

Rapid Identification of Clinically Relevant and Class A BioThreat Bacterial Pathogens using Universal PCR coupled with High Resolution Melting Profile Analysis.

PACER

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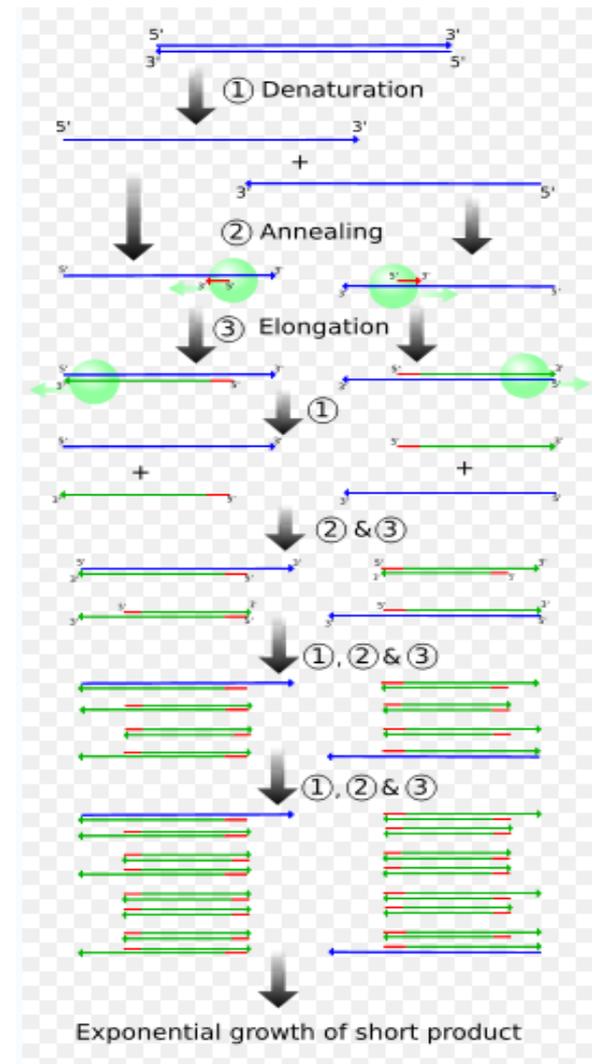
BACKGROUND

- **Bacteria culture (Gold Standard)**
 - Time consuming (18-24 hrs)
 - Lack sensitivity
 - Requires large laboratory facility

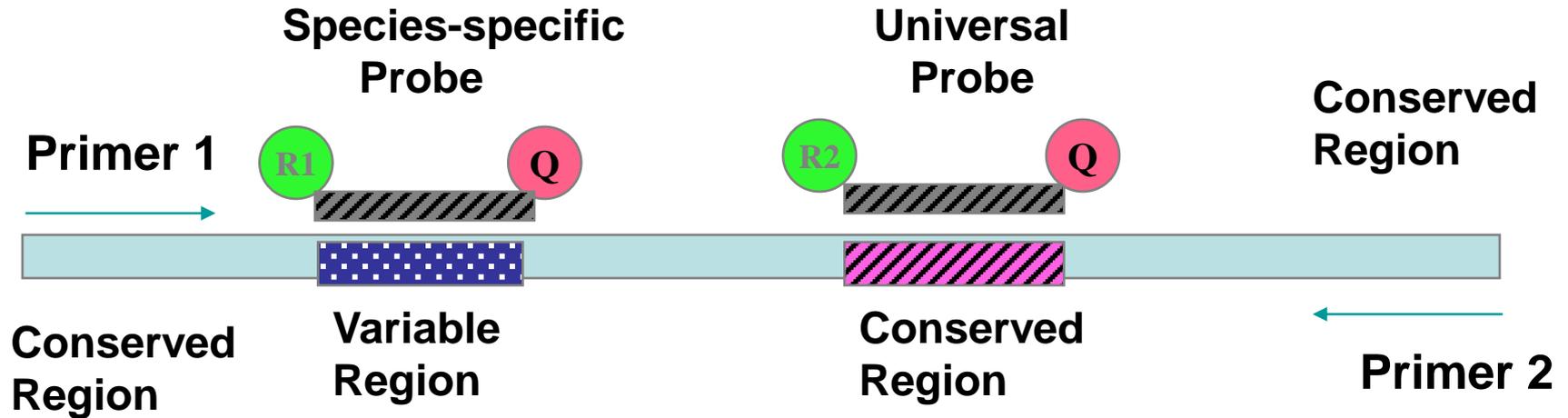
- **Universal PCR (Polymerase chain Reaction) targeting 16S rRNA gene**
 - Rapid
 - High sensitivity and specificity
 - Reduce biohazard risk

Polymerase Chain Reaction- PCR

- **PCR** – A technique that allows isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA.
- **Real-time PCR**- Established tool for DNA quantification that measures the accumulation of DNA product after each round of PCR amplification.

