PFGE: Tips and Tricks to Success and Interpretation of Results for Foodborne Outbreak Investigations

BioNumerics Workshop for PulseNet Participants

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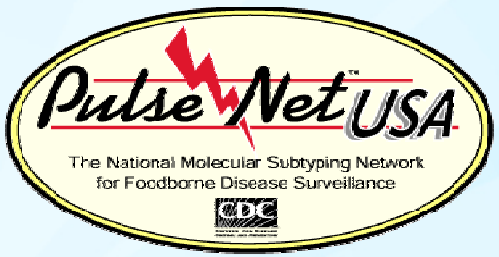


National Center for Emerging and Zoonotic Infectious Diseases

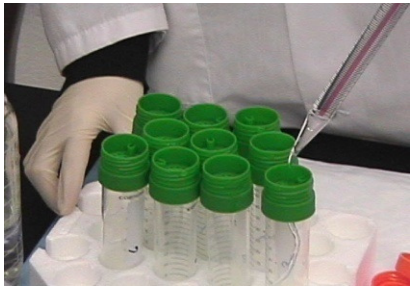
Division of Foodborne, Waterborne, and Environmental Diseases

PulseNet Standardized Protocol for PFGE

patient specimen collection

pure culture

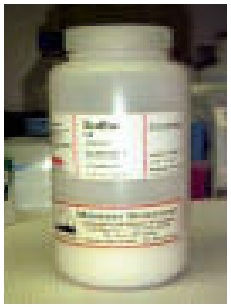
grow isolated colony



cell lysis and plug washing

cells trapped in plug

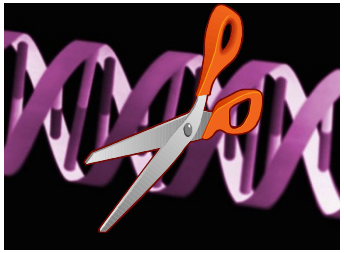
=



agarose

+

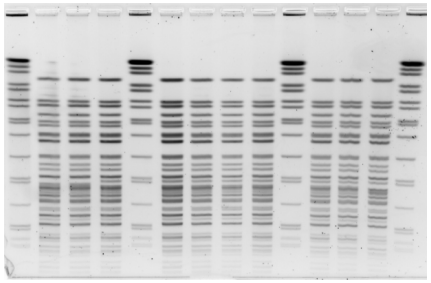
cell suspension



restriction

electrophoresis

imaging



tiff for analysis

Interpretation of PFGE Data

- Technical artifact vs. genetic variation
  - Technical artifact
    - Effect of reproducibility
    - “Ghost” or “shadow” bands
    - Effect of resolution
    - Pattern analysis
  - Genetic variation
    - Expected degree of variability
    - Dependent on the organism being studied

Garbage In → Garbage Out

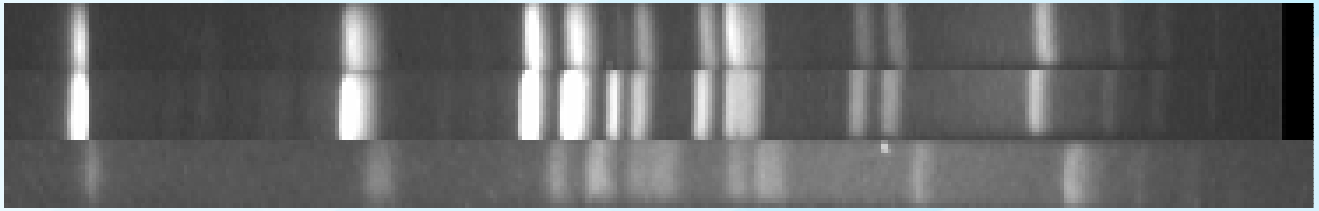
- Follow the most current protocol
- Start with a pure cell culture
  - grow 14 – 18 hours
  - use non-selective media
  - do NOT vortex cell suspension
- Use quality reagents
  - purchase molecular grade or QC in-house, sterile when necessary
  - throw out contaminated or expired reagents
  - track vendors, lot numbers, dates, etc…
- More is not better – more is just more
  - units of enzyme, enzyme incubation time, agarose
  - exception: washes

- Use good equipment
  - confirm temperature (H_2O bath, fridge/ freezer, etc…)
  - use clean glassware, plasticware
  - discard rusty/nicked spatulas, blades
  - use only aloe- and powder-free gloves
  - CHEF Mapper/DRIII maintenance
    - level, temperature, pump, tubing, electrodes, decontaminate
  - imager maintenance
    - clean lens, clean platform, focus

Reproducibility

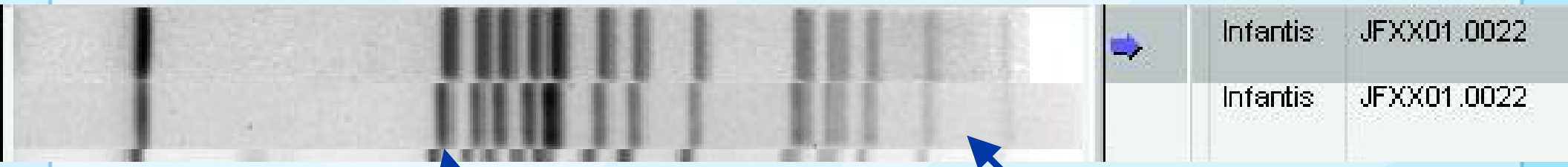
3 isolates of *Salmonella* Schwarzengrund digested by *Xba*I and run in two different laboratories

Lab 1



Lab 2

Pattern Differences: Artifact vs. Reality

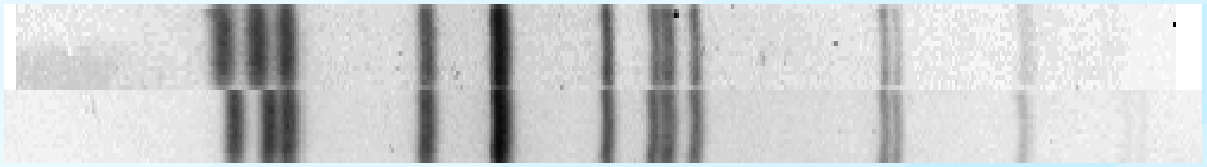


Artifacts?

