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TECHNICAL NOTE

CRIMINALISTICS; PATHOLOGY/BIOLOGY

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Pathogen Detection Using a Liquid Array Technology*

ABSTRACT: Low concentrations of microbial pathogens in pure and mixed samples were detected using a bead-based, liquid array technology. A 20-bp sequence in the 23S rRNA gene, *rrl*, was amplified in four microorganisms: *Bacillus cereus*, *Escherichia coli*, *Salmonella enterica* and *Staphylococcus aureus*. PCR products were positively identified with the Luminex® 100™ system. The system could detect very low amounts of DNA and the instrument response was proportional to the input concentration. The lower limit of detection (LLD) was determined to be 0.5 ng for *B. cereus* and *E. coli* and 2 ng for *S. enterica*. The LLD for *S. aureus* was not determined as the instrument response was still above the threshold when quantities of DNA as low as 0.25 ng were used. The platform positively identified organisms present in mixed samples even when the minor component was overshadowed by a 10-fold excess of the major component.

KEYWORDS: forensic science, pathogen detection, *Bacillus cereus, Escherichia coli, Salmonella enterica, Staphylococcus aureus,* liquid array technology

Pathogenic species of microorganisms pose a considerable threat to public health in the United States (1). Diseases caused by foodborne and waterborne bacteria, viruses, and parasites affect as many as 80 million people in the United States each year and cost an estimated 7–17 billion dollars for containment, treatment, and lost productivity in the workforce (1). Since the World Trade Center terrorist attacks and the Anthrax bioterrorism scare which followed in 2001, the subject of bioterrorism has become one of great concern. In addition to the intentional spread of dangerous biological agents by terrorists, pandemics could also be caused by accidental release of such agents, as in the 2005 scare when a dangerous influenza strain was accidentally included in kits used by laboratories all over the world.

Several biological organisms have a tremendous potential for destruction based on their ability to cause disease and death in humans. Microorganisms that could be used in a terrorist event include *Variola major* (smallpox), *Clostridium botulinum* (botulism), *Yersinia pestis* (plague), *Franciscella tularensis* (tularemia), and *Bacillus anthracis* (anthrax). Owing to their characteristic morphological and metabolic profiles, these pathogenic microorganisms have historically been identified by light microscopy and/or a battery of biochemical tests. Although such identifications may be somewhat accurate, they are tedious and time consuming. In addition, these methods offer nothing more than a presumptive identification because they cannot differentiate closely related strains. Identification of the microorganism's strain is of particular importance to microbial forensics, mainly because it enables possible source attribution.

Many of the traditional identification techniques based on microscopy or enzymatic testing are slow and imprecise. A rapid, cost-effective, and specific method of pathogen detection/identification is sorely needed. To that end, many recent detection systems have focused on DNA-based methods of identification. Owing to the inherent variability of DNA from organism to organism, DNA-based techniques have great potential to make rapid and irrefutable identifications. Additionally, many DNA-based techniques are amenable to the simultaneous detection of multiple analytes, thus accelerating the entire identification process. These multiplexing technologies are very sensitive, highly precise, easy to use, and relatively inexpensive (2,3). Limiting a reaction series to a single tube also reduces the cost of consumables and chances for error and contamination.

Liquid array analyses offer quantitative and multiplexing abilities, high specificity, and high sensitivity. One of these platforms is a bead-based liquid array system, which represents a convergence of microsphere technology and flow cytometry. The Luminex® 100™ (Luminex, Austin, TX) multi-analyte profiling system with xMAP™ technology and MasterPlex Analysis (MiraiBio, San Francisco, CA) software is a bead-based liquid array system that relies on small (5.6 μm diameter) polystyrene microspheres internally labeled with a unique dye combination. The microspheres are coated with thousands of copies of a probe (oligonucleotides, peptides, antibodies, or other ligands) unique for a particular target (http://www.invitrogen.com/site/us/en/home/brands/BioSource.html, accessed on November 1, 2009). Hybridization/binding between the probes and the target (DNA, RNA, or protein) is detected via fluorescent emission. The software classifies the microspheres by using a red diode laser (635 nm) to detect the fluorescence emitted by the internal dyes, and a green diode laser (532 nm) to detect and quantify the target analyte by measuring the intensity

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of the fluorescence emitted by a reporter. With this technology up to 100 different analytes per sample can be rapidly and reproducibly detected in real time (4,5).

