Presentation 6:
PFGE Troubleshooting

Everything you wanted to know about PFGE, but were afraid to ask…

OR

Yes, in fact, we've done that experiment!

Trouble shooting philosophy

- Can or do we **test** every issue / variable?
  - Most
- Can or do we **solve** every issue / variable?
  - No, only consistent issues, but there are exceptions…
- Our role at PFGE Reference lab at the CDC
  - Test the big issues
  - Make recommendations

Your expectations of us:

- ✓ get trained
- ✓ receive prompt answers to questions
- ✓ get feedback on gels
- ✓ receive occasional trouble shooting assistance
- ✓ read about reviews and reports on reagent options

Our expectations of labs:

- ✓ **follow protocols closely**
- ✓ perform trouble shooting
- ✓ provide feedback
- ✓ **follow protocols closely**

Trouble shooting approach

- **What has changed since the last "good" gel?**
  - Equipment, reagent, person, isolate / strain, etc…
  - Change in lab procedures / new SOPs, new vendor, contamination / decontamination event, etc…
- **Examine the gel closely**
  - Ask if the problem is:
    - in all lanes, only the isolates, only the standards, only one enzyme?
    - apparent with multiple lab personnel?
    - apparent with multiple enzymes or multiple organisms?
    - associated with a specific mapper?
- **Maintain notebook of troubleshooting findings, gels**

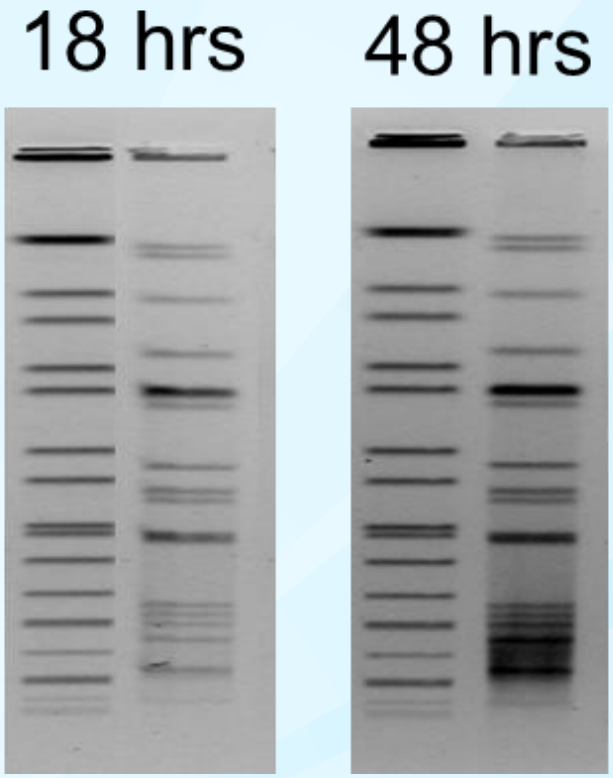
→ What is in common and what has changed?

Trouble shooting approach, continued

- **Reagents: garbage in → garbage out**
  - Always track vendors or individuals (if in-house), dates, lots, etc…
  - Make and store properly – room temperature, 4°C / -20°C, protect from light, dilute in TE or water, consider making aliquots, etc…
  - If it looks suspicious → do not use it!
    - cloudy, stringy, precipitates, etc…
    - do not be superstitious
  - Adverse affects may be gradual or sudden, may be pronounced in some organisms and not others or H9812

- **Difficulty repeating these effects and "bad gels"**
  - Complex experiment design, interaction of variables, protocol interpretation, unique lab environment, too many variables!!!
  - All organisms, all strains, all enzymes not affected equally