- *Salmonella* Saintpaul alfalfa sprout outbreak, multi-state (OR) 2003

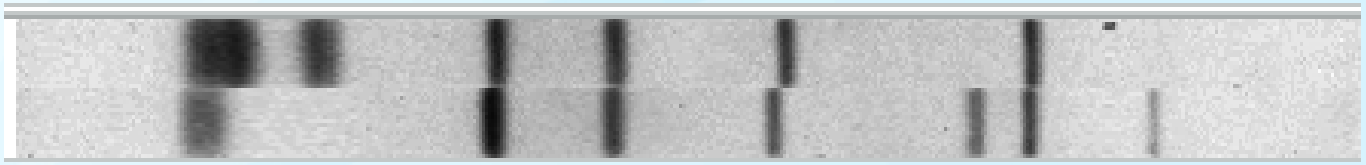
Artificial band shifts? **No!**

***Xba*I**



Outbreak?
Outbreak

***Bln*I**

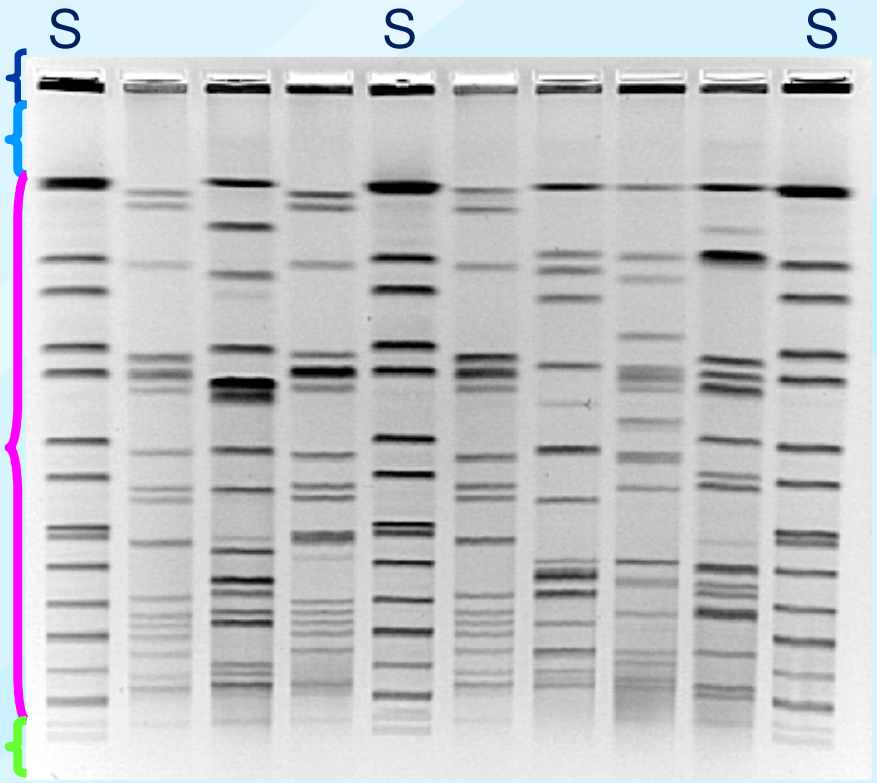


Non-outbreak
Outbreak

Troubleshooting PFGE Gels

Consider all steps of the protocol

- Cell suspension preparation
- Preparation of PFGE plugs
- Lysis of cells in PFGE plugs
- Washing of PFGE plugs
- Restriction digestion of DNA
- Gel electrophoresis of restricted DNA
- Documentation of PFGE gel
- Procedural / processing steps

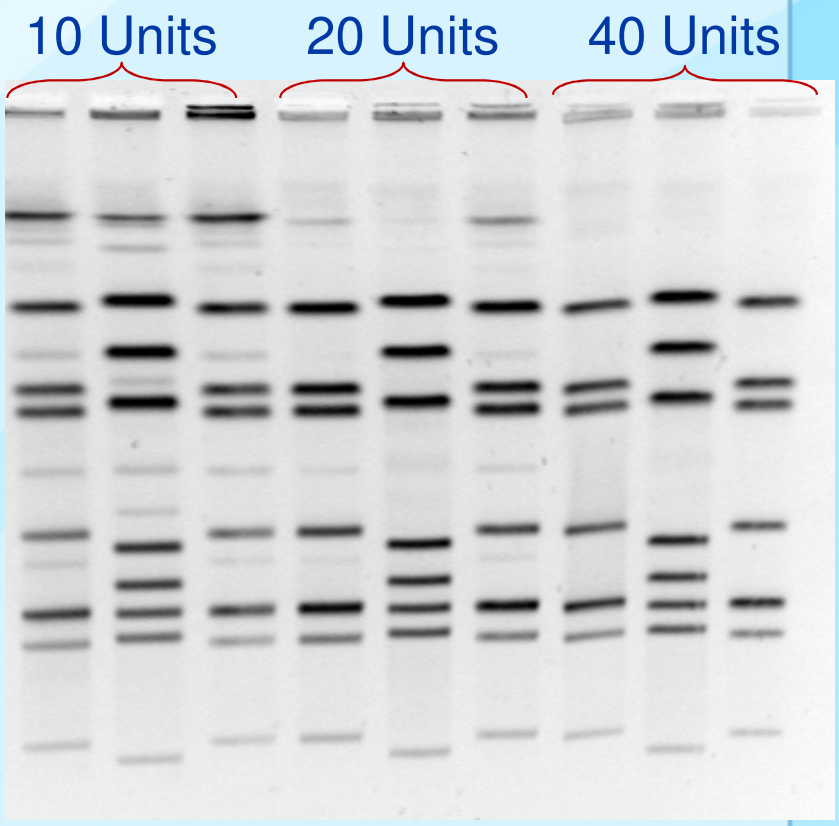


Determine if anything changed since the last “good” gel.

“Ghost” or “Shadow” Bands

- Due to incomplete digestion or star activity
- May be the result of:
  - Poor plug quality
    - proteinase K not washed out of plug
    - enzyme inhibitors not washed out of plug
    - cell concentration too high (DNA and debris)
  - Poor enzyme quality
    - bad lot, change in manufacturing process
    - expired or vial opened frequently
  - Enzyme digestion not optimal
    - old/bad BSA or BSA not included in master mix
    - not enough units of enzyme
    - too many units of enzyme (star activity)
    - incubation time too short
    - incubation time too long (star activity)
    - incorrect incubation temperature
    - incorrect buffer

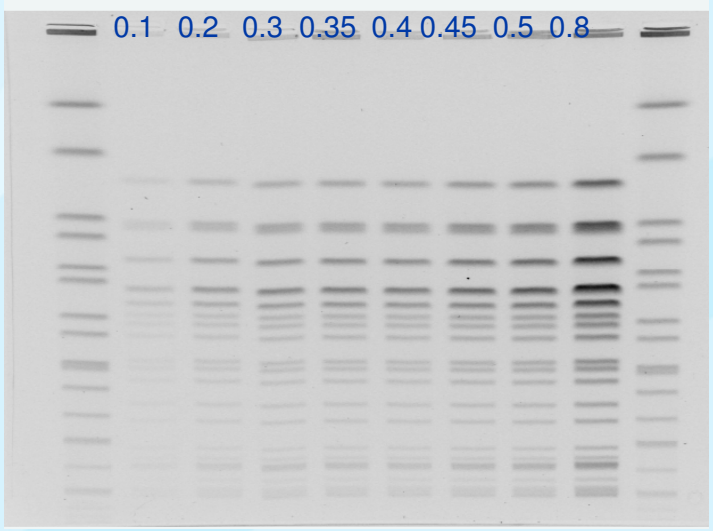
Incomplete digestion of *Campylobacter* DNA due to insufficient units of enzyme



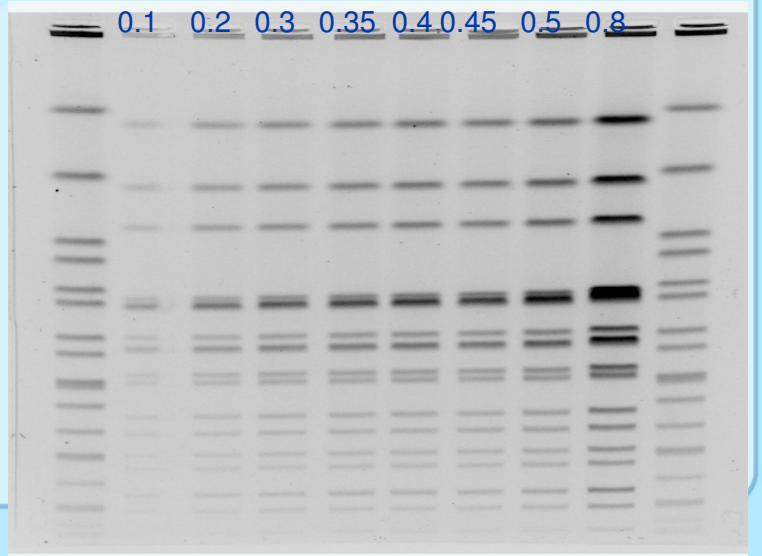
Cell suspension

- Cell suspension concentration
  - Band intensity is relatively similar from cell suspensions of 0.3 to 0.5 (as measured with Dade Microscan turbidity meter)
  - Fewer cells = more efficient lysis = similar band intensity
  - Benefits of lower cell suspension:
    - sharper bands
    - increased resolution of closely migrating bands
    - potential to lower the units of enzyme used