Within the past decade, researchers have begun to use the Luminex® liquid array technology for pathogen detection (5). The platform has been employed to detect microorganisms in environmental samples (6) and clinical samples (2). The small size of the Luminex® instrument offers the possibility of constructing a field-deployable pathogen detection system. A portable system was comparable to a laboratory bench-top system in its ability to detect *B. anthracis* and *Y. pestis*, thereby demonstrating that field-deployable platforms need not suffer from an inferior performance (7,8).

Thus, development of multiplex pathogen detection arrays will have a significant impact on the ability to detect bacterial and viral agents. The development of an oligonucleotide direct-hybridization multiplex system for detection and identification of bacteria is described herein.

Materials and Methods

Bacterial Strains

The capabilities of the Luminex® liquid-array technology were tested with four microorganisms of minimum pathogenicity: *Bacillus cereus* (ATCC number 10987D), *Escherichia coli* (ATCC number 10798D), *Salmonella enterica* (ATCC number 10832D), and *Staphylococcus aureus* (ATCC number 49284D). Lyophilized genomic DNA (gDNA) samples were purchased from American Type Culture Collection (ATCC, Manassas, VA) and rehydrated in 250 µL molecular grade water. This provided a 20 ng/µL stock of *B. cereus*, *E. coli*, and *S. aureus* and a 40 ng/µL stock of *S. enterica* gDNA.

Bioinformatics—Designing Probes and Primers

The US National Center for Biotechnology Information site was accessed (http://www.ncbi.nlm.nih.gov/, accessed November 1, 2009) to obtain the genomic sequences of *B. cereus*, *E. coli*, *S. enterica,* and *S. aureus*. A genetic marker in the 23S ribosomal subunit, the multi-copy *rrl* gene, was chosen (5).

Sequences within the *rrl* gene of the four species were identified based on prior research. These "probe" sequences were all 20 nucleotides in length (Table 1) and appear several times (Table 2) in their respective bacterial genomes (5). The BLAST algorithm (http://www.ncbi.nlm.nih.gov/, accessed November 1, 2009) was used to search for these sequences, determine their loci in the bacterial genomes, and confirm that they are unique to each microorganism.

PCR primers were designed by examining the nucleotide sequences flanking the probes. Sequences were aligned in ClustalW (http://align.genome.jp/, accessed on November 1, 2009) to search for an appropriate set of degenerate primers that would amplify the region of interest in all four of the microorganisms (Table 1). As the probe sequences appear several times in each genome, the primers were designed to amplify all areas of the genome where the probe sequence appears. For example, there were seven amplicons from the *B. cereus* genomic sample, because the 20 nucleotide probe sequence appears seven times in the *B. cereus* genome. The *rrl* gene appears more times within the organism's DNA (Table 2), but those gene copies without the specific probe sequence were irrelevant to this study as they were not detected by the assay.

PCR Amplification of the Target Sequences

A set of degenerate primers (Table 3) was used for amplification of the target sequences. Oligonucleotides (1 µmol, salt-free; Fisher Scientific, Pittsburgh, PA) were reconstituted in molecular grade

TABLE 2—*Bioinformatics data.*

Organism	Genome (bp)	*rrl* Copies	Probe Copies	Amplicon (bp)
Bacillus cereus	5,224,283	12	7	111
Escherichia coli	4,639,675	7	5	108
Salmonella enterica	4,755,700	7	4	108
Staphylococcus aureus	2,820,462	6	3	112

