Next Gen Sequencing based Biodefense Assays: The Need for Standardized Alternate Reference Materials

Presented to:
NIST workshop on standards for pathogen detection-2017
Disclaimer

• The views expressed in this presentation are those of the author and do not necessarily represent the views or official position of Defense Biological Product Assurance Office (DBPAO), Joint Project Manager for Guardian (JPM G), Joint Program Executive Office (JPEO), U.S. Department of Defense (DoD)
Who are we?

1998
CRP stood up at the JPEO-CBD

2007
CRP begins funding UCC

2003
CRP opens antigen repository at Dugway

2010
CRP obtains ISO 9001:2008 accreditation

2015
- April - DPG ships inadequately irradiated BA samples
- May - DoD moratorium on inactivated agents
- May - AT&L creates Review Committee for DoD procedures involving BA
- June - CDC issues new guidance for testing iBA spores for growth
- July - Review Committee releases report on BA shipments
- September - SECARMY closes CRP

2016
- March - JPEO-CBD renamed CRP to DBPAO, aligns it to JPM-Guardian
- July - SECARMY describes new policies/procedures for BSAT-related materials
- July - SECARMY describes DBPAO BSAT-related requirements

2017
- June - Noblis delivers BCA report and recommendation
DBPAO: CAR and TARMAC

Material Products

CAR

- OSCAR (ORDERING SYSTEM FOR CRITICAL ASSAYS AND REAGENTS)
  - Lateral Flow Immunoassays
  - Electrochemiluminescence Assays
  - Polymerase Chain Reaction Assays
  - Antibodies
  - Surrogates (Non-BSAT)

Knowledge Products

TARMAC

- UCC Pathogen Data
- Microbial Data Index
- Microbial Metadata
- Characterization Data
- TARMAC Data
- CAR Product Data
- Assay Data
- DHIS 2 Data

Earo

- JIBS/Defense Business System
- Inactivated Organisms (BSAT)
- Genomic Material
Outline - modifications to standards

- Standards for Reference Materials
  - Surrogates as alternate reference materials for Select Agents
- Standards for Strain Characterization
  - End to end characterization
- Standards for Assay Development
  - Incorporation of extensive *in silico* analyses of assay signatures and minimize wet lab testing with risky reference materials
The problems with select agent reference materials or derivatives for Biodefense Assay Development

• 2015 DPG debacle and the ensuing moratorium on working with select agents and shipping

• New samples/nstrains availability (e.g., Ebola Zaire Makona, Lassa)
  • Potential signature erosion with new sequences and the need for assay redesign

• Time line for optimization of assay for new sequences?

• Is there a need to reevaluate all steps in assay development and FDA approval?
Traditional pathway for development of a nucleic acid based molecular assay

Reference Materials: live or inactivated organisms or genomic materials

1 prototype strain  Multiple strains

A Primer Design
SYBR Green Testing

Assay Optimization
Probe Down Selection
Standard Curve and LoD

B Inclusivity and Exclusivity

C Mock Clinical Trials
Document Submission

D FDA Vetting

1–2 days  2–3 days  1–2 days  2–3 days  4–5 days  Weeks to Months (Use Case Contingent)

Courtesy:
New Standards for Select Agent Reference Materials
Why were these products needed?

• What is the purpose for the inactivated Spores and other inactivated Select Agents?
  • The inactivated agent materials serve several critical needs
    • Positive controls in assays used to detect these pathogens in suspected samples
    • Improvement of currently existing detection methods or development of new methods/platforms for detection of Ba (develop and validate assays)
    • Quality assurance and Proficiency testing and Training activities
    • End to End Validation of systems
a) Ba Spore Surrogate to Replace Inactivated Virulent Spores

a) Ba Spore Inactivation Studies
Alternate Risk Mitigated Reference Materials for Ba

‘Genetically inactivated/modified (attenuated)’
‘Non-pathogenic’ Ba Strains with Assay Targets

Construction, Testing and Production
# Technical Aspects - What are we trying to do?

