Microbiological diagnosis of *Bacillus anthracis*

The first bacterium shown to be the cause of a disease: 1877 by Robert Koch

Member of the “Bacillus cereus group”

- B. cereus
- B. anthracis
- B. thuringiensis
- B. mycoides
- B. weihenstephanensis
Differentiation of *B. cereus* group members

- *B. cereus*: environmental clone, opportunistic pathogen: food poisoning- emetic type and diarrheal type
- *B. anthracis*: causative agent of anthrax, mammalian pathogen, non-hemolytic, gamma phage sensitivity, virulence plasmids pXO1, pXO2
- *B. thuringiensis*: insect pathogen, plasmid encoded crystal proteins, opportunistic pathogen
- *B. mycoides*: psychrotolerant (*cspA*), plant growth promoting bacterium, rhizoid growth
- *B. weihenstephanensis*: psychrotolerant (*cspA*), occasionally food poisoning
“Bacteria were originally classified largely on the basis of phenotype, morphology, ecology and other associated metabolic characteristics. Bacterial taxonomy has been a tedious, esoteric and uncertain discipline.”

Porwal S. et al. PLoS One 2009 e4438
Genetic relatedness of *B. cereus* group members

- Identical 16S and 23S rDNA
- Not differentiable by DNA-DNA hybridization
- Molecular characteristics differ from phenotypic characteristics
- Phenotypic features are located on plasmids
MLST/ AFLP based phylogenetic tree of the B. cereus group


Priest FG et al. J Bact 2004; 186:7959-7970

Bacillus cereus

- Suggestion that *B. cereus*, *B. anthracis*, and *B. thuringiensis* are members of a single species

*B. cereus sensu lato:*
- *pv. anthracis*
- *pv. cereus*
- *pv. thuringiensis*
Correct and rapid identification of pathogenic clones is important: specific \textit{rpoB}, \textit{gyrB} sequences, pXO1 and pXO2 plasmids.

Establishment of a discriminative method to differentiate \textit{B. anthracis} strains in an outbreak situation or in case of intentional release.
Identification and typing of *B. anthracis*

SNP-based typing:

**Strain-Specific Single-Nucleotide Polymorphism Assays for the**

*Bacillus anthracis* Ames Strain


*Journal of Clinical Microbiology, Jan. 2003, p. 47-53*

MLVA:

**Global Genetic Population Structure of Bacillus anthracis**

• Melting curve analysis using real-time PCR was introduced in 1997:
  • Lay MJ, Wittwer CT. Clin Chem 1997;43:2262-2267

• The technique requires the usual unlabeled primers and a „Novel intercalating dye” (LCGreen I) to identify heteroduplex DNA saturating, non-inhibitory dsDNA binding without redistribution during melting added before PCR for amplicon genotyping.

• Precise Instrument to allow genotyping and/or mutation scanning of whole PCR products.
  • homogenous temperature profile and temperature control
  • high sensitivity optical system

• Analysis software generating normalized and temperature shifted difference plot (instead of melting curves derivatives used with probes)

• Advantages of genotyping by melting analysis:
  • multiple alleles can be analyzed, whereas other methods require one probe for each allele
  • cheaper
Melting Curve Analysis

Established & New Applications

SYBR Green I for Product Identification
High Resolution Melting Dye for Gene Scanning
Fluorescence labeled Probes for Genotyping

© Roche Diagnostics, kindly provided by Dr. P. Hufnagl
Preparing a Gene Scanning Experiment

Requirements for a successful HRM experiment:

- high quality DNA
- an optimized PCR system
- small amplicon - not larger than 200bp
- no primer dimers or unspecific products
- every reaction amplified into the plateau phase

Amplification

High resolution melting

Temperature Shift

Normalization

© Roche Diagnostics, kindly provided by Dr. P. Hufnagl
## SNP-Classes (Venter, Science 2001)

<table>
<thead>
<tr>
<th>SNP Class</th>
<th>Base Exchange</th>
<th>typical $T_M$ melting curve shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C/T and G/A</td>
<td>$&gt; 0.5 \degree C$</td>
</tr>
<tr>
<td>2</td>
<td>C/A and G/T</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>C/G</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>A/T</td>
<td>$&lt; 0.2 \degree C$</td>
</tr>
</tbody>
</table>
Table 1: Database results for the identification of dangerous pathogens through 16S rDNA sequence homologies. Search results with the highest homology are shown for each database.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>GenBank/e-Value</th>
<th>RIDOM*</th>
<th>MicroSeq 1.4.1</th>
<th>MicroSeq V1.0/100% Identity</th>
<th>RDP-II</th>
<th>Origin, References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td><em>Bacillus cereus</em> DSM 4312</td>
<td>+1/0.0</td>
<td>-</td>
<td>+</td>
<td>+/100</td>
<td>*</td>
<td>DSMZ</td>
</tr>
<tr>
<td>3</td>
<td><em>Bacillus anthracis</em> BH</td>
<td>+1/0.0</td>
<td>-</td>
<td>-</td>
<td>-/100</td>
<td>*</td>
<td>Military strain of Austria (Allerberger et al., 2002)</td>
</tr>
<tr>
<td>4</td>
<td><em>Bacillus anthracis</em> steme 34F2</td>
<td>+1/0.0</td>
<td>-</td>
<td>-</td>
<td>-/100</td>
<td>*</td>
<td>Onderstepoort Biologic Products (spore vaccine)</td>
</tr>
<tr>
<td>5</td>
<td><em>Bacillus anthracis</em> 9080G</td>
<td>+1/0.0</td>
<td>-</td>
<td>-</td>
<td>-/100</td>
<td>*</td>
<td>Georgia</td>
</tr>
<tr>
<td>6</td>
<td><em>Bacillus anthracis</em> 3520</td>
<td>+1/0.0</td>
<td>-</td>
<td>-</td>
<td>-/100</td>
<td>*</td>
<td>Isolate from cattle, Austria</td>
</tr>
<tr>
<td>7</td>
<td><em>Bacillus anthracis</em> 4675</td>
<td>+1/0.0</td>
<td>-</td>
<td>-</td>
<td>-/100</td>
<td>*</td>
<td>Isolate from cattle, Austria</td>
</tr>
<tr>
<td>8</td>
<td><em>Bacillus anthracis</em> 6782</td>
<td>+1/0.0</td>
<td>-</td>
<td>-</td>
<td>-/100</td>
<td>*</td>
<td>Isolate from cattle, Austria</td>
</tr>
<tr>
<td>9</td>
<td><em>Bacillus anthracis</em> 411-G</td>
<td>+1/0.0</td>
<td>-</td>
<td>-</td>
<td>-/100</td>
<td>*</td>
<td>Georgia</td>
</tr>
</tbody>
</table>
## Table 1: Sequences of primers and TaqMan MGB probes