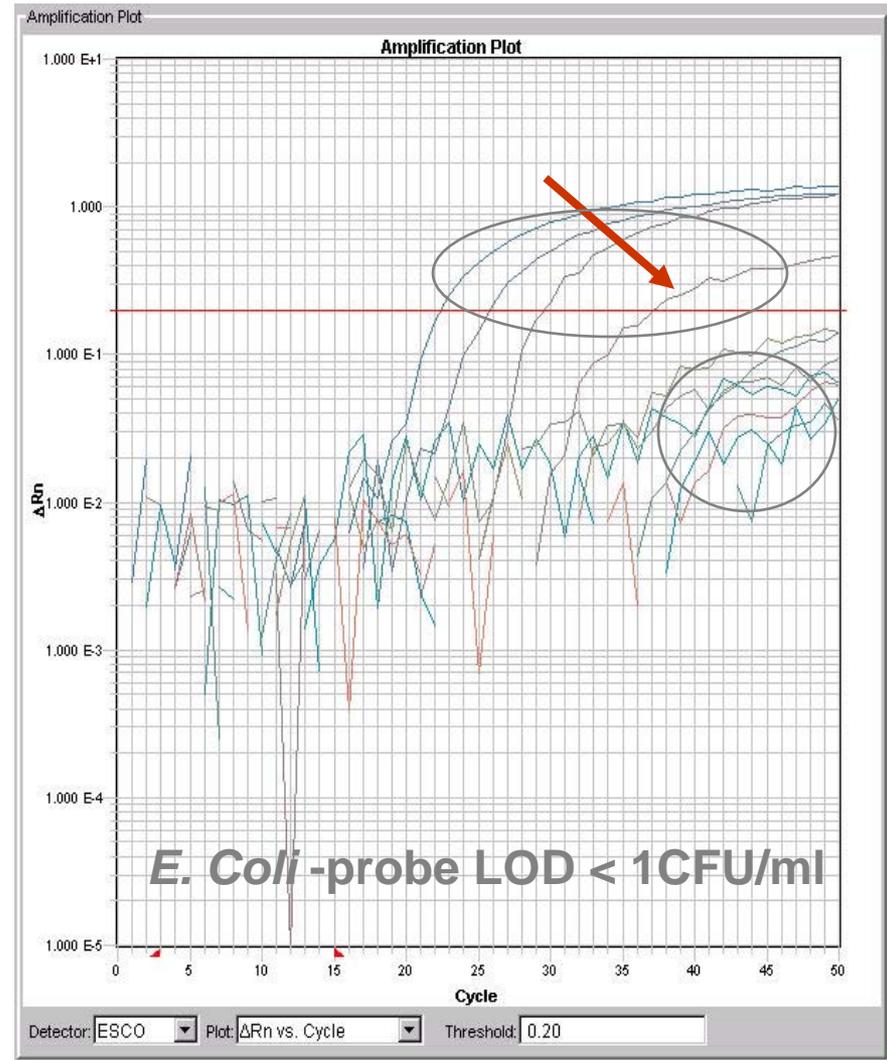
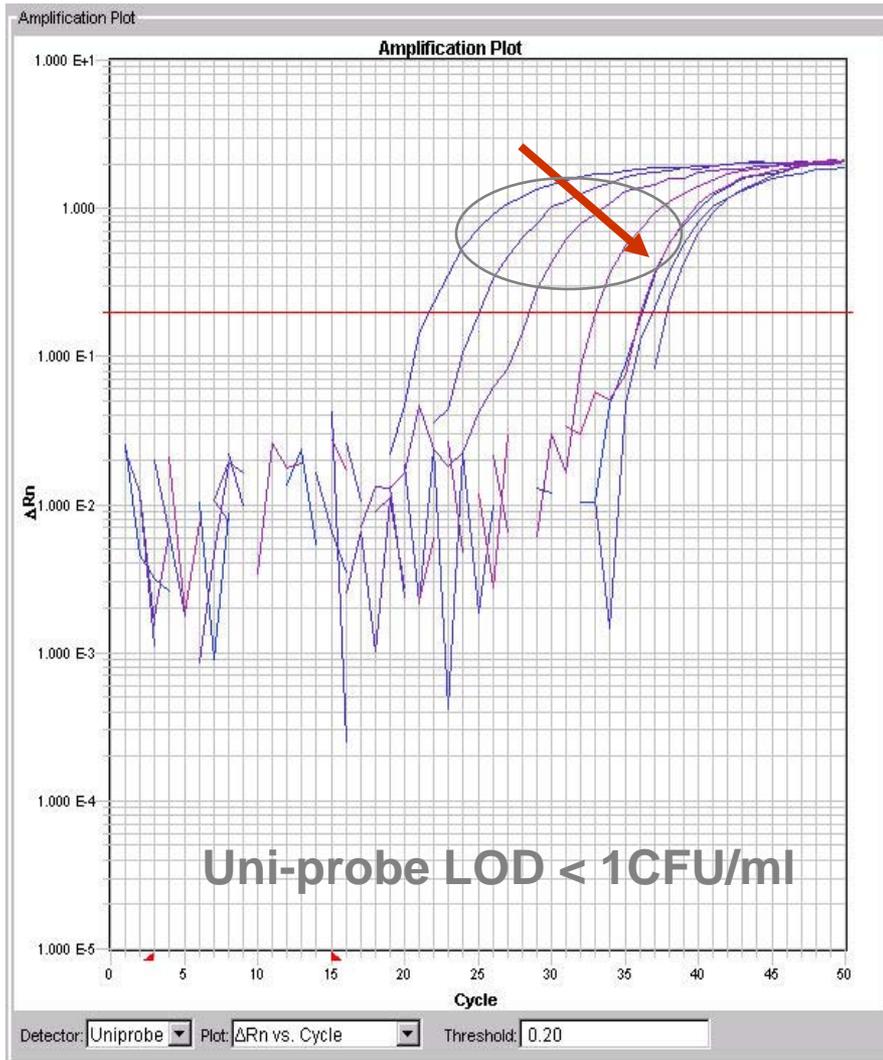


