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Rapid Polymerase Chain Reaction-based Screening Assay for Bacterial Biothreat Agents

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Abstract

Objectives—To design and evaluate a rapid polymerase chain reaction (PCR)-based assay for detecting Eubacteria and performing early screening for selected Class A biothreat bacterial pathogens.

Methods—The authors designed a two-step PCR-based algorithm consisting of an initial broad-based universal detection step, followed by specific pathogen identification targeted for identification of the Class A bacterial biothreat agents. A region in the bacterial 16S rRNA gene containing a highly variable sequence flanked by clusters of conserved sequences was chosen as the target for the PCR assay design. A previously described highly conserved region located within the 16S rRNA amplicon was selected as the universal probe (UniProbe, Integrated DNA Technology, Coralville, IA). Pathogen-specific TaqMan probes were designed for *Bacillus anthracis, Yersinia pestis,* and *Francisella tularensis.* Performance of the assay was assessed using genomic DNA extracted from the aforementioned biothreat-related organisms (inactivated or surrogate) and other common bacteria.

Results—The UniProbe detected the presence of all tested Eubacteria (31 / 31) with high analytical sensitivity. The biothreat-specific probes accurately identified organisms down to the closely related species and genus level, but were unable to discriminate between very close surrogates, such as *Yersinia philomiragia* and *Bacillus cereus*.

Conclusions—A simple, two-step PCR-based assay proved capable of both universal bacterial detection and identification of select Class A bacterial biothreat and biothreat-related pathogens. Although this assay requires confirmatory testing for definitive species identification, the method has great potential for use in ED-based settings for rapid diagnosis in cases of suspected Category A bacterial biothreat agents.

Keywords

polymerase chain reaction; bioterrorism; infectious disease; molecular diagnostics; bacteria; Bacillus anthracis; Yersinia pestis; Francisella tularensis

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The emergency department (ED) is now recognized by many in the infectious disease and public health community as the frontline for early identification of biothreat and emerging infections. Accordingly, capacity for rapid and accurate diagnosis of infectious disease outbreaks in the ED is critical both for individual patient care and for initiation of timely public health countermeasures.

Unfortunately, rapid recognition of infections caused by new or unexpected pathogens, which frequently present with nonspecific clinical syndromes, is extremely difficult, and reliance on either the astute clinician or the syndromic surveillance methods may prove inadequate. [1] Current traditional hospital diagnostic assays, which are culture-based, have limited to no utility in ED settings in the event of a suspected outbreak due to prolonged wait times required for bacterial growth. Furthermore, although specialized assays are available at centralized public health laboratories, the utility of such tools for ED care is limited due to inherent delays associated with transporting specimens to outside laboratories, as well as the design of the assays themselves, which are pathogen-specific. A diagnostic platform that has the capacity for both rapid broad-based detection of any bacterial agent and specific pathogen identification would thus be highly desirable for the acute care setting.[2–4] Applicability in the ED includes not only early detection of biothreat or emerging pathogens, but also, potential for everyday use in expediting diagnosis of systemic eubacterial infections.[2]

We describe a novel adaptation of a previously described real-time polymerase chain reaction (PCR) assay that could be used in a hospital or ED satellite laboratory to detect a potential biothreat.[5,6] It consists of an initial broad-range detection of Eubacteria using primers and probes (UniProbe, Precision Electrolysis Inc., Barrington, RI) targeting conserved regions within the 16S rRNA. In instances in which the initial eubacterial detection is positive, simultaneous parallel PCR analyses using multiple panels of both common organisms and biothreat-specific probes follow. For those that are positive by UniProbe and that cannot be identified by any of the specific probe panels, sequencing would be required.

As proof of concept, we designed pathogen-specific probes against the select Class A eubacterial biothreat agents based on the variable sequences within our target region. We report the limit of detection (LOD) and analytical specificity of the PCR assay, using both Uni- Probe and pathogen-specific probes.

METHODS

Study Design

This study was a two-step PCR-based algorithm consisting of an initial broad-based universal detection step, followed by specific pathogen identification targeted for Class A bacterial biothreat agents. This research study and protocol was approved by the institutional review boards at Johns Hopkins University, the Mid-Atlantic Center of Excellence for Emerging Diseases at the University of Maryland, and the National Institutes of Health.

