A Multiplex Polymerase Chain Reaction Microarray Assay to Detect Bioterror Pathogens in Blood

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Heightened concern about the dangers of bioterrorism requires that measures be developed to ensure the safety of the blood supply. Multiplex detection of such agents using a blood-screening DNA microarray is a sensitive and specific method to screen simultaneously for a number of suspected agents. We have developed and optimized a multiplex polymerase chain reaction microarray assay to screen blood for three potential bioterror bacterial pathogens and a human ribosomal RNA gene internal control. The analytical sensitivity of the assay was demonstrated to be 50 colony-forming units/ml for *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pseudotuberculosis* (surrogate for *Yersinia pestis*). The absence of any false-positives demonstrated high analytical specificity. Screening *B. anthracis*-infected mouse blood samples and uninfected controls demonstrated effectiveness and specificity in a preclinical application. This study represents proof of the concept of microarray technology to screen simultaneously for multiple bioterror pathogens in blood samples. (*J Mol Diagn* 2005, 7:486–494)

In response to concerns about potential bioterror attacks and the occurrence of intentional *Bacillus anthracis* contaminations that resulted in fatalities, there has been active development of new diagnostics for detection of potential bioterror agents. However, diagnostic tests for category A (those presenting the greatest danger of use as bioweapons¹) bacterial pathogens such as *B. anthracis*, *Francisella tularensis*, and *Yersinia pestis* have not been optimized for detection in blood. These agents can be disseminated in the environment resulting in human infections. Most of these agents reach a bacteremic stage late in the infection when standard donor question-

naire screening would prevent donation of contaminated blood. Concerns would remain, however, about blood from potentially exposed individuals requiring methods to test such suspect blood units. Additionally, if a widespread environmental release occurred, effective means of testing blood products would be required to ensure a safe and adequate supply. Effective diagnosis is also important because deferral of potentially exposed, but uninfected donors in the area of a bioterror incident would result in unnecessary loss of blood donors.

In a potential contamination event, the identity of the pathogen will not be known with certainty. Simultaneously screening for multiple bioterror agents would be desirable. A number of multiplex real-time polymerase chain reaction (PCR) assays have been tested that perform highly sensitive and rapid detection of conventional pathogens in blood.^{2–4} However, in multiplexing PCR, as the number of primer pairs in a reaction is increased there is a trend to generate more nonspecific PCR products due to inappropriate primer pairing.^{5,6} The nonspecific PCR products make identification of the diagnostic PCR product by gel electrophoresis difficult to impossible.⁷ Techniques such as Southern blotting or DNA sequencing to identify the multiplex PCR products are mostly considered too time consuming for screening purposes.

An additional technology with even greater potential to screen for a multitude of pathogens simultaneously is the DNA microarray. A DNA microarray is a miniature device with many unique oligonucleotide probes printed on a solid surface. These oligonucleotide probes would be able to simultaneously discriminate, by hybridization, many specific PCR products amplified from pathogens using a small amount of sample. The microarray platform has the potential to screen blood for bioterror pathogens. Microarray-based diagnostic techniques already have proven effective in matrices other than blood.^{6–8}

In our study, a microarray was developed for detecting three bioterror pathogens and a human sequence for internal positive control. Sensitivity and specificity of the multiplex PCR microarray assay for detection of three bacterial agents in blood was evaluated by spiking a

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Table 1. Pathogens, Targets, and Primer Sequences

Category	Organism	Gene target (Accession no.)	Primer sequences	Primer pair usage
Bacteria	<i>Bacillus anthracis</i> (Gram positive rod and spore forming)	Protective antigen gene on plasmid pXO1 (M22589)	BAF TCGAATTCAAGTACGGTCGC BAR GTTGTAGATTGGAGCCGTCC BAR2 GATTGGAGCCGTCCCAGTAT BAL TTGGAGCCGTCCCAGTATTT	Primary: BAF, BAR Secondary: BAF, BAR2 PET*: BAL
	<i>Francisella tularensis</i> (Gram negative coccobacillus)	17 kDa lipoprotein TUL4 gene (M32059)	TUL4-438 GCTGTATCATCATTTAATAAACTGCTG TUL4-863 TTGGGAAGCTTGTATCATGGCACT FTf2 CGCAGGTTTAGCGAGCTGTTC FTL GAAGCTTGTATCATGGCACTTAGA	Primary: TUL4-438, TUL4-863 [22] Secondary: Ftf2, FTL PET: FTL
	<i>Yersinia pestis</i> (Gram negative and non-spore forming)	16S Ribosomal RNA gene (Z21939)	YPTF GTAGTTTACTACTTTGCCGGCG YPTR GATTGAGCGTATTAACTCAACCC YPTF2 CGGACGGGTGAGTAATGTCTG YPTL GCGTATTAACTCAACCCCTTC	Primary: YPTF, YPTR [23] Secondary: YPTF2, YPTL PET: YPTL
Internal control	Human	18S Ribosomal RNA gene (K03432)	HrRNAF CGAAGACGATCAGATACCGT HrRNAR CAGCTTTGCAACCATACTCC HrRNAL TTGCAACCATACTCCCCCG	Primary: HrRNAF, HrRNAR Secondary: HrRNAF, HrRNAL PET: HrRNAL

* PET, primer extension thermocycling, the first step in the labeling reaction.

known amount of pathogens into whole blood. In addition, the performance of the assay was evaluated by screening blinded blood samples from mice infected with *B. anthracis*.

