Multiplex Detection of Three Select Agents Directly from Blood by Use of the GeneXpert System

Padmapriya P. Banada,^a Srinidhi Deshpande,^a Sukalyani Banik,^a Darshini Shah,^b Ranie Koshy,^c Bhavana Patel,^b Robert Kwiatkowski,^d David Persing,^d David Alland^a

^aCenter for Emerging Pathogens, Division of Infectious Diseases, New Jersey Medical School, Rutgers Biomedical and Health Sciences University, Newark, New Jersey, USA

^bDepartment of Pathology and Laboratory Medicine, University Hospital, Newark, New Jersey, USA

^cBlood Bank/Transfusion Service, University Hospital, Newark, New Jersey, USA

^dCepheid, Sunnyvale, California, USA

ABSTRACT *Francisella tularensis*, *Bacillus anthracis*, and *Yersinia pestis* are tier 1 select agents with the potential to rapidly cause severe disease. Rapid detection of these bacteria from patient samples at the point of care could contribute to improved clinical outcomes in the event of a bioterrorism attack. A multiplex nested PCR assay for detection of *F. tularensis*, *B. anthracis*, and *Y. pestis* directly from patient blood samples was developed using the GeneXpert system. The multiplex GeneXpert cartridge-based assay includes all necessary sample processing and amplification reagents. Blood samples spiked with different numbers of CFU were used to measure the analytical limit of detection (LOD) and dynamic range. Sensitivity was determined by testing spiked blood samples and negative-control blood in a blind manner. Specificity was determined by testing against nontarget pathogens and blood samples from clinical patients. The assay LOD was 8.5 CFU/ml for *F. tularensis*, 10 CFU/ml for *B. anthracis*, and 4.5 CFU/ml for *Y. pestis*. The sensitivity was 100% at the LOD for all three select agent bacteria in spiked patient blood samples. The assay specificity was 100% when it was tested against both nontarget pathogens and clinical patient blood samples. The total assay time was approximately 100 min. This automated assay, which is suitable for use at the point of care, identifies three select agents directly in blood without the need for enrichment with a high sensitivity within 100 min. This assay may enable rapid detection and treatment of patients infected with the target organisms in the event of a bioterrorism attack.

KEYWORDS anthrax, *Bacillus anthracis*, diagnostic, *Francisella tularensis*, GeneXpert, multiplex PCR, plague, tularemia, whole blood, *Yersinia pestis*

The public health threat posed by the accidental or deliberate release of bacterial select agents remains a serious concern. *Francisella tularensis*, *Bacillus anthracis*, and *Yersinia pestis* are among the top biothreat agents because of their potential to rapidly cause severe disease and death and their potential to be weaponized (1, 2). Ongoing sporadic or epidemic cases of disease caused by each of these agents also continue to occur (3–5). The failure to rapidly recognize infection with these agents can lead to increased mortality and might delay recognition of a bioterrorism attack. Blood culture remains the diagnostic gold standard; however, cultures can take 24 to 48 h to become positive and are not reliable (6–9). Nucleic acid-based amplification (NAA) methods have the potential to achieve a more rapid and sensitive detection of bacterial pathogens (6, 9–11). However, most of the NAAs described to detect these three agents are not sensitive enough to detect sepsis early in the course of disease, when the pathogen count can be as low as 1 to 10 CFU/ml (12–16). Our group previously

Citation Banada PP, Deshpande S, Banik S, Shah D, Koshy R, Patel B, Kwiatkowski R, Persing D, Alland D. 2019. Multiplex detection of three select agents directly from blood by use of the GeneXpert system. *J Clin Microbiol* 57:e00036-19. <https://doi.org/10.1128/JCM.00036-19>.

Editor Karen C. Carroll, Johns Hopkins University School of Medicine

Copyright © 2019 American Society for Microbiology. All Rights Reserved.

Address correspondence to Padmapriya P. Banada, Priya.banada@rutgers.edu, or David Alland, allandda@njms.rutgers.edu.

Received 7 January 2019

Returned for modification 29 January 2019

Accepted 25 February 2019

Accepted manuscript posted online 6 March 2019

Published 26 April 2019

described highly sensitive and rapid assays to detect *Staphylococcus aureus* (17), *F. tularensis* (6), and *B. anthracis* (9) directly from a patient blood sample using the GeneXpert system at cell concentrations at or below 10 CFU/ml. This diagnostic platform uses prefilled assay cartridges that contain all necessary reagents for sample processing, PCR amplification, and target detection. Assays are performed automatically in the GeneXpert instrument in a modular format that can run between 1 and 48 tests simultaneously depending on the instrumentation used. Here, we describe the results of our successful effort to multiplex a tularensis-anthraxis-pestis (TAP) assay for the detection of *F. tularensis*, *B. anthracis*, and *Y. pestis* into a single assay cartridge.

MATERIALS AND METHODS

Ethics statement. The Rutgers-Newark Institutional Review Board (IRB) approved this study under protocol numbers P20150002274 and Pro20170000818.

