by all microsporidian species. Because *E. cuniculi*, *E. hellem*, and *E. intestinalis* belong to one genus, three genus-specific probes (Ence1 to Ence3) which are common to three *Encephalitozoon* species were chosen to distinguish between *Encephalitozoon* and *Enterocytozoon* spp. In addition to homologous regions, there were many variable regions identified within the SSU rRNA

TABLE 1. Oligonucleotide probes used in this study

Name	Sequence	Length (nt)[b]	T_m[c] (°C)	% GC	Position[a]
Microsporidia					
Msp1	GATTCTGCCTGACGTGGATGCTATT	25	58	48	10
Msp2	ATTCCGGAGAGGGAGCCTGAGAGAT	25	61	56	257
Msp3	ATTGACGGAAGGACACTACCAGGA	24	57	50	794
Msp4	GTGCGGCTTAATTTGACTCAACGCG	25	58	48	825
E. bieneusi					
Eb1	ACGGCTCAGTAATGTTGCGGTAATT	25	56	44	85
Eb2	CCTATCAGCTTGTTGGTAGTGTAAA	25	54	40	172
Eb3	TCATGAGACGTGAGTATAAGACCTG	25	56	44	326
Eb4	ATCGAATACGTGAGAATGGCAGGAGT	26	58	46	856
Eb5	CTAAAAGCGGAGAATAAGGCGCAAC	25	58	48	147
Eb6	CGTTGTTCAATAGCGATGAGTTTGC	25	56	44	474
Eb7	GGTGAAACTTAAAGCGAAATTGACGG	26	56	42	762
Eb8	AGCCTGTGTGTGAGAATACGTGG	23	56	52	691
Encephalitozoon					
Ence1	ACGGCTCAGTGATAGTACGATGATT	25	56	44	86
Ence2	TATCAGCTGGTAGTTAGGGTAATGG	25	56	44	192
Ence3	GAGTGAAACTTGAAGAGATTGACGG	25	56	44	777
E. cuniculi					
Ec1	TGTGGGGTTGGCAAGTAAGTTGTGG	25	59	52	163
Ec2	ATGAGAAGTGATGTGTGTGCGAGTG	25	58	48	344
Ec3	GCCTGTGAGTGCATGGCATGAG	22	59	59	875
Ec4	GGGAAACTGCAGATAGTGGTCTGC	24	59	54	136
Ec5	GGATGTAGTGATGTGTGTGGCAGAG	25	59	52	516
Ec6	CTGGACGGGACAGTGTGTGTTGT	23	59	57	706
E. hellem					
Eh1	TAAGTTCTGGGGGTGGTAGTTTGTA	25	56	44	149
Eh2	GCGGTTATGAGAAGTAAGATGTTTAGCA	28	57	39	338
Eh3	CTGAAGTGAGTGTGAGAGTGTTTTTAC	27	57	41	878
Eh4	ATTGGGAGCCTGGATGTAACTGTGG	25	59	52	113
Eh5	GGATGTAGTTTTATTGTAGCAGAGG	25	54	40	527
Eh6	TGGACGGGACTGTTTTAGTGTTGTC	25	58	48	717
E. intestinalis					
Ei1	TTGACACGAGCCAAGTAAGTTGTAG	25	56	44	166
Ei2	TTTCGAGTGTAAAGGAGTCGAGATTGA	27	57	41	362
Ei3	GGCAGGAGAACGAGGACGGGAT	22	60	64	871
Ei4	CAGGTAGGGGGCTAGGAGTGTTTTT	25	59	52	143
Ei5	TATGTCCTGATGTGGATGTAAGAGG	25	56	44	502
Ei6	TGGACGGGACTATATAGTGTTGTG	24	56	46	703

[a] The first nucleotide of the probe corresponding to the position within the primary amplicon starting from the 5′ end. Positions of *Microsporidia* family-specific probes and *Encephalitozoon* genus-specific probes correspond to the amplicon sequence of *E. cuniculi*.
[b] nt, nucleotides.
[c] T_m, melting temperature.