Study Protocol

PCR Probe Design—The target site selection within the 16S rRNA gene, which encompasses conserved primers and probe (UniProbe) regions as well as a hypervariable region, was used to design the biothreat-specific probes for this study.[5] Biothreat-specific probes for *Bacillus anthracis* (5′ FAM-CCTCTGACAACCCTAGAGATAGGGCTTCTC-MGB 3′), *Yersinia pestis* (5′ FAMCACAGAATTTGGCAGAGATGCTAAAGTGCC-MGB 3′), and *Francisella tularensis* (5′ FAM-

CGAACTTTCTAGAGATAGATTGGTGCTTCGGAA- MGB 3′) were designed based on 16S rRNA sequence data obtained from GenBank[7] and aligned with sequences from a variety of other bacterial species using the program ClustalW.[8] As part of the design process, our primers and probe sequences were analyzed against all known published genetic sequences in the GeneBank database to determine the degree of similarity using the software program, National Center for Biotechnology Information (NCBI), Basic Local and Alignment Search Tool (BLAST).[9]

Selection of Test Organisms for PCR Assay and DNA Isolation Procedure—As proof of concept, 31 common (30 Eubacteria and one yeast) or biothreat-related or biothreat-surrogate organisms were selected for specificity evaluation and consisted of either American Type Culture Collection strains, clinical isolates, or inactivated biothreat agents (Table 1). *Candida albicans* was included as a negative control. All water used for resuspension and dilution of bacterial colonies and PCR reagents was PCR-grade water (Roche Diagnostics, Indianapolis, IN), treated with DNase (Invitrogen, Carlsbad, CA) at a concentration of 0.1 U/ μL. Genomic DNA from organisms was extracted from a suspension of 10–15 bacterial colonies in 200 μL of water utilizing the Roche MagNA Pure LC robotic instrument (Roche Molecular Diagnostics) using the "DNA I Blood Cells High Performance Serum" protocol. Isolated DNA from each biothreat-related organism was quantified based on optical density at 260 nm, and then equal quantities of DNA for each organism were serially diluted to extinction to determine the LOD.

To determine the LOD of our assay in "mock-up" samples, *Y. pestis*, which was chosen as our model organism, was serially diluted in 500 μL of healthy human whole blood. Fifty microliters from each dilution were subjected to plating on blood agar to determine colony-forming units (CFUs), and the remaining 450 μL underwent DNA isolation for subsequent testing using our PCR assay.

PCR Procedures—All PCR procedures were performed in triplicate, and each reaction was carried out in a final volume of 89 μL, composed of 56 μL of PCR master mix and 33 μL of sample. The PCR master mix consisted of 1 × TaqMan universal PCR mix (Applied Biosystems, Foster City, CA), 1.96 μM each of primers p1033r and p891f, and 0.28 μM TaqMan probe. As a decontamination measure to remove background eubacterial DNA from PCR reagents, the TaqMan universal PCR mix and primers were filtered using Centricon 100,000 nominal molecular weight limit centrifugal filter units (Millipore, Billerica, MA) for 65 minutes at 4,000 rpm. The master mix was supplemented with the additional 0.05 U/ μL polymerase, since ultrafiltration resulted in partial loss of polymerase premixed in the TaqMan universal PCR mix and thereby compromises PCR efficiency (data not shown). Unlike other *Taq* polymerases, which are well known to contain background bacterial DNA, AmpliTaq Gold LD (Applied Biosystems) was used as additional enzyme because it has been shown to contain minimal copies of background bacterial DNA. PCR-grade DNase-treated water was used as the no-template control in place of DNA and run in triplicate in every experiment. The PCR was performed using a Prism 7900 HT sequence detection system (Applied Biosystems) under the following conditions: 50°C for 2 minutes and 90°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Data Analysis

Postamplification analysis was performed using the supplied SDS software (Applied Biosystems). A positive reaction was defined as a reaction having an averaged cycle threshold (Ct) at least 3 standard deviations lower than the mean Ct of the no-template controls.

RESULTS

The UniProbe component of the PCR assay amplified and detected DNA isolated from the common eubacterial organisms tested ($n = 30$), including nine biothreat- related organisms, and not that from DNA isolated from *C. albicans* (negative control; Table 1). The biothreat-specific probes were able to phylogenetically discriminate down to the genus level and closely related species level of target biothreat organisms ($n = 11$), but was unable to discriminate between very closely related (or surrogate) biothreat species.

The LOD is defined as the lowest concentration of target analyte detectable by the assay; the lower the LOD, the higher the analytical sensitivity of the assay. Given the technical constraint in cultivating some of our biothreat organisms that were inactivated, the LOD of our PCR assay was calculated based on serially diluted DNA extracted from selected Class A bacterial biothreat agents. As described under Methods, all PCR procedures were performed in triplicate and the results reported are based on their rounded average value. As shown in Table 2A, the LOD (in genomic equivalents) of our assay was determined to be quite low. For proof of concept, we also attempted to determine the LOD of our target analyte in sample matrix by spiking healthy human blood samples with serially diluted *Y. pestis* as a model organism. The LOD (in CFUs/mL of blood) of our assay using these mock-up samples was also found to be comparably low, in the range of single CFUs/mL (Table 2B). Time to detection based on our assay algorithm was less than 4 hours.