Materials and Methods

Design of Microarray Oligonucleotide Probes and Target Gene Primers

All amplicons, primers, and probes were optimized by the following criteria: 18 to 24 nucleotides in length, 40 to 60% GC content, melting temperature of 52 to 55°C, 1- to 2-nucleotide GC clamp at the 3' end, avoidance of dimers, hairpins, long stretches of single base repeats, false priming sites with a total amplicon size to be 150 to 400 bp in length by using Vector NTI 5 on a desktop PC and Prime3 software in the GCG Wisconsin Package (Accelrys, San Diego, CA) running on a server with a web

interface. All amplicons were searched using BLAST (<http://www.ncbi.nlm.nih.gov/blast>) against the complete GenBank database to ensure they represented unique sequences that would only hybridize with the selected microarray probes, avoiding cross-hybridization with other species. The lengths of microarray probes were 33 to 45 nucleotides in our study. Three nonoverlapping microarray probes were selected for each pathogen amplicon so that the labeled amplicon would need to hybridize to three independent sequences to achieve positive detection. The primer sequences are shown in Table 1 and probe sequences in Table 2.

Array Design and Fabrication

Three different amino-modified oligonucleotides for each bioterror pathogen and the ribosomal RNA region of the blood-borne parasites of the family *Trypanosomatidae* were printed in a grid (Table 3 and Figure 2). The parasite

Table 2. Sequences of Oligonucleotide Probes Printed on the Microarray

Type	Name	Sequence	Length (nt)	GC (%)
<i>B. anthracis</i>	BAp1	CAAGTACGGTCGCAATTGATCATTCATATCTCTAGCAGG	40	45
	BAp2	GGAAAGAAGTTGGGCTGAAACAATGGGTTTAAATACCGCT	40	43
	BAp3	GATACAGCAAGATTAAATGCCAATATTAGATATGTAAATA	40	25
<i>F. tularensis</i>	FTp1	CGCAGGTTTAGCGAGCTGTCTACTCTAGGGTTAGGTGGC	40	55
	FTp2	GCTGCTGCTCAGACAGCTACTACTGAGCAAGCTGCTGCTG	40	58
	FTp3	GGAAGTGTAAGATTACAATGGCAGGCTCCAGAAGGTTTC	38	47
<i>Y. pestis</i>	YPp1	GGGGATCTGCTGATGGAGGGGGATAACTACTGG	34	59
	YPp2	GGTGGGGTAATGGCTCACCTAGGCGACGATCCC	33	64
	YPp3	CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG	33	70
<i>Leishmania sp./Trypanosoma sp.</i>	LTp1	CGGAATGGCACCACAAGACGTGGAGCGTGCGGTTTA	36	70
	LTp2	CGGGGAACTTTACCAGATCCGGACAGGATGAGGATTGACAG	41	54
	LTp3	CTCGATTCCCTGAATGGTGGTGCATGGCCGCTTTTGGTCCG	41	72
Control	HrRNA	AGTTCCGACCATAAACGATGCCGACCGCGATGCGCGCGCGTTAT	45	60
QC	Cy5-QC	CCCATATCGTTTCATAGCTTCTGCCA	26	46
	QCprobe	TTGGCAGAAGCTATGAAACGATATGGG	27	44

Table 3. Microarray Probe Layout

Position (Rows:columns)	Organism
BCD:1	<i>B. anthracis</i>
EFG:1	<i>F. tularensis</i>
HIJ:1	<i>L. sp./T. sp.</i>
ABC:2	<i>Y. pestis</i> and <i>Pseudotuberculosis</i>
Internal control	
A:1, J:2	Human 18S ribosomal RNA gene

See Figure 2.