sequence alignment. At least six oligonucleotide probes were designed to represent each species. To avoid cross-hybridization, each species-specific probe had at least four nucleotide mismatches with the same regions in other species. To determine the specificity of the designed oligonucleotide probes, we downloaded SSU rRNA genes of each microsporidian species from GenBank (five from *E. bieneusi*, seven from *E. cuniculi*, eight from *E. hellem*, and three from *E. intestinalis*) and aligned them with ClustalW. Overall, rRNA genes within each species had much higher homology compared with interspecies genes and had occasionally scattered polymorphic sites. More than half of the species-specific probes were found to match perfectly all of the corresponding SSU rRNA genes. A few probes had only one or two nucleotide mismatches with one of the rRNA genes, and such small mismatches would potentially decrease hybridization intensities when hybridized with diverse strains.

Microarray analysis of microsporidian reference strains. To validate the selected oligonucleotide probes, four microsporidian species were used as references. Prior to this, their identities were confirmed using established species-specific PCR (data not shown). Extraction-free, FTA filter-based template preparation and PCR amplification with primers MICRO-F and MICRO-R was first used to directly amplify 1,300-bp fragments from all isolates, followed by primer extension, fluorescent labeling, and hybridization to the array. The unique hybridization profile of each of representative species is shown in Fig. 1B. As expected, all four Msp probes were simultaneously hybridized by different species, confirming that these probes can served as general microsporidian recognition markers. It