DISCUSSION

While the potential applicability of PCR-based technology for detecting infectious pathogens in clinical settings has been recognized for several decades,[2] application of such techniques in the ED has been slow. The threat of a bioterrorist event, along with recognition of the ED as the likely first site for patient presentation, highlights the need for translating advances in PCR diagnostics to the bedside for use during acute care. PCR assays, in general, offer significant advantages over conventional culture-based methods, including rapidity, accuracy, reproducibility, and reduced biohazard risk associated with culture-based propagation of potentially highly contagious pathogens.[2] Our findings with a multiprobe PCR assay serve as proof of concept that a broad-based PCR-based platform could be used for both biothreat and universal bacterial detection.

Real-time PCR methods have been developed by others for select biothreat pathogens by amplifying specific genetic targets unique to the pathogen of interest.[10,11] However, even in multiplexed testing formats with broadened capacity to screen multiple pathogens simultaneously, these assays are constrained by the fact that they can only detect a suspected pathogen in the sample. [12] We chose a broad-based PCR approach for eubacterial detection targeting evolutionarily conserved sequences within the 16S rRNA gene to serve as a safeguard against emerging or unsuspected bacterial pathogens. In theory, as an essential housekeeping gene with high degree of sequence conservation, the 16S rRNA gene should also be less susceptible to genetic alteration, making it an ideal target for detecting potentially engineered bacterial agents. In addition, our target region was carefully selected to contain a highly variable intervening sequence for phylogenetic discrimination of the detected pathogen. The two-step algorithm is proposed as a cost-saving measure to reduce unnecessary additional PCR testing in UniProbe-negative cases.

The algorithm we designed involves first performing broad-based amplification employing the conserved 16S rRNA primers and UniProbe to “rule-in” or “rule-out” eubacterial infection in an otherwise sterile specimen. In cases where a sample is positive by UniProbe and in which a biothreat event is suspected, multiple simultaneous parallel PCR procedures

using a panel of biothreat- specific probes can then be performed for early pathogen identification. Our preliminary data demonstrate that our probes do not cross-react with any of the common bacterial agents, but do detect biothreat and related eubacteria to the genus level, with low limits of detection, in the range that might be expected in an infected patient.[13]

The clinical applicability of our UniProbe assay algorithm as a “molecular triage tool” in the ED can extend beyond biothreat detection to common pathogens in otherwise sterile body fluids and is the subject of a future report. Prompt recognition and characterization of systemic bacterial infection in acute febrile patients would be equally invaluable in routine clinical care, leading to early directed therapy, reduced unnecessary hospitalizations, and decreased rates of antibiotics overuse and drug resistance.

LIMITATIONS

Although our biothreat-specific probes did cross-react with other surrogate and closely related members of the same genus, this was an expected finding based on the fact that identical sequences exist even within the hypervariable region of the 16S rRNA gene, an inherent limitation of our methodology. Nonetheless, the assay would be invaluable as an initial screening tool for potential biothreat agents. If positive reactions are obtained, these results would alert the laboratory to perform specific confirmatory molecular assays by testing for other genetic loci, when combined with sufficient clinical suspicion. This result would then prompt final specific identification and definitive characterization of the detected pathogen. In UniProbe-positive but biothreat-specific probe-negative cases (and a suspected biothreat or emerging infectious outbreak), sequencing of the amplicons would serve to definitively identify another etiologic agent. Of course, these results would complement culture-based methods from the clinical microbiology laboratory.

Ideally, multiplex detection of all classes of biothreat agents in a single PCR procedure would be more costeffective than performing individual PCR procedures for several agents, but may lower the sensitivity of each probe. Additionally, current technical constraints of the assay limit the ability to detect more than four fluorescent labels at once, making multiple parallel PCR probe panels a rational alternative. Alternative methods of product detection, including use of biothreat-specific molecular beacons for direct enzyme-independent amplicon hybridization and melting curve analysis of the amplicons, are under active investigation and may represent future time- and cost-saving improvements. Finally, it is important to note that evaluation of our assay in various mocked-up sample matrices (e.g., whole blood, cerebral spinal fluid) using both biothreat and nonbiothreat common agents is a necessary next step in assay development. This will be followed by testing real clinical samples from patients with suspected sepsis as well as animals infected with biothreat agents.