E. coli



Salmonella

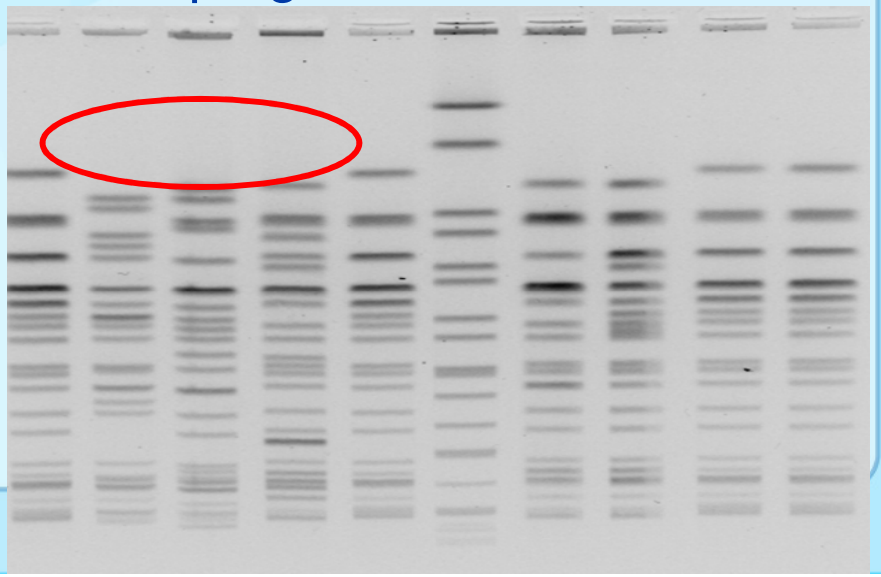


Washing PFGE plugs

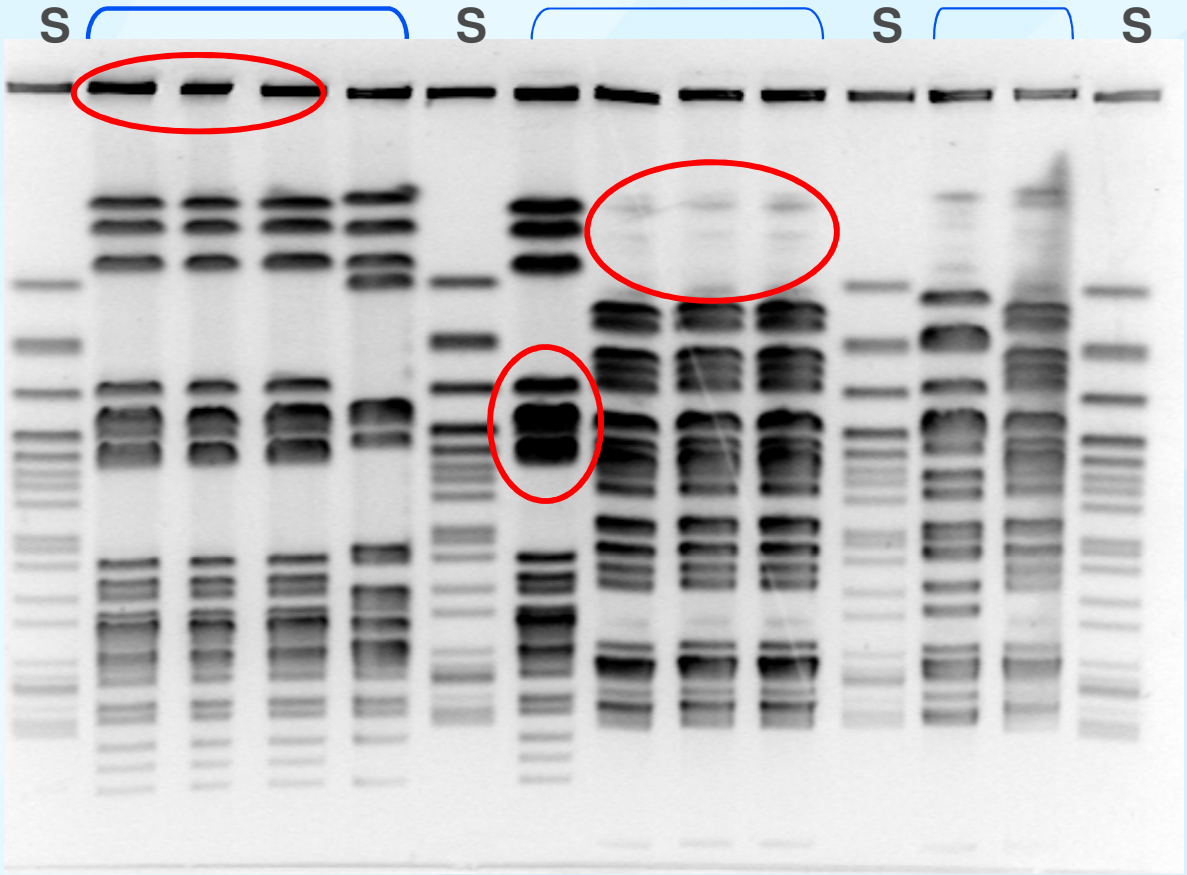
- Washes
  - wash in 10 – 15 ml at 50 – 54◦C for 10 – 15 min with constant agitation (~170 rpm)
  - 2X with sterile clinical laboratory reagent grade water
  - 4X with TE buffer (10 mM Tris:1 mM EDTA, pH 8.0)
- Inadequate washing typically results in incomplete digestion (i.e. ghost bands) and/or smearing
- If your gel has ghost bands
  - wash plugs 2X more with TE buffer
  - cut a new plug slice, digest it, run it

Plugs washed with TE 4X

Same plugs washed with TE 6X

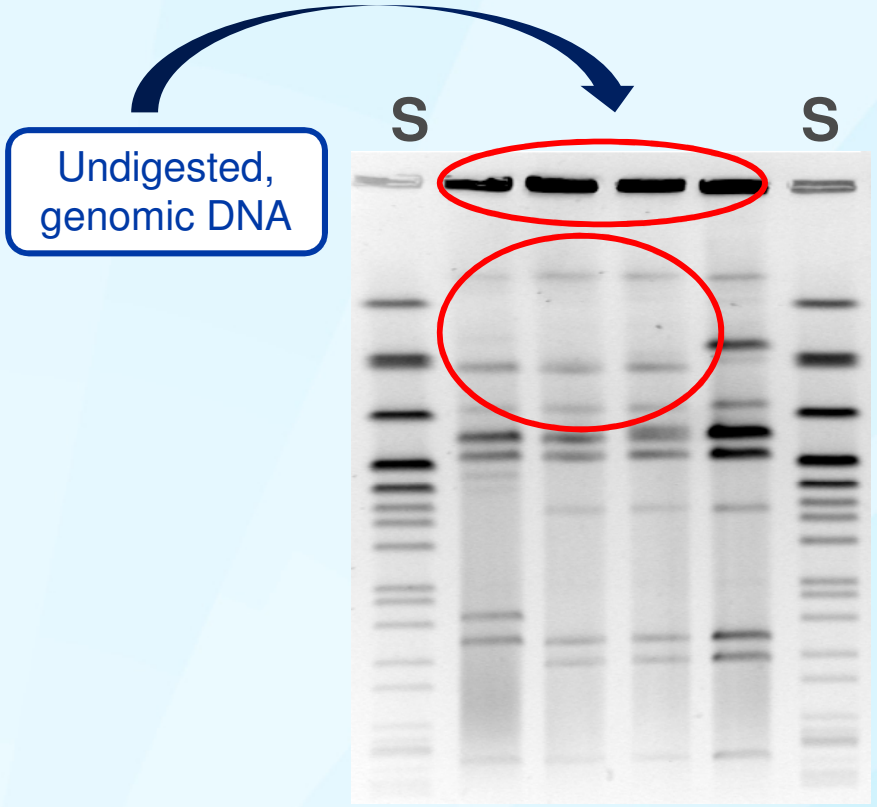


Problems with cell suspensions



- Cell concentration is too high
  - DNA (dark bands) in wells → incomplete cell lysis
  - thick “blurry” bands in lanes
  - more cell debris and more enzyme inhibitors → requires more washing and more proteinase K
  - more DNA → requires more units of enzyme and/or more time for complete digestion