TABLE 1—*ClustalW alignment of genome sequences flanking the probe.*

Organism	Genome Sequence	
Bacillus cereus	AAGGGCGCACGGTGGATGCCTTGACACTAGGAGTCGATGAAGGACGGGAC	50
Escherichia coli	AAGCGTACACGGTGGATGCCCTGGCAGTCAGAGGCGATGAAGGACGTGCT	50
Salmonella enterica	AAGCGTACACGGTGGATGCCCTGGCAGTCAGAGGCGATGAAGGGCGTGCT	50
Staphylococcus aureus	AAGGGCGCACGGTGGATGCCTTGGCACTAGAAGCCGATGAAGGACGTTAC	50
B. cereus	TAACGCCGATATGCTTCGGGGAGCTGTAAGTAAGCTTTG-ATCCGAAGAT	99
E. coli	AATCTGCGATAAGCGTCGGTAAGGTGATATGAACCGTTATAACCGGCGAT	100
S. enterica	AATCTGCGATAAGCGCCGGTAAGGTGATATGAACCGTTATAACCGGCGAT	100
S. aureus	TAACGACGATATGCTTTGGGGAGCTGTAAGTAAGCTTTG-ATCCAGAGAT	99
B. cereus	TTCCGAATGGGGAAACCCACCATACGTAATGGTATGGTATCCTTATCTGA	149
E. coli	TTCCGAATGGGGAAACCCA--GTGTGTTTCGACACACTATCATTAACTGA	148
S. enterica	ACCCGAATGGGGAAACCCA--GTGTGACTCGTCACACTATCATTAACTGA	148
S. aureus	TTCCGAATGGGGAAACCCAGCATGAGTTATGTCATGTTATCGATATGTGA	149
B. cereus	ATACATAGGGTAA-GGAAGACAGACCCAGGGAACTGAAACATCTAAGTAC	198
E. coli	ATCCATAGGTTAA-TGA-GGCGAACCGGGGGAACTGAAACATCTAAGTAC	196
S. enterica	ATCCATAGGTTAA-TGA-GGCGAACCGGGGGAACTGAAACATCTAAGTAC	196
S. aureus	ATACATAGCATATCAGAAGGCACACCCGGAGAACTGAAACATCTTAGTAC	199
B. cereus	CTGGAGGAAGAGAAAGCAAATGCGATTTCCTGAGTAGCGGCGAGCGAAAC	248
E. coli	CCCGAGGAAAAGAAATCAACCGAGATTCCCCCAGTAGCGGCGAGCGAA-C	245
S. enterica	CCCGAGGAAAAGAAATCAACCGAGATTCCCCCAGTAGCGGCGAGCGAA-C	245
S. aureus	CCGGAGGAAGAGAAAGAAAATTCGATTCCCTTAGTAGCGGCGAGCGAAAC	249

The probe sequences are highlighted above (5). The forward primer is underlined, beginning in the second row, *B. cereus* at position 90. The reverse primer is also underlined, beginning in the fourth row, *B. cereus* at position 180.

TABLE 3—*Degenerate primers for bacterial amplification. T_m = 62°C forward primer; T_m = 55°C reverse primer.*

Forward Primer	5′-AWCCRRMGATWYCCGAATGG-3′
Reverse Primer	5′-Biotin-RGGTACTWAGATGTTTCAGTTC-3′

W = A/T, R = A/G, M = A/C, Y = C/T.

dH_2O to a 100 μM stock, then diluted 20-fold to a 5 μM (10×) working solution. Primers for the anti-sense strands (the reverse complements of the probes) were biotinylated at the 5′ end to facilitate detection.

Four separate PCR reactions were performed. Target DNA was amplified in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) using the Qiagen HotStarTaq Plus PCR kit (Valencia, CA) under the following conditions: 95°C 5 min, 25 cycles of 94°C 30 sec, 55°C 30 sec, 72°C 30 sec, final extension 72°C 3 min.

PCR products were confirmed by agarose gel electrophoresis.

Coupling of Probes to Microspheres

The four probe sequences (50 nmol, HPLC; Fisher Scientific) were synthesized with a 5′ C12 amine modification. Probes were coupled to the carboxylated microspheres (Luminex Corp., Austin, TX) according to the manufacturer's instructions. The concentration of beads in each microsphere set was determined by counting a 1:100 dilution of each in a hemacytometer. Each set was then diluted in 1.5× tetramethylammonium chloride (TMAC) hybridization solution (4.5 M TMAC, 0.15% sarkosyl, 75 mM Tris–HCl, pH 8, 6 mM EDTA, pH 8) to a concentration of 600 beads/μL. The light-sensitive microspheres were stored at 4°C, wrapped in aluminum foil. The *B. cereus*, *E. coli*, *S. enterica,* and *S. aureus* probes were coupled to bead sets 110, 140, 146, and 155, respectively.

Coupling was confirmed using reverse complement oligonucleotides (5′ biotin, 0.2 μmol, HPLC; Fisher Scientific). It has been empirically determined that *c.* 460,000 identical probes are attached to a single bead in the process (6). Each microsphere set was diluted to a working concentration of 150 beads/μL in 1.5× TMAC hybridization solution. Approximately 200 fmol reverse complement and 33 μL of the respective microsphere set were combined and brought to a total volume of 50 μL with TE. Samples were incubated at 95°C for 3 min to denature the PCR amplicons and hybridized at 45°C for 15 min. Twenty-five microliters of the reporter (10 μg/μL Streptavidin-R-Phycoerythrin [SAPE] in 1× TMAC) was added to each reaction. The reactions were incubated for 5 min at 45°C and analyzed on the Luminex® 100™ at 45°C.