Bacteria and genomic DNA. The bacterial cultures used in this study are listed in Table 1, along with their respective sources. All of the strains used were biosafety level 2 (BSL-2)-level low-pathogenic surrogate bacteria. The select agent surrogate bacterial cells used in this study were *Francisella tularensis* subsp. *holarctica* live vaccine strain (LVS), which is a Centers for Disease Control and Prevention (CDC) live vaccine strain deposited in the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA) by the CDC reference collection. *Bacillus anthracis* Ames 35 is a strain devoid of the pX02 plasmid, and *Yersinia pestis* Yokohama derivative 11 (Yokohama D11) lacks the pCD1 plasmid, which is essential for virulence, as well as the unstable chromosomal *pgm* locus. All of these strains are listed as appropriate for study at the BSL-2 level by BEI Resources and were approved for handling in BSL-2-level laboratories by the Rutgers Institutional Biosafety Committee (IBC). Additionally, we tested all the *Yersinia pestis* strains and DNA in our collection for the *pgm* locus targeting the *hmsH*, *hmsF*, *hmsR*, and *irp2* genes using the assay published by Gaddy et al. (18). Genomic DNA from the select agent bacteria used in this study was procured from BEI Resources or Batelle (Columbus, OH). This DNA is approved for work in BSL-1 laboratories. The genomic DNA from other bacteria used in this study was obtained either from BEI Resources or from the American Type Culture Collection (ATCC; Manassas, VA) or was extracted at the laboratory from BSL-2-level pathogenic cultures using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA).

For inoculum preparation, *F. tularensis* subsp. *holarctica* LVS was grown at 37°C in Mueller-Hinton II (MH) broth (BD, Sparks, MD) supplemented with 10% dextrose (Sigma, St. Louis, MO), 2.5% ferric pyrophosphate (Sigma), 2% IsoVitalEx (BD), and 2.5% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) for 18 to 24 h. Both *B. anthracis* Ames 35 and *Y. pestis* Yokohama D11 were grown at 37°C in brain heart infusion (BHI) broth (BD, Sparks, MD) for 16 to 18 h. The bacteria were serially diluted and plated in their respective growth media to enumerate the CFU per milliliter, and concurrent dilutions were used in analytical experiments.

Nested PCR and multiplexing in the filter-based GeneXpert cartridge. Molecular beacon-based nested PCR assays were designed for multiplex detection of the three pathogens, minimizing cross-reactivity (17). The details of the primer sequences, amplicon size, and amplification conditions are described in Table 2. All assay mixtures were designed using the PrimerSelect (DNASTar Lasergene, version 8.1.3) and/or Primer3 (19) program. Molecular beacons were designed using the Mfold web server (20). The *F. tularensis* assay targeted the multicopy gene *ISFtu*, and the *pag* gene was targeted in the *B. anthracis* assay as described previously (6, 9). The *Y. pestis* assay targeted the *pla* gene, which encodes a plasminogen activator unique to *Y. pestis* (21). The assay was designed to specifically detect *Y. pestis*, avoiding the conserved regions in *Citrobacter koseri* and *Escherichia coli* (22). All three assays were further optimized for nested PCR to achieve higher sensitivity and multiplexing. An internal sample processing and PCR amplification control (IC) assay that targeted a DNA sequence within *Bacillus globigii* was also included as described previously (9) (Table 2). The PCR mix for the outer and inner PCRs was similar to that described previously (9), with some modifications. In the current study, we added all three sets of target-specific outer and inner primers for multiplexing. The inner PCR assay mixture also included target-specific molecular beacon probes of different colors targeting *ISFtu* (*F. tularensis*-6-carboxyfluorescein [FAM]), *pag* (*B. anthracis*-CF3), and *pla* (*Y. pestis*-CF5), added at concentrations of 497 nM, 125 nM, and 322.5 nM, respectively. The IC molecular beacon with a different color (CF11) was added at a 690 nM concentration. All master mixes were added to defined chambers within a GeneXpert filter-based (FB) cartridge (17), and the assay was performed in a GeneXpert instrument (version 4.7b; Cepheid, Sunnyvale, CA) with a custom program written for this assay, such that the in-cartridge procedures described previously were followed (17).

Whole-blood sample processing. The GeneXpert system offers the advantage of completely hands-free processing of blood samples, as described previously (6, 9, 17, 23). Similar to these earlier studies, we performed analytical experiments of the TAP multiplex assay by spiking the respective bacteria into deidentified human blood (expired blood from the Rutgers University Hospital [UH] Blood Bank, Newark, NJ) and/or blood collected in K₂-EDTA tubes obtained from deidentified samples in the UH Hematology Laboratory.

Blood bank blood was prepared as described previously (9). Briefly, the blood was diluted in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) to ensure hematocrit values of 40% to simulate normal adult blood. Blood was spiked with either *F. tularensis* subsp. *holarctica* (LVS), *B. anthracis* (Sterne), or *Y. pestis* (Yokohama D11) at the desired number of CFU, and 1 ml of this preparation was added to an open