PROBE-BASED PCR



- **Universal PCR for Eubacterial detection followed by specific PCRs.**
- **Blood spiked with Class A BT agents- LOD (CFU/ml) using *Y. pestis* and *F. philomiragia* was <10 CFU/ml.**
- **Clinically validated - Septic Arthritis (n=121)**
 - **Sensitivity 95%; Specificity 97%**

Amplification curves- PCR



Limitations of using Probe-based PCR for pathogen identification

- **Unable to differentiate closely related species with highly homologous sequences**
 - *B. anthracis* and *B. cereus*
 - *F. tularensis* and *F. philomiragia*
 - *Y. pestis* & *Y. pseudotuberculosis*
- **Unable to identify emerging infectious pathogens**
- **Limited number of pathogens can be screened.**

POST-PCR AMPLICON ANALYSIS

- **High density microarray chip**
- **DNA sequencing**
- **Mass spectrometry**

- **Limitations of post PCR analysis**
- **Costly, laborious**
- **Resource demanding**

HIGH RESOLUTION MELTING ANALYSIS

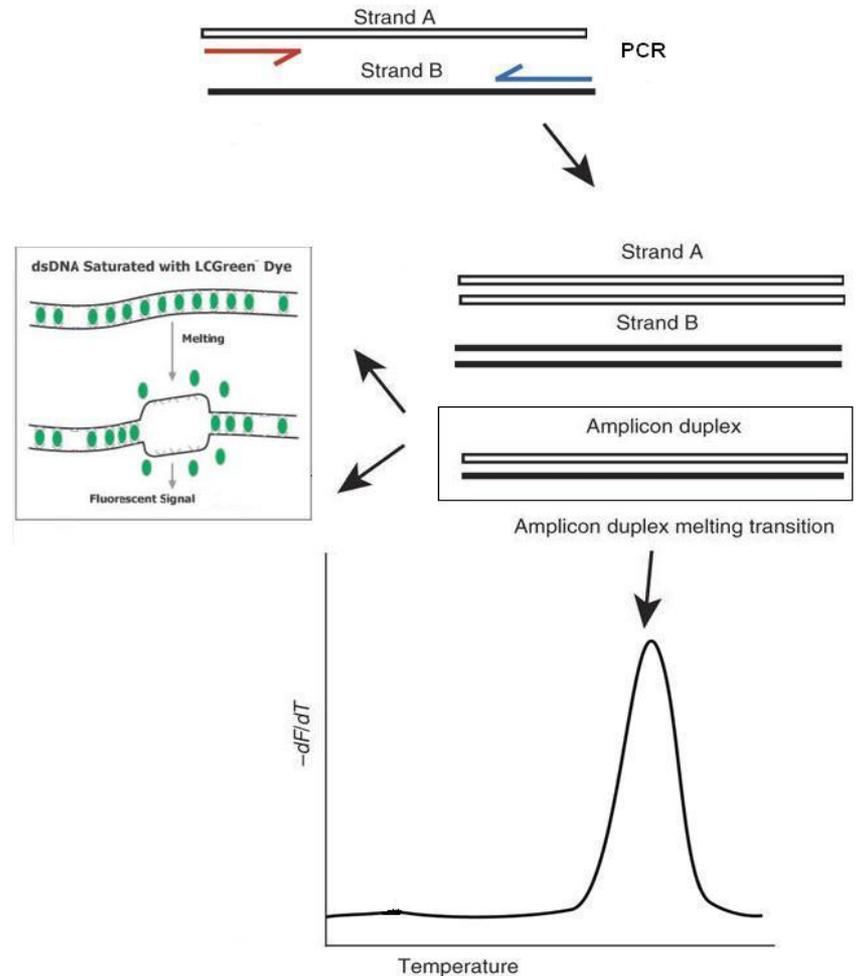
- **Promising Post PCR amplicon analysis method**
- **Differentiate sequence variations based on differences in melting profile**
- **Resolves single nucleotide differences in sequences**
- **Easy to integrate with PCR**
- **High throughput (<2 minutes)**
- **Relatively low cost**
- **Allows mass screening of sequence variations.**

OBJECTIVE

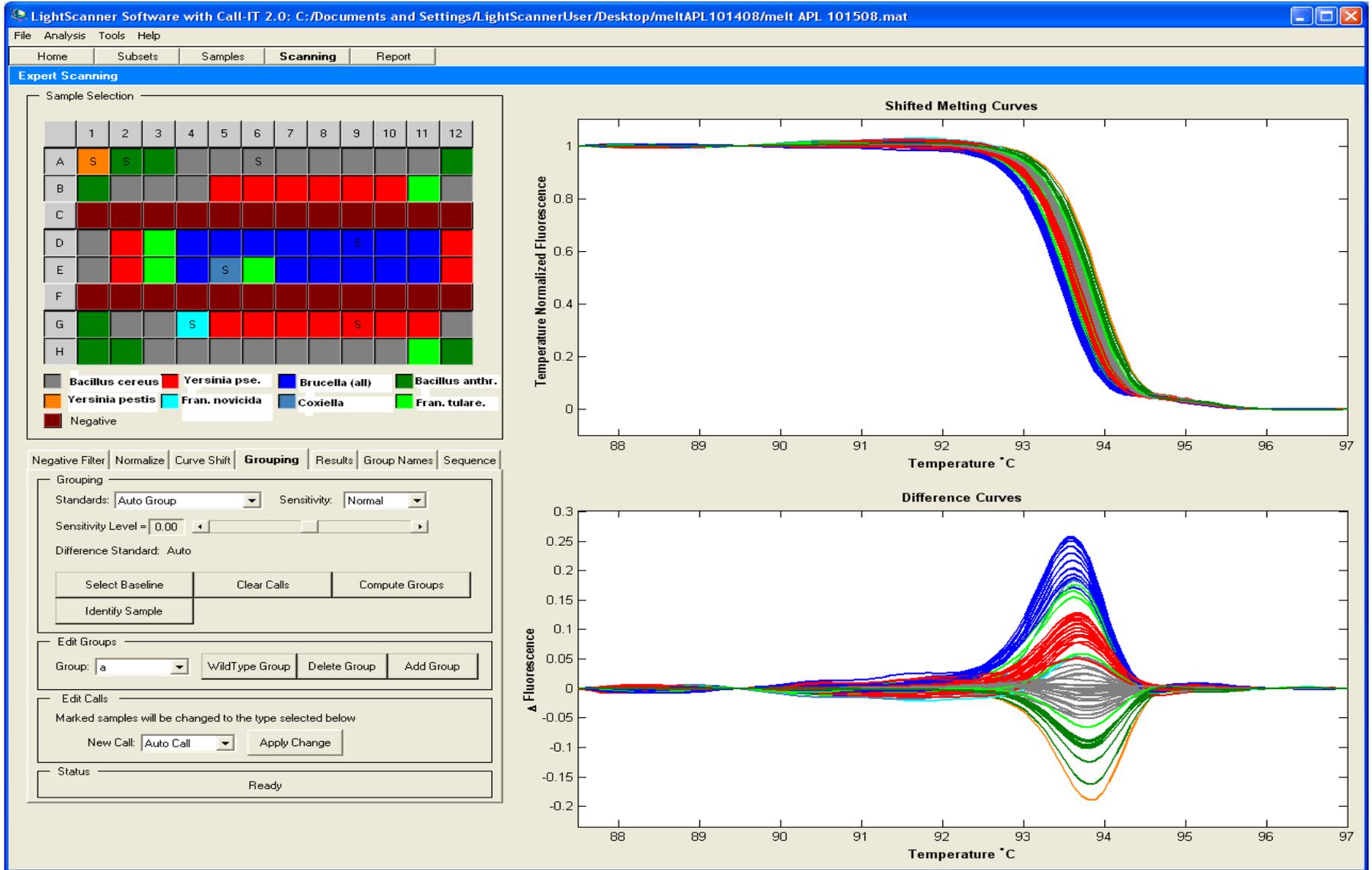
To determine if clinically relevant and biothreat related bacterial pathogens can be identified using Universal PCR coupled with High Resolution Melting Profile Analysis

