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Molecular Beacons for Multiplex Detection of Four Bacterial Bioterrorism Agents, *Mandira Varma-Basil,[1†] Hiyam El-Hajj,[2] Salvatore A.E. Marras,[2] Manzour Hernando Hazbón,[1] Jessica M. Mann,[1] Nancy D. Connell,[1] Fred Russell Kramer,[2] and David Alland[1*]* ([1] Department of Medicine, Division of Infectious Disease, New Jersey Medical School, The University of Medicine and Dentistry of New Jersey, Newark, NJ; [2] Department of Molecular Genetics, The Public Health Research Institute, Newark, NJ; † current affiliation: Department of Microbiology, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India; * address correspondence to this author at: Division of Infectious Disease, New Jersey Medical School, 185 South Orange Ave., MSB A920C, Newark, NJ 07103; fax 973-972-0713, e-mail allandda@umdnj.edu)

The advent of bioterrorism has highlighted the need for rapid, simple, and robust diagnostic assays to detect select agents. Mortality from select agents may be greatly reduced by prompt treatment *(1)*; however, treatment may be delayed if diagnostic assays are outsourced to reference laboratories. Most bacterial species that would likely be used as bioterrorism agents infect the blood stream during the course of life-threatening disease. Furthermore, even "nonseptic" syndromes may produce hematogenous bacterial DNA that could be detected by a sensitive assay *(2)*. This means that a rapid "molecular" version of a blood culture would fulfill many of the rapid diagnostic needs for biodefense.

Bacteria can be detected in blood and other sterile body sites by the identification of species-specific DNA sequences in their 16S rRNA genes. These species-specific sequences are flanked by conserved sequences, permitting most rRNA targets to be amplified by PCR using a limited set of "universal" primers *(3)*. Real-time PCR is well suited for sensitive and specific pathogen detection because it is performed in hermetically sealed wells, which greatly reduces the risk of cross-contamination, and it does not require post-PCR analysis *(4)*. Real-time PCR assays have been developed for some select agents, most of which use fluorogenic 5′-nuclease (TaqMan) probes *(5–7)*. However, TaqMan probes are difficult to use in multiplex PCR assays *(8, 9)*. In contrast, molecular beacons are real-time PCR probes that are particularly amenable to multiplexing *(10)*. They can be labeled with differently colored fluorophores *(11)*, use a common nonfluorescent quenching moiety *(9)*, and have thermodynamic properties that favor highly specific detection of nucleic acid sequences *(12)*.

Here we describe a real-time PCR assay that simultaneously detects four bacterial agents that could be used in bioterrorism. This assay is specifically designed to test sterile body fluids, where a rapid and simple assay would be beneficial. We developed a flexible assay format that can easily be adapted to the wide range of spectrofluorometric thermal cyclers that are in common use, including thermal cyclers that have only one- or two-color capabilities, and others that can detect four or more colors simultaneously.

Phenol–chloroform extraction of DNA from standard strains of *Bacillus anthracis* Vollum and Sterne, *Yersinia pestis* CO92, and *Burkholderia mallei* (ATCC 23344) was performed as described previously *(13)* in a biosafety level III laboratory certified to work with select agents (registration number 20011016-798; entity number C20031123-0125). A 180-bp amplicon for use as template in *Francisella tularensis* assays was also constructed in vitro from two overlapping oligonucleotides (Invitrogen). DNA was also extracted from clinical isolates of *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, and *Serratia marcescens* to serve as controls.

The conservation of 16S rRNA gene sequences among bacteria enabled us to design primers FUHP (5′-GTGGACTTAGATACCCTGGTAGTCCAC-3′; underlined sequence indicates additional nucleotides added to create a hairpin structure) and RUP (5′-GCGTTGCATCGAATTAA-3′) to amplify short segments of the 16S rRNA genes of *Y. pestis*, *F. tularensis*, and *B. mallei* by PCR. The benefits of hairpin-shaped primers have been noted previously *(14–16)*. A second primer pair, FBa (5′-TGACGACAACCATGCACC-3′) and RBa (5′-ATGTGGTTTAATTCGAAGCAA-3′), was designed to amplify a segment of the 16S rRNA gene of *B. anthracis.*

We designed molecular beacons (Table 1) that bound to amplicons generated from *F. tularensis*, *B. mallei*, and *B. anthracis* and that could not bind to amplicons generated by other significant human pathogens. However, the *Y. pestis* molecular beacon also bound to amplicons gener-

Table 1. Molecular beacons used in the study.