Example of bad cell treatment – plates grown for too long



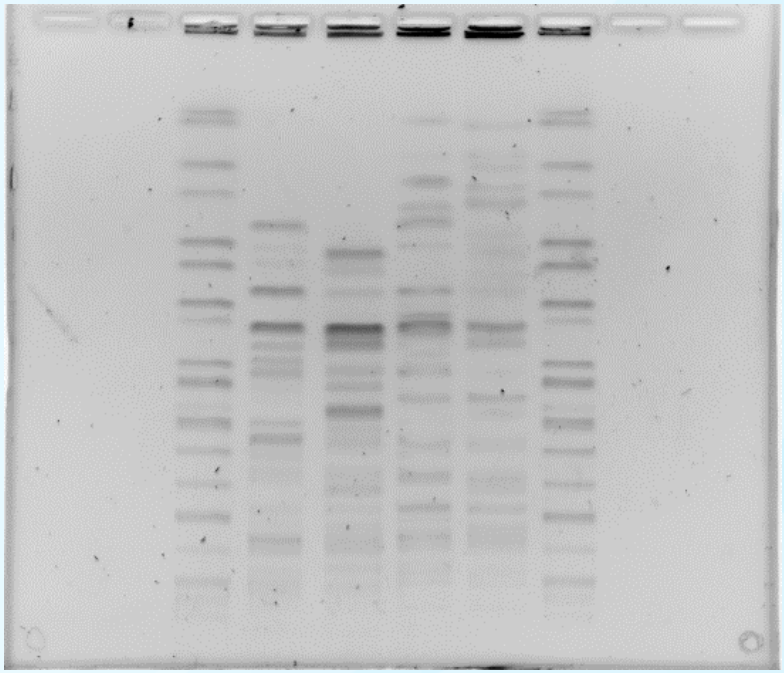
yes

no

- Plates should be grown for **14 – 18 hours**
- **Fresh** cell growth
- Do not keep plates at 4°C, grow on bench, let sit out at room temperature, etc…
- Treat cells carefully
- Not all strains / serotypes similarly affected

Example of bad cell treatment – plates stored improperly

E. coli O157 cultures stored at 4C for 3 days before making cell suspensions



no

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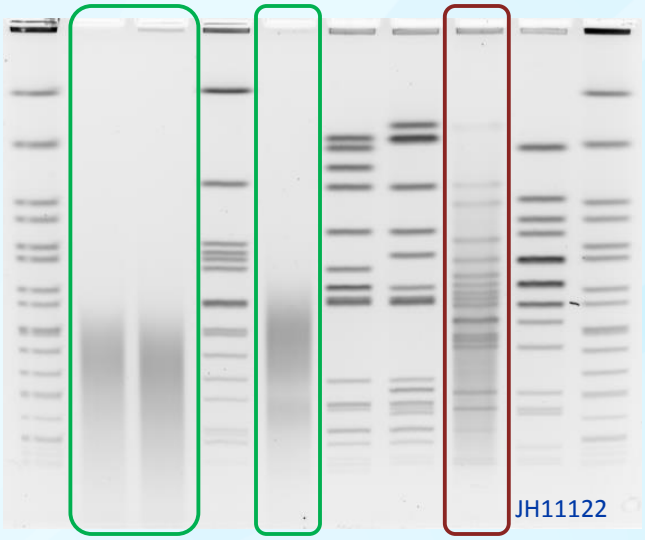
Thiourea-dependent or “Untypeable” Strains

- **Typical smear – DNA degraded in bottom third of gel, no DNA in wells**
- **Add thiourea to the running buffer**
  - 1g thiourea in 100ml dH_2O (10mg/ml)
  - wrap bottle in foil and store in dark
  - add 837μL to 2.2L 0.5X TBE at start of run (50mM)
- **Do not use thiourea routinely or add directly to plugs because it is toxic**
- **Thiourea does not improve poor quality plugs or poor patterns**
- **Include a positive control on gel**
  - digested plug slice of “untypeable” strain that “typed” previously when thiourea was added to buffer

Example of bad plug – thiourea will not help

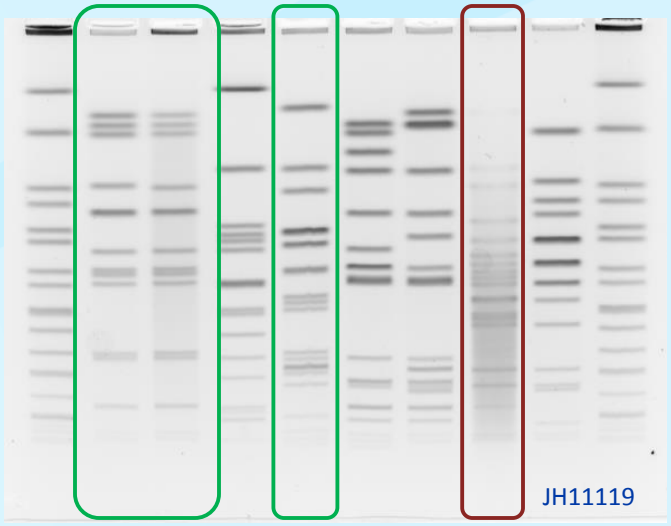
- **Are these thiourea-dependent isolates?**
  - Is there DNA in the plug slice?
  - Is the smear in the whole lane or just towards the bottom?
  - Are there any bands present?