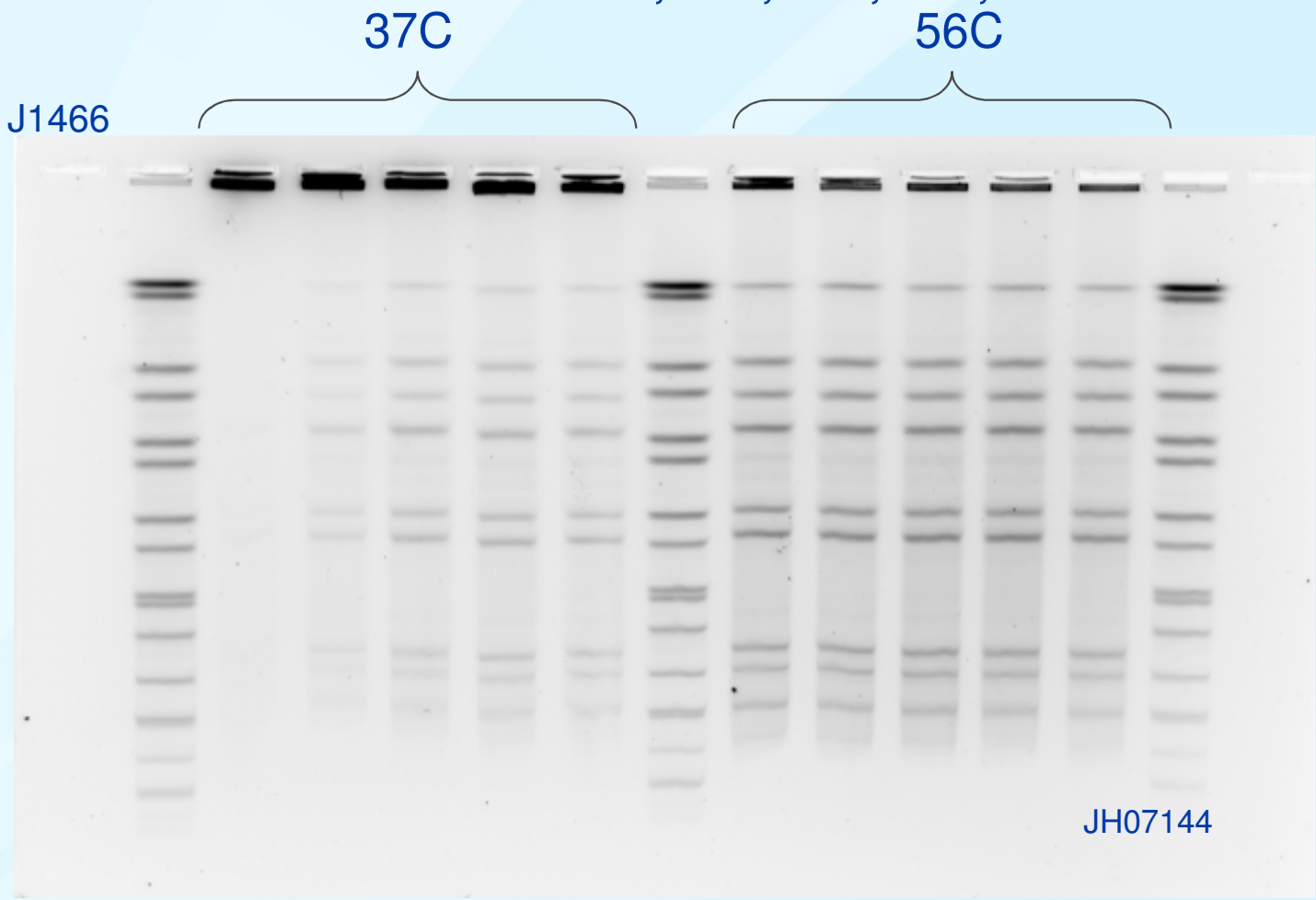
Incomplete cell lysis



- Lyse in 5ml cell lysis buffer (50 mM Tris: 50 mM EDTA, pH 8.0; 1% sarcosyl; 100 µg/ml Proteinase K per sample) at 54ºC with constant and vigorous agitation
- Some organisms lyse better than others
  - lysis times may vary, typically 1 – 4 hours
  - Gram+ organisms more difficult to lyse than Gram- organisms
- Plugs typically clear as cells lyse
- Incomplete lysis indicated by incomplete restriction, smearing, and significant fluorescence in the plug slice

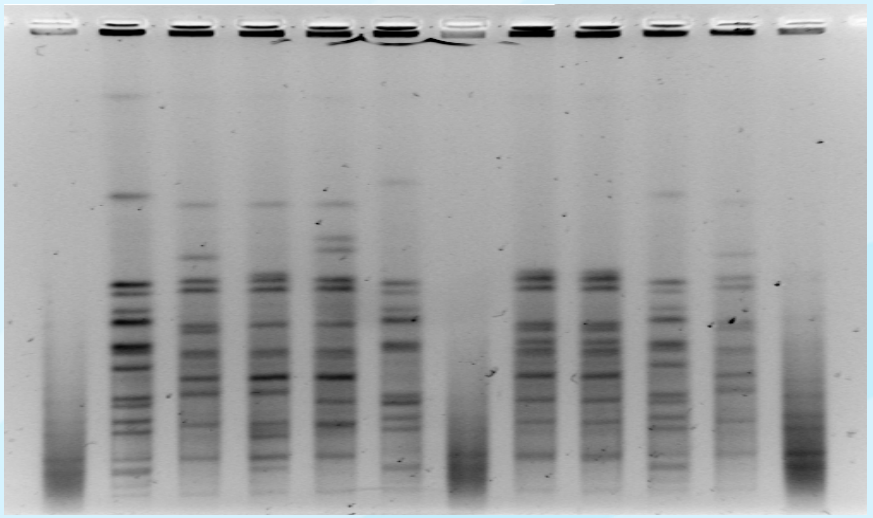
Lysozyme Incubation (only *Listeria*)

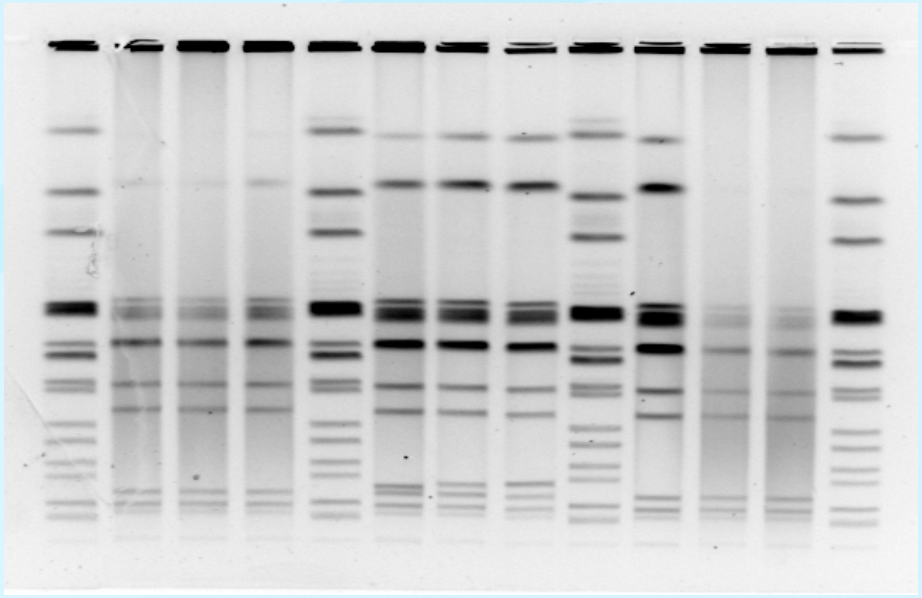
- Wells 2-7: 37℃ for 5, 10, 15, 20, 30 minutes
- Wells 8-13: 56℃ for 5, 10, 15, 20, 30 minutes