TABLE 1 Microbial cultures used in the study^a

Panel and strain	Source	GeneXpert TAP assay result
Inclusivity panel		
<i>Francisella tularensis</i> subsp. <i>tularensis</i> Schu S4 (DNA)	Carl H. Gelhaus, Batelle, Columbus, OH	Positive
<i>Francisella tularensis</i> subsp. <i>tularensis</i> Schu S4 (DNA)	BEI DD-201 DNA-FRAN016	Positive
<i>Francisella tularensis</i> subsp. <i>holarctica</i> (DNA)	BEI DNA-FRAN024	Positive
<i>Francisella tularensis</i> subsp. <i>novicida</i> GA99-3548 (DNA)	BEI DD-720 DNA-FRAN133	Positive
<i>Francisella tularensis</i> subsp. <i>holarctica</i> LVS (DNA)	BEI DD-445 DNA-FRAN004	Positive
<i>Francisella tularensis</i> subsp. <i>holarctica</i> LVS	BEI NR-646	Positive
<i>Bacillus anthracis</i> Ames 35 DNA	BEI NR10450	Positive
<i>Bacillus anthracis</i> Ames 35	BEI NR10355	Positive
<i>Bacillus anthracis</i> Weybridge	BEI NR10350	Positive
<i>Bacillus anthracis</i> UM23	BEI NR10351	Positive
<i>Bacillus cereus</i> G9241 DNA	BEI NR-10050	Positive
<i>Yersinia pestis</i> Yokohoma11	BEI NR-4693	Positive
<i>Yersinia pestis</i> Kimberly13	BEI NR-4695	Positive
<i>Yersinia pestis</i> Kuma 7 (DNA)	BEI NR-4714	Positive
<i>Yersinia pestis</i> Nepal 516 (DNA)	BEI NR-2720	Positive
<i>Yersinia pestis</i> PEXU2 (DNA)	BEI NR-2716	Positive
Exclusivity panel		
Gram-positive bacteria		
<i>Bacillus cereus</i>	ATCC 13061	Negative
<i>Bacillus cereus</i> DNA	BEI NR2544	Negative
<i>Bacillus cereus</i> DNA	BEI NR-2545	Negative
<i>Bacillus cereus</i> DNA	BEI NR-10353	Negative
<i>Bacillus cereus</i> DNA	BEI NR-12314	Negative
<i>Bacillus cereus</i> FDA5	BEI NR-608	Negative
<i>Bacillus cereus</i> Gibson 971	BEI NR-606	Negative
<i>Bacillus megaterium</i> DNA	D. Alland lab	Negative
<i>Bacillus megaterium</i> DNA	BEI NR2543	Negative
<i>Bacillus licheniformis</i> DNA	D. Alland lab NCIB9375	Negative
<i>Bacillus licheniformis</i> DNA	BEI NR-2546	Negative
<i>Bacillus samarii</i> DNA	BEI NR-12239	Negative
<i>Bacillus sphaericus</i> DNA	BEI NR2548	Negative
<i>Bacillus subtilis</i>	N. Connell lab	Negative
<i>Bacillus circulans</i> strain Ford 26	BEI NR-681	Negative
<i>Bacillus thuringiensis</i>	BEI NR-610	Negative
<i>Bacillus thuringiensis</i>	BEI NR-611	Negative
<i>Clostridium botulinum</i> DNA	D. Alland lab VPI4404	Negative
<i>Staphylococcus aureus</i>	UH clinical isolate	Negative
<i>Staphylococcus epidermidis</i> SK-135 DNA	BEI HM-118D	Negative
<i>Staphylococcus epidermidis</i> DNA	D. Alland lab SK135	Negative
<i>Streptococcus agalactiae</i>	D. Alland lab	Negative
<i>Streptococcus pyogenes</i>	D. Alland lab	Negative
<i>Streptococcus pneumoniae</i> DNA	D. Alland lab TCH8431	Negative
Gram-negative bacteria		
<i>Acinetobacter baumannii</i>	ATCC 19606	Negative
<i>Acinetobacter cloacae</i>	UH clinical isolate	Negative
<i>Campylobacter jejuni</i> DNA	D. Alland lab D3071	Negative
<i>Citrobacter koseri</i> 14804	ATCC 27028	Negative
<i>Citrobacter koseri</i>	UH clinical isolate	Negative
<i>Escherichia coli</i>	UH clinical isolate	Negative
<i>Escherichia coli</i> O157:H7	UH	Negative
<i>Escherichia coli</i>	ATCC 25922	Negative
<i>Escherichia coli</i>	ATCC 35150	Negative
<i>Escherichia coli</i>	ATCC 35218	Negative
<i>Escherichia coli</i> DNA	D. Alland lab 83972	Negative
<i>Kingella kingae</i> DNA	D. Alland lab clinical isolate	Negative
<i>Klebsiella pneumoniae</i> DNA	D. Alland lab clinical isolate	Negative
<i>Klebsiella pneumoniae</i>	ATCC 35657	Negative
<i>Providencia stuartii</i>	UH clinical isolate	Negative
<i>Pseudomonas aeruginosa</i>	UH clinical isolate	Negative
<i>Yersinia pseudotuberculosis</i> DNA	D. Alland lab IP2515	Negative
<i>Yersinia enterocolitica</i> WAC	BEI NR211	Negative
<i>Yersinia enterocolitica</i> NCTC 11175	BEI NR212	Negative
<i>Candida albicans</i> (yeast)	ATCC 470547	Negative

^aATCC, American Type Culture Collection; BEI, Biodefense and Emerging Infections Research Resources Repository; UH, University Hospital, Newark, NJ.