<table>
<thead>
<tr>
<th>Ba Strain Name</th>
<th>Genetic make up</th>
<th>Status/Risk</th>
<th>Toxin Genes</th>
<th>Capsule Genes</th>
<th>All Assay Targets?</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus anthracis</em> Ames (exists)</td>
<td><img src="image1" alt="Diagram" /></td>
<td>Select Agent</td>
<td>X</td>
<td>X</td>
<td>Yes (3)</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> Sterne (exists)</td>
<td><img src="image2" alt="Diagram" /></td>
<td>Exempt (pathogenic for animals)</td>
<td>X</td>
<td>-</td>
<td>No (2)</td>
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<tr>
<td><em>Bacillus anthracis</em> Sterne ΔpXO1 (aka TKO exists)</td>
<td><img src="image3" alt="Diagram" /></td>
<td>Exempt (non-pathogenic)</td>
<td>-</td>
<td>-</td>
<td>No (1)</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> Sterne ΔpXO1 plus (to be constructed rBaSwAT)</td>
<td><img src="image4" alt="Diagram" /></td>
<td>Exempt (non-pathogenic)</td>
<td>-</td>
<td>-</td>
<td>Yes (3)</td>
</tr>
</tbody>
</table>

Assay target sequences: 🟢 - Gene deletion: [ ] TKO- Triple Knock Out
Safety Aspects of the Parent Strain Sterne (TKO)

TKO- Triple Knock Out

Toxin gene region of parent strains

Gene to Signature Fragment Ratio

Gene 1

Gene 2

Gene 3

Gene 4

Signature

Drawn to scale
Schematic of the introduced DNA

- Different constructs for different assays
- Barcodes for complete traceability and bio forensics
- “Stop Cassette” as an extra measure to prevent fortuitous translation of insert from neighboring transcriptional read through

Courtesy: Dr. Mark Munson-NMRC
End to End Characterization of rBaSwAT

• Vegetative cells
  • Phenotypic assays
    ✓ Microbiological tests
    ✓ Phage sensitivity (\(\gamma\) and AP50c)
    ✓ Antimicrobial resistance
    ✓ Spore formation (@NSWC)
    ✓ Immunological tests (LFI)
  • Genotypic assays
    ✓ Whole genome sequencing
    ✓ Molecular tests for signatures (PCR)

• Spores
  • Production, purification (@NSWC) and irradiation inactivation (using new protocol established by the working group @NMRC)
    ✓ Molecular and Immuno assays and bridging studies (@NSWC)
    ✓ Animal studies
PCR verification of toxin gene deletions in rBaSwAT
Whole Genome Sequencing of rBaSwAT

Toxin region in rBaSwAT strain compared to grant parent (34F2, 34F2 DKO, 34F2 TKO strains)
Spore Characteristics

Coulter Counter
Mean (µm) ± S.D. (µm)
1.153 ± 0.122

Spore titer: Plate counts: \(1.5 \times 10^{10}\); Coulter Counter: \(1.9 \times 10^{10}\); GE: TBD
<table>
<thead>
<tr>
<th>ASSAY</th>
<th>STRAIN</th>
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<tr>
<td></td>
<td>Sterne</td>
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<tr>
<td></td>
<td>BA663</td>
</tr>
<tr>
<td></td>
<td>Grand Parent</td>
</tr>
<tr>
<td>Chr</td>
<td>+</td>
</tr>
<tr>
<td>Sig1</td>
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<td>Sig2</td>
<td>+</td>
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<tr>
<td>Sig3</td>
<td>+</td>
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<tr>
<td>Sig4</td>
<td>-</td>
</tr>
<tr>
<td>Sig5</td>
<td>+</td>
</tr>
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</table>
Demonstration of non-lethality of constructs in A/J mice