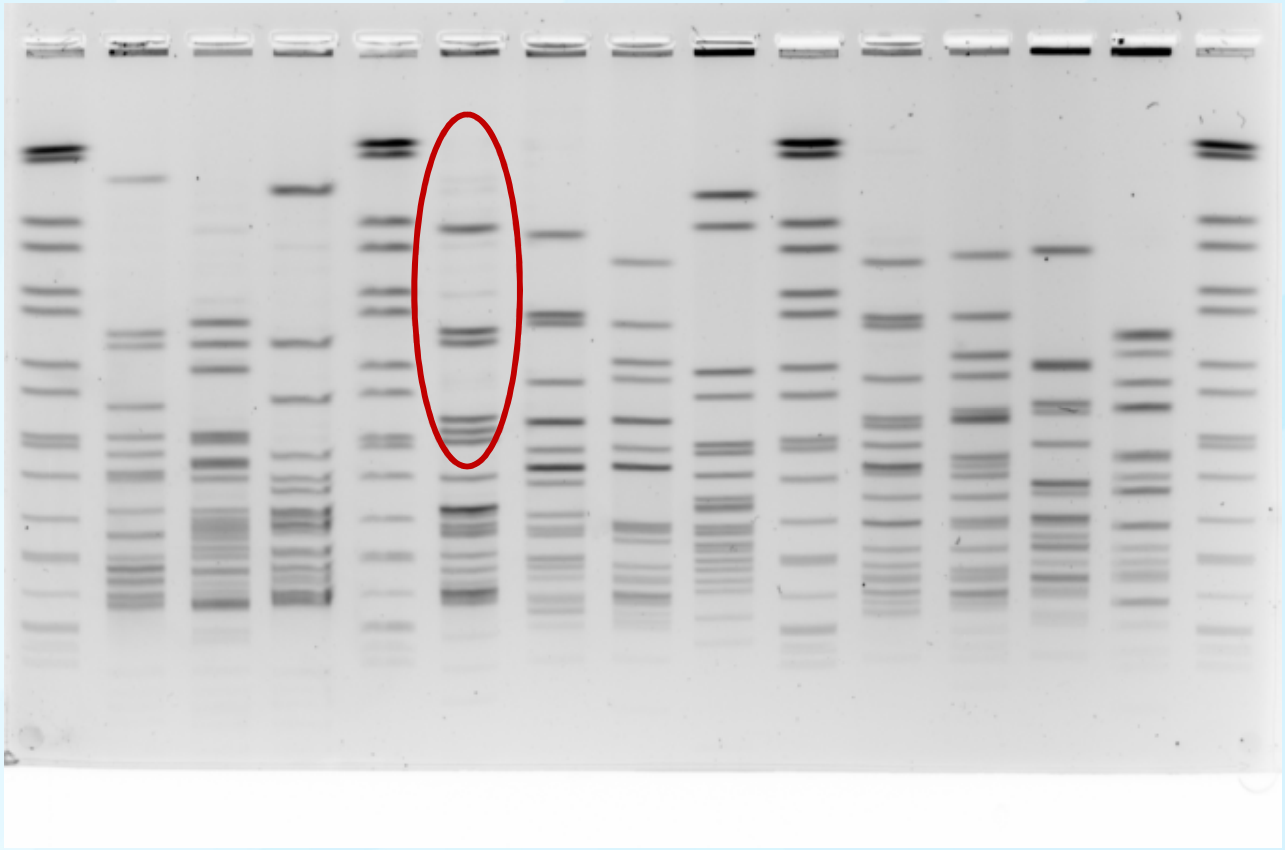
High background

- DNA degraded
- Cell concentration was too high
- Incomplete lysis
- Inadequate washing
- Incomplete restriction