METHODS

- Conserved primers were designed to target 3 hypervariable regions in 16S rRNA gene. (V1, V3 and V6)
- Universal PCR reactions were performed in thermocycler with LC green dye.
- High-resolution melting analysis of the amplicons were performed using the Light Scanner instrument (Idaho Technology).
- Melting profiles were generated and grouped based on their similarity.



RESULTS



Unique grouping of the melt profile based on three hyper-variable regions for all 36 clinically relevant bacterial pathogens tested

DNA Tested	V1	V3	V6
<i>Acinetobacter</i> sp. ATCC 5459	b	b	a
<i>Bacteriodes fragilis</i>	b	a	e
<i>Bordetella pertussis</i>	c	c	f
<i>Brucella ovis</i>	g	a	f
<i>Campylobacter jejunii</i>	c	a	e
<i>Cornebacterium</i> sp.	c	c	e
<i>Chlamydia pneumoniae</i>	g	c	a
<i>Chlamydia trachomatis</i>	f	a	b
<i>Citrobacter freundii</i>	a	c	a
<i>Enterobacter faecalis</i> ATCC 29212	u	u	a
<i>Escherichia coli</i> ATCC 25927	e	d	c
<i>Helicobacter pylori</i>	g	b	a
<i>Haemophilus influenzae</i> ATCC 49247	a	g	d
<i>Klebsiella pneumoniae</i>	h	c	a
<i>Legionella pneumophila</i> ATCC 33495	b	a	b
<i>Listeria monocytogenes</i> ATCC 7648	a	e	a

Unique grouping of the melt profile based on three hypervariable regions for all 36 clinically relevant bacterial pathogens tested

DNA Tested	V1	V3	V6
<i>Micrococcus sp.</i> ATCC 14396	a	b	b
<i>Mycoplasma pneumoniae</i>	a	d	g
<i>Mycoplasma hominis</i>	b	b	e
<i>Neisseria meningitidis</i> ATCC 6250	d	f	c
<i>Neisseria gonorrhoeae</i>	b	c	a
<i>Pseudomonas aeruginosa</i> ATCC 10145	a	b	c
<i>Proteus mirabilis</i> *	a	a	f
<i>Proteus vulgaris</i>	c	a	u
<i>Salmonella sp.</i> ATCC 31194	c	e	a
<i>Serratia marscecens</i> (ATCC 8101)	a	u	c
<i>Treponema pallidum</i>	f	b	e
<i>Staphylococcus aureus</i> ATCC 25923	b	b	h
<i>Staphylococcus epidermidis</i> ATCC 12228	b	a	h
<i>Streptococcus pneumoniae</i> ATCC 49619	g	d	g
<i>Streptococcus pyogenes</i> *	a	e	b
<i>Streptococcus agalactiae</i> ATCC 13813	a	e	d
<i>Viridans Group Streptococci</i> ATCC 10556	c	e	f