TABLE 2 PCR primers and conditions^a

PCR	Species	Primer	Sequence	Product size (bp)	Mg2+ concn (mM)	Annealing temp (°C)
Outer PCR	<i>F. tularensis</i>	ISFtu_OF	TTAAATCTGGAGAGTACGGAGGTAGA	150	2.5	65
		ISFtu_OR	CACTTATGCCCATCATTAACCT			
	<i>B. anthracis</i>	Pag-OF	GGGAAAGAACTTGGGCTGAA	122		
		Pag-OR	CGAAGTCGTTGGTAACACGTTG			
	<i>Y. pestis</i>	Pla-OF	CGGGGAGGATGCTGTGGAA	122		
		Pla-OR	CGTGGAGAATGTCAAGGCAAAA			
	<i>B. globigii</i>	IC_F	AGCGCTTGC GGCAAACACGGAGAAA	246		
		IC_R	GCGACACCGGCGAATACAGAGATACC			
Inner PCR	<i>F. tularensis</i>	ISFtu_IF	TGGCTATTTCAATCAGCTTAGAGG	80	6	60
		ISFtu_IR	CCCATCATTAACCTAAACTAC			
	<i>B. anthracis</i>	Pag-IF	CCGCTGATACAGCAAGATTAATGC	81		
		Pag-IR	TCGTTGGTAACACGTTGTAGA			
	<i>Y. pestis</i>	Pla-IF	AGGAGGTTGGTGTGGACCA	74		
		Pla-IR	AATGAGCGCCCCGTCATTAT			
	<i>B. globigii</i>	IC_IF	AGCGCTTGC GGCAAACACGGAGAAA	159		
		IC_IR	GCTCCACCGAACAATCCGATCTTACGCCC			
Probes	<i>F. tularensis</i>	ISFtu-bcn	FAM-ccgcgGAAACTAGCATTATCCATTACTATAGTAGcgcgg-CDQ38			
	<i>B. anthracis</i>	Pag-bcn	CF3-ccgcgGATATGTAAATACTGGGACGGCTCCcgcgg-CDQ38			
	<i>Y. pestis</i>	Pla-bcn	CF5-ccgcgACCTTCAGATGTGTGAAAAATCACCTcgcgg-CDQ38			
	<i>B. globigii</i>	IC-bcn	CF11-cggccGACAGACAAGC(T42)CCGTCATTGA(U01)cggcc-DABCYL			

^aPag, *Bacillus anthracis* protective antigen; OF, outer PCR forward primer; OR, outer PCR reverse primer; IF, inner nested PCR forward primer; IR, inner nested PCR reverse primer; bcn, molecular beacon. Pla, plasminogen activator; ISFtu, insertion sequence of *Francisella tularensis*; CF3, CF5, CF11, and FAM, fluorophores; CDQ38 and DABCYL, quenchers; T42 and U01, nucleotide modifications. Lowercase letters in the probe sequences indicate the nontarget sequence used in the molecular probe.

filter-based (FB) GeneXpert cartridge for sample processing as described previously (17). A positive test occurred when the real-time cycles versus fluorescence units for one or more of the bacterium-specific molecular beacons reached a value above 20. A negative test required a positive IC reaction. All negative tests with negative IC reactions were judged to be invalid.

LOD. The analytical limit of detection (LOD) was determined by testing serial dilutions of the respective number of bacterial CFU or genomic DNA. Bacteria were spiked into human whole blood as indicated above. Genomic DNA was tested using a cartridge processing protocol similar to that used with blood, except that Tris (50 mM)-EDTA (0.1 mM)-Tween (0.1%) (TET) buffer, pH 8.4, buffer was used instead of blood and DNA was added directly to the outer PCR mix. For this study, the LOD was defined as the lowest number of spiked DNA genomic equivalents (GE) or CFU at which 100% of the samples tested were positive.

Analytical dynamic range. The dynamic range of the assay was tested analytically for each target by spiking blood samples ($n = 3$) with 1, 10, 10², 10³, and 10⁴ CFU/ml of *B. anthracis* Sterne and *Y. pestis* Yokohama D11. *F. tularensis* LVS was spiked at 4, 40, 400, 4,000, and 4 × 10⁴ CFU/ml. *F. tularensis* could not be prequantitated and frozen due to the fragile nature of the cells. Freshly grown cells were spiked by assuming a certain initial cell number, and the quantitated numbers were available only after the experiments. The cell numbers were estimated by plating as mentioned above.

Inclusivity and exclusivity. Analytical inclusivity testing was carried out at 4 times the LOD for each strain (12 GE/reaction mixture or 34 CFU/ml of *F. tularensis*, 20 GE/reaction mixture or 40 CFU/ml of *B. anthracis*, and 32 GE/reaction mixture or 18 CFU/ml of *Y. pestis*) (Table 1). Exclusivity was determined by spiking 10⁶ CFU/ml of different bacteria or yeast species into blood, as mentioned in Table 1. Where cells were not available, DNA from various bacterial species was spiked at concentrations of ~10⁶ copies into the outer PCR master mix. A total of 44 different bacterial species, including 24 Gram-positive, 19 Gram-negative, and 1 *Candida* species, were tested (Table 1). The test isolates were obtained from different sources, as listed in Table 1. BEI Resources strains were obtained through the Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. All clinical isolates were from deidentified patient collections either from the D. Alland laboratory collection, Rutgers University, or from UH.