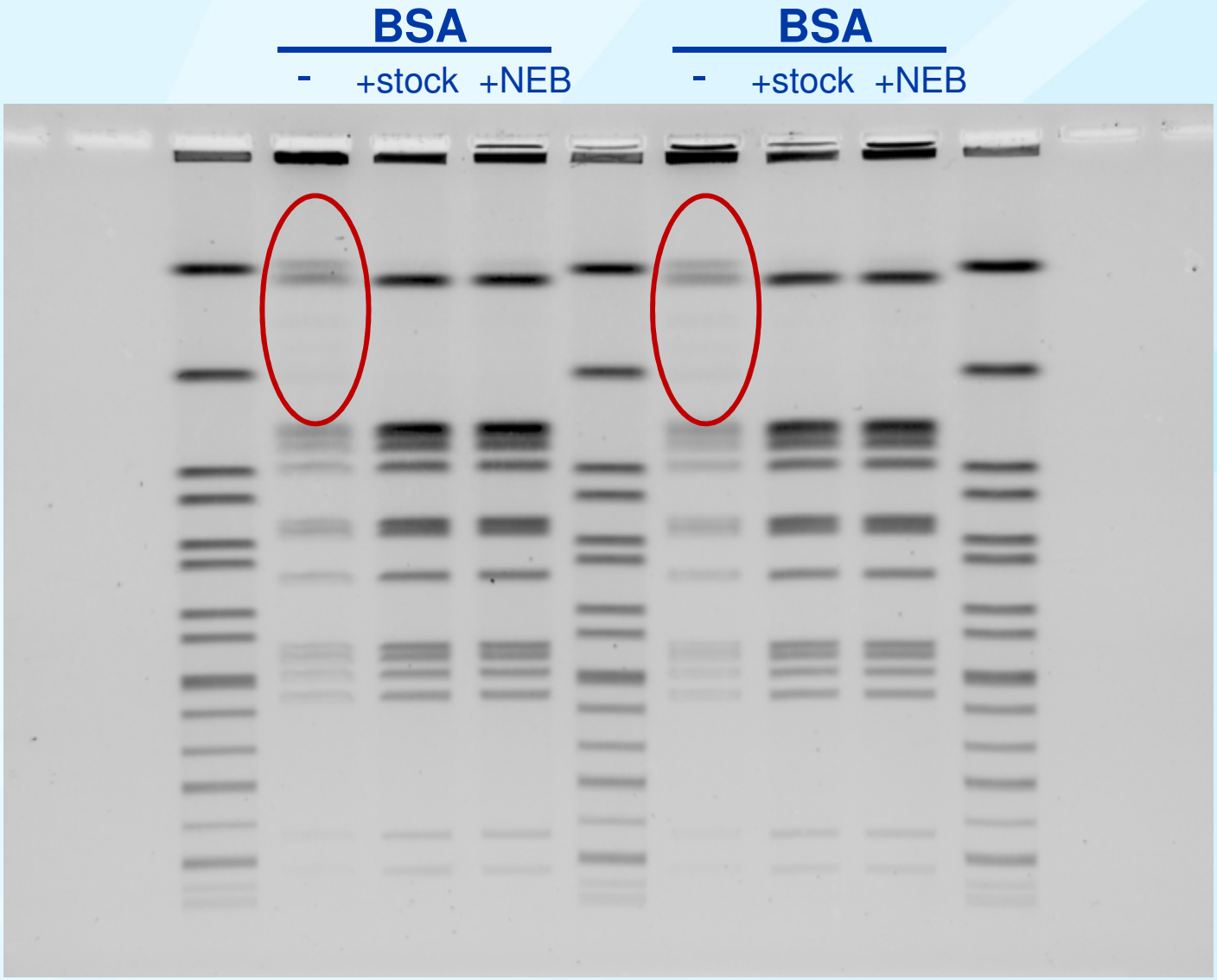


Enzyme digestion without BSA



Listeria monocytogenes digested with *Apa*I (NEB), but without additional BSA

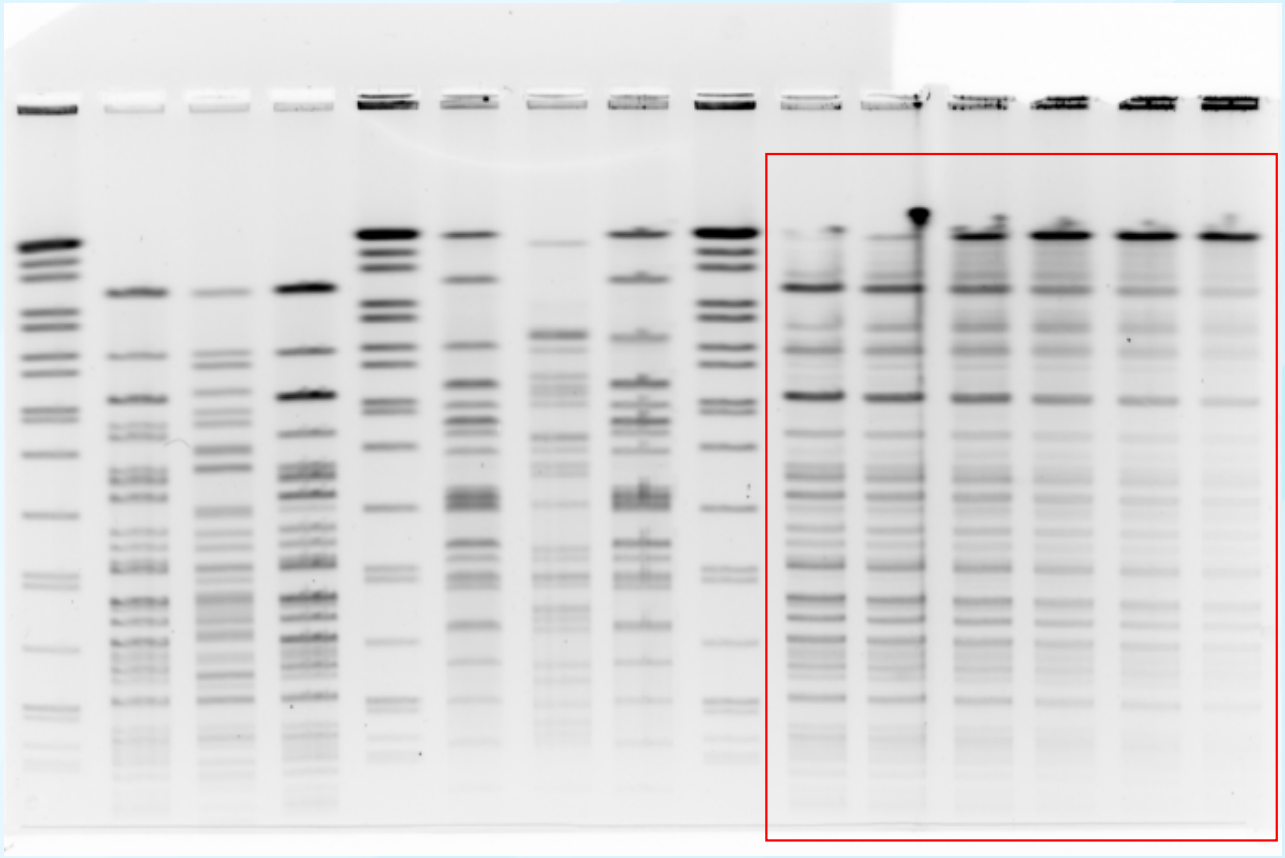
Enzyme digestion without BSA



Two different strains of *Salmonella* Newport digested with *Bln*I.

Image courtesy of Carol Sandt and David Faucette (PA)

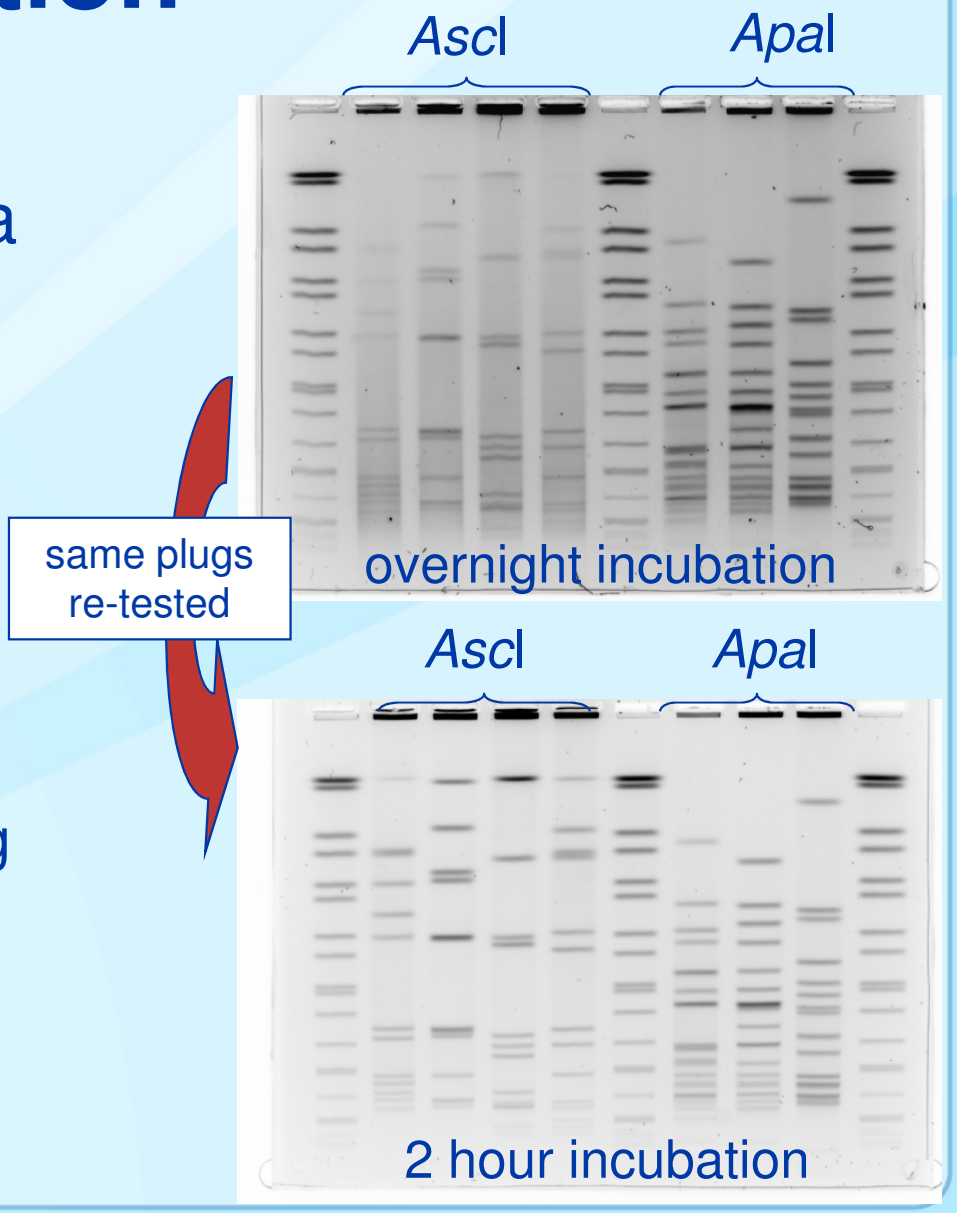
Poor / bad enzyme



Vibrio cholerae digested with high fidelity *Not*I enzyme (NEB); not recommended for use with PFGE.

