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Molecular Diagnostics and Interpretation used in the 2001 Amerithrax Case

Sarah E. Schmedes, M.S., Ph.D. Candidate
Institute of Applied Genetics
Department of Molecular and Medical Genetics
University of North Texas Health Science Center
Fort Worth, TX, USA

ESCMID Postgraduate Technical Workshop
Application of Molecular Diagnostics in Forensic Microbiology
Leuven, Belgium 2-3 June 2016

In the Aftermath of 9/11



7 DAYS LATER



Consequences of Attack

- The anthrax letter attack resulted in 22 infections and five deaths
 - Florida, New York, Washington, D.C.
- Major health scare
- Close in time to 9-11 attack – now a bioterrorist attack which might still be under way
- First major bioterrorism investigation
- Demonstrated that dissemination of a bioweapon did not require sophisticated technology
- Changed view regarding our nation's security system

Confirmed Anthrax Cases

Following are cases of anthrax confirmed by the Centers for Disease Control and Prevention. Other suspected cases remain unconfirmed by the CDC. They include two workers at the New York Post and a second NBC worker.



| VICTIM | KIND | LOCATION | CONFIRMED | STATUS |
|--------------------------|---------|--------------------------------------|-----------|---------------|
| Bob Stevens, 63 | Inhaled | American Media, Boca Raton | Oct. 4 | Died Oct. 5 |
| Erin O'Connor, 38 | Skin | NBC in Manhattan | Oct. 12 | Recovering |
| Boy, 7 months | Skin | ABC in Manhattan | Oct. 15 | Recovering |
| Ernesto Blanco, 73 | Inhaled | American Media, Boca Raton | Oct. 15 | Left hospital |
| Claire Fletcher, 27 | Skin | CBS in Manhattan | Oct. 18 | Recovering |
| Teresa Heller, 32 | Skin | West Trenton post office | Oct. 18 | Recovering |
| Patrick O'Donnell, 35 | Skin | Hamilton Township mail center, N.J. | Oct. 19 | Recovering |
| Leroy Richmond, 57 | Inhaled | Brentwood mail center, D.C. | Oct. 21 | Hospitalized |
| Unnamed man | Inhaled | Brentwood mail center, D.C. | Oct. 22 | Hospitalized |
| Thomas L. Morris Jr., 55 | Inhaled | Brentwood mail center, D.C. | Oct. 23 | Died Oct. 21 |
| Joseph Curseen Jr., 47 | Inhaled | Brentwood mail center, D.C. | Oct. 23 | Died Oct. 22 |
| Unnamed worker, 59 | Inhaled | State Department mail center, D.C. | Oct. 25 | Hospitalized |
| Unnamed woman, 56 | Inhaled | Hamilton Township mail center, N.J. | Oct. 28 | Hospitalized |
| Non-postal worker | Skin | Works near Trenton, N.J. | Oct. 29 | Left hospital |
| Unnamed worker | Inhaled | Hamilton Township mail center, N.J. | Oct. 30 | Left hospital |
| Kathy Nguyen, 61 | Inhaled | Manhattan Eye, Ear & Throat Hospital | Oct. 30 | Died Oct. 31 |

SOURCES: Centers for Disease Control and Prevention, staff and wire reports

THE WASHINGTON POST

Demonstrated the need for a coordinated national response for threat assessments countermeasures and forensics for biological agents

Covert Attack

- First victim, Robert Stevens, became ill on October 2, 2001
 - Employee of American Media in Boca Raton, Florida
- Presumed bacterial meningitis
 - Examination of the Gram stain of the cerebrospinal fluid sample revealed gram-positive bacilli singly and in chains
 - A diagnosis of anthrax was considered
- Presumptively identification of *Bacillus anthracis* within 18 hours of inoculating the cerebrospinal fluid onto bacterial culture plates
 - Confirmed by LRN laboratory within the Florida Department of Health the next day