Probe	Target	Molecular beacon sequence[a]
1	*Y. pestis*	FAM[b]-5′-CGCTGCCCCTTGAGGCGTGGCTGCAGCG-3′-D
2	*F. tularensis*	FAM/TET-5′-CGCTCGTGGAGTCGGTGTAAAGGCTCCGAGCG-3′-D
3	*B. mallei*	FAM/Texas red-5′-CGCTGCGTTGGGGATTCATTTCCTTAGTAAGCAGCG-3′-D
4	*B. anthracis*	FAM/Cy5-5′-CCGACGAGGGTTGTCAGAGGATGCGTCGG-3′-D/BHQ2

[a] Underlined sequences form the stem of each molecular beacon. If more than one fluorophore or quencher moiety is indicated, the second is used in multiplexed assays.

[b] FAM, 6-carboxyfluorescein; D, DABCYL; TET, tetrachlorofluorescein; BHQ2, Back Hole Quencher 2.

ated from *K. pneumoniae*, *E. coli*, *E. cloacae*, and *S. marcescens*, which share an identical 16S rRNA gene target sequence with *Y. pestis*. To standardize assay operating conditions, we also adjusted the probe and arm sequences of each molecular beacon so that all had similar melting temperatures in the presence of perfectly complementary targets.

We tested the ability of each molecular beacon to specifically identify its target in a single-color assay using multiple wells of a 384-well assay plate. The molecular beacons designed to detect *Y. pestis* (Table 1, probe 1), *F. tularensis* (probe 2), *B. mallei* (probe 3), and *B. anthracis* (probe 4) were labeled with the fluorophore fluorescein for these experiments. A 384-well assay plate was prepared containing a series of identical four-well assays with four different probes (200 nM) in each of the four wells. In addition, the wells contained 1× PCR buffer (Applied Biosystems); 4 mM $MgCl_2$; 250 μM each of dATP, dCTP, dGTP, and dTTP; 0.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems); 2.5 pmol each of both sets of primers; and 1 μL of template DNA in a final volume of 5 μL. Real-time PCR was performed with a 7900HT Prism spectrofluorometric thermal cycler (Applied Biosystems). The reaction mixtures were incubated

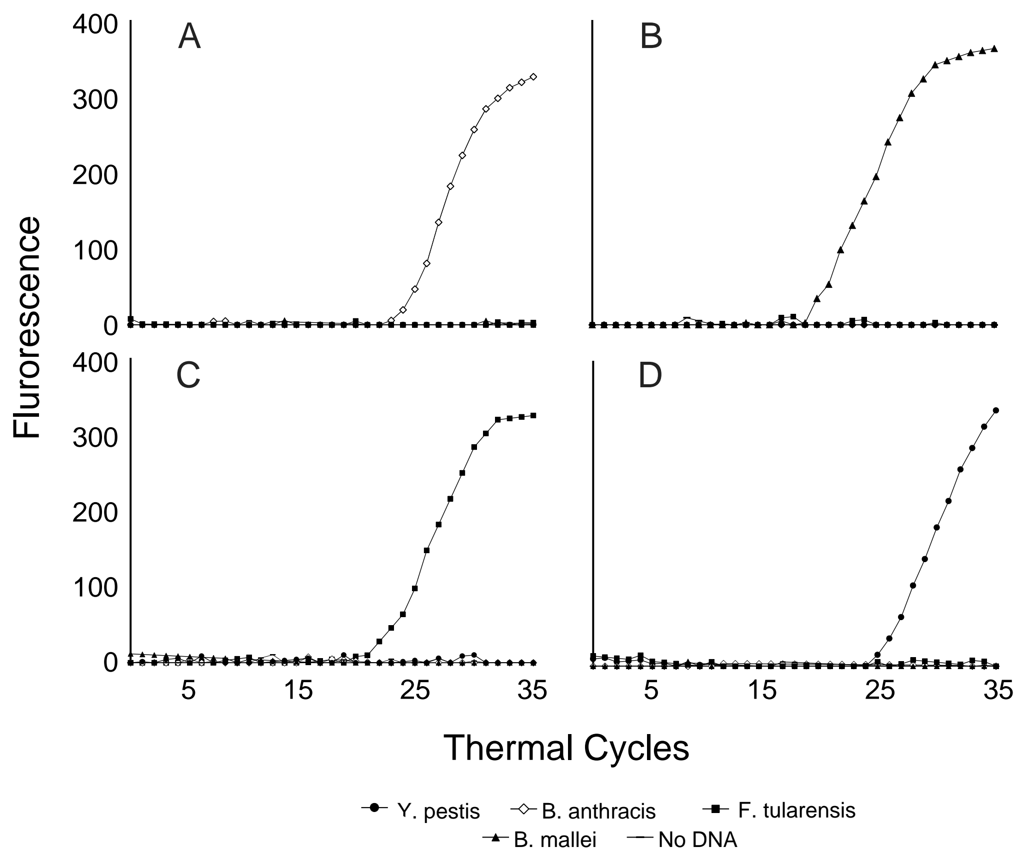

Fig. 1. Four-color multiplex assay.