Based upon a One-sided Fisher exact test $P=0.0003$ for groups compared to 34F2 (N=5 for that control)
Partners

• DBPAO Performers
  ▪ Drs. Joan Gebhardt and Mark Munson
    ➢ Naval Medical Research Center, Ft. Detrick, MD
  ▪ Drs. Chris Cote, Dave Rozak and Terry Abshire
    ➢ USAMRIID, Fort Detrick, MD
  ▪ Drs. Cory Bernhards and Nicole Rosenzweig, Rebecca Rossmaier and Tracey Biggs
    ➢ ECBC, Edgewood, MD
  ▪ Drs. Tony Buhr, Linda Beck and Andrea Staab
    ➢ NSWC, Dahlgren, VA

• FDA Collaborators
  ▪ Drs. Roger Plaut and Scott Stibitz
    ➢ FDA, Silver Spring, MD
Traditional pathway for development of a molecular assay

Reference Materials: live or inactivated organisms or genomic materials
1 prototype strain

Multiple strains

A
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Weeks to Months (Use Case Contingent)

Courtesy:
Stakeholder Panel on Agent Detection Assays (SPADA)

- List of Inclusivity and Exclusivity Panel strains decided by SMEs for each organism
  - Ba
  - Yp
  - Ft
  - Burk
  - Brucella
  - Toxins (Various toxins)
- Any assay needs to be wet lab tested against this panel- expectation- the assay will hit all inclusivity panel and will not hit exclusivity panel
On the need for *in silico* analyses to replace inclusivity/exclusivity testing

Heat map of signature sequence hits in various genomic sequences

```
<table>
<thead>
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<th>Sequence</th>
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<tr>
<td>Perfect</td>
</tr>
<tr>
<td>BA</td>
</tr>
<tr>
<td>YP</td>
</tr>
<tr>
<td>FT</td>
</tr>
<tr>
<td>BK-P</td>
</tr>
<tr>
<td>BK-M</td>
</tr>
<tr>
<td>BK-NN</td>
</tr>
</tbody>
</table>

Perfect: Perfect match
BA: Best alignment
YP: Y chromosome polymorphism
FT: Forensic trace
BK-P: BK chromosome polymorphism
BK-M: BK chromosome monomorphism
BK-NN: BK chromosome neutral
```

Legend:
- Green: 100%
- Yellow: 85%-99%
- Red: 50%-84%
- Orange: 0%-49%

9/11/2017 NIST Workshop_Aug_2017
Genome Sequence Explosion with the advent of Next Gen Sequencing

- Sequences of SPADA panels were published in 2015
- Assays were designed earlier

CRP Assay design time frame

- accum # of virus
- Acc # of Francisella tularensis
- acc # of Yersinia pestis
- accum # of Burkholderia mallei
- accum # of Flavi

- accum # Prok
- Accum # of Bacillus anthracis
- Accum # of Burkholderia pseudomallei
- accum # of Brucella
Standards for *in silico* analyses of assay signatures (rapidly evolving pathogen)

*In silico* signature evaluation of 2014 EBOV outbreak strains before we obtained samples
How do we make the call?

• Parameters
  
  • Assay Hit- A positive match between the assay primer/probe set sequences and sequences from the NCBI databases @ > 90% identity over 90% of the primer length (2 mismatches allowed for a 20 nt primer)

  • Amplicon Hit- A positive match between an amplicon sequence from an assay and sequences from the NCBI databases @ > 85% identity over 90% of the amplicon length (15 mismatches allowed for a 100 bp amplicon)
## PSET Results (Ebola Assays)