CONCLUSIONS

Development of timely, cost-effective, and accurate diagnostic assays is of fundamental importance for timely identification of a biothreat agent or emerging pathogen. Availability of such technologies could be critical in preventing a disease outbreak from becoming a public health crisis. The proof-of-concept data presented here shows that a simple PCR-based assay algorithm has the potential to provide rapid, sensitive universal eubacterial detection, as well as early identification capacity for biothreat and closely related pathogens. Although our assay requires final confirmatory tests for definitive confirmation of a biothreat agent, it should prove useful as an initial screen in the ED in the setting of a suspected biothreat event; further development of this and similar rapid diagnostic assays

will significantly enhance the ability of ED clinicians to diagnose and respond to biothreats and emerging outbreaks. Further evaluation of our assay in patient samples may demonstrate utility for routine infectious disease diagnostic testing as well.

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Table 1

Analytical Specificity of the UniProbe and Biothreat-specific Probes

		Biothreat-specific Probes		
Clinically Relevant Organisms	**UniProbe**	***B. anthracis***	***Y. pestis***	***F. tularensis***
Serratia marcescens (ATCC 8101)	+	−	−	−
Pseudomonas aeruginosa (ATCC 10145)	+	−	−	−
Staphylococcus epidermidis (ATCC 12228)	+	−	−	−
Staphylococcus aureus (ATCC 25923)	+	−	−	−
Corynebacterium sp.*	+	−	−	−
Enterococcus fecalis (ATCC 29212)	+	−	−	−
Streptococcus viridans (ATCC 10556)	+	−	−	−
C. albicans (ATCC 90029)	−	−	−	−
*Proteus mirabilis**	+	−	−	−
Enterobacter sp.*	+	−	−	−
Acinetobacter sp. (ATCC 5459)	+	−	−	−
*Streptococcus pyogenes**	+	−	−	−
Micrococcus sp. (ATCC 14396)	+	−	−	−
Neisseria meningitidis (ATCC 6250)	+	−	−	−
Listeria monocytogenes (ATCC 7648)	+	−	−	−
Legionella pneumoniae (ATCC 33495)	+	−	−	−
Haemophilus influenzae (ATCC 49247)	+	−	−	−
Streptococcus agalactiae (ATCC 13813)	+	−	−	−
Escherichia coli (ATCC 25927)	+	−	−	−
Streptococcus pneumoniae (ATCC 49619)	+	−	−	−
*Citrobacter freundii**	+	−	−	−
Salmonella sp. (ATCC 31194)	+	−	−	−
BT-related organisms				
B. anthracis†	+	+	−	−
*Bacillus subtilis**	+	+	−	−
*Bacillus cereus**	+	+	−	−
Y. pestis (P14−)	+	−	+	−
F. tularensis (LVSB)	+	−	−	+
Yersinia pseudotuberculosis (PB1/+)	+	−	+	−
Francisella filomiragia (GAO1–2810)‡	+	−	−	+
Coxiella burnetii§	+	−	−	−
Brucella ovis¶	+	−	−	−

Note: + = indicate detected by probe; − = indicate not detected by probe.

ATCC = American Type Culture Collection (http://www.atcc.org/Home.cfm), Manassas, VA; LVSB = live vaccine strain type; PB1/+ = a wild-type strain; P14− = depigmented and virulence plasmid pCD1-negative.

* Clinical isolate.

[†] Inactivated nonpathogenic strain.[14]

[‡] GAO1-2810 = nonpathogenic strain obtained from Centers for Disease Control and Prevention, Fort Collins, CO, via the Walter Reed Army Medical Hospital, Washington, DC.

[§] *Coxiella burnetii* DNA from Steve Dumler, MD, Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD.

[¶] *Brucella ovis* DNA obtained from Joany Jackman, PhD, Applied Physical Laboratory, Johns Hopkins University, Baltimore, MD.

Table 2A

Averaged Limits of Detection Based on Serially Diluted DNA Extracted from Category A Bacterial Biothreat Agents Using Either UniProbe or Biothreat-specific Probes

	Genomic Equivalents	
Organism	**UniProbe**	**Biothreat-specific**
B. anthracis[*]	3	1
Y. pestis (P14)	3	1
F. tularensis (LVSB)	8	6

Genomic equivalents = (weight sample molecular weight of genome) × Avogadro's number.

LVSB = live vaccine strain type B; P14 = depigmented and virulence plasmid pCD1-negative.

[*] Inactivated nonpathogenic strain.

Table 2B

Averaged Limits of Detection Based on Serially Diluted *Y. pestis* Spiked in Healthy Human Whole Blood

	Colony-forming Units/mL of blood	
Organism	**UniProbe**	**Biothreat-specific**
Y. pestis (P14)	1	1

P14 = depigmented and virulence plasmid pCD1-negative.