Simulated clinical sensitivity and specificity. We created simulated clinical samples by spiking the three test bacteria at three different concentrations (below the LOD, at the LOD, and at 4 times the LOD of the respective bacteria; Table 3) into patient K₂-EDTA blood samples in a blinded study. The patient K₂-EDTA blood samples were collected from UH, as described in previous studies (17). Briefly, K₂-EDTA-anticoagulated blood tubes submitted for a complete blood count (CBC) to the Department of Hematology at UH were saved in a refrigerator for 4 to 6 days after they had been used for testing. In coordination with the UH Clinical Microbiology Laboratory, patients with positive/negative blood cultures were identified along with the date of the blood draw. Tubes for a CBC with blood drawn from these patients from the same day as positive blood cultures were then identified and collected from the UH Hematology Laboratory. Patient identifiers were removed before these tubes were removed from the clinical laboratory and transported to the research laboratory. These tubes had about 1 to 3 ml blood

TABLE 3 Simulated clinical sensitivity and clinical specificity

Target pathogen	Sensitivity ^a			Specificity ^b
	≤0.5× LOD	1× LOD	4× LOD	
<i>F. tularensis</i>	3/7 (42.8)	10/10 (100)	7/7 (100)	0/39 (100)
<i>B. anthracis</i>	2/6 (33.3)	5/5 (100)	8/8 (100)	
<i>Y. pestis</i>	6/7 (85.7)	8/8 (100)	12/12 (100)	

^aThe data represent the number of samples positive/total number of samples tested (percent sensitivity).

^bDetermined with blood from K₂-EDTA tubes from patients concurrently testing blood culture negative or positive for nontarget pathogens. The data represent the number of samples false positive/total number of samples tested (percent specificity).

remaining after their routine testing. To evaluate clinical specificity, 1 ml of blood from these samples was used directly in the assay. To establish clinical sensitivity, 1 ml of the blood sample was spiked with the target pathogens as described above. All samples were coded, and the testing scientist was blind to the identity of the samples. These blood samples were processed using the same procedures described above for banked blood, except that 1 ml of blood was mixed with 200 μ l of proteinase K (PK; 20 mg/ml) in optimized PK buffer (10 mM Tris-HCl, 2 mM EDTA, 10 mM KCl, 1% Sarkosyl) inside the sample chamber of a GeneXpert cartridge and run with the automated GeneXpert protocol.

Statistics. Standard statistical analyses (average, standard deviation, and *t* test) were performed using Microsoft Excel 2000 software for Windows. Linear regression curve fit was performed using SigmaPlot (version 13) software.

RESULTS

Analytical LOD. The limit of detection (LOD) of the TAP assay for each bacterial species was first tested using genomic DNA obtained from BEI Resources, *F. tularensis* subsp. *holarctica* LVS, *B. anthracis* Ames 35, and extracted DNA from *Y. pestis* Yokohama D11 (Table 1). The LOD was 3, 6, and 8 genomic equivalents (GE) per reaction mixture for *F. tularensis*, *B. anthracis*, and *Y. pestis*, respectively (Fig. 1A). We then spiked defined numbers of CFU of each of these species (*F. tularensis* subsp. *holarctica* LVS, *B. anthracis* Sterne, and *Y. pestis* Yokohama D11) into human banked blood and tested them in the same assay. The LOD in this more realistic sample was 8.5 CFU/ml of blood for *F. tularensis* (Fig. 1B), 10 CFU/ml of blood for *B. anthracis* (Fig. 1C), and 4.5 CFU/ml of blood for *Y. pestis* (Fig. 1D).

Dynamic range studies in whole blood. The linear dynamic range (24) of the TAP assay for detecting each bacterial species was tested, to ensure that detection was not adversely affected by a higher target input. Each of the three bacterial strains was tested in blood at 10-fold increasing concentrations from approximately their LOD to 10,000 times their LOD. Assay linearity (*R*²) was 0.9893, 0.9981, and 0.9848 for *F. tularensis* (Fig. 2A), *B. anthracis* (Fig. 2B), and *Y. pestis* (Fig. 2C), respectively. Consistent with the LOD studies, the TAP assay was able to detect each species in 100% of the samples tested at their LOD and above (Fig. 2).

Inclusivity and exclusivity. The inclusivity of the assay was tested on all available DNA and cultures of *F. tularensis*, *B. anthracis*, and *Y. pestis* species, as shown in Table 1. All tests were performed at 4 times the LOD of each species. Five different DNA samples of *F. tularensis*, five different cultures or DNA of *B. anthracis* plus one culture of *Bacillus cereus* G9241 (which carries the PX01 plasmid and which is therefore expected to cause a clinical syndrome similar or identical to that caused by *B. anthracis* infection), and four cultures of *Y. pestis* were tested. All samples tested positive by the TAP assay. Furthermore, each species was specifically identified only by its species-identifying probe. Thus, the overall assay inclusivity was 100% at 4 times the assay LOD. Assay exclusivity was then explored by testing 10⁶ CFU/ml (or 10⁶ GE/reaction mixture) of an extensive collection of Gram-positive and Gram-negative bacteria and a yeast species, including species closely related to the TAP test organisms (Table 1). The assay did not detect any of the 44 nontarget pathogens tested, indicating an analytical exclusivity of 100%.