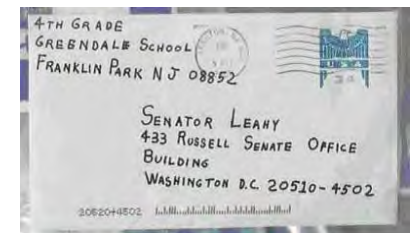
Covert Attack

- On October 4th the Florida Department of Health informed the public
 - A case of inhalational anthrax had been confirmed
- This case immediately raised concern epidemiologically because the last reported case of inhalational anthrax in the United States occurred in 1976
- During the subsequent investigation, extensive environmental sampling detected the presence of *B. anthracis* spores on the Robert Steven's computer keyboard at his workplace and in the mailroom at American Media
 - Uncommon place to find spores of *B. anthracis*
 - Likely an act of bioterrorism or biocrime
- *B. anthracis* spores in regional and local postal centers that processed mail destined for the American Media building, implicated one or more mailed letters or packages as the probable source of exposure

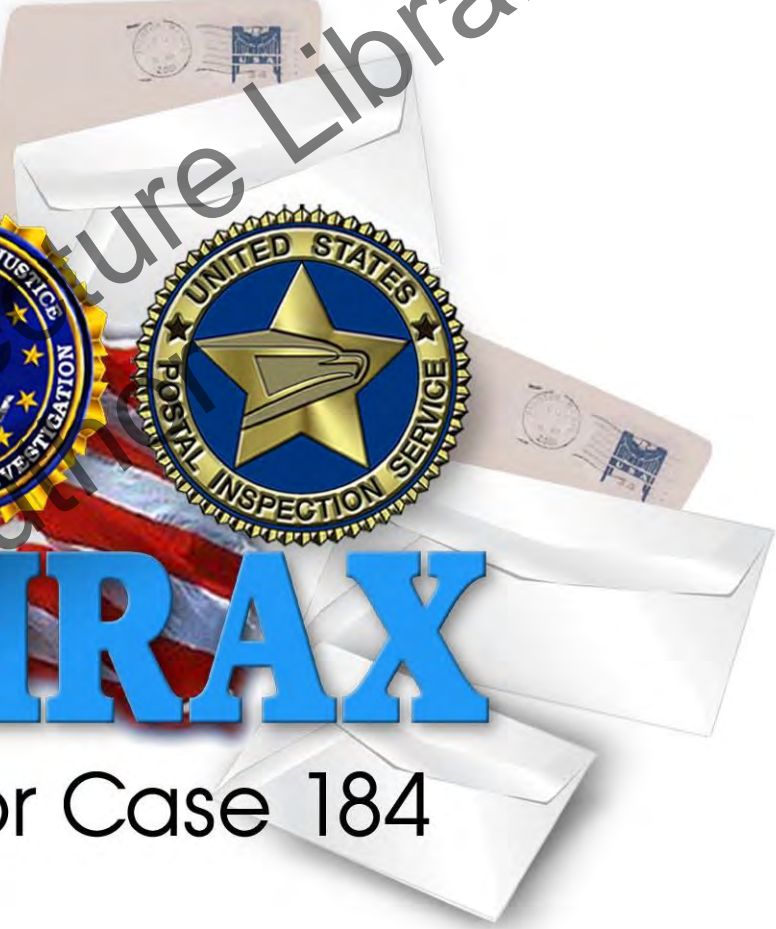


Overt Attack

- Subsequently, letters containing anthrax spores were discovered at the offices of three major television broadcasting companies in New York City (NBC, CBS, and ABC), at the newspaper headquarters of the *New York Post*, and in the Washington, DC, offices of Senator Patrick Leahy



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AMERITHRAX

Major Case 184

Microbial Forensics Work

- The technologies and methodologies were still nascent, laborious, time-consuming, and costly
 - Limited sequencing technology
 - NGS had yet to be invented
- Little expectation that the level of individualization enjoyed for human DNA analyses would be possible when applied to microbial genetic evidence
- Any reduction in possible sources of the spores in the letters was important
 - Eliminate unlikely leads
- Any signature markers could provide possible leads
- Strain of *B. anthracis* was determined to be the Ames Strain
- More likely obtained from a laboratory source than from the environment