Shown are the results of real-time PCRs containing DNA from different bacterial species, both primer pairs, and a mixture of four differently colored molecular beacons designed to detect all four select agents. Fluorescence signals are shown separately for the Cy5-labeled molecular beacon designed to detect *B. anthracis* (*A*), the Texas red-labeled molecular beacon designed to detect *B. mallei* (*B*), the tetrachlorofluorescein-labeled molecular beacon designed to detect *F. tularensis* (*C*), and the fluorescein-labeled molecular beacon designed to detect *Y. pestis* (*D*). These results show that in a mixture of the four molecular beacons, only the appropriate molecular beacon generates a fluorescence signal in the presence of its complementary DNA. Wells containing DNA from control organisms did not develop measurable fluorescence in any color.

for 10 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 55 °C for 60 s, and 72 °C for 30 s. Fluorescence was measured in every well or tube during each annealing step throughout the course of each reaction. The "threshold cycle" was automatically determined by the computer program controlling the spectrofluorometric thermal cycler. Significant fluorescence appeared only in the wells containing a molecular beacon complementary to the target sequence of the bacterial DNA added to that well.

We also studied whether the assay could be multiplexed into a single-color, single-well screening assay to identify the presence of a select agent in a simplified format but not to distinguish among them. All four fluorescein-labeled molecular beacons and both primer pairs were combined in a single reagent mixture. DNA from one of the select agents or control organisms was added to each well, and real-time PCR was performed. As expected, a detectable fluorescence signal developed in every well that contained DNA complementary to one of the four molecular beacons present in the well (data not shown). In both these assay formats, no fluorescence signals appeared in any well when DNA from *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *B. cereus*, or a no-DNA control was added. Wells containing probe 1 fluoresced in the presence of *Y. pestis*, *K. pneumoniae*, *E. coli*, *E. cloacae*, and *S. marcescens* (all of which share the same molecular beacon target sequence).

We also estimated the lower limit of detection of the single-color multiplex assay. Triplicate DNA samples from each select agent were serially diluted in water and tested using the above protocol. We found positive PCR signals in all three replicates containing DNA extracted from the equivalent of ≥50 bacilli for *B. mallei* and ≥20 bacilli for the other bacteria.

The optimum biodefense assay should be able to both detect and distinguish among all select agents in a single assay well or tube. Each molecular beacon in the multiplex assay would have to be individually distinguishable to accomplish this goal. We met these design criteria by labeling each molecular beacon with a different fluorophore and performed the assay in a SmartCycler II (Cepheid) that could independently monitor the fluorescence generated by each fluorophore in an assay tube containing all four fluorophores. We selected each of the four fluorophores so that their emission maxima were well spaced from each other across the visible spectrum. All four molecular beacons and both primer pairs were multiplexed into a single reagent mixture to a final volume of 25 μL. DNA from one of the select agents or control organisms was added to each well, and real-time PCR was performed. A detectable fluorescence signal of the appropriate color developed in every well that contained DNA complementary to one of the four molecular beacons present in the well (Fig. 1). Each experiment was repeated at least three times to test its reproducibility.

The importance of bacterial diagnostics in sterile body fluids is supported by current medical practice in which blood cultures are performed on virtually all ill patients with fevers and a suspected bacterial source *(17)*. The ability of molecular beacons to be labeled with differently colored fluorophores, and the development of instruments that are able to detect them, raises the possibility that highly multiplexed PCR assays can be designed to serve as "molecular blood cultures", replacing current culture-based techniques. The availability of PCR screening assays would greatly advance our ability to rapidly detect a broad range of infections, including those introduced by bioterrorism. Although the *Y. pestis*-specific molecular beacon was also able to hybridize to other *Enterobacteriaceae*, we do not view this as a disadvantage. This feature expands the range of the assay, enabling the detection of other common agents that cause sepsis. Importantly, the assay did not detect pathogens from skin flora that can occasionally contaminate a blood draw (in contrast, the presence of *Enterobacteriaceae* in a sterile body site is almost always indicative of disease). Future assays can be designed to specifically detect *Y. pestis* by including a third primer pair and a molecular beacon that differentiates between *Y. pestis* and other *Enterobacteriaceae*.