Clinical sensitivity and specificity. The analytical testing described above was performed in blood bank blood spiked with bacteria. To further evaluate the TAP assay with more realistic clinical samples, we obtained deidentified blood drawn from

FIG 1 Assay limit of detection (LOD). Percent positivity rate for tests of blood samples spiked with the specified genomic DNA of *F. tularensis* Schu4 (FT), *B. anthracis* Ames 35 (BA), and *Y. pestis* (YP) (A) and defined numbers of CFU of *F. tularensis* (B), *B. anthracis* (C), and *Y. pestis* (D). Between 6 and 32 replicates were tested for each concentration. The logistical regression fit, Gompertz fit curve, and 95% confidence band were generated in SigmaPlot (version 13) software.

hospitalized patients into K₂-EDTA-anticoagulated tubes for complete blood count analysis. Either 1 ml of blood left over from these tubes was tested as is for specificity, or 1 ml of the blood was spiked with one of the three test bacterial species. A total of 109 patient K₂-EDTA blood samples confirmed to be blood culture negative for the test species were used in this study. *F. tularensis*, *B. anthracis*, and *Y. pestis* cells were randomly spiked into 70 different blood samples at the LOD, ≤ 0.5 times the LOD, and 4 times the LOD. Thirty-nine additional samples were left uninoculated. All samples were coded without any species-identifying information and then tested by another scientist who was blind to the identity of each sample. This blind testing correctly identified 100% of the samples spiked with the three target species at the LOD and above (Table 3). At levels below the LOD, *Y. pestis* was identified in 85.7% (6/7), *B. anthracis* was identified in 33.3% (2/6), and *F. tularensis* was identified in 42.8% (3/7) of the samples. The assay was negative for all blood culture-negative samples tested, indicating 100% clinical specificity.

Time to result. The TAP assay did not require any sample processing time before blood was added to the cartridge. The on-instrument time to a result averaged 1 h 40 min.

DISCUSSION

The bacterial causes of anthrax, plague, and tularemia pose a substantial bioterrorism risk, and these bacteria have been classified as tier 1 select agents by the Centers for Disease Control and Prevention (CDC). In 2001, the spread of *B. anthracis* spores in unopened mail in the United States killed 5 people and sickened 17 (25, 26). The rapid detection of select agents from patient blood samples can help to identify new sources

FIG 2 Assay dynamic range. Assay results are shown for serial dilutions of *F. tularensis* (A), *B. anthracis* (B), and *Y. pestis* (C) spiked into blood at the indicated numbers of CFU per milliliter. The linearity of the fit curve (R^2) is displayed for each data set.

of infection and more rapidly treat those infected. Rapid detection methods implemented by the World Health Organization (WHO) for plague detection in Madagascar led to a dramatic reduction in the severity and scope of the outbreak (27). These diagnostic activities may be performed more quickly at the point of care.

Here, we demonstrate the first integrated completely hands-free diagnostic multiplex assay for *F. tularensis*, *B. anthracis*, and *Y. pestis*. The assay detected each bacterial species at an LOD of <10 CFU/ml of blood, with a linear dynamic range of up to 10^4 CFU/ml, which is likely above the upper limit for bloodstream infections (28, 29). The multiplexed TAP assay LOD was not compromised by the presence of multiple sets of primers and probes for all three targets, in contrast to our earlier studies on a single target (6, 9). It was not possible to acquire patient samples infected with these pathogens for this study. However, we were able to simulate real-world testing by spiking bacteria in a blind manner into blood samples drawn from hospitalized patients and outpatients for other purposes. The assay sensitivity was 100% for each bacterial species spiked at the LOD. These results suggest that the assay may have the capacity to detect bloodstream infections at the early stages of bacteremia, when the bacterial load is shown to be 1 to 10 CFU/ml (12–16), and often, traditional culturing methods fail to detect these low numbers (6, 11). Indeed, a uniplex version of the *F. tularensis* assay was able to detect this species directly from the blood of infected macaques on the first day of fever in a subset of animals; and overall, the assay was positive for blood samples drawn sooner after infection than those samples that ultimately became positive by standard blood culture or conventional real-time PCR (6). Our assay also detected a *B. cereus* strain (G9241) which is classified as a tier 1 agent (30) and contains the same pX01 plasmid and *pag* gene as *B. anthracis* strains. This was an expected and desired result since

this *B. cereus* strain also causes an anthrax-like illness. In contrast, our assay did not cross-react with other bacillus species that are devoid of the pX01 plasmid.

Our assay took <100 min from the start to the availability of results and was performed directly from patient blood. The assay uses completely hands-free integrated sample processing and detection, requiring only that 1 ml of whole patient blood be added directly to the cartridge, making it ideal for point-of-care use. The United States Food and Drug Administration (FDA)-cleared FilmArray BioSurveillance system has an LOD of 250 to 2,500 CFU/ml for *F. tularensis*, *Y. pestis*, and *B. anthracis* (31, 32), and the FDA-approved Joint Biological Agent Identification and Diagnostic System (JBAIDS), which detects individual select agent pathogens, has a limited LOD, for example, 48 copies for *B. anthracis* per reaction mixture. This limited LOD is the likely reason why JBAIDS assays are not recommended for direct use on whole blood. Rather, both of these assays are recommended for testing only blood culture-positive samples, which may take from 24 h to 3 days and requires additional sample manipulation steps (33). Previous studies have demonstrated the promising biocontainment properties of the GeneXpert system (34). The ability to contain aerosols during sample preparation suggests that this system is suitable for handling unknown samples that may contain select agent bacteria.

In conclusion, the multiplex TAP assay offers a hands-free, integrated automated testing method for blood samples containing three tier 1 select agent pathogens, making it ideal for pathogen detection at the point of care. In the event of a bioterrorism attack, this assay has the potential to be rapidly deployed to thousands of health care facilities in the United States and around the world that already use existing GeneXpert systems for other diagnostic needs. The widespread availability of these systems, plus their easy portability to new locations, should support the needs for a rapid response to biothreat emergencies.