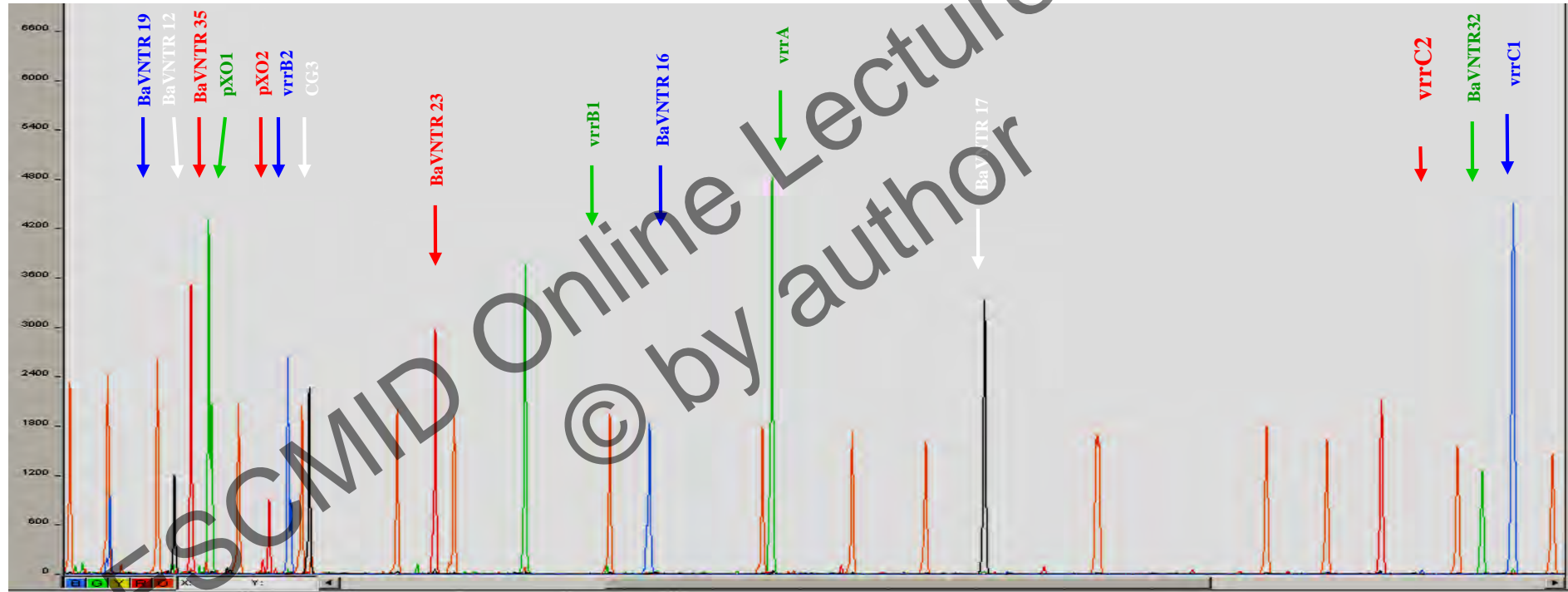
Bacillus anthracis

- A global pathogen
- Highly genetically homogeneous
- Methods to discriminate among isolates had little success until molecular genetic markers were applied
- High resolution of multiple locus variable number tandem repeat (VNTR) analysis (MLVA)
 - First developed for *B. anthracis* and *Mycobacterium tuberculosis*

MLVA Test

- An eight-locus system (MLVA8)
- A large database was being used actively at both the CDC and NAU
- The allelic profiles between the isolate from the index case and the Ames strain were consistent at all eight loci
- The profile was not unique
 - A Texas goat isolate from 1997

A 15 locus 4-color Genotyping System (VNTRs and STRs)