Multiplex Analysis of PCR Amplicons

Equal parts of all four microsphere stock (600 beads/μL) bead sets were combined to create a working bead mixture in which each set was present at a concentration of 150 beads/μL. In all assays the reverse complement oligonucleotides were used as positive controls for each microorganism. Seventeen microliters of TE was used as a negative control. All samples and controls were combined with 33 μL working microsphere mixture to yield a total volume of 50 μL. Reactions were incubated under the following conditions: 95°C 5 min, 45°C 15 min. The beads were pelleted by centrifugation at 3000 × *g* for 3 min and the supernatant was discarded. Seventy-five microliters 4 μg/μL SAPE in 1× TMAC hybridization solution was added to all reactions. Samples were incubated at 45°C for 5 min and analyzed on the Luminex® 100™ at 45°C.

Testing Sensitivity

To test the sensitivity of the Luminex® assay, various concentrations of PCR product were studied. Samples of *B. cereus*, *E. coli*, *S. enterica,* and *S. aureus* from 0.25 to 100 ng were prepared and analyzed as described previously.

Testing Mixed Samples

Two mixture studies were conducted. Twenty nanograms of each PCR amplicon was combined to create mixtures of two, three, and four microorganisms to study how competition affects the hybridization of targets to their probes.

Different binary combinations of bacterial samples in various ratios—10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10—were prepared and analyzed. The value of "1" was designated as 2 ng, the lower limit of detection (LLD) for *S. enterica,* and all other concentrations were proportional to this.

Results and Discussion

The Luminex® instrument determines the fluorescence of at least 100 beads for each set present and the analysis software reports the results as the median fluorescent intensity (MFI). The spectral address identifies the bead and, thereby, the probe sequence that the sample has hybridized to. The amount of reporter fluorescence provides quantitation, as it is proportional to the amount of target that hybridized to the probe. The results were compared to background fluorescence. In this study, the threshold for a positive result was defined as a net MFI value that was at least two times greater than the background MFI (5).

Confirmation of PCR Products

The yield gel confirmed that all the PCR products were between 100 and 150 base pairs in length (data not shown). Amplicon concentrations (20 ng/μL) were determined by visual comparison to the standard (data not shown).

The *B. cereus*, *E. coli*, *S. enterica*, and *S. aureus* PCR products contained 1.75×10^{11}, 1.80×10^{11}, 1.80×10^{11}, 1.74×10^{11} *rrl* "probe" copies/μL, respectively.

Probe Coupling

The singleplexed assay to confirm that the probes had been coupled to the microspheres was successful. In this assay, the negative control wells contained all the bead types, but no reverse complement; the sample wells contained one reverse complement and its corresponding microsphere set. The high MFI values (Table 4) indicated that there was hybridization between the microspheres and the PCR products in the sample wells, thus confirming that the 20 base pair probes were successfully coupled to the beads.

There was some microsphere carry-over from well to well during the analysis. For example, there were no "BC Probe" beads present in the *E. coli*, *S. enterica,* or *S. aureus* sample wells, yet a low MFI was reported (Table 4). After observing this, it was decided to place "wash wells" with 75 μL 1× TE in between samples on the 96-well plate to reduce inter-sample carry-over. Two washes were sufficient to reduce MFI values to levels below the threshold (>2× background MFI [5]) for a positive result.

TABLE 4—*Raw MFI values for verification of coupling probes to the microspheres. The combinations of designed complementarity are highlighted.*

Sample	BC Probe	EC Probe	SE Probe	SA Probe
Bacillus cereus	1412	0	0	0
Escherichia coli	17	1130	0	0
Salmonella enterica	3	27	868	0
Staphylococcus aureus	1	0	10	579
Neg. control	180	116	104	100

Sensitivity

Several sensitivity assays were performed to determine the LLD for each of the species. Results from an initial study that analyzed samples ranging from 10 to 100 ng of PCR amplicon indicated positive identifications of all genera for the entire range of input DNA (data not shown). A second study repeated this analysis on samples ranging from 0.25 to 25 ng of PCR product. As expected, the MFI decreased proportional to the decreasing concentration of the samples. For all species, the decrease was fairly linear down to 5 ng (Fig. 1). The MFI then dropped more drastically for PCR amplicons below 5 ng.