ACKNOWLEDGMENTS

This study was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number R01AI098713.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank Michele Burday and Qing Wang for their permission to store and use the patient blood from the pathology laboratories. We thank Nancy Connell and her lab and Carl H. Gelhaus, Batelle, Columbus, OH, for kindly lending us the strains used in this study.

D.A. is one of a group of investigators who invented the molecular beacon technology and who receive income from licensees, including Cepheid. R.K., and D.P. are employees of Cepheid, which manufactures and sells the GeneXpert system and assays.

REFERENCES

1. Federal Select Agents Program. 2018. Select agents and toxins list. <https://www.selectagents.gov/SelectAgentsandToxinsList.html>.
2. Goel AK. 2015. Anthrax: a disease of biowarfare and public health importance. *World J Clin Cases* 3:20–33. <https://doi.org/10.12998/wjcc.v3.i1.20>.
3. Pedati C, House J, Hancock-Allen J, Colton L, Bryan K, Ortbahn D, Kightlinger L, Kugeler K, Petersen J, Mead P, Safranek T, Buss B. 2015. Notes from the field: increase in human cases of tularemia—Colorado, Nebraska, South Dakota, and Wyoming, January–September 2015. *MMWR Morb Mortal Wkly Rep* 64:1317–1318. <https://doi.org/10.15585/mmwr.mm6447a4>.
4. Stidham RA, Freeman DB, von Tersch RL, Sullivan PJ, Tostenson SD. 2018. Epidemiological review of *Francisella tularensis*: a case study in the complications of dual diagnoses. *PLoS Curr* 10:ecurrents.outbreaks.8eb0b55f377abc2d250314bbb8fc9d6d. <https://doi.org/10.1371/currents.outbreaks.8eb0b55f377abc2d250314bbb8fc9d6d>.
5. WHO. 2017. Madagascar plague: mitigating the risk of regional spread. <https://www.who.int/en/news-room/feature-stories/detail/madagascar-plague-mitigating-the-risk-of-regional-spread>.
6. Banada PP, Deshpande S, Chakravorty S, Russo R, Occi J, Meister G, Jones KJ, Gelhaus CH, Valderas MW, Jones M, Connell N, Alland D. 2017. Sensitive detection of *Francisella tularensis* directly from whole blood using the GeneXpert system. *J Clin Microbiol* 55:291–301. <https://doi.org/10.1128/jcm.01126-16>.
7. Aloni-Grinstein R, Schuster O, Yitzhaki S, Aftalion M, Maoz S, Steinberger-Levy I, Ber R. 2017. Isolation of *Francisella tularensis* and *Yersinia pestis* from blood cultures by plasma purification and immunomagnetic separation accelerates antibiotic susceptibility determination. *Front Microbiol* 8:312. <https://doi.org/10.3389/fmicb.2017.00312>.
8. Dunning J, Morgan D, Walsh A. 2017. Plague: interim guidance for clinical laboratories in England for diagnosing potential plague cases. PHE Publications, London, UK. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/656164/Plague_laboratory_guidance.pdf.
9. Banada PP, Deshpande S, Russo R, Singleton E, Shah D, Patel B, Burday M, Koshy R, Wang Q, Jones M, Gall A, Lokhov S, Kwiatkowski R, Persing D, Connell N, Alland D. 2017. Rapid detection of *Bacillus anthracis* blood stream infections using a novel assay in the GeneXpert system. *J Clin Microbiol* 55:2964–2971. <https://doi.org/10.1128/jcm.00466-17>.
10. Mölsä M, Hemmälä H, Katz A, Niemimäa J, Forbes KM, Huitu O, Stuart P,