probes were included as a negative control and because one of the genera in the family, *Leishmania*, has been a long-standing research organism in our laboratory, presenting a need to monitor its contamination. Two replicate copies of an oligonucleotide for the human internal control (Table 3 and Figure 2) were included and the whole set of probes printed within a 2×10 grid (total area, $1 \text{ mm} \times 4.95 \text{ mm}$) to form a subarray. Each subarray was printed eight times on a CSS-100 silylated slide (CEL Associates, Inc., Pearland, TX) by a microspotting pin (Telechem, Inc., Sunnyvale, CA) with a PIXSYS5500 contact microspotting robotic system (Cartesian Technologies, Inc., Irvine, CA). Each oligonucleotide probe solution was a mixture that contained a $10 \mu\text{mol/L}$ concentration of quality control oligo (QCprobe, Table 2),⁶ and a $100 \mu\text{mol/L}$ concentration of the pathogen oligo in 50% dimethyl sulfoxide. In addition, spots for marking the array position on the slide were made by using $1 \times$ microspotting solution (Telechem). The printed slides were dried for 10 minutes at 80°C and soaked in freshly prepared 0.25% sodium borohydride solution for 10 minutes at room temperature. The slides were washed with 0.2% sodium dodecyl sulfate in water for 5 minutes once and washed with distilled water for 5 minutes three times to eliminate unbound oligonucleotides. A sample-printed slide was hybridized with Cy5-QC-complement oligo (Table 2 and Figure 2A) to confirm the quality of the print.

Bacterial Species and Culture Conditions

The vaccine strain of *B. anthracis* (strain 34F2) (Colorado Serum Co., Denver, CO) was grown in vegetative culture, induced to sporulate in SG sporulation medium,⁹ and spore suspensions were prepared. *Y. pseudotuberculosis*, which has the identical sequence in the region of the target gene to *Y. pestis*, serving as a surrogate for the select agent, was cultured in Luria-Bretani (LB) liquid medium¹⁰ and stored at -80°C according to standard procedure. To calculate cell concentration of the stored cultures, an aliquot of the culture was used to count colony-forming units (CFU) by serial dilution and plating on LB agar plates. *F. tularensis* cells (no. 29684; American Type Culture Collection, Manassas, VA) were grown and quantitated essentially as described¹¹ with the following modifications: the bacterial stock was streaked on a Mueller-Hinton broth (M-H; Difco, Detroit, MI) agar plate containing 1% ferric pyrophosphate, 10% glucose, and 2.5% fetal calf serum and incubated at 37°C in 5% CO_2

for 4 days. A single colony was used to inoculate M-H broth and incubated with gentle shaking at 37°C under 5% CO_2 for ~ 8 to 10 hours. The culture was monitored at OD_{600} and stopped when it reached 0.15 to 0.2 ($\sim 1 \times 10^8$ cells per ml.) indicating early to mid-log phase. Aliquots of 0.5 ml were frozen at -80°C for future use. Aliquots were thawed and quantitated by serial dilution and plate count.

Pathogen Spiking into Whole Blood and Total DNA Isolation

A liquid culture or spore suspension of each pathogen at known concentration was used to make a series of 10-fold dilutions in phosphate-buffered saline and $10 \mu\text{l}$ of each dilution was spiked into $200 \mu\text{l}$ of research use whole blood, which was obtained from the Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD. Total DNA was prepared from the spiked blood by using a QIAamp DNA blood minikit (Qiagen Inc., Valencia, CA) according to the DNA blood kit's protocol. The final eluted volume was $100 \mu\text{l}$ in Qiagen's Buffer AE.

Multiplex PCR Amplification

The three different pathogen primer pairs and an internal control primer pair (Table 1) were combined in the standard PCR mixture ($25 \mu\text{l}$), which contained 1.5 U of TaqDNA polymerase with $1 \times$ TaqMaster reagent, $1 \times$ Eppendorf PCR buffer, 2.0 mmol/L $\text{Mg}(\text{OAc})_2$, $0.6 \times$ Applied Biosystems (Roche, Indianapolis, IN) PCR buffer (300 mmol/L KCl, 60 mmol/L Tris-HCl, pH 8.3), $200 \mu\text{mol/L}$ concentration of each dNTP (Invitrogen, Carlsbad, CA), 320 nmol/L concentration of each pathogen forward and reverse primers, 160 nmol/L each internal control primer, and $5 \mu\text{l}$ of DNA template. Thermocycling was performed using a T gradient 96 PCR machine (Whatman Biometra, Goettingen, Germany) following the program of an initial 94°C for 4 minutes, and 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 40 seconds, finally 72°C for 10 minutes. A negative control tube containing water in place of the DNA template was used to monitor for contamination. An internal control primer pair was used that targeted the 18S human ribosomal RNA gene to monitor the performance of PCR, labeling, and hybridization. PCR products were evaluated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Nested PCR Amplification

Each of the three pathogen-nested primer pairs was combined with the internal control primer pair in three separate nested PCR reactions. The total PCR volume of each was $40 \mu\text{l}$. The PCR mixture contained 2.0 U TaqDNA polymerase, $1 \times$ Eppendorf PCR buffer, 2.0 mmol/L $\text{Mg}(\text{OAc})_2$, $0.6 \times$ Applied Biosystems (Roche) PCR buffer (300 mmol/L KCl, 60 mmol/L Tris-HCl, pH

8.3), 200 $\mu\text{mol/L}$ dNTP, 300 nmol/L of the pathogen forward and reverse primers, and 100 nmol/L internal control primers. The primary PCR products were diluted 1 to 10. Three μl of the diluted primary PCR products were added to each of the three nested PCR tubes. The thermocycle conditions were the same as the first PCR and also negative controls were performed. The synthesis of PCR products was confirmed by gel electrophoresis. The three tubes of nested PCR were pooled and purified using a MinElute PCR purification kit (Qiagen, Inc.) following the manufacturer's instructions with 10 μl as the final elution volume.