As seen in Fig. 1, the LLD was reached for three of the organisms. *B. cereus* and *E. coli* samples were detected by the Luminex® 100 down to a concentration of 0.5 ng in 50 μL. *S. enterica* was only identified when at least 2 ng of sample in 50 μL was present. The MFI for *S. aureus* samples remained very high, even for the lowest amount of input DNA (0.25 ng) and thus, the LLD could not be determined. Studies are planned for the future to determine if these effects are because of a difference in the coupling efficiency for the respective probes and/or the stringency of the hybridization reaction.

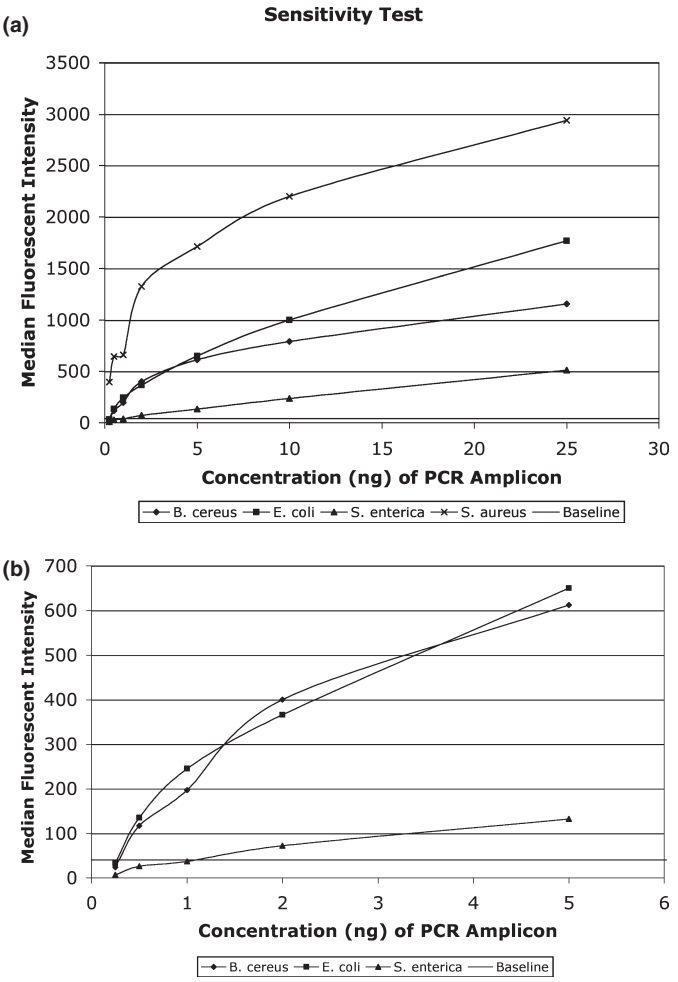

FIG. 1—*MFI form singleplexed samples (0.25–25 ng) of PCR amplicons from* Bacillus cereus, Escherichia coli, Salmonella enterica, *and* Staphylococcus aureus *(top) and closer view of* B. cereus, E. coli, *and* S. enterica *(bottom). Baseline is indicated by a horizontal line at 30 MFI in both graphs.*

Mixtures

The mixture study, using combinations of two, three, and four microorganisms, was fairly successful. As seen in Table 5, the MFI values for *B. cereus* and *E. coli* amplicons were moderately proportional to each other. The *S. aureus* amplicons produced much higher MFI values than other amplicons present in equal quantities in the same sample. With the exception of the "BC + EC + SE + SA" sample, the *S. aureus* amplicon generally produced an MFI twice that of the other amplicon(s) present. On the other hand, the *S. enterica* amplicons generally produced very low MFI values. In fact, *S. enterica* was only positively identified in one case ("BC + EC + SE").

The data show that competition between organisms' DNA sequences negatively affects the instrument's capability to detect the presence of *S. enterica* in mixed samples. The sensitivity assay defined the LLD for *S. enterica* as 2 ng. The mixture study samples, at 20 ng, were 10 times more concentrated, yet there was a failure to detect *S. enterica* amplicons in all but one mixture sample. The MFI values also show that *S. aureus* target DNA hybridized to its probe with a great degree of efficiency, producing a much higher fluorescent response than that observed for other probe-amplicon duplexes when similar quantities of input DNA were used.