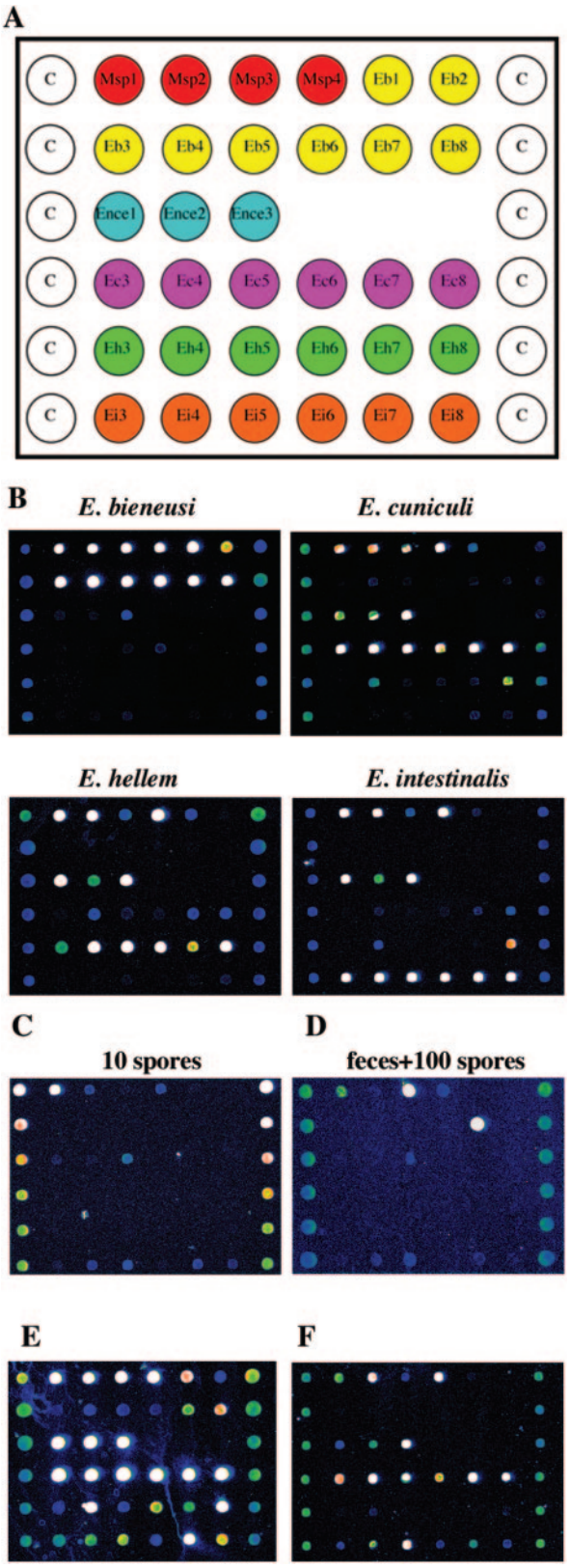

FIG. 1. Specificity and sensitivity of the microarray for four microsporidian species. (A) Microarray template showing the identity of each probe (Table 1). The spots at the left and right ends of the array represent internal positive controls. (B) Hybridization analysis of amplified SSU rRNA sequences from four reference species. (C and D) Sensitivity testing of the microarray with 10 pure spores of *E. intestinalis* and 100 spores spiked into 100 μl fecal material. (E) Hybridization of mixed amplified SSU rRNA fragments from all four reference species. (F) Hybridization of SSU rRNA PCR products amplified from the mixture of *E. cuniculi* (10^5 spores) and *E. intestinalis* (100 spores).

was noticed that Msp3 had a relatively weaker signal when hybridized with *Encephalitozoon* species. This is not surprising, because Msp3 perfectly matches the *E. bieneusi* sequence but has two mismatches with all *Encephalitozoon* sequences examined here. Eight *E. bieneusi* probes were only hybridized by *E. bieneusi*, which clearly differentiated itself from *Encephalitozoon* species as illustrated in the top two lines of the microarray. Apart from sharing three genus-specific spots in the middle line, *Encephalitozoon* species had their representative microarray patterns distinguished by the last three lines. Two false-positive spots were observed for Eh2 and Eh6 probes when hybridized with *E. cuniculi* and *E. intestinalis* target DNAs, respectively. However, the few instances of cross-hybridizations within the array did not interfere with our interpretation of microarray results. Furthermore, when genomic DNAs from other protozoa, such as *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum*, were amplified with the same primers and applied to microsporidian arrays, no signals were obtained from the hybridizations (data not shown), further suggesting the specificity of this array.

Testing of microarray sensitivity. The sensitivity of this microsporidian microarray was determined by using decreasing concentrations of microsporidian spores as templates. A series of diluted *E. intestinalis* spores were directly spotted onto FTA filters, and another series were spiked into 100 μl of fecal material before spotting onto FTA filters. All these samples were subjected to the hybridization protocol as already described. Although intensities of positive signals dramatically decreased, the microarray was able to clearly detect 10 pure spores (Fig. 1C). However, in spiked samples, 50 spores were barely visible and 100 spores were reliably detected (Fig. 1D) on the microarray. It was noticed that a false-positive spot for Eb7 was always present in all spiked samples (including 0 spore), indicating that the fecal material used for spiking contained a homolog of the Eb7 sequence.

Microarray identification of microsporidia from mixed samples. As microsporidian coinfections are not uncommon, we sought to determine the ability of the microarray to identify microsporidian species from a mixed population by combining 1,300-bp primary amplicons from different species in one tube, labeling with fluorescence, and hybridizing onto the microarray. The result showed that the microarray could efficiently and specifically identify all species present in the mixture (Fig. 1D). Also, we sought to test whether the simultaneous amplification of two microsporidian species would result in a lower detection sensitivity for the less-abundant microsporidia in the mixture. One hundred thousand *E. cuniculi* spores and 100 *E. intestinalis* spores were applied to an FTA filter, amplified, and hybridized onto the microarray. Hybridization profiles revealed the presence of both *E. cuniculi* and *E. intestinalis*. The intensity of the latter was apparently weaker, as expected (Fig. 1E). This result suggested that the microarray was able to simultaneously detect multiple microsporidian species and that detection of

coinfectants with a different ratio did not adversely affect the detection sensitivity for less-abundant species.