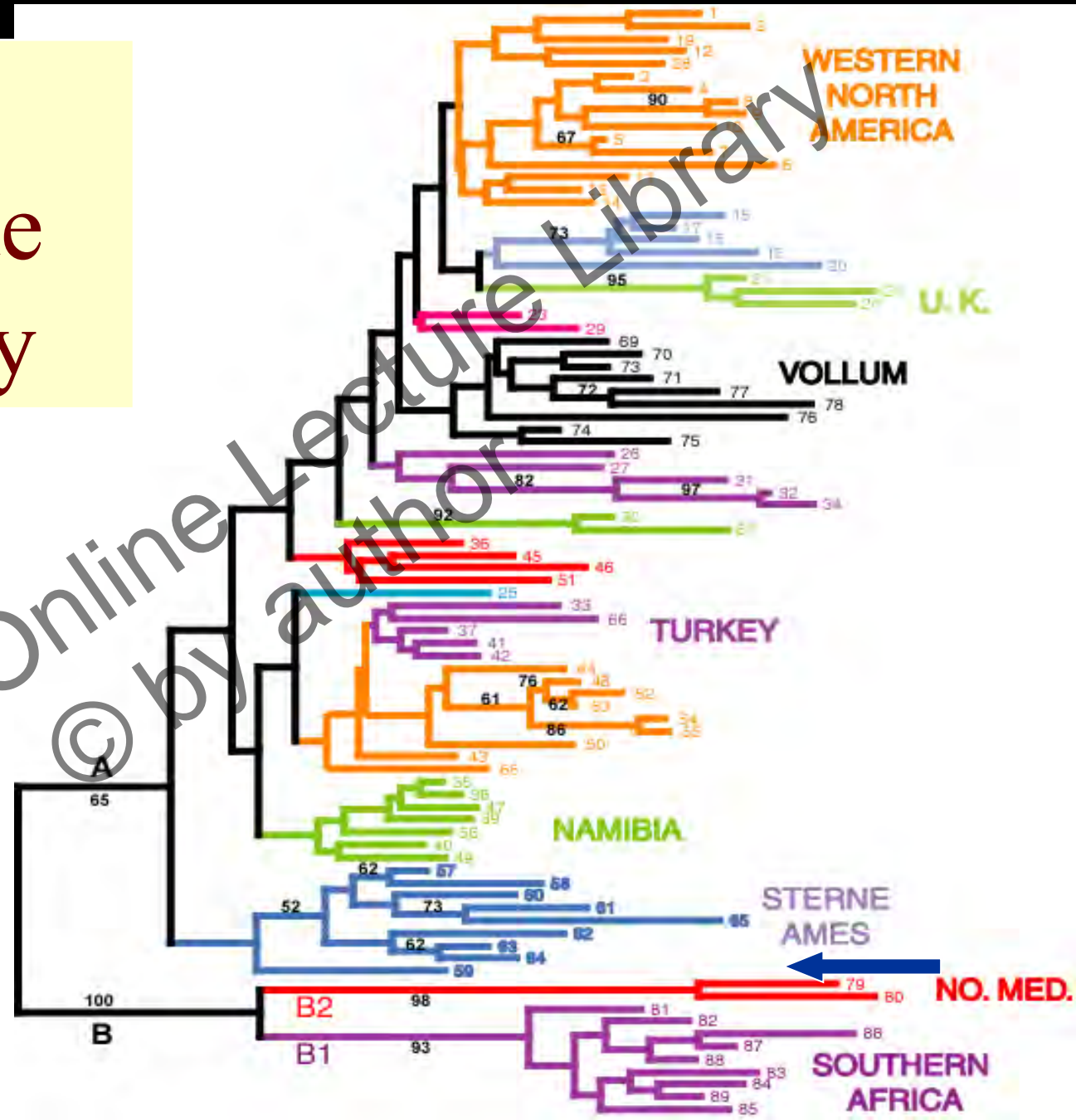


B. Anthracis
Ames Strain Identification

Anthrax Worldwide Phylogeny

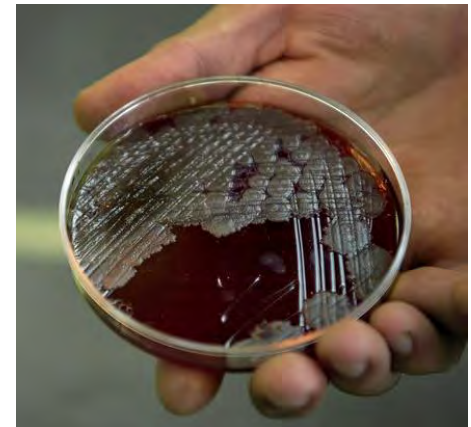
What lead may this provide?