- Henttonen H, Nikkari S. 2015. Monitoring bioterror agents (*Francisella tularensis*, *Bacillus anthracis* and *Yersinia pestis*) with a portable real-time PCR instrument. *J Microbiol Methods* 115:89–93. <https://doi.org/10.1016/j.mimet.2015.05.026>.
11. Scerbo MH, Kaplan HB, Dua A, Litwin DB, Ambrose CG, Moore LJ, Murray CC, Wade CE, Holcomb JB. 2016. Beyond blood culture and Gram stain analysis: a review of molecular techniques for the early detection of bacteremia in surgical patients. *Surg Infect (Larchmt)* 17:294–302. <https://doi.org/10.1089/sur.2015.099>.
12. Zasada AA, Formińska K, Zacharczuk K, Jacob D, Grunow R. 2015. Comparison of eleven commercially available rapid tests for detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. *Lett Appl Microbiol* 60:409–413. <https://doi.org/10.1111/lam.12392>.
13. Weller SA, Cox V, Essex-Lopresti A, Hartley MG, Parsons TM, Rachwal PA, Stapleton HL, Lukaszewski RA. 2012. Evaluation of two multiplex real-time PCR screening capabilities for the detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* in blood samples generated from murine infection models. *J Med Microbiol* 61:1546–1555. <https://doi.org/10.1099/jmm.0.049007-0>.
14. Kellogg JA, Ferrentino FL, Goodstein MH, Liss J, Shapiro SL, Bankert DA. 1997. Frequency of low level bacteremia in infants from birth to two months of age. *Pediatr Infect Dis J* 16:381–385. <https://doi.org/10.1097/00006454-199704000-00009>.
15. Schelonka RL, Chai MK, Yoder BA, Hensley D, Brockett RM, Ascher DP. 1996. Volume of blood required to detect common neonatal pathogens. *J Pediatr* 129:275–278. [https://doi.org/10.1016/S0022-3476\(96\)70254-8](https://doi.org/10.1016/S0022-3476(96)70254-8).
16. Hutt JA, Lovchik JA, Dekonenko A, Hahn AC, Wu TH. 2017. The natural history of pneumonic tularemia in female Fischer 344 rats after inhalational exposure to aerosolized *Francisella tularensis* subspecies *tularensis* strain SCHU S4. *Am J Pathol* 187:252–267. <https://doi.org/10.1016/j.ajpath.2016.09.021>.
17. Banada P, Chakravorty S, Shah D, Burday M, Mazzella FM, Alland D. 2012. Highly sensitive detection of *Staphylococcus aureus* directly from patient blood. *PLoS One* 7:e31126. <https://doi.org/10.1371/journal.pone.0031126>.
18. Gaddy CE, Cuevas PF, Hartman LJ, Howe GB, Worsham PL, Minogue TD. 2014. Development of real-time PCR assays for specific detection of *hmsH*, *hmsF*, *hmsR*, and *irp2* located within the 102-kb pgm locus of *Yersinia pestis*. *Mol Cell Probes* 28:288–295. <https://doi.org/10.1016/j.mcp.2014.08.004>.
19. Krawetz S, Misener S (ed). 2000. Primer3 on the WWW for general users and for biologist programmers. Humana Press, Totowa, NJ.
20. Zuker M. 2003. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 31:3406–3415. <https://doi.org/10.1093/nar/gkg595>.
21. Sebbane F, Jarrett CO, Gardner D, Long D, Hinnebusch BJ. 2006. Role of the *Yersinia pestis* plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague. *Proc Natl Acad Sci U S A* 103:5526–5530. <https://doi.org/10.1073/pnas.0509544103>.
22. Hänsch S, Cilli E, Catalano G, Gruppioni G, Bianucci R, Stenseth NC, Bramanti B, Pallen MJ. 2015. The *pla* gene, encoding plasminogen activator, is not specific to *Yersinia pestis*. *BMC Res Notes* 8:535. <https://doi.org/10.1186/s13104-015-1525-x>.
23. Banada PP, Koshy R, Alland D. 2013. Detection of *Mycobacterium tuberculosis* in blood by use of the Xpert MTB/RIF assay. *J Clin Microbiol* 51:2317–2322. <https://doi.org/10.1128/JCM.00332-13>.
24. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 55:611–622. <https://doi.org/10.1373/clinchem.2008.112797>.
25. Kman NE, Nelson RN. 2008. Infectious agents of bioterrorism: a review for emergency physicians. *Emerg Med Clin North Am* 26:517–547, x–xi. <https://doi.org/10.1016/j.emc.2008.01.006>.
26. Inglesby TV, O'Toole T, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Gerberding J, Hauer J, Hughes J, McDade J, Osterholm MT, Parker G, Perl TM, Russell PK, Tonat K. 2002. Anthrax as a biological weapon, 2002: updated recommendations for management. *JAMA* 287:2236–2252. <https://doi.org/10.1001/jama.287.17.2236>.
27. WHO. 2018. Revamp of the plague detection in Madagascar yields quick and sustainable wins. <https://www.who.int/csr/disease/plague/laboratory-detection-madagascar/en/>.
28. Yagupsky P, Nolte FS. 1990. Quantitative aspects of septicemia. *Clin Microbiol Rev* 3:269–279. <https://doi.org/10.1128/CMR.3.3.269>.
29. Puttaswamy S, Lee BD, Sengupta S. 2011. Novel electrical method for early detection of viable bacteria in blood cultures. *J Clin Microbiol* 49:2286–2289. <https://doi.org/10.1128/JCM.00369-11>.
30. Price TE. 2017. Possession, use, and transfer of select agents and toxins—addition of *Bacillus cereus* biovar anthracis to the HHS list of select agents and toxins. *Fed Regist* 82:17569–17570. <https://www.govinfo.gov/content/pkg/FR-2017-04-12/pdf/2017-07210.pdf>.
31. Seiner DR, Colburn HA, Baird C, Bartholomew RA, Straub T, Victry K, Hutchison JR, Valentine N, Bruckner-Lea CJ. 2013. Evaluation of the FilmArray system for detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*. *J Appl Microbiol* 114:992–1000. <https://doi.org/10.1111/jam.12107>.
32. Wagar E. 2016. Bioterrorism and the role of the clinical microbiology laboratory. *Clin Microbiol Rev* 29:175–189. <https://doi.org/10.1128/CMR.00033-15>.
33. Emanuel PA, Hankla LW. 2016. Assessment of the capability of the ngds prototype to replace the JBAIDS for environmental sample analysis. Edgewood Chemical Biological Center, U.S. Army Research, Development and Engineering Command, Aberdeen Proving Ground, MD. <https://apps.dtic.mil/dtic/tr/fulltext/u2/1020951.pdf>.
34. Banada PP, Sivasubramani SK, Blakemore R, Boehme C, Perkins MD, Fennelly K, Alland D. 2010. Containment of bioaerosol infection risk by the Xpert MTB/RIF assay and its applicability to point-of-care settings. *J Clin Microbiol* 48:3551–3557. <https://doi.org/10.1128/JCM.01053-10>.