Primer Extension Thermocycling (PET) for Synthesis of Amino Allyl-Labeled Single-Strand DNA (ssDNA)

The purified pooled nested PCR products were used as DNA templates in primer extension reactions for incorporation of amino allyl-dUTP. The reaction contained one primer for each of the three pathogens and the human control (PET primers listed in Table 1), chosen to generate single DNA strands that were complementary to the probe on the microarray for each amplicon. The PET mixture (50 μl) contained 3.0 U of *Taq*DNA polymerase, 1 $\times$ *Taq* Master, 1 $\times$ Eppendorf PCR buffer, 0.6 $\times$ Applied Biosystems (Roche) PCR buffer (300 mmol/L KCl, 60 mmol/L Tris-HCl, pH 8.3), 2.0 mmol/L $\text{Mg}(\text{OAc})_2$, 200 $\mu\text{mol/L}$ dGTP, dCTP, dATP, 40 $\mu\text{mol/L}$ dTTP, 40 $\mu\text{mol/L}$ amino allyl-dUTP (AA-dUTP) (Sigma Aldrich, St. Louis, MO) with 320 nmol/L of each primer and 10 μl of the purified PCR product. The cycling program was initially 95°C for 1 minute, then 35 cycles (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds) and finally, 72°C for 10 minutes. The PET product was purified using the MinElute PCR purification kit. The final eluted solution was dried in a Speed Vac (ThermoSavant, Holbrook, NY) for 15 minutes.

Fluorescent Dye Coupling to the Amino Allyl Groups Incorporated into ssDNA

The dried ssDNA was resuspended with 5 μl of 0.2 mol/L NaHCO_3 , coupling buffer, and 5 μl of Cy3 or Cy5 monofunctional dye solution [the dry contents of one tube, Q15108 (Amersham Biosciences, NJ), was resuspended in 62 μl of dimethyl sulfoxide] was added, mixed, and incubated in a dark box for 1 hour at room temperature. Subsequently the fluorescent ssDNA was purified using the MinElute PCR purification kit. The final eluted solution was dried in Speed Vac for 10 minutes.

Hybridization

The dried fluorescent DNA was resuspended in 5 μl of distilled water and mixed with 5 μl of a 2 $\times$ hybridization buffer containing 10 $\times$ Denhardt's solution, 12 $\times$ standard saline citrate, and 0.2% Tween 20 in distilled water. The sample was denatured at 96°C for 1 minute and placed in

ice. Seven μl of the hybridization solution was placed on two subarrays and covered with a plastic coverslip (5.5 mm $\times$ 20 mm) (PGC Scientifics, Frederick, MD). Hybridization was conducted at 55°C for 30 minutes in a hybridization chamber (Telechem Inc.) immersed in a water bath. After incubation, the slide was washed with a solution of 2 $\times$ standard saline citrate and 0.1% sodium dodecyl sulfate for 10 minutes. Next, it was washed with 2 $\times$ standard saline citrate solution for 10 minutes. Finally, it was washed with 0.2 $\times$ standard saline citrate solution for 10 minutes. All three washes were done at room temperature. Subsequently, the slide was dried by a centrifugation at 1000 rpm for 5 minutes.

Microarray Scanning and Analyzing

The processed slides were scanned on a GenePix 4000B array scanner, and fluorescent images were analyzed by GenePix Pro 5.0 software (Axon Instruments, Union City, CA). The mean fluorescent signal intensities minus background were calculated and recorded for each spot with signal value that was more than two times the background value. Each detection experiment resulted in an image with two arrays. The average of the four internal control mean spot intensities contained in these arrays was calculated. Each pathogen had six spots total on the two arrays. The average of the pathogen mean spot intensities was divided by the average of the mean intensities of the four internal control spots because the positive control signal was the most consistent measure of the performance of the assay. Using this ratio as the measure of a pathogen signal normalized to the internal control eliminated the variations in signal intensity from assay to assay due to variables other than the quantity of the pathogen. This value, termed the relative pathogen signal (RPS), is expressed by the formula:

$$\text{RPS} = \frac{\text{Average of pathogen mean spot intensities}}{\text{Average of internal control mean spot intensities}}$$

Based on evaluation of known positives and known negatives described below, if the RPS was greater than 0.3 and all of the pathogen spots had a mean intensity greater than 8% of the average internal control mean intensity, the assay was scored as positive for that pathogen. If the RPS was less than 0.3 or any one spot was less than 8%, the assay was scored as negative. The internal control spots were used as valid run criteria, which meant that an assay was considered invalid and repeated unless the mean spot intensity of all four control spots was greater than two times the background.