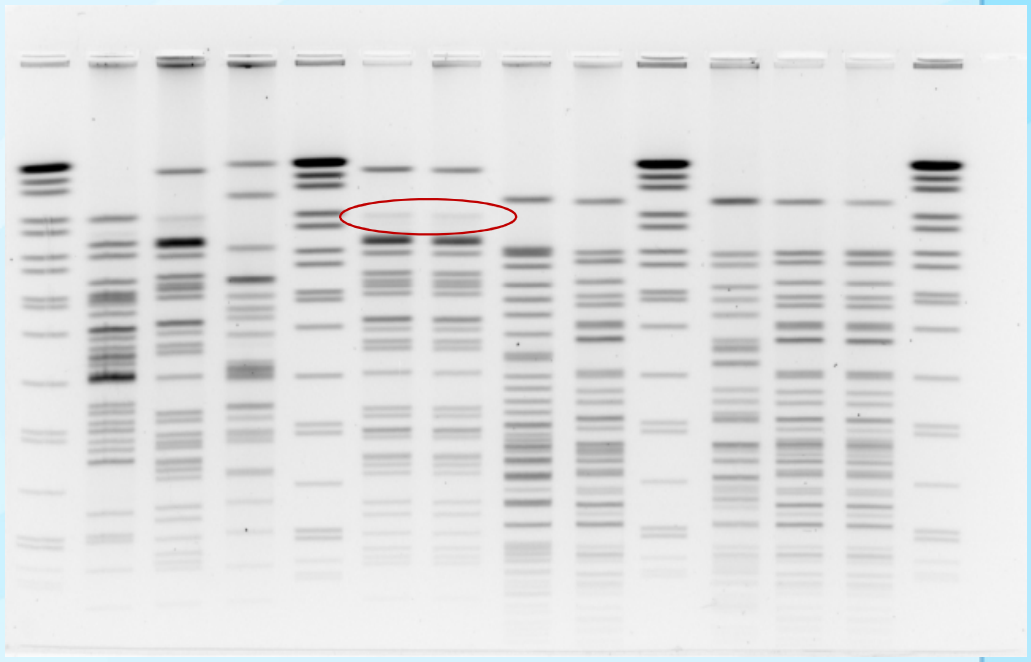
Star activity looks like incomplete digestion

- Star activity – a relaxation or alteration of the specificity of a restriction enzyme
- Conditions that can lead to star activity
  - prolonged reaction time
  - suboptimal buffer or buffer concentration
  - high (> 5% v/v) glycerol concentrations
  - high concentration of enzyme/µg of DNA ratio
- Star activity is often misidentified as a lysis issue



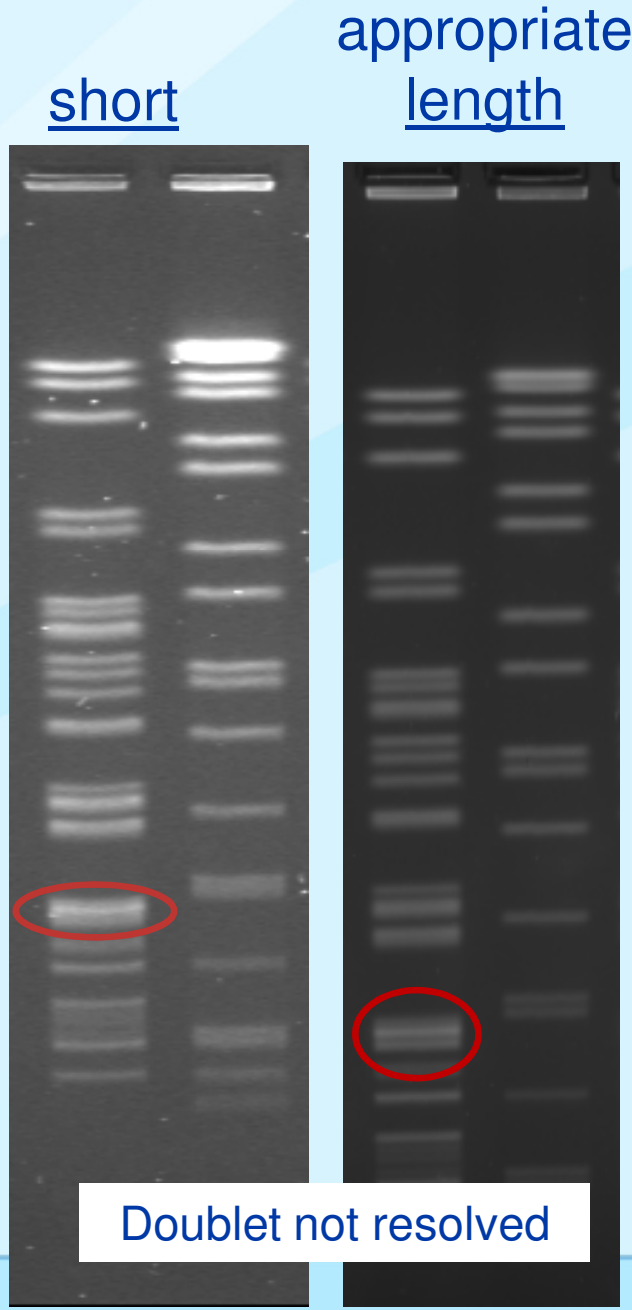
Troubleshooting "ghost" bands

- Possible solutions
  - decrease cell suspension concentration
  - wash plugs 2X more with TE buffer
  - include BSA (0.1mg/ml) in enzyme master mix
    - only use molecular grade BSA
    - make aliquots to reduce freeze/thaw cycles
  - use concentrated (40 U/µl vs. 10 U/µl) enzyme to decrease the amount of glycerol in the master mix
  - confirm water bath temperature is correct
  - follow protocol closely – varies for organism and/or enzyme
    - use appropriate buffer
    - use suggested units of enzyme
    - use suggested incubation time
  - new vial and/or lot of enzyme and/or buffer
- **Do not mark during analysis**

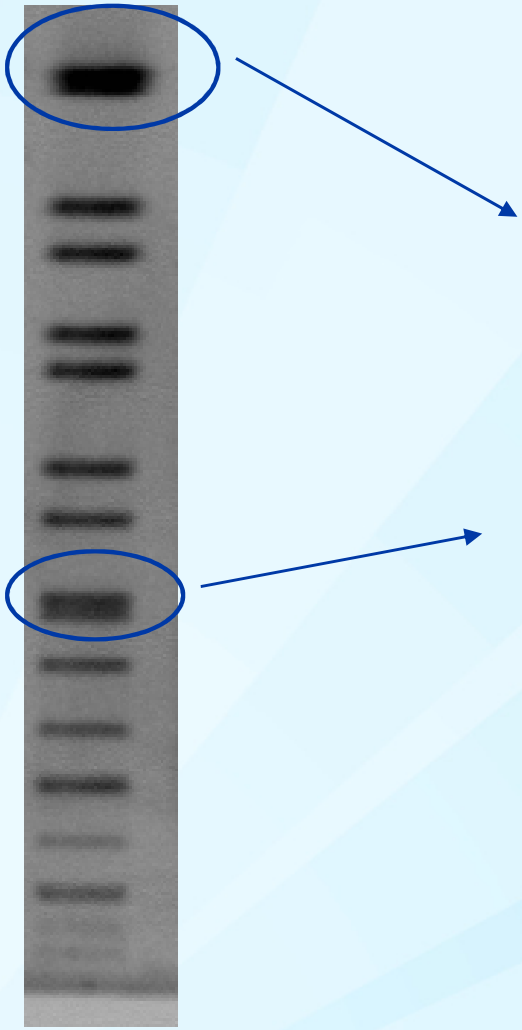


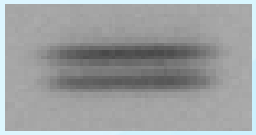
Impact of run length on resolution

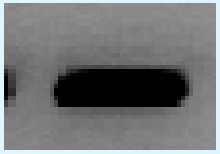
- Bottom band (20.5 Kb) of the standard should be 1 – 1.5 mm from the bottom of the gel
- If the run time is too short
  - pattern is compressed
  - decreased resolution of closely migrating bands
  - normalization of the pattern may be compromised
- If the run time is too long
  - bottom band of the standard runs off the gel
  - unable to perform normalization



Review General Band Marking Rules







Singlet vs. Doublet

If there is **indentation**, then it is a **doublet**.

