Development of a rapid, high sensitivity, direct from blood assay for identification of biothreat organisms

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Introduction

Historically biological agents have been used to inflict severe economic, agricultural, or health consequences. In 2001, the use of *Bacillus anthracis*, the causative agent of anthrax, in the United States demonstrates the effectiveness of these agents. The release of *B. anthracis* spores through delivery of contaminated mail resulted in the reporting of 22 illnesses, 5 deaths, and widespread psychological and economic effects[1]. The use of biological agents to carry out a bioterrorism attack, such as the 2001 Anthrax attacks, poses a public health emergency requiring rapid identification and clinical diagnosis of patients.

The Center for Disease Control and Prevention (CDC) has classified biological agents identified as biothreat organisms as Category A, B, or C depending on the transmissibility, mortality rates, and potential for public panic and social disruption[2]. Rapid identification of biothreat organisms poses a unique challenge due to the low incidence of infection by biothreat organisms in the United States and non-specific symptoms of the diseases. Studies have demonstrated poor diagnosis and management of care for biothreat organisms[3]. The difficult identification of biothreat organisms was recently evidenced by a CDC bulletin that raised awareness of the identification of four melioidosis cases (caused by *Burkholderia pseudomallei*) in the United States[4] that led to two deaths. Development of a rapid *in vitro* diagnostic assay with high sensitivity and specificity for the identification of biothreat organisms as the causative agent of infection may aid in the diagnosis and implementation of effective therapy for patients.

T2 Biosystems selected six bacterial species identified by the CDC as either Category A or B biothreat organisms capable of producing blood stream infections. The six species selected, *Bacillus anthracis, Francisella tularensis, Yersinia pestis*, *Burkholderia mallei, Burkholderia pseudomallei,* and *Rickettsia prowazekii,* respond to effective antibiotic treatment but require rapid intervention to prevent long-term consequences or death of the patient. Patients with illnesses caused by these organisms present with broad, generic symptoms that can be commonly mistaken for other illnesses in non-endemic areas. During an exposure event, rapid identification of patients exposed to these organisms would be required to understand the scope and magnitude of the event. The development of the T2Biothreat Panel, a multiplex amplification reaction utilizing T2MR detection for identification of these biothreat organisms, will provide a strong diagnostic tool to aid public health and emergency physicians in identifying and treating patients effectively.

Here we discuss the development of a high sensitivity assay for the identification of six biothreat bacterial species direct from a patient blood sample without a positive blood culture to reduce the time to identification of biothreat organisms.

Time to Result with T2MR Detection

In an analysis of over 1500 patient samples during the T2Bacteria Panel clinical trial the mean time to a T2MR result was 3-5 hours, while the mean time to a positive blood culture species identification was 78 hours and negative blood culture was 123 hours. These data demonstrate a substantial reduction in time compared to blood culture for the identification of a blood stream infection and ultra high sensitivity capable with T2MR detection direct from patient sample. T2Biothreat Panel has a similar time to result and will presumably provide a similar reduction in time to identification of the bacterial species identified by the panel compared to positive blood culture and subsequent species identification.

Sample processing on T2Dx

A multiplex T2MR assay was designed to identify target sequences in 6 biothreat bacterial species (*Bacillus anthracis, Burkholderia mallei and Burkholderia pseudomallei, Francisella tularensis, Yersinia pestis, and Rickettisa prowazekii*). Available BSL-2 isolates of the biothreat bacterial species were obtained from BEI Resources and BSL-3 isolates were obtained from clinical collections, BEI Resources, and MRIGlobal strain collection. Samples were prepared with K_2EDTA-treated whole blood from healthy donors at clinically relevant concentrations for testing or genomic DNA as indicated. Assay performance was developed with a manual assay with equipment and materials consistent with the fully automated version of the assay on the T2Dx Instrument.

References

1. S Das and VK Kataria. MJAFI 201;66:255-260
2. The Center for Disease Control and Prevention. https://emergency.cdc.gov/agent/agentlist-category.asp
3. Cosgrove S.E, et al. Arch Intern Med.(2005);165:2002-2006
4. CDCHAN-00448. New Case Identified: Multistate Investigation of Non-travel Associated *Burkholderia pseudomallei* Infections in Four Patients: Georgia, Kansas, Minnesota, and Texas -2021.