<table>
<thead>
<tr>
<th>Assay #</th>
<th>Assay ID</th>
<th>Intended Target Species</th>
<th>Gene Target</th>
<th>Species/Strain of Hips</th>
<th>Amplicon length (bps)</th>
<th>Assay Hit</th>
<th>Percentage of perfect amplicon hit</th>
<th>Report*</th>
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<tr>
<td>1</td>
<td>Sig 1</td>
<td>EBOV NP EBOV</td>
<td>124 135 132</td>
<td>97.8 115 11</td>
<td>8.1 135 0</td>
<td>0 0</td>
<td>Pass</td>
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<tr>
<td>2</td>
<td>EbolaZaire-MGB</td>
<td>EBOV NP EBOV</td>
<td>76 135 134</td>
<td>99.3 135 124</td>
<td>91.9 135 0</td>
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<td>3</td>
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<td>EBOV NP EBOV</td>
<td>49 135 135</td>
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<td>92.6 135 0</td>
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<td>EBOV NP EBOV</td>
<td>80 136 22</td>
<td>16.2 136 19</td>
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<td>ZAI-NP</td>
<td>EBOV NP EBOV</td>
<td>268 148 123</td>
<td>83.1 148 10</td>
<td>6.8 148 0</td>
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<td>Ebola MGB-EBOV</td>
<td>EBOV NP EBOV</td>
<td>79 148 23</td>
<td>15.5 148 11</td>
<td>7.4 148 0</td>
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<td>7</td>
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<td>21.6 148 32</td>
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<td>Ebola Zaire-TM</td>
<td>EBOV GP EBOV</td>
<td>579 152 0</td>
<td>0 0 152 5</td>
<td>3.3 152 0</td>
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<td></td>
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<tr>
<td>9</td>
<td>Ebola GP-1</td>
<td>EBOV GP RESTV</td>
<td>579 12 0</td>
<td>0 0 0 0</td>
<td>0 12 0</td>
<td>0 0</td>
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<td></td>
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<tr>
<td>10</td>
<td>Ebola GP-1</td>
<td>EBOV GP RESTV</td>
<td>64 153 13</td>
<td>8.5 153 13</td>
<td>8.5 153 0</td>
<td>0 0</td>
<td>Pass</td>
<td></td>
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<tr>
<td>11</td>
<td>Ebola Zaire-TM</td>
<td>EBOV GP EBOV</td>
<td>80 144 13</td>
<td>9.0 153 13</td>
<td>8.5 144 9</td>
<td>0 0</td>
<td>Fail</td>
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<tr>
<td>12</td>
<td>Filo AB</td>
<td>pan-Filo L EBOV</td>
<td>419 135 0</td>
<td>0 0 135 12</td>
<td>8.9 135 0</td>
<td>0 0</td>
<td>Pass</td>
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<tr>
<td>13</td>
<td>Filo AB</td>
<td>pan-Filo L MARYV</td>
<td>419 55 0</td>
<td>0 0 0</td>
<td>0 55 0</td>
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<td>Pass</td>
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<td>Filo AB</td>
<td>pan-Filo L SUDV</td>
<td>419 12 0</td>
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<td>0 12 0</td>
<td>0 0</td>
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<tr>
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<td>12.6 135 12</td>
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<tr>
<td>16</td>
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<td>BDBV NP BDBV</td>
<td>74 5 1</td>
<td>20.0 5</td>
<td>20.0 5 0</td>
<td>0 0</td>
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</tr>
<tr>
<td>17</td>
<td>Ebola BDBV-TM</td>
<td>BDBV NP BDBV</td>
<td>74 5 1</td>
<td>20.0 5</td>
<td>20.0 5 0</td>
<td>0 0</td>
<td>Pass</td>
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</tr>
<tr>
<td>18</td>
<td>Ebola TAFV-MGB</td>
<td>TAFV GP TAFV</td>
<td>64 2 1</td>
<td>50.0 2</td>
<td>50.0 2 0</td>
<td>0 0</td>
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</tr>
<tr>
<td>19</td>
<td>Ebola TAFV-TM</td>
<td>TAFV GP RESTV</td>
<td>79 2 2</td>
<td>100.0 2</td>
<td>100.0 2 0</td>
<td>0 0</td>
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</tr>
<tr>
<td>20</td>
<td>Reston-GP-RESTV</td>
<td>RESTV NP RESTV</td>
<td>337 8 8</td>
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<tr>
<td>21</td>
<td>Ebola-GP-RESTV</td>
<td>RESTV GP EBOV</td>
<td>97 8 2</td>
<td>25.0 8</td>
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<td>Ebola RESTV-GP</td>
<td>RESTV GP EBOV</td>
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<td>0 0 9</td>
<td>0 0 0 0</td>
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<td>23</td>
<td>Ebola Reston-TM</td>
<td>RESTV VP40 RESTV</td>
<td>80 8 2</td>
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<td>25.0 8 0</td>
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<tr>
<td>24</td>
<td>Ebola Sudan-MGB</td>
<td>SUDV NP SUDV</td>
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<td>90.9 12 10</td>
<td>83.3 11 1</td>
<td>0 0</td>
<td>Fail</td>
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<tr>
<td>25</td>
<td>Ebola Sudan-MGB</td>
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<td>80 11 10</td>
<td>90.9 11 10</td>
<td>90.9 11 0</td>
<td>0 0</td>
<td>Pass</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Ebola Sudan-MGB</td>
<td>SUDV NP SUDV</td>
<td>80 0 0</td>
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<td>0 0 0 0</td>
<td>0 0</td>
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<td>27</td>
<td>Ebola Sudan-MGB</td>
<td>SUDV NP SUDV</td>
<td>81 11 7</td>
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<td>Pass</td>
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<tr>
<td>28</td>
<td>Ebola Sudan-MGB</td>
<td>SUDV NP SUDV</td>
<td>89 11 10</td>
<td>90.9 11 10</td>
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<td>0 0</td>
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<tr>
<td>29</td>
<td>Sudan</td>
<td>SUDV NP SUDV</td>
<td>74 14 9</td>
<td>64.3 14 9</td>
<td>64.3 14 0</td>
<td>0 0</td>
<td>Pass</td>
<td></td>
</tr>
</tbody>
</table>