If there is a **difference in color** (light and dark can be resolved), then it is a **doublet**.

If there is **clear separation**, then it is a **doublet**.

If **none of the above** cases hold true, then it should be marked as a **single band**

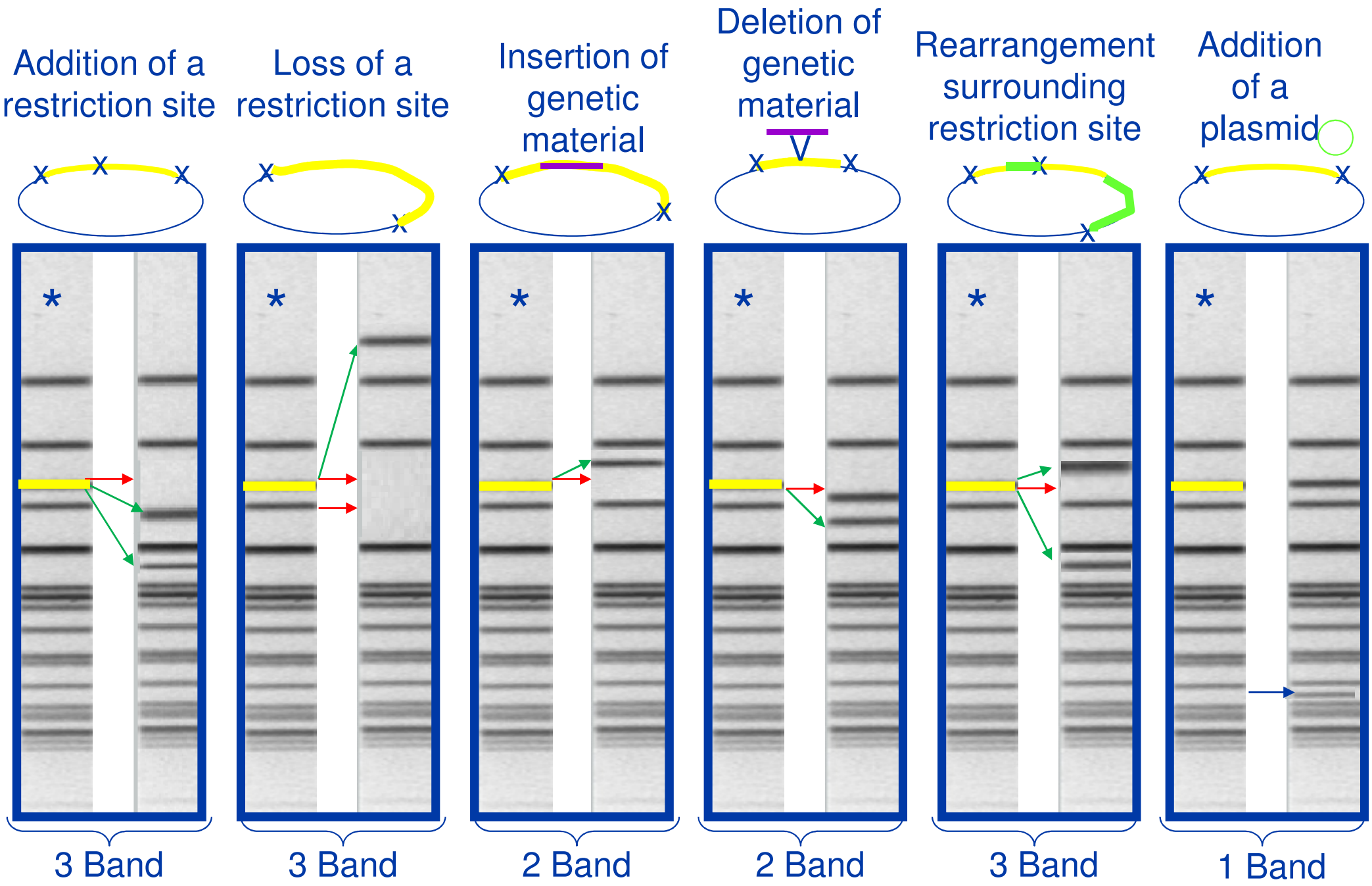
Interpretation of PFGE Data

- Technical artifact vs. genetic variation
  - Technical artifact
    - Effect of reproducibility
    - Incomplete restriction
    - Effect of resolution
    - Pattern analysis
  - Genetic variation
    - Expected degree of variability
    - Dependent on the organism being studied

Genetic Variation

- Types of genetic changes affecting PFGE patterns
  - point mutations (enzyme recognition sites)
  - insertions (small mobile elements and larger phages)
  - deletions (small mobile elements and larger phages)
  - plasmids (gain or loss, not genomic insertions)
  - rearrangements
- Can occur in vivo (person-to-person, person-to-environment-to-person) or in vitro (lab passages)
- A single genetic event can result in 0 – 3 band differences

Genetic Variation



Factors Influencing the Variability of an Outbreak Pattern

- **Point source outbreak**
  - single contamination event
  - everyone (or everything) exposed to same strain(s)
  - variation in PFGE patterns is minimal
- **Ongoing transmission**
  - person-to-person, infected herd, contaminated facility, environmental reservoir
  - *in vivo* propagation = more changes
  - more time = more changes

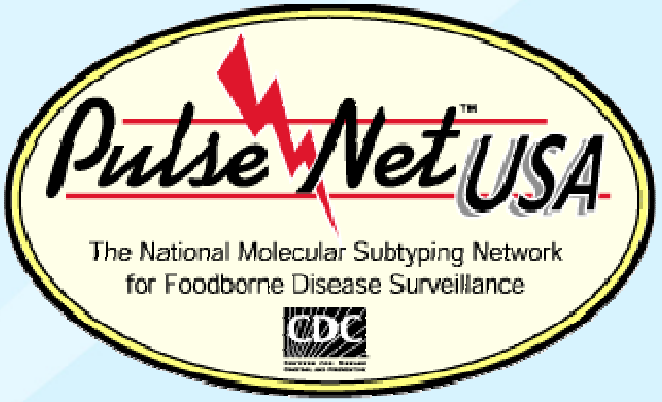
Mode of Transmission

Contrasting *Shigella sonnei* outbreaks 1999

- Person-to-person
  - 3 cases with same pattern in June
  - 2nd pattern in July
  - 21 patterns by December
  - PFGE alone not sufficient
- Point source foodborne outbreak (bean dip)
  - 25 cases, 2 food isolates
  - 21 cases and food were indistinguishable
  - 3 other highly similar patterns

The Big Picture

- DNA tests are only part of picture
  - isolates from same source don't always match
  - isolates with same patterns don't always have the same source (common patterns)
- Results needs to be considered along with epidemiological evidence and result from environmental investigations



Acknowledgements

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The findings and conclusions in this presentation are those of the author and do not necessarily represent the views of the Centers for Disease Control and Prevention

For more information please contact Centers for Disease Control and Prevention
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