Microarray diagnosis of clinical samples. We next tested the ability of the microarray to efficiently diagnose microsporidian infections in suspected clinical specimens. Twenty fecal samples collected from AIDS patients presenting with chronic diarrhea but with unknown identities were treated as described in Materials and Methods and screened by microarray. Twelve samples were detected as microsporidium positive, and five hybridization profiles were identified as shown in Fig. 2. It is surprising that there was a high incidence of coinfections; 10 samples contained two species, and 1 even had a triple infection. *E. intestinalis* was the most common species found in 11 of these positive samples, and *E. bieneusi* was the next most common in 7 samples. These microarray results were subsequently confirmed using the species-specific PCR as shown in Fig. 2F and a conventional multiplex PCR assay (data not shown). These results indicate that the microarray has the ability to detect microsporidian infections and differentiate multiple species in clinical samples.

DISCUSSION

This research represents another successful example of the application of oligonucleotide microarrays in the detection of pathogenic protozoan parasites. By integrating extraction-free, FTA filter-based template preparation and amplification of homologous SSU rRNA sequences with universal primers, this microarray analysis strategy allowed us to generate unique profiles so as to rapidly detect and differentiate four major species of microsporidia with greater sensitivity and specificity and high-throughput capability.

Traditional methods for the diagnosis of microsporidia in clinical laboratories are still reliant on histochemical staining and light microscopy (14, 15). Due to the small size and nondistinct shape of the spores, these methods lack sensitivity, reproducibility, and species differentiation capability. Although visualization of ultrastructures by transmission electron microscopy can be used to identify microsporidia to the species level, low sensitivity, laborious sample preparation, and the necessity of professional training make this technique less attractive and more costly in practice (2, 7). PCR-based diagnostic methods have become increasingly popular for pathogen identification and are particularly suitable for those microbial organisms that are hard to culture in vitro, morphologically similar, and present in low infectious doses. With a limited database of the microsporidian genome, the SSU rRNA gene is still a main target for current PCR assays.

PCR-based methods, such as conventional PCR, restriction fragment length polymorphism, and real-time PCR, have greatly improve sensitivity and specificity to detect and differentiate microsporidian species in clinical and environmental samples (14, 15). However, the PCR method usually requires setting up multiple reactions with different specific primer pairs so as to discern a specific species. It is more difficult when testing a large amount of unknown samples. In this oligonucleotide microarray assay, we combined a PCR amplification strategy with subsequent hybridization to multiple specific probes immobilized on the microchip as a means to simultaneously detect multiple species of microsporidia. The FTA filter-based template preparation strategy not only eliminates labor-intensive steps, such as sonication, freeze-thawing, and glass bead disruption, used to extract DNA from microsporidian spores but also prevents significant DNA loss due to smaller sample sizes and effectively removes inhibitors from fecal samples (27). Compared with the commercial stool DNA