The results of the ratio mixture study are indicated in Table 6. No false positives were obtained. However, several false negatives were observed involving *S. enterica*. These MFI values were often too low to provide a positive identification (i.e., "BC:SE 10:1," "BC:SE 2:1," and "EC:SE 2:1"). These particular MFI ratios were drastically skewed from the actual sample ratios. For example, in a sample combining *B. cereus* and *S. enterica* in a 10:1 ratio, the MFI value of *B. cereus* was 24 times as large as that of *S. enterica*. The fluorescent intensity even remained stronger for the former when the sample contained a higher concentration of *S. enterica* than *B. cereus*. It was not until a sample ("BC:SE 1:10") contained 10 times more *S. enterica* than *B. cereus* amplicon that a 1:1 MFI ratio was achieved. A clinical sample would have to contain 10 times more *S. enterica* than *B. cereus* to provide a comparable MFI value. A similar phenomenon was seen in the "EC:SE 1:5" sample, where five times more *S. enterica* than *E. coli* was necessary to achieve a 1:1 MFI ratio.

The high efficiency of *S. aureus* hybridization seen in the 1:1 combination mixture study was also observed in the ratio mixture study. The MFI value for *S. aureus* amplicon was often higher than that of the other amplicon in the same sample, even in cases where the latter was present in a much higher proportion (i.e., all "SE:SA" samples).

Again, future studies will be conducted to determine if these effects are because of a difference in the coupling efficiency for the respective probes and/or the stringency of the hybridization reaction. Still, with the exception of three *S. enterica* false negatives, the Luminex® instrument was able to identify both

TABLE 5—*Net MFI values* for the mixture assay. All samples contained 20 ng of each PCR amplicon. The combinations of designed complementarity are highlighted. Values two times the average background (81) indicate a positive identification and are denoted in bold.*

Sample**	BC Probe	EC Probe	SE Probe	SA Probe
BC + EC	**486**	**599**	6	−2
BC + SE	**586**	−6	75	−9
BC + SA	**537**	−10	7	**1643**
EC + SE	−8	**665**	62	7
EC + SA	−7	**713**	−6	**1733**
SE + SA	−8	−1	76	**1750**
BC + EC + SE	**603**	**644**	**83**	−1
BC + EC + SA	**532**	**707**	10	**1672**
BC + SE + SA	**508**	10	79	**1642**
EC + SE + SA	11	**718**	78	**1362**
BC + EC + SE + SA	**502**	**519**	80	**722**

*The net MFI values are equivalent to the raw MFI value less one background of the specific microsphere set.

**BC, *Bacillus cereus;* EC, *Escherichia coli;* SE, *Salmonella enterica;* SA, *Staphylococcus aureus.*

TABLE 6—*Net MFI values* for the ratio mixture study. The combinations of designed complementarity are highlighted. Values two times the average background (46) indicate a positive identification and are denoted in bold. False negative values are underlined.*