- Ames genotype (hence strain) is not common
- The lineage is ecologically established in West Texas
- Nearest relatives are a few Texas isolates, then Chinese isolates
- Suggested laboratory source



Ames Strain

- Common in many research laboratories
 - Used as a vaccine challenge strain due to its high virulence
- Involvement of a laboratory strain in the index case reinforced suspicions that this was a nefarious event and not a case of naturally acquired anthrax
- Focused on laboratory source
 - Not as likely to have been found in nature by terrorist



Sequencing the Genome

- The Institute for Genomic Research (TIGR) in Rockville, Maryland (2002)
- Sequence the genome of the evidence and compare with a reference sample
- Genome sequence of the isolate from the index case, aka the Florida Ames strain
 - Completed in ~3 months
- **Provided little investigative value**
 - No genome database existed for comparison
- The only other *B. anthracis* genome available was that of an attenuated strain
 - Porton Down Ames isolate
 - Which was partially completed
 - Was found to be highly similar to that of the Florida Ames strain
 - A few SNPs and indels were identified
 - Little value because the Porton Down strain had been cured of its plasmids by mutagenic treatments and the differences could have been the results of this treatment



Sequencing the Genome

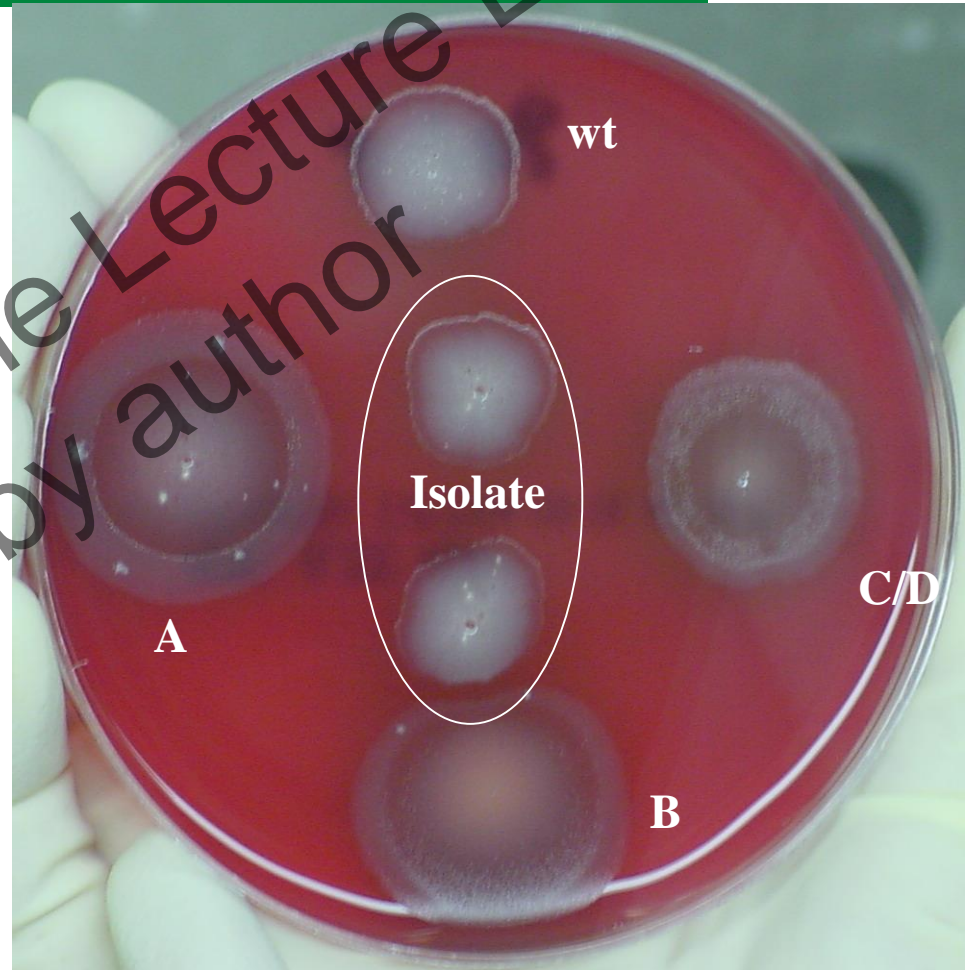
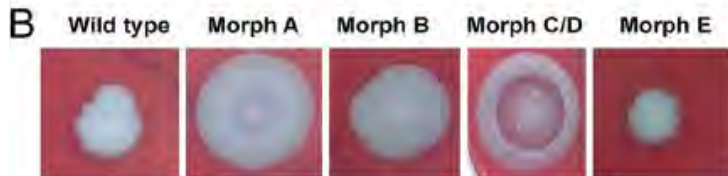
- Subsequently, the whole genome sequence of *B. anthracis* Ames Ancestor (Texas Goat) demonstrated that the differences in the Porton Down sample provided no investigative value
 - They were unique to the Porton Down sample
 - No insights into the origin of the Florida Ames sample
- Florida Ames strain showed no differences from the ancestral Ames isolate