*Testing Unknown *B. tularensis*-Infected Mouse Blood Samples*

The performance of the multiplex assay was tested on the blood obtained from mice infected with *B. anthracis* (Sterne). The mice were inoculated intraperitoneally with a target dose of 2 to 2.5 $\times 10^7$ spores of bacteria essentially as described previously.¹² Blood samples were col-

lected in the presence of anti-coagulant from these infected mice at different time points after challenge. Samples from noninoculated mice were included in the analysis as negative controls. Each mouse blood sample (5 to 10 μ l) was brought up to 200 μ l with human whole blood and then DNA was extracted from the sample with the DNA blood mini kit (Qiagen Inc.). The extracted DNA (5 μ l) was mixed into the first multiplex PCR mixture using the amplifying conditions described above. All subsequent assay procedures were as above.

Results

Optimization of Multiplex PCR for the Microarray Assay with Bioterror Pathogens

As a prototype for development of a multiplex PCR microarray assay to detect bioterror pathogen contamination of blood, we selected three category A pathogens, *B. anthracis*, *F. tularensis*, and *Y. pestis*. To avoid handling select agents, the animal vaccine strain was used as a surrogate for *B. anthracis*, the live vaccine strain was used for *F. tularensis* and *Y. pseudotuberculosis* for *Y. pestis*. Each of these surrogates have DNA sequence identical to the select agent in the area of the target amplicon and have physical cell characteristics that mimic the bioterror pathogen presenting all of the same challenges for sample preparation. The following targets were selected for specific identification of these pathogens: protective antigen gene on pXO1 plasmid for *B. anthracis*, 17-kd lipoprotein TUL4 gene for *F. tularensis* and the 16S rRNA gene for *Y. pestis*. Subsequently, primer pairs were designed to uniquely amplify a segment (the amplicon) of the target gene (Table 1). In addition, an internal control amplicon and appropriate primers from human 18S rRNA genes (Table 1) were selected and added into this PCR system for evaluating the efficiency of both multiplex PCR and microarray hybridization.

To test the ability of the selected primer pairs to detect pathogens, bacteria cells were grown in laboratory culture and spiked into fresh blood in a series of 10-fold dilutions for each pathogen. DNA was extracted from the spiked pathogen blood samples and from a sample of blood alone as a negative control to assess the potential for false-positives due to contamination during PCR amplification or cross-hybridization to the microarray.

The first multiplex PCR contained three pathogen primer pairs, a primer pair for the internal control and DNA extracted from one of the spiked blood samples. Each pathogen could generate a PCR fragment of a unique size in this primary multiplex PCR reaction: 150 bp for *B. anthracis*, 407 bp for *F. tularensis*, 399 bp for *Y. pestis*, and 141 bp for the human rRNA gene. At this stage, the multiplex PCR sensitivity ranged from 500 to 50,000 CFU/ml based on visualizing an ethidium bromide-stained band on an agarose gel. An example, shown in Figure 1A, contains PCR products resulting from multiplex amplification of serial dilutions in blood of *Y. pseudotuberculosis* from 5000 CFU/ml to 5 CFU/ml. The

Figure 1. Agarose gels of multiplex PCR products stained with ethidium bromide. **A:** Gel-analyzing PCR products from primary multiplex reactions containing primer pairs for the human rRNA gene as well as all three pathogen primer pairs for detection of *B. anthracis*, *F. tularensis*, and *Y. pseudotuberculosis* (surrogate for *Y. pestis*). **Lane M** is the 50-bp GeneRuler (Fermentas) with selected band sizes in bp at left. In **lane 1**, the template for the reaction was no DNA as a negative control, the remaining lanes contain products from reactions with template DNA from a blood sample spiked with the following amounts of *Y. pseudotuberculosis* bacteria: **lane 2**, 5 CFU/ml; **lane 3**, 50 CFU/ml; **lane 4**, 500 CFU/ml; **lane 5**, 5000 CFU/ml; **lane 6**, blood alone. The **arrow** indicates the position of the *Y. pseudotuberculosis* amplicon DNA and the **arrowhead** indicates the position of the human rRNA amplicon DNA. **B:** Gel-analyzing PCR products from secondary multiplex reactions each using PCR products from a primary reaction as template, the primer pair for the human rRNA gene and one pathogen primer pair. The lanes are sets of three using the same template in which the first lane has the *B. anthracis* primer pair, the second lane has the *F. tularensis* primer pair, and the third has the *Y. pestis* primer pair. **Lanes 1 to 3**, template is negative control reaction shown in **A**, **lane 1**. **Lanes 4 to 6**, template is primary PCR reaction with blood alone. **Lanes 7 to 9**, template is primary PCR reaction with *Y. pseudotuberculosis*, 5 CFU/ml. **Lanes 10 to 12**, template is primary PCR reaction with *Y. pseudotuberculosis*, 50 CFU/ml. **Lanes 13 to 15**, template is primary PCR reaction with *Y. pseudotuberculosis*, 500 CFU/ml. **Arrows** indicate the position of DNA fragments that correspond to the *Y. pseudotuberculosis* amplicon and the human rRNA amplicon as in **A**.