Results

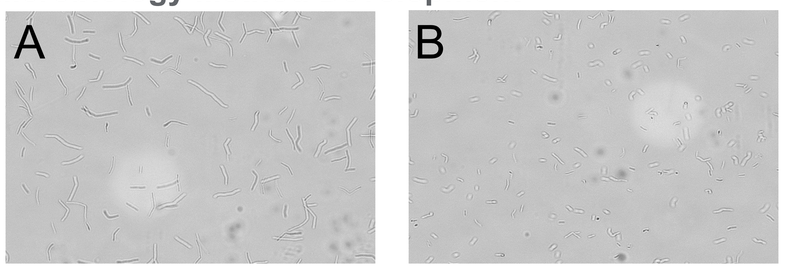
Panel Targets

Species	Disease	Panel Channel
Bacillus anthracis	Anthrax	pXO1[1]
		pXO2[1]
Burkholderia mallei	Glanders	Bu[2]
Burkholderia pseudomallei	Melioidosis	
Francisella tularensis	Tularensis	Ft
Rickettsia prowazekii	Epidemic Typhus	Rp
Yersinia pestis	Plague	Yp

[1] Both pXO1 and pXO2 plasmids need to be detected for identification of *B. anthracis*
[2] The panel cannot distinguish between *B. mallei* and *B. pseudomallei*

Panel Targets - Six bacterial species identified on the CDC Category A and Category B select agents were included in the panel design and genetic targets were selected to allow for sensitive and specific detection with a multiplex amplification reaction. The species represent biothreat agents that are difficult to identify with current diagnostics and that respond to early use of effective therapy. The multiplex reaction was designed to amplify the target sequences direct from a patient blood sample without the requirement of a positive blood culture result.

Microbiology Method Development



Microbiology Development- Growth methods and quantification methods were developed to provide accurate titer determination for sample preparation. Growth medium, culture timing, and plating medium were optimized for each bacterial species to achieve consistent cultures and minimize differences between cell equivalents and colony forming units. Images show the *Bacillus anthracis* strain A0463 grown with **A)** conventional culture methods and **B)** optimized culture methods (shown at 10X magnification). Optimized culture methods produce a higher percentage of single cells and short chains compared to conventional methods that produce long filamentous, multi-nucleated cells. Development of growth methods required to ensure accurate titer for samples and ensure reproducible results in assay performance were done for all species on the T2Biothreat Panel.

Inclusivity

Species	Wet tested	*In silico*
Bacillus anthracis	**A0463**, **A1085**, Sterne (pXO1 only), Weybridge (pXO1 only), Ames (pXO1 only)	2002013094, 14RA5914, 170D930, A0248, A1144, A16, Ames, BA1035, BA10515, BF1, Canadian Bison, CDC684, CZC5, H9401, HYU01, K3, London499, Ohio ACB, PCr, RA3, Shikan-NIID, SK-102, Stendal, SVA11, Tangail-1, Turkey32, Tyrol 4675, Vollum, Vollum 18
Burkholderia mallei	**Human 1964**, **NCTC 12938, NCTC-10260,** NR-23	6, 11, 200031063, 2002721276, 2002734299, 200273406, 23344, ATCC 23344, Bahrain1, BMQ, FMH, FMH 23344, India86-567-2, JHU, NCTC 10229, NCTC 10247, SAVP1, Turkey1, Turkey2, Turkey 3, Turkey4, Turkey5, Turkey6, Turkey7, Turkey8, Turkey9, , Turkey10,
Burkholderia pseudomallei	**1026b**, **K96243**, NR-51280	Pasteur 52237, MSHR668, K96243, PB08298010, PHLS 112, vgh16R, vgh 16W 982, Bp1651, MSHR840, TSV 48, vgh07, 406e, M1, MSHR491, MSHR2543, 7894, 2002721184, MSHR3763, 3000465972, 2002721123, 2002721171, MSHR4083, 2002734728, 2008724734, 2008724758, Burk178-Type2, Burk178-Type2, MS, VB976100, K42, MSHR7929, MSHR6755, MSHR5864, MSHR520, NAU20B-16, NCTC 13178, MSHR511, MSHR146, Bp3921g, 576,1710b, 668, BPC006, BGR, MSHR305, NCTC13179, A79A, BSR, BDP, NAU35A-3, MSHR62, B03, TSV202, MSHR5848, MSHR1655, Mahidol-1106a, MSHR5855, MSHR5858, UKMPMC2000
Francisella tularensis	**Scherm**, **SchuS4**, **MA002987,** NR-14, NR-585	WY96, WY-00W4114, NR-28534, DPG 3A-IS, PA10-7858 NIH B-38, U112, D9876, LVS, FTT_1, F6168, F92, Fx1, OSU18, FSC200, 425, VT68, PHIT-FT0049, NDE061598, FSC147, FTNF002-00, WY96-3418, U112, COLL, KU1, 2015315990, 2015321842, 201317779, 2014313438, 2017314593, 2016320786, 15NIIEG, NR-28537, NVF1, A271_1, S4_249, TI0902, TIGB03, TCH2015, AZ06-7470, AL97-2214, B-8364, B-8365, B-8366, B-8367
Rickettsia prowazekii	**83-2P**, **103-2P**, **ZRS**	Naple-1, Breinl, NMR Madrid E, BuV67-CWPP, Katsinyian, Chernikova, GvF24, GvV257, Rp22,
Yersinia pestis	**KIM**, **Pestoides B**, NR-639	Shasta, A1122, El Dorado, Harbin 35, Nicholisk 41, 91001, PBM19, Angola, Antiqua, Nepal516, CO92, Z176003, D182038, D106004, SCPM-O-B-6291, SCPM-O-B-6530, CPM-O-B-5935, SCPM-O-B-6899, S19960127, 2944, SCPM-O-B-5942, FDAARGOS_603, 195/P, Cadman