- Most of the assays passed with the relaxed criteria (90/90; 85/90 rule) (3 failed-false positive or false negative)
- Only 3 passed 100/100 rule
Heat map of assay hits to GenBank entries

- Most of the EBOV assays have less than perfect matches to many GenBank entries.
- Species specific assays are mostly specific with relaxed criteria.
- Cross reactivity is seen with pan assays and specific assays.
- Assay positive but amplicon negative hits are due to extensive variation and pan assays.
- Amplicon positive but assay negative hits are usually genetic drift.
Summary

• Assessed the performance of the existing EBOV assays using in vitro and in silico (PCR Signature Erosion Tool) approach-most assays work despite mismatches between signatures and target.

• Periodic monitoring of assay performance in silico will facilitate better assay designs or improvements.

• PSET can be a valuable tool to determine whether a wet lab testing of new sequences is needed or not.

• FDA reevaluation?
Evaluation of Signature Erosion in Ebola Virus Due to Genomic Drift and Its Impact on the Performance of Diagnostic Assays

Shanmuga Sozhamannan 1,2,*, Mitchell Y. Holland 3, Adrienne T. Hall 4, Daniel A. Negrón 3, Mychal Ivancich 3, Jeffrey W. Koehler 4, Timothy D. Minogue 4, Catherine E. Campbell 5, Walter J. Berger 3, George W. Christopher 6, Bruce G. Goodwin 1 and Michael A. Smith 1
Summary

• Limited wet lab testing using risk mitigated recombinant surrogates, or synthetic constructs, or VLPs for viral agents
• New pathway for assay design using *in silico* approaches
• Well characterized reference materials
• Well defined assays
  • Move existing panels of PCR assays to amplicon sequencing based assays with increased genomic content as a first step to “Microseq”
  • Eventually move to UHTP sequencing later (pathogen discovery)
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