

The Role of Public Health Laboratories in Supporting Investigations of Food-borne Illness Outbreaks



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Presentation Outline

- Basic concepts of molecular epidemiology
- Summary of testing activities at the MD DHMH Laboratories that are performed in support of epidemiological investigations of outbreaks
- Overview of sub-typing bacterial pathogens using PFGE techniques
- What newer molecular sub-typing technologies will eventually supplement or replace PFGE?



Molecular Epidemiology Basic Concepts

- Clonal Nature of Microorganisms
 - Generally asexual reproduction: binary fission
 - Clones of microorganisms are genetically identical or (nearly identical)
 - Common- source microorganisms of outbreaks in generally are clonal (the progeny of a single cell)
- At the bacterial species level there is sufficient genetic diversity to identify genetically different clones or clonal groups (strains that have a high degree of genetic relatedness) among isolates of that species that were collected from different sources, locations and at different times.



Sub-typing of Microorganisms

- Sub-typing refers to laboratory methods that are used to examine several characteristics of a group of bacterial isolates that allows for discrimination below a species level to identify clones or clonal groups.
- Phenotypic markers: bio-typing, serotyping, bacteriophage typing, bacteriocin typing, anti-microbial resistance etc.).
- Molecular markers: based on the physical characterization of bio-molecules (nucleic acids, proteins, LPS, fatty acids). Most common nucleic acid based sub-typing methods: genotyping.



Molecular Epidemiology Operational Definition

- Molecular epidemiology (as applied to the investigations of infectious disease outbreaks): is the physical characterization of bio-molecules of different isolates of pathogenic microorganisms of the same species to determine their degree of genetic relatedness . Ultimately to identify highly related organisms as the potential source of an outbreak or to recognize isolates from subsequent cases that are associated with a discrete outbreak .

DHMH Public Health Microbiology Laboratories: Sources of Foodborne Outbreak Specimens

- Patient isolates from hospital or commercial labs are sent directly for confirmation and further characterization as part of routine pathogen surveillance
- Clinical specimens from outbreak associated individuals are submitted directly from LHD's for isolation and identification
- Isolates of bacterial pathogens from food items collected during outbreak investigations



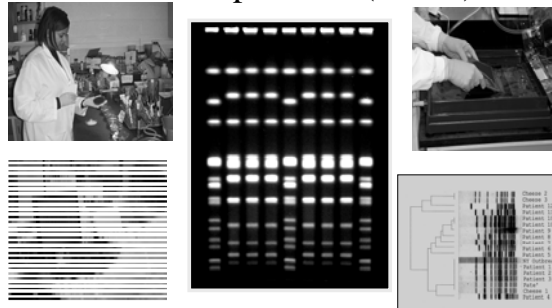


Confirmation and Serotyping of Salmonella Isolates

- Sub-culture and re-isolate putative Salmonella cultures
- Biochemically confirm and characterize to sub-species
- Serotype Salmonella isolates
 - “O” Somatic Antigen (slide agglutination)
 - “H” monophasic or biplasic flagular Antigens (tube agglutination)
- *Salmonella St. Paul* (Serotype): (Kauffmann-White scheme (Subspecies I, [Group B: O Ag] 4,5,1,2:[1st. Phase H Ag] e,h,[2nd. Phase H Ag]:1,2)
- 5-10 days to complete confirmation and typing of isolates

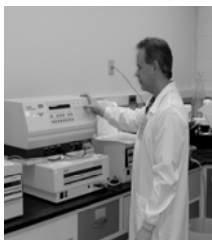


Pulsed Field Gel Electrophoresis (PFGE)



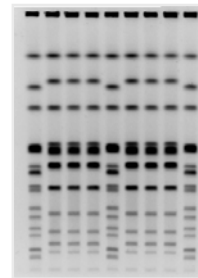
Laboratory Investigations: Molecular Epidemiology/PFGE

- PFGE: Molecular “fingerprinting” technique to establish the degree of genetic relatedness between isolates of the same species or serotype
- CDC provides: nationally standardized procedures, proficiency testing programs, pattern data bases and data sharing between State Federal and Labs via the Pulse –Net USA

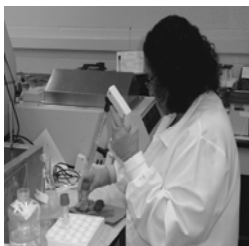


Standardized PFGE Procedures for Bacterial Pathogens

- *E.coli* 0157(H7)
- Non-0157 (H7) STEC's
- *Salmonella* sp.
- *Listeria monocytogenes*
- *Shigella* sp.
- *Campylobacter*
- *Neisseria meningitidis*



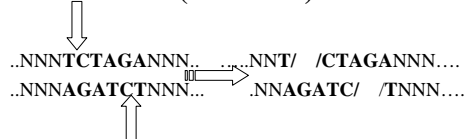
PFGE Basic Principles:



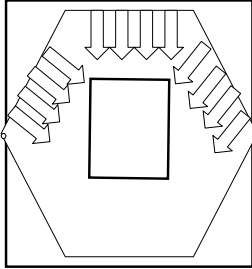
- 1.) “Pure Culture” of Bacterial Pathogen Isolated and Typed
- 2.) Bacteria Imbedded into Gel Plugs
- 3.) Gently Lyse Bacteria in the Plug
- 4.) Cut Bacterial Chromosome(DNA) into (10-20) Fragments using Specialized Enzymes (Restriction Endonucleases)

Restriction Endonucleases

- Cut (ds)DNA at specific genetically determined sites along the chromosome (ex. Xba-I)



PFGE Basic Principals



- Computer Controlled/ alternating electrical fields (pulses)
- DNA fragments snake through agarose gel
- Large DNA fragments (2-10Mb) can be resolved
- Most bacterial PFGE DNA fragments 700KB-30KB

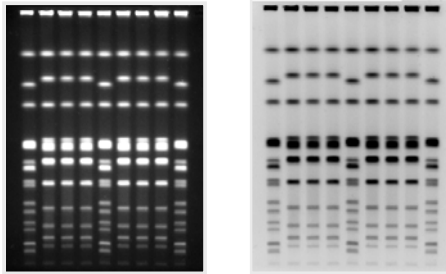
PFGE Basic Principles:

- 5.) Separate the DNA Fragments in an Agarose Gel by PFGE
- 6.) Stain DNA with a Fluorescent Dye and Visualize the Separated Fragments within the Gel
- 7.) Electronically capture an image of the PFGE fragment pattern



Imaged PFGE Gel

Example: *Salmonella serotype* Enteritidis

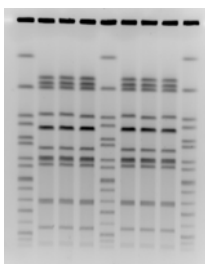


PFGE Basic Principles:

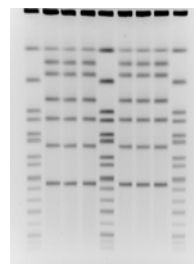
- 9.) Utilize Computer Software to Analyze and Compare PFGE Patterns
- 10.) Electronically store PFGE patterns in local databases and transmit patterns to national databases (Pulse Net) for future reference



Salmonella St. Paul : Tomato Outbreak Patterns



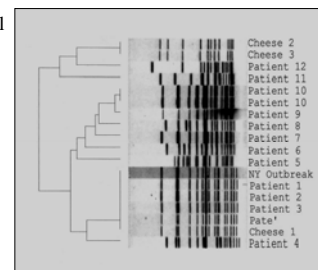
XbaI Digestion

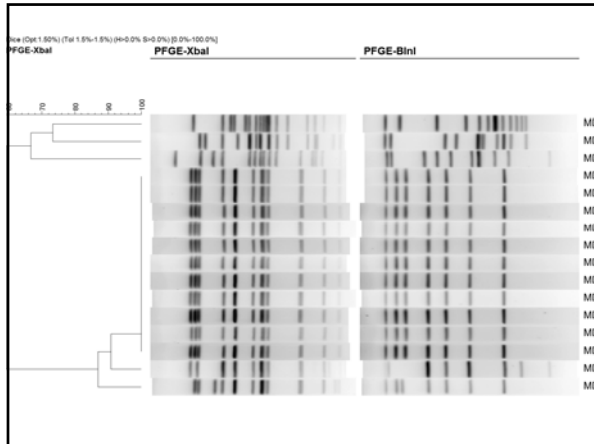


BlnI Restriction

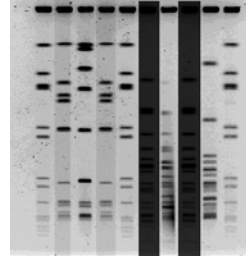
PFGE Data Analysis

- Comparison of PFGE patterns to local and national databases
- Dendrogram of PFGE Patterns:
 - Indistinguishable Outbreak associated PFGE patterns in "red"
- Total PFGE testing turn around-time (TAT) 3-4 working days for receipt of "pure culture"



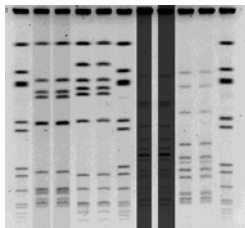


Identical PFGE Patterns: Epidemiologically Linked Patients?