141-bp human rRNA gene band is visible with all samples, as expected, except lane 1 which is the no template control. The 399-bp band for *Y. pseudotuberculosis* is only visible with the 5000 CFU/ml sample (lane 5). The ~50-bp band in all lanes may be due to some primer dimer formation and the faint 500-bp band was a non-specific product of some of the eight primers in this reaction, which do not amplify in the nested reaction described below. Attempts were made to detect the PCR products from the lower dilutions by fluorescent labeling and hybridization to the microarray, but this approach did not reach sufficient sensitivity (data not shown). To improve the sensitivity of the assay, a secondary, nested PCR reaction was added.

Nested primers (Table 1) were designed following the criteria described above to generate a secondary PCR product of smaller size using the primary PCR product as template. The secondary reactions generated new unique fragments: 144 bp for *B. anthracis*, 265 bp for *F. tularensis*, 396 bp for *Y. pestis*, and 136 bp for the human internal control. To overcome the interference that is increased in a nested, multiplex reaction, the secondary reactions were separated into a set of three reactions for each primary reaction. Each tube contained a small amount of the primary PCR products and two primer

Figure 2. Layout of the pathogen detection microarray probes and hybridization results. **A:** Image of one array hybridized with the Cy5-QC oligo. **B:** Image of one of the two replicate arrays in a microarray assay of blood spiked with *B. anthracis* (50 CFU/ml) labeled with Cy3. **C:** *F. tularensis* (50 CFU/ml) labeled with Cy5. **D:** *Y. pseudotuberculosis* (50 CFU/ml) labeled with Cy5.

pairs, one of the three pathogen primer pairs and the internal control pair. The secondary PCR achieved a sensitivity of 50 CFU/ml for some pathogens by gel electrophoresis. Figure 1B shows one example of a gel containing the PCR products from sets of three nested PCR reactions using PCR products from reactions shown in Figure 1A as templates. Although detection of *Y. pseudotuberculosis* at 50 CFU/ml was possible in this example, identifying the PCR products from all pathogens consistently on agarose gels was not practical due to low yield at times and size similarity of many of the fragments, such as the 144-bp *B. anthracis* band and the 136-bp internal control band. Therefore PCR products were identified by hybridization to a microarray. To achieve multiplex detection using a microarray, fluorescent labeled, single-strand copies of the PCR products were made in an indirect PET reaction and subsequent chemical coupling reaction attaching either a Cy5 or Cy3 fluorophore to the amino groups incorporated into the DNA.

Identification of PCR Products by Microarray Hybridization

The labeled PET products were mixed in a hybridization solution and applied to two replicate arrays containing oligonucleotide probes for the three bioterror pathogens, a negative control pathogen and two replicate copies of the probe for the internal control (Table

3 and Figure 2). After hybridization, an image of the washed slide was captured with a laser scanner. The fluorescent images revealed a hybridization pattern that correctly identified the spiked pathogen and showed strong signals on the two human rRNA gene internal control spots (Figure 2; B to D). To establish rigorous criteria for distinguishing a positive microarray result from a negative result, any spot on the microarray with signal above background was quantitated and a mean pixel intensity was calculated for each spot. The RPS was calculated as described in the Materials and Methods section.

To evaluate the analytical sensitivity and specificity of the multiplex PCR microarray assay for bioterror pathogens, cultured pathogens were spiked into normal donor whole blood as described above in 10-fold dilution series from 5000 to 5 CFU/ml for *B. anthracis* animal vaccine strain, *F. tularensis* live vaccine strain and *Y. pseudotuberculosis*. Each dilution series was prepared three times from independent cultures to test the consistency of the assay. Primary and secondary PCR reactions, labeling, hybridization, and image analysis were performed for each pathogen at each dilution as well as unspiked blood in three separate experiments on 3 separate days to show reproducibility. From the results of known positives and known negatives, a cutoff value of RPS = 0.3 allowed separation of almost all positives from negatives. However, some of the probes printed on the array were particularly cross-reactive and in a few cases resulted in an RPS > 0.3 for a pathogen not present in the sample. An example can be seen in Figure 2B in which a visible signal appears on spot E1, the first probe for *F. tularensis* in the assay of a *B. anthracis*-spiked sample. Therefore, a second criterion for positive detection was applied that required all three probes for a pathogen to have a spot mean intensity greater than 8% of the average mean intensity of the internal control spots. When both criteria were met, all known positives could be discriminated from all known negatives (Table 4). All three pathogens were consistently detectable in this assay at a concentration of 50 CFU/ml. At 5 CFU/ml, the assay could detect bacteria only intermittently (Table 4). Applying the dual criteria for detection resulted in no false-positives. The specificity of the assay was also challenged with common bacteria that could be present in samples submitted for testing. DNA extracted from *Bacillus cereus* (1.9 μ g or 3×10^8 genome equivalents) was spiked into the standard amount of human blood DNA and assayed as above resulting in strong signals for the internal control, but no positive RPS for any pathogen (data not shown). Simi-