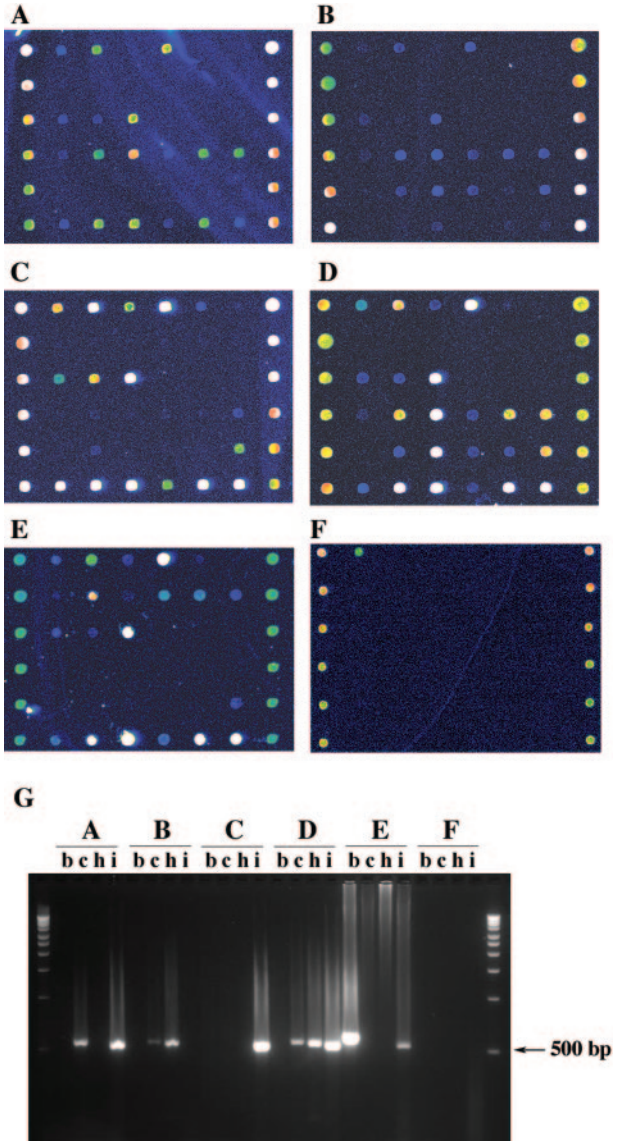

FIG. 2. Microarray hybridization results from clinical samples. SSU rRNA PCR products from 20 clinical samples were hybridized to microarrays. Twelve samples were positive, and eight were negative, which are represented by six hybridization profiles. Panels: A, *E. cuniculi* and *E. intestinalis*, two samples; B, *E. cuniculi* and *E. hellem*, one sample; C, *E. intestinalis*, one sample; D, *E. cuniculi*, *E. hellem*, and *E. intestinalis*, one sample; E, *E. bieneusi* and *E. intestinalis*, seven samples; F, negative, eight samples; G, species-specific PCR analysis of six representative clinical samples. Groups A to F in panel G correspond to the six hybridization profiles shown above. Lanes b, c, h, and i show designated amplification of clinical samples with primer pairs specific for *E. bieneusi*, *E. cuniculi*, *E. hellem*, and *E. intestinalis*, respectively. The expected amplicon sizes are 607 bp (b), 556 bp (c), 554 bp (h), and 528 bp (i).

extraction kit, this method has a lower cost, requires less technical training and equipment, and can handle a larger number of samples at one time (29). By taking advantage of conserved sequences of SSU rRNA genes shared by all microsporidian species, we were able to amplify 1,300-bp fragments from microsporidium-containing samples, followed by a second reaction using one degenerate primer to synthesize and fluorescently label single-stranded DNA from every primary PCR product. These products were then hybridized to specific oligonucleotide probes on the microarray. DNA-DNA hybridization limited nonspecific PCR fragments and yielded reliable sensitivity limits to a level of 10 *E. intestinalis* spores. Using the microarray as designed, identification of each microsporidian species was dependent not on a single amplicon but on a defined composite hybridization profile. Since each species has its own unique array pattern, it can be easily distinguished from another, as shown in Fig. 1B. Genetic diversities have been frequently found in many microsporidian species by analyzing the rRNA gene internal transcribed spacers and other genes with repetitive sequences (16, 21, 39). However, the targets in this study are 18S SSU rRNA genes, which are more conserved within a species than between species based on ClustalW alignment analysis. Searching the designed oligonucleotide probes against all of the SSU rRNA genes in GenBank confirmed that they are species specific and are able to represent the diversity of clinical isolates. Few probes having one or two mismatches with SSU rRNA genes of other genotypes might result in less-intense or false-negative hybridization spots. However, designing redundant probes from one gene not only increases confidence in and accuracy of results but also reduces the vulnerability of potential polymorphism or spontaneous mutations that might occur in circulating clinical or environmental isolates. In order to improve the ability of microarrays to genotype clinical and environmental isolates, which was demonstrated in our previous study (37), more oligonucleotide probes targeting highly variable regions, such as internal transcribed spacers and polar tube genes, could be immobilized on the chip with an increasing number of sequences available. As the microarray consists of probes derived from multiple species, it was able to simultaneously analyze multiple organisms in one experiment so as to greatly increase the assay throughput. Based on the ClustalW alignment of SSU rRNA, 1,300-bp fragments from four microsporidia, of both conserved and variable regions, were chosen to design hybridization probes at three taxonomic levels (family, genus, and species). Accordingly, printing these probes by levels on the chip apparently aided the recognition of hybridization profiles and identification of organisms. Also, targeting conserved region probes would potentially identify other genera and species in the microsporidian family based on signals at the top two levels. Furthermore, in this study, four identical arrays were printed on each slide so that four independent samples could be hybridized under individual chambers at the same time. Since one slide is capable of holding more than 10 arrays, depending on the size of printing grid, the throughput of microarrays to analyze multiple samples will be greatly increased and will also dramatically reduce reagent costs and processing time.