No thiourea in running buffer



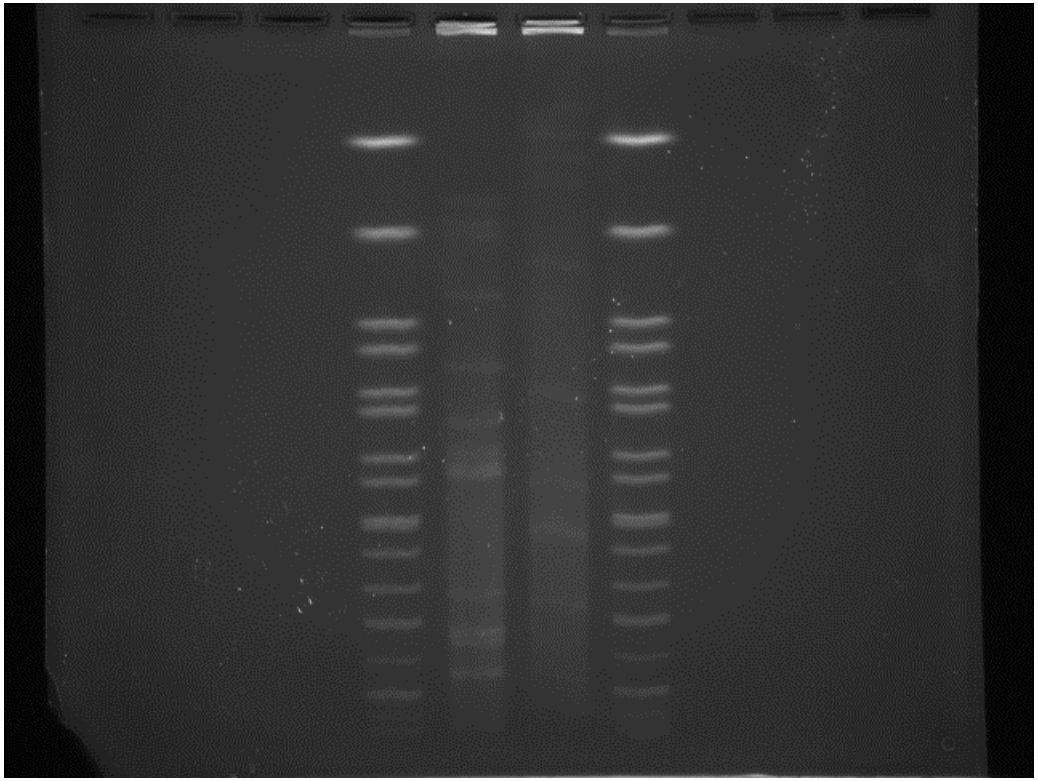
thiourea-dependent

50 μM thiourea in running buffer



bad plug

Incorrect stopping point – plugs stored before lysis

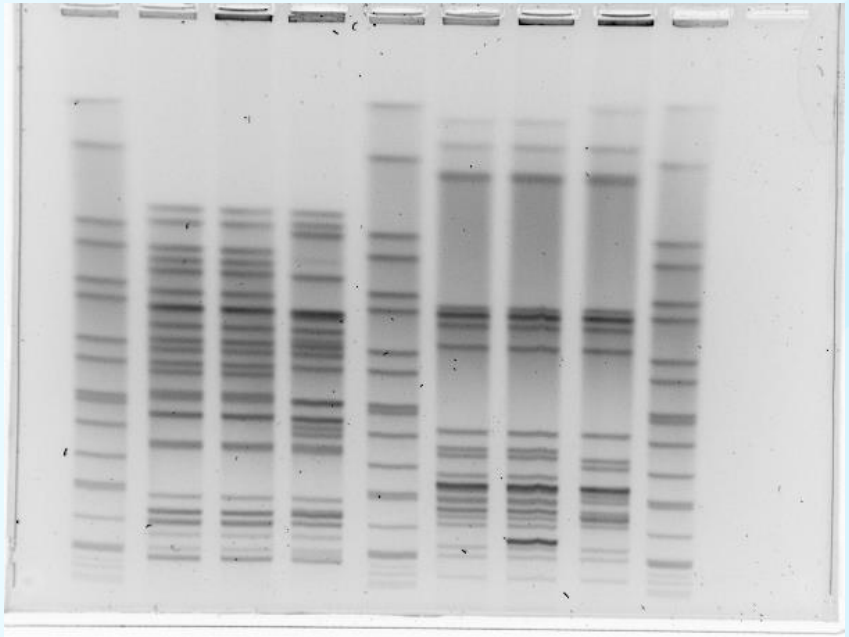


What to consider → lysis buffer, cell suspension buffer, reagent contamination, reagent storage, cell "happiness," alteration of standardized protocol