Table 4. Results of Microarray Detection of Pathogens Spiked into Whole Blood

Number of CFU/ml	<i>B. anthracis</i> experiment number				<i>F. tularensis</i> experiment number				<i>Y. pseudotuberculosis</i> experiment number			
	1	2	3	P/T	1	2	3	P/T	1	2	3	P/T
500	+	+	+	3/3	+	+	+	3/3	+	+	+	3/3
50	+	+	+	3/3	+	+	+	3/3	+	+	+	3/3
5	—	—	+	1/3	+	—	—	1/3	+	—	+	2/3
Blood Alone	—	—	—	0/3	—	—	—	0/3	—	—	—	0/3

+, positive; —, negative. P/T, number of positive/total experiments.

Table 5. Microarray Detection of *B. anthracis* in Blood from Infected Mice

Time after inoculation (hours)	Number of mice		Total
	Positive	Negative	
0	0	6	6
6	0	6	6
12	0	6	6
24	0	4	4
36	2	1	3
48	1	1	2

larly, DNA extracted from *Escherichia coli* did not result in a positive signal for any of the bioterror pathogens (data not shown).

Screening of Blood Samples from Mice Infected with *B. anthracis*

To evaluate the performance of the multiplex PCR microarray assay, we obtained blood samples from mice in a *B. anthracis* (Sterne) challenge study. Five groups of six mice were infected intraperitoneally with *B. anthracis* spores and blood samples were collected in anti-coagulant at various times after infection. In the groups sampled 24 hours or later, some mice died before blood could be collected, this accounts for the smaller sample sizes in those groups. Blood samples also were collected from six mice before infection. A total of 27 samples (5 to 10 μ l) were coded for blind testing and brought up to a total volume of 200 μ l each with normal human blood. DNA extraction, multiplex PCR, and hybridization were performed as above. Mixing the mouse blood sample with human blood allowed the human rRNA gene internal control to indicate successful amplification and hybridization. No sample was declared negative unless adequate signal appeared on the internal control spots. The RPS for pathogens detected was calculated as above and each mouse sample scored as positive or negative. No pathogens other than *B. anthracis* scored positive on the microarray with these samples. After breaking the blinding code, the assay results were compared with the time after infection (Table 5). Time 0 represented blood samples collected before injection (negative controls) and were all negative for *B. anthracis*. In three of the five mouse blood samples taken 36 hours after infection or later, *B. anthracis* organisms were detected, although all of the mice that were challenged eventually developed symptoms of anthrax and died from infection.

Discussion

The current study serves as a proof of concept demonstrating the potential to use a microarray to screen for three category A potential bioterror agents, *B. anthracis*, *F. tularensis*, and *Y. pestis*, simultaneously from a blood sample. We demonstrated that the detection assay was sensitive, specific, and reproducible with whole pathogen-spiked blood. Existing, nucleic acid-based methods

have achieved rapid and sensitive detection of both blood-borne¹³ and bioterror^{4,14,15} pathogens. Assays also have been developed that use nested multiplex PCR reactions to increase the information gained,^{16,17} but the vast potential for multiplicity of the microarray-based detection assay in blood will mean that a minimal amount of sample can be screened for a large number of pathogens and even the specific pathogen genotype at the same time.