Unique grouping of the melt profile based on three hyper-variable regions for Category A BT agents and surrogates tested

Category A BT agents and surrogates	v1	v3	v6
<i>Bacillus anthracis</i>^d	c	a	a
<i>strain 3001</i>	<i>c</i>	<i>a</i>	<i>a</i>
<i>Bacillus cereus</i>^a	a	a	d
<i>strain BC 9634</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 12480</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 27877</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 7064</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC B33</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 1410-1</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 1410-2</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC T</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 2599</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 2464</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 7687</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 10329</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 11143</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 11145</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 1414</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 7089</i>	<i>a</i>	<i>a</i>	<i>d</i>

Category A BT agents and surrogates	v1	v3	v6
<i>strain BC 6464</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 6474</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 7004</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 10987</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 23674</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 9189</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 246</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>strain BC 13472</i>	<i>a</i>	<i>a</i>	<i>d</i>
<i>Bacillus subtilis 110 NA</i>	a	a	g
<i>strain SB168</i>	<i>a</i>	<i>a</i>	<i>g</i>
<i>strain W168</i>	<i>a</i>	<i>a</i>	<i>g</i>
<i>strain W23</i>	<i>a</i>	<i>a</i>	<i>g</i>
<i>strain her 148</i>	<i>a</i>	<i>a</i>	<i>g</i>
<i>strain T6</i>	<i>a</i>	<i>a</i>	<i>g</i>
<i>strain ATCC 27505</i>	<i>a</i>	<i>a</i>	<i>g</i>
<i>strain ATCC 15841</i>	<i>a</i>	<i>a</i>	<i>g</i>

Unique grouping of the melt profile based on three hyper-variable regions for Category A BT agents and surrogates tested

Category A BT agents and surrogates	v1	v3	v6
<i>Coxiella brunetti</i>^c	d	b	g
strain "9 mile"	d	b	g
<i>Franscicella phylomiragia</i> (GAO1-2810) ^e	a	g	g
<i>Franscicella tularensis</i> (LVSB) ^f	b	h	g
strain Fran 0001	b	h	g
<i>Yersinia pseudotuberculosis</i> (PB1/+)^g	a	g	c
strain Schutze's group type B/ ATCC 6903	a	g	c
strain Schutze group II/ ATCC 27802	a	g	c
strain CDC P62 / ATCC 29910	a	g	c
strain Schutze's group III/ ATCC 13980	a	g	c
strain raffinose positive ATCC 4284	a	g	c
strain ATCC 13979	a	g	c
<i>Yersinia enterocolitica</i>, strain 0:9 Serotype	a	g	d
strain WA.C	a	g	d
<i>Yersinia pestis</i> (P14 -)^h	a	b	d
strain 1122	a	b	d

a Clinical isolates

b *Brucella ovis* DNA obtained from Joany Jackman, PhD, Applied Physics Laboratory, Johns Hopkins University, Baltimore, MD.

c *Coxiella brunettei* DNA from Steven Dumbler, MD, Department of Pathology, School of Medicine, Johns Hopkins University, Baltimore, MD.

d Inactivated non-pathogenic strain.
e Non pathogenic strain obtained from Centre for Disease Control and Prevention, Fort Collins, Colorado, via Walter Reed Army Medical Hospital, Washington, D.C.

f LVSB- Live vaccine strain type.

g Wild type strain.

h De-pigmented and virulence pCD1-negative.

CONCLUSIONS

- **High resolution melting analysis can be easily integrated with 16S Universal PCR**
- **Unique species-specific “melting curve signature”**
- **By querying multiple targets, even closely related bacterial pathogens can be differentiated**
- **High throughput with < 2min of Post-PCR analysis time. Total assay time is 2 hrs.**

FUTURE DIRECTION

- **Expand our melt curve database to include all clinically relevant bacterial pathogens.**
- **Extend this method Category A, B, C bacterial biothreat agents.**
- **Evaluating animal models**
- **Clinical validation of the assay- diagnosis of Sepsis, Meningitis, Septic Arthritis**