Panel Inclusivity. To ensure the breadth of coverage of the species detected by the T2Biothreat Panel an *in silico* analysis of the primers and probes contained as part of the panel was performed. Sequenced and curated strains contained in the NCBI Assembly database that had a 100% primer and probe identify were considered to be inclusive of the panel and have a high probability of detection. To confirm the ability of the panel to identify each species a minimum of two strains of each species were wet tested. Samples were prepared using BSL-3 (**bold**) or BSL-2 strains grown with the optimized growth conditions, quantified, and spiked into K_2EDTA treated whole blood at clinically relevant concentrations. Samples were processed and detected on the T2Dx instrument to ensure performance of the panel..

Panel Exclusivity

Species	Titer	Hit Rate
Bacillus cereus	1500 CFU/mL	No species detected
Bacillus circulans	1500 CFU/mL	No species detected
Bacillus thuringiensis	1500 CFU/mL	No species detected
Burkholderia cepacia	1500 CFU/mL	No species detected
Burholderia thailandensis	1500 CFU/mL	No species detected
Francisella philomargia	1500 CFU/mL	No species detected
Rickettsia parkeri	1000 cp/rxn	No species detected
Rickettsia rickettsia	1000 cp/rxn	No species detected
Rickettsia typhi	1000 cp/rxn	No species detected
Yersinia enterocolticia	1500 CFU/mL	No species detected
Yersinia pseudotuberculosis	1500 CFU/mL	No species detected

Exclusivity- Specificity of the panel primer and probes was determined using species that are genetic near neighbors to the panel target species. Species were tested either as high titer samples using bacteria cells in K_2EDTA-treated whole blood or as purified genomic DNA (*Rickettsia spp.*) in a buffer reaction. A minimum of N=4 samples were tested using a multiplex amplification reaction and showed no detection for any target panel species.

Panel Cross-reactivity

1000 copies/mL	Detection Channel						
Target	**pXO1**	**pXO2**	**Bu**	**Rp**	**Ft**	**Yp**	**Control**
B. anthracis pXO1	3/3	0/3	0/3	0/3	0/3	0/3	3/3
B. anthracis pXO2	0/3	3/3	0/3	0/3	0/3	0/3	3/3
Burkholderia spp.	0/3	0/3	3/3	0/3	0/3	0/3	3/3
R. prowazekii	0/3	0/3	0/3	3/3	0/3	0/3	3/3
F. tularensis	0/3	0/3	0/3	0/3	3/3	0/3	3/3
Y. pestis	0/3	0/3	0/3	0/3	0/3	3/3	3/3
Negative	0/3	0/3	0/3	0/3	0/3	0/3	3/3

Cross-reactivity- To determine if any of the target sequences were detected in an off-channel the target sequence were cloned into the chromosome of a host organism, genomic DNA purified, and 1000 genomic copies added as template to a multiplex reaction. No off-channel detections were seen demonstrating the targets are specific and there is no cross-reactivity of the panel.

Preliminary Analytical Sensitivity

	pXO1	pXO2	Bu (Bp)	Bu (Bm)	Rp	Ft	Yp
Titer (CFU/mL)	**5**	**5**	**20**	**10**	**3***	**4**	**2**
Positive	19	19	21	20	39	20	20
Total	20	20	21	21	39	21	20
Hit Rate	**95%**	**95%**	**100%**	**95.2%**	**100%**	**95.2%**	**100%**

**R. prowazekii* sample concentrations are in Cell associated genomic equivalents per mL (CAGe/mL) due to the inability of *R. prowazekii* to form colonies on solid agar

Analytical Sensitivity. To test the analytical sensitivity of the panel, samples were prepared in K_2EDTA whole blood at the sample titers shown and processed on the T2Dx instrument. The titer of Rp was quantified by a modified infection assay to determine a cell-associated genomic equivalents. Each species demonstrated a positive detection for the intended target of greater than 95% establishing a preliminary limit of detection for each species equal to or below 20 CFU/mL There were no off-target positive detections in positive samples or in matched negative blood samples (data not shown) producing a 100% specificity.

Conclusions

- The T2Biothreat Panel is designed to identify 6 CDC Category A or Category B bacterial biothreat species
- T2Biothreat Panel can detect target organisms in K_2EDTA-treated whole blood at 20 CFU/mL or less
- The panel has been shown capable of detecting multiple strains for each species and *in silico* analysis shows detection of all strains sequenced and curated in the NCBI database
- T2Biothreat Panel shows high specificity for the target species and does not detect genetic near-neighbors of panel target species

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