Like our previous protozoan microarray (37), we also chose short oligonucleotide probes ranging from 22-mer to 28-mer, which proved to be ideal for distinguishing closely related species and even recognizing polymorphic loci. Such short probes were able to detect one- or two-nucleotide mismatches. For example, Msp3 perfectly matches the sequence of *E. bieneusi* but has two nucleotides different from sequences of *Encephalitozoon* spp. close to its 3′ end. Therefore, the intensity at this spot dramatically dropped with hybridizations of target DNAs from *Encephalitozoon* species. In this study, the majority of genus- and species-specific probes contained at least four mismatches in the corresponding regions, so cross-hybridizations were avoided. However, Eh2 and Eh6 probes have three mismatches in the corresponding regions of *E. cuniculi* and *E. intestinalis*, so we saw two false-positive spots in preclinical microarray results. This observation suggested that three nucleotide mismatches are thresholds to prevent cross hybridization under the hybridization conditions used in this study.

Microsporidian infections have been frequently reported in AIDS patients. In order to validate the microarray method in a clinical setting, we used this protocol to examine 20 archived stool samples collected from AIDS patients suffering from chronic diarrhea. The results showed that 12 of these samples were microsporidian positive and the majority showed mixed infections. Eleven of the positive samples contained *E. intestinalis*, and seven contained *E. bieneusi*, which are the most frequently microsporidian species detected in AIDS patients. Our data not only demonstrated the ability of microarrays to simultaneously detect multiple species but also further suggested that coinfection of microsporidia is not an uncommon event (24). However, it will be more interesting to investigate why this batch of archived samples had such a high incidence of dual infections. Since the frequency of global prevalence of microsporidiosis is increasing, this microarray methodology needs to be further validated with a large number of samples collected from diverse resources and geographic locations. Compared with microarray profiles generated from reference strains, the intensities of array signals from those clinical samples were relatively weaker. There were two possibilities. Either (i) the concentrations of organisms in the samples were low and resulted in weak amplification, or (ii) some unknown inhibitors still present in the stool samples even after filter extraction decreased the efficiency of PCR and primer extension labeling. The previous study reported that as few as 10 *E. intestinalis* spores seeded in 100 μl fecal material could be detected by nested PCR (27), which was 5- to 10-fold more sensitive than the microarray used in this study. Compared with exponential amplification of primary PCR products by second-round nested PCR, primer extension labeling during microarray processing linearly generated Cye5-labeled single strands. On the other hand, the universal primer used for primer extension labeling was degenerated, which might not efficiently anneal to the target sequence, particularly under the influence of PCR inhibitors. Therefore, the efficiency of primary PCR and single-strand labeling needs to be further improved so as to increase microarray sensitivity. Meanwhile, in each of those arrays tested with clinical samples, a few spots (Enceph1, Ec1, Ec4, Eh1, Eh4, Ei1, and Ei4) showed false-negative results or extremely low intensities. All these probes were found to be located on the 5′ end of the target amplicons, as shown in Table 1. Since the amplicons were labeled with Cy5-dCTP by primer extension from the 3′ end and produced 900-bp single-stranded DNA, it is possible that polymerization

and dye incorporation were prematurely terminated and thus resulted in truncated single-stranded DNA labeled with dye. Therefore, probabilities for these probes to be hybridized by the fully labeled target DNA declined.

In conclusion, we have developed a microarray for simultaneous detection and discrimination of four major microsporidian species and have successfully applied it to investigate clinical samples. Therefore, with its sensitive, specific, rapid, and high-throughout characteristics, this method will be an attractive diagnostic tool for microsporidian diagnosis and epidemiological studies.

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