<table>
<thead>
<tr>
<th>SNP</th>
<th>5' to 3' sequences for primers</th>
<th>TaqMan MGB probe</th>
<th>SNP change and genome position</th>
<th>Genbank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS-1</td>
<td>F-TGATGTTTTGATTTTTTAACTTTT</td>
<td>FAM-AAGGTCAGAAGTC</td>
<td>C=H, 7452</td>
<td>NC_003820</td>
</tr>
<tr>
<td></td>
<td>R-CAGACGTTTTGATTTTTTAACTTTT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS-52</td>
<td>F-ATATGCTGAAATAAAAAGAAATAAAAATAGA</td>
<td>VIC-ATGGACAATCTGTTGTT</td>
<td>A=C, 72924</td>
<td>NC_003821</td>
</tr>
<tr>
<td></td>
<td>R-GATTGCTGAAATAAAAAGAAATAAAAATAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branch1-7</td>
<td>F-TCATGTCGACAGAATCGGCA</td>
<td>FAM-CAGACCAATACGCTTTT</td>
<td>C=A, 433277</td>
<td>NC_003997</td>
</tr>
<tr>
<td></td>
<td>R-TTCTGUGAAGAAGGAAATACTTTT</td>
<td>VIC-CAGACCAATACGCTTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branch1-28</td>
<td>F-GACGCGAGCGAAACAGAA</td>
<td>FAM-GAAGCAGTTATTTAGTCTCTTTGCTT</td>
<td>T=C, 4624132</td>
<td>NC_003997</td>
</tr>
<tr>
<td></td>
<td>R-CGGCTGAAATAAACGAGATAGAATTTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Branch1-31</td>
<td>F-AATATTTTTTATCCACAGCAGACTAC</td>
<td>FAM-CGTTGAAAGATTTAC</td>
<td>T=G, 4923186</td>
<td>NC_003997</td>
</tr>
<tr>
<td></td>
<td>R-GATAATTTTTTATCCACAGCAGACTACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F-GAAGAAGGAAGAAGGAGAAGCCTC</td>
<td>VIC-CGTTGAAAGATTTAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R-GTTATTTTTTATCCACAGCAGACTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note that all probes have a minor-groove binder, nonfluorescent quencher attached to the 3’ end. FAM, 6-carboxyfluorescein. The Ames probes were labeled with FAM except in the case of PS-1, which was labeled with VIC. Underlined letters indicate SNP location.

**SNP changes are expressed as "Ames = non-Ames."**
HRM results for Branch 1-31

B. anthracis G AAGAACAAGCGAAAGACGTACCTGTCGGTTCACATGGCATTATCCCTACTTTTTCAAACGTTATGAACTAC
B. a. Ames GAAGAACAAGCGAAAGACGTACCTGTCGGTTCACATAGCATTATCCCTACTTTTTCAAACGTTATGAACTAC
B. cereus G AAGAACAAGCGAAAGACGTACCTGTCGGCTCACATGGGATTATCCCTACTTTTTCAAACGTAATGAACTAC

B. anthracis non Ames
B. anthracis Ames
B. cereus

Genotyping of *Bacillus anthracis* strains based on automated capillary 25-loci Multiple Locus Variable-Number Tandem Repeats Analysis

Florigio Lista*1,2, Giovanni Faggioni1, Samina Valjevac3,4, Andrea Ciammaruconi1, Josée Vaissaire5, Claudine le Doujet5, Olivier Gorgé3,4, Riccardo De Santis1, Alessandra Carattoli6, Alessandra Ciervo6, Antonio Fasanella7, Francesco Orsini1, Raffaele D’Amelio2,8, Christine Pourcel4, Antonio Cassone6 and Gilles Vergnaud3,4
• The 2001 postal bag isolate is a B. anthracis Ames strain.
• 9080G from Georgia is identical to the vaccine strain 34F2 Sterne
• 411-G from Georgia is an A3a isolate
Three isolates from the Austrian province Tyrol were shown to be European B2 strains, extremely similar to French and Italian B2 isolates.
• HRM curve analysis of single chromosomal SNPs is a fast, simple and cost effective screening method that may allow the differentiation of *B. cereus* group members

• HRM curve analysis allows the differentiation of the Ames strain from other *B. anthracis* strains using a single PCR assay

• 25 –loci MLVA analysis revealed that Austrian *B. anthracis* isolates are European B2 strains

• These MLVA data provide a basis for comparison of future Austrian *B. anthracis* isolates
Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH


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