Sample**	BC Probe	EC Probe	SE Probe	SA Probe	MFI Ratio***
BC:EC 10:1	**1035**	**254**	−10	−2	4:1
BC:EC 5:1	**817**	**313**	21	9	2.5:1
BC:EC 2:1	**533**	**283**	−2	−6	2:1
BC:EC 1:1	**341**	**303**	−2	2	1:1
BC:EC 1:2	**315**	**470**	1	5	1:1.5
BC:EC 1:5	**331**	**907**	8	7	1:2.5
BC:EC 1:10	**303**	**1276**	18	23	1:4
BC:SE 10:1	**1074**	−3	45	−8	24:1
BC:SE 5:1	**901**	−10	**56**	−12	16:1
BC:SE 2:1	**560**	−8	37	−6	15:1
BC:SE 1:1	**383**	−4	**56**	−11	7:1
BC:SE 1:2	**420**	1	**97**	7	4.5:1
BC:SE 1:5	**275**	−5	**189**	−9	1.5:1
BC:SE 1:10	**291**	11	**340**	−5	1:1
BC:SA 10:1	**1169**	−6	1	**462**	2.5:1
BC:SA 5:1	**795**	−9	−11	**297**	2.5:1
BC:SA 2:1	**619**	−7	−2	**822**	1:1.5
BC:SA 1:1	**424**	−4	−1	**925**	1:2
BC:SA 1:2	**408**	0	2	**1332**	1:3.5
BC:SA 1:5	**335**	5	−9	**1815**	1:5.5
BC:SA 1:10	**242**	−4	5	**2075**	1:8.5
EC:SE 10:1	2	**1648**	**115**	33	14.5:1
EC:SE 5:1	0	**1036**	**64**	26	16:1
EC:SE 2:1	−4	**571**	45	9	12.5:1
EC:SE 1:1	−6	**380**	**49**	3	8:1
EC:SE 1:2	0	**292**	**89**	3	3.5:1
EC:SE 1:5	−6	**249**	**207**	1	1:1
EC:SE 1:10	−2	**232**	**374**	−1	1:1.5
EC:SA 10:1	−4	**1592**	43	**1226**	1.5:1
EC:SA 5:1	5	**1109**	3	**1009**	1:1
EC:SA 2:1	−13	**672**	−4	**1002**	1:1.5
EC:SA 1:1	1	**442**	6	**1050**	1:2.5
EC:SA 1:2	0	**472**	9	**190**	2.5:1
EC:SA 1:5	−2	**469**	10	**1987**	1:4
EC:SA 1:10	−4	**499**	−3	**2261**	1:4.5
SE:SA 10:1	6	10	**436**	**1012**	1:2.5
SE:SA 5:1	8	4	**252**	**983**	1:4
SE:SA 2:1	−2	6	**124**	**986**	1:8
SE:SA 1:1	1	0	**84**	**1028**	1:12
SE:SA 1:2	−5	14	**76**	**1386**	1:18
SE:SA 1:5	4	14	**103**	**1938**	1:19
SE:SA 1:10	5	15	**87**	**2398**	1:27.5

*The net MFI values are equivalent to the raw MFI value less one background of the specific microsphere set.

**BC, *Bacillus cereus;* EC, *Escherichia coli;* SE, *Salmonella enterica;* SA, *Staphylococcus aureus.*

***An approximate ratio of MFI values was determined between the two organisms present in the sample by dividing the larger MFI value by the smaller one and rounding to the nearest 0.5.

contributors in binary mixtures, even when the minor component was overshadowed by a 10-fold excess of the major component.

Summary

We have verified that Luminex® is capable of detecting microbial DNA present in a sample when probes are well designed to be complementary to a PCR amplicon. The sensitivity study indicated that the instrument was able to detect DNA samples present in very low quantities. All four microorganisms were positively identified when at least 2 ng of target DNA was present. Mixture studies showed that competition did not suppress positive identification in most cases. Neither mixture study resulted in any false positives. False negatives were limited to a few *S. enterica* samples, in which hybridization of target DNA to probe was probably reduced by competition with other oligonucleotides.

Luminex® liquid array technology requires a substantial commitment in terms of background research that must precede the benchwork. The research must be carried out using probe and target sequences that have underdone extensive studies prior to their use. For example, it must be confirmed that the target DNA is derived from highly polymorphic, thermodynamically stable, and nonhairpin-forming regions of the DNA (9). The research and preparation involved (especially if 100 analytes will be screened) can be quite laborious. Nevertheless, the data output mirrors the work that preceded it, so it is important to invest the appropriate amount of time in the early phases of the research. Once the initial background work is completed, many assays can be completed without the need for any further bioinformatics research.

Techniques such as T-RFLP and real time PCR (RT-PCR) have been used for profiling microbial communities. However, the generation of artifacts ("pseudo" peaks and background noise) is problematic for the T-RFLP method (10) and RT-PCR has limited multiplexing ability (11).

The advantages of pathogen detection with Luminex® liquid array technology are clear—small sample sizes, rapid assay time, multiplexing capabilities, sensitivity, and robustness. The identification of a pathogen in the field or in clinical samples can be accomplished with celerity and specificity, thus helping to contain possible threats and administer the appropriate treatment.

Although a high level of specificity was observed in the work reported here, future research is necessary, because the single marker studied does not offer enough information to positively identify a microorganism. Development of an assay with additional probes from independent genes will provide more discriminative identification. Overall, this work demonstrates the extraordinary potential for the Luminex® liquid array technology to serve as the paradigm for multiplexed pathogen detection systems.

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