Investigation

- FBI collected information from anthrax research laboratories concerning their collections of strains
 - Under subpoenas requesting detailed inventories of strain collections
- 16 laboratories in the US working with and/or housing the Ames strain
- 1077 samples collected
 - May have represented every single culture of the Ames strain in the United States
 - Although it cannot be verified that this repository was 100% complete, certainly was the most comprehensive collection possible

Anthrax from Mailings Contained >99% Wild Type

- Wild-type, A, B, and C/D around
- Perimeter of plate were isolated from the Leahy letter
- A,B, C/D are Morphological Variants



Signatures

- All of the anthrax-letter variants studied were poorly sporogenic compared with the wild-type Ames ancestor
 - *oligosporogenic phenotype* has secondary effects upon other phenotypes, including colony morphology
- Four morphological variants were purified, studied extensively, and used to attempt to eliminate potential sources of the letter spores
 - Sequenced at TIGR
 - Compared to sequence of the Ames ancestor strain
 - This sequence was identical to the Florida Ames isolate
 - All the letter isolates were all identical to the Ames ancestor

Signatures

- The variants were not observed in original sample
- Genome sequences of the morphological variants were very similar
- However, each contained one minor difference
 - Likely to be the basis of the phenotypic variations
 - Included SNPs, indels, and large duplications
- In three of the four variants, the genetic variation was in or near genes involved in sporulation
 - Phosphorylation of *spoOF*/*spoOA*, dephosphorylation of *spoOF*, and near the *spoOF* gene itself
- One case, the mutation was on the plasmid pXO1

Testing of Repository

- PCR-based assays for these variants were developed
 - TIGR, Midwest Research Institute, and Commonwealth Biotechnologies Inc.
- Assays were performed on DNA from the 1077 FBI repository samples
- All analyses were done blindly and independently by the different contractors
- Data interpretation and conclusions were then made by FBI scientists, independent of the performance laboratories

Testing of Repository

- 8 repository samples contained signatures for all four of the morphological variants
- All derived from the spore stock at USAMRIID known as RMR1029 including RMR1029
 - False negatives may increase number



Points for Discussion

- What are important considerations when dealing with a particular pathogen? What should be known?
- What are important considerations about using/selecting a proper reference sample?
- What would have been done today with NGS? And why?
- What approach would you propose to characterize evidence and repository/reference samples for the 4 morphological variants?

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ACKNOWLEDGMENTS

#1

- Paul Keim
- Jacques Ravel
- Bruce Budowle

- ESCMID
Workshop
Organizers and
Course
Coordinators

Research Team



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