- *Listeria* PFGE patterns for patients (#1 & #2) were indistinguishable possibly indicating a common source of infection
- Asc-I patterns: highlighted in yellow
- Apa-I patterns: highlighted in purple

PFGE Patterns: Linking Human to Food Isolates

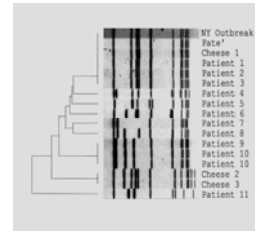


- Mousse Truffee `Pate` and Port Salut cheese had indistinguishable patterns with 3 of the patient isolates
- Asc-I patterns: highlighted in yellow
- Apa-I patterns: highlighted in purple



PFGE Communications and Cooperation with Epidemiology

- Lead Scientist in the PFGE Lab monitors the PulseNet WEB board postings daily
- Immediately informs DHMH EDCP epidemiologists of matches to local patterns via E-mail
- Posts local patterns on PulseNet WEB board when necessary for possible associations with outbreaks in other states



Limitations of PFGE

- PFGE is laborious, it has relative low through-put, and requires precise standardization.
- Some organisms are highly clonal with only limited number of PFGE patterns(ex. *Salmonella serotype* Enteritidis) resulting in lack of discrimination between isolates to effectively detect clusters and outbreaks
 - Two PFGE patterns represent 48% of *S. Enteritidis* isolates in the USA
 - In Maryland approx. 30% (257/873) of the *Salomonella* isolates sent to the PFGE lab for testing in 2007 were *S. Enteritidis*.
 - Yet only 3 distinct PFGE patterns represent 74% of *S. Enteritidis* isolates in Maryland



Newer Molecular Sub-typing Technologies: MLVA

- Multiple-Locus, VNTR (Variable Number Tandem Repeat) Analysis [MLVA]
 - PCR amplification and fragment size analysis of polymorphic regions of DNA containing VNTR's
 - Quantitative analysis of small number of selected repetitive regions of DNA (VNTR) that vary in frequency and copy number between strains
 - Knowledge of the complete genome sequence of organism and software are needed to select candidate VNTR loci that are most suitable for sub-typing
- VNTR approaches have been used by forensic laboratories to identify individual humans in criminal investigations



MVLV Basic Principles

- VNTR : Variable –Number Tandem Repeat

Locus 1

StrainA: VRNT array4X3

atgggtnaatccgctg[ACgC][ACgC][ACgC]gccatcgat

StrainA: VRNT array4X5

atgggtnaatccgctg[ACgC][ACgC][ACgC][ACgC][ACgC]gccatcgat

Locus 2

StrainA: VRNT array 3X4

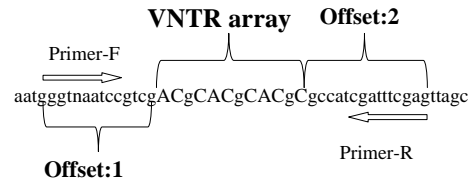
ggtaccggtaaagcgc[ACC][ACC][ACC][ACC] gcacggtg

Strain B: VRNT array 3X5

ggtaccggtaaagcgc [ACC][ACC][ACC][ACC][ACC]gcacggtg



MLVA Terminology



Amplicon size = offset-1+ offset-2 +VNTR array

VNTR copy number = $\frac{\text{Amplicon Size} - (\text{offset:1} + \text{offset:2})}{\text{VNTR repeat size (e.g. 4)}}$

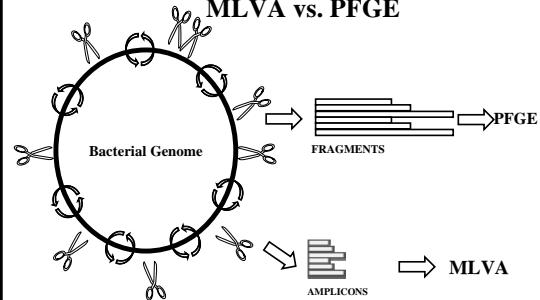


MLVA Procedures

- Amplify predetermined VNTR loci by PCR with dye labeled forward primers
- Separate PCR VNTR amplicons on the basis of size using capillary electrophoresis and fragment analysis software on an automated nucleic acid sequencer
- Assign variant score to each fragment based on size . Score amplicons from several selected VRNT loci
- Use software to establish phylogenetic relationships between isolates based on the difference of MLVA fragment analysis

Fragment-Based Subtyping Methods

MLVA vs. PFGE



In order to compare data across multiple runs, fragment-based methods require careful sizing and normalization.



PulseNet Implementation of MLVA

- CDC in collaboration with selected PulseNet Labs have developed MLVA protocols for; *E. coli* O157(H7), *S. Enteriditis* and *S.Typhimurium*
- Wide spread use of MLVA by PulseNet Labs has not yet occurred
- In comparison to PFGE MLVA does not always demonstrate superior discriminatory power
- MLVA results have not always been reproducible between laboratories using different automated nucleic acid sequencers



Newer Molecular Sub-typing Technologies: SNP's

- Single Nucleotide Polymorphisms (SNP's)
- SNP's are single nucleotide differences in a genetic sequence
- They typically are selected to have only two different variants or alleles
 - One of the alleles usually occurs rarely at a frequency of <1%
- SNP's can occur in both coding and non-coding DNA sequences
- At the protein level the mutations can result in synonymous (silent mutation) amino acid changes, non-synonymous amino acid changes or the termination of protein elongation (stop codons)