The development of this assay design involved optimization at many steps. Primer design relied heavily on computer-based methods, but ultimately trial and error still played a role, because some primer sequences failed to perform well and were replaced with others that were superior. Choice of primer sequence also involved optimizing amplicon length, a property that must balance efficiency of amplification (shorter length) with specificity of detection (sufficient length to accommodate multiple probes of up to 45 nucleotides). Reaction optimization involved choosing annealing temperatures, Mg⁺⁺, and primer concentrations that were a compromise between all eight primers in the multiplex reactions. The addition of a second, nested PCR reaction to improve sensitivity increased the danger of contamination as reported by others.⁵ However, care in handling, separation of PCR setup and PCR product manipulation into different rooms, and monitoring each reaction with a no template control tube eliminated false-positives. Reaction optimization leading to increased sensitivity also occurred in the fluorescent-labeling step. Blending the single-strand primer extension method⁶ with indirect labeling resulted in greater sensitivity. Single-strand primer extension improved the hybridization efficiency over double-stranded thermocycle labeling because the extension products generated were all complementary to the probes on the array thereby avoiding competitive hybridization. Indirect labeling enhanced the detection sensitivity because the small amino-allyl group is more readily incorporated into DNA than a bulky molecule such as the Cy5-dUTP used in direct labeling. Thus, the direct labeling would likely cause early chain termination and incomplete elongation of the labeled single-strand product, with a corresponding loss of pathogen-specific fluorescent signal. The indirect labeling method may also contribute to specificity by reducing the amount of short, premature termination products that would be more likely to cross hybridize. The inclusion of a human internal control target sequence was fundamentally important to the performance of the assay. Previous multiplex pathogen detection PCR assays have demonstrated the importance of an internal control^{18,19} to verify extraction of DNA from the tissue and performance of all of the components in the enzyme reaction. In our assay, the human rRNA gene target verifies the efficiency of the labeling and hybridization as well. Thus, absence of fluorescence signal at the four internal control spots forces a repetition of the assay and avoids false-negatives. Additionally, the internal control is a reference to normalize the quantitation of the fluorescent signal from the pathogen probes. This normalization provides the basis to set criteria for positive detection in the assay. A crucial step in optimizing the use of the internal control in

the multiplex PCR was adjusting the primer concentration. The samples of whole blood have a constant and abundant quantity of human DNA, whereas the pathogen DNA becomes vanishingly rare at the limit of detection. Excess amounts of the human rRNA gene PCR product inhibited the amplification of the rare pathogen DNA. Therefore lower concentrations of the internal control primers permitted greater sensitivity of pathogen detection.

The multiplex PCR microarray assay was able to detect *B. anthracis*, *F. tularensis*, and *Y. pseudotuberculosis* with a sensitivity of 50 CFU/ml of whole blood. This sensitivity was reproducible in replicate spiked samples. This limit of detection is only slightly higher than reports of single target PCR for select agents²⁰ and is comparable to recently published multiplex assays.¹⁵ The limit of detection compares well to real-time multiplex PCR when samples are prepared from pathogen-spiked blood.^{2,4} Our assay was highly specific because no false-positive detection occurred in 45 assays. At pathogen concentrations of 500 CFU/ml or more, this assay is a simple, qualitative assay. The microarray images appear as in Figure 2, showing signal at the detected pathogen spots, the internal control spots, and no signal at all of the other spots. However, as the target DNA becomes rare at lower pathogen concentrations, false priming events are more likely to give rise to unwanted PCR products that can cross hybridize with probes on the array. This assay achieved a sensitivity of 50 CFU/ml while retaining high specificity by quantitating the scanned image. The software provided with all models of microarray scanners is equipped with tools that allow calculation of the mean signal intensity at each spot. Once this value is collected, simple manipulation on a calculator, or in any spreadsheet program for higher throughput, allows calculation of the RPS as described above. The high specificity of this assay is also due to the multiplicity of probes that are exposed to hybridization simultaneously. There are three unique probes printed on the array for each of the pathogen targets. Amplification of a target present in a sample results in hybridization to all three probes, while spurious amplification products do not hybridize evenly to all three probes preventing false-positive detection.

The multiplex PCR microarray assay was demonstrated capable of detecting a bioterror pathogen from peripheral blood of an infected organism. The majority of mouse blood samples collected 36 hours or more after *B. anthracis* infection tested positive. Although all of the mice after time 0 can be presumed infected because they all died of anthrax within 5 days, the level of bacteria in the blood at the time of collection is uncertain. Bacteremia occurs late in *B. anthracis* infection in humans,²¹ and it is not known at what hour after inoculation of mice it will rise more than 50 CFU/ml (the analytical sensitivity of this assay). Some loss of sensitivity may have occurred with the mouse samples due to the very low blood volume. Human samples tested in the analytical study were 200 μ l in volume. The mouse blood samples were 5 to 10 μ l representing at least a 20-fold lower sampling of pathogen DNA. Larger sample volumes or modification of the sample preparation procedure that would result in a

higher concentration of infected sample DNA in the PCR reaction could improve the sensitivity. The results suggested a high preclinical specificity because none of the time 0 samples showed *B. anthracis*-positive and no positive signals were seen for the other pathogens detectable by this microarray.

In conclusion, we have demonstrated a proof of the concept of a DNA microarray for simultaneously screening blood for contamination with three bioterror bacteria. An analytical sensitivity of 50 CFU/ml was demonstrated with no false-positives. Effectiveness in screening infected blood was demonstrated with *B. anthracis*-infected mouse samples. Although considerable work remains in simplifying and consolidating the assay procedures, this work is the necessary first step toward application of the microarray to high-throughput blood screening.

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