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Ischemia-Modified Albumin during Skeletal Muscle Ischemia, *Edgar Zapico-Muñiz,*[1] *Miquel Santaló-Bel,*[2] *Javier Mercé-Muntañola,*[1] *José A. Montiel,*[2] *Antonio Martínez-Rubio,*[3] *and Jordi Ordóñez-Llanos*[1,4*] ([1] Biochemistry, [2] Emergency, and [3] Cardiology Departments, Hospital de la Santa, Creu i Sant Pau, Barcelona, Spain; [4] Biochemistry and Molecular Biology Department, Universitat Autònoma, Barcelona, Spain; * address correspondence to this author at: Servei de Bioquímica, Hospital de la Santa Creu i Sant Pau, Avinguda Sant Antoni Maria Claret 167, 08025 Barcelona, Spain; fax 34-93-2919196, e-mail jordonez@hsp.santpau.es)

Ischemia-modified albumin (IMA) has been proposed as a biological marker of myocardial ischemia *(1, 2)*. Exposure to ischemic myocardium modifies circulating albumin at its NH_2 terminus by different mechanisms, and this modification is the basis of IMA measurement by the albumin cobalt binding (ACB) test *(3)*. The tissue-specific nature of the mechanism by which ischemia modifies albumin remains undetermined. Together with a nondiagnostic electrocardiogram and negative troponin values, IMA concentrations within the reference interval have high negative predictive value of myocardial ischemia in patients with suspected acute coronary syndromes *(1, 2)*. However, IMA cardiospecificity has not been validated and needs an evidence base before routine clinical use. A recent report showed significant IMA increases 24–48 h after a marathon race, with exercise-promoted gastrointestinal and/or delayed skeletal muscle ischemia being evoked as possible causes of such increases *(4)*. However, because IMA has shown rapid kinetics of increase (in minutes) and return to baseline no longer than 12 h after angioplastic procedures *(5)*, long-duration skeletal muscle ischemia (i.e., occurring during marathons) does not appear to be the most appropriate model to investigate the effect of such ischemia on IMA values or the kinetics of IMA occurring during acute coronary syndromes. The aim of this work was to analyze the possible contribution of skeletal muscle ischemia to IMA by investigating its short-term kinetics in an isolated skeletal-muscle ischemia model. Because lactate and ammonia concentrations increase sharply after a forearm ischemia test, their possible influence in the ACB assay was studied.

Ten healthy volunteers (4 men and 6 women) from our laboratory staff (age range, 48–61 years; median, 53 years) with no personal or family history of cardiovascular disease and no known cardiovascular risk factors after a medical examination underwent a forearm ischemia test *(6)*. Briefly, after an overnight fast (10–12 h) and 30 min of previous rest, a preexercise (0 min) blood sample was drawn, and blood systolic pressure was recorded twice within a 5-min interval. Thereafter, forearm ischemia was produced by inflating the blood pressure cuff up to 20–30 mmHg higher than the maximum systolic pressure registered. Under these ischemic conditions, a hand-grip exercise at maximum possible strength was performed for 1 min. Thereafter, the cuff was removed, and serial blood samples were drawn at 1, 3, 5, 10, 15, and 30 min. Serum for IMA, creatine kinase, and potassium; EDTA plasma for ammonia; and fluoride plasma for lactate and glucose were collected at each time point into Vacutainer® Tubes (Becton Dickinson). To establish reference values, IMA was tested in a group of 86 fasting (10–12 h), ambulatory (median age, 57 years; 38 women) sedentary individuals who underwent blood sampling after health examinations or before minor surgical procedures. Individuals with cardiovascular risk factors or past or present signs or symptoms of cardiovascular disease recorded during the medical examination were excluded. Volunteers and reference individuals gave written informed consent. All procedures were in accordance with our Institutional Review Board protocols.

Serum IMA was measured with the ACB test (Ischemia Technologies Inc.) adapted to a Roche Cobas Mira analyzer (ABX Diagnostics) according to the manufacturer's instructions for specimen and reagent handling. The principle of the test has been described previously *(3)*. In individuals undergoing the forearm ischemia test, ammonia, lactate, and glucose (all samples), and creatine kinase and potassium (basal and 5 min postexercise) were measured in a Vitros 250 analyzer (Ortho Diagnostics). Concentrations of L-(+)-lactic acid (lactic acid free acid; 300 g/L solution in water; Sigma, cat. no. L-1875; lot no. 052K1278; M_r 90.08) ranging from 50 to 900 mmol/L and ammonia (from ammonium chloride salt; Merck; cat. no. 1145; lot no. 7448183; M_r 53.49) ranging from 2 to 18 mmol/L were dissolved separately in 20 mmol/L MOPS buffer and added to a serum pool (IMA = 106 kilounits/L) at a constant ratio of 1/100 of the final sample volume. Lactate and ammonia concentrations of the enriched pool were measured by the above-described methods. All enriched samples were measured in quadruplicate. The Wilcoxon paired *t*-test and correlation equations were calculated with GraphPad Prism, Ver. 3.0 (GraphPad Software Inc.).

Between-batch imprecision (CV) of the ACB test was assessed at IMA concentrations of 69 and 114 kilounits/L and was $<3\%$ (n = 8) for both concentrations. The 95th percentile for IMA in the reference population was 101 kilounits/L (nonparametric). Results of the forearm ischemia test are shown in Table 1. Increases in lactate and ammonia were five- and sevenfold over basal values, respectively, during the first 5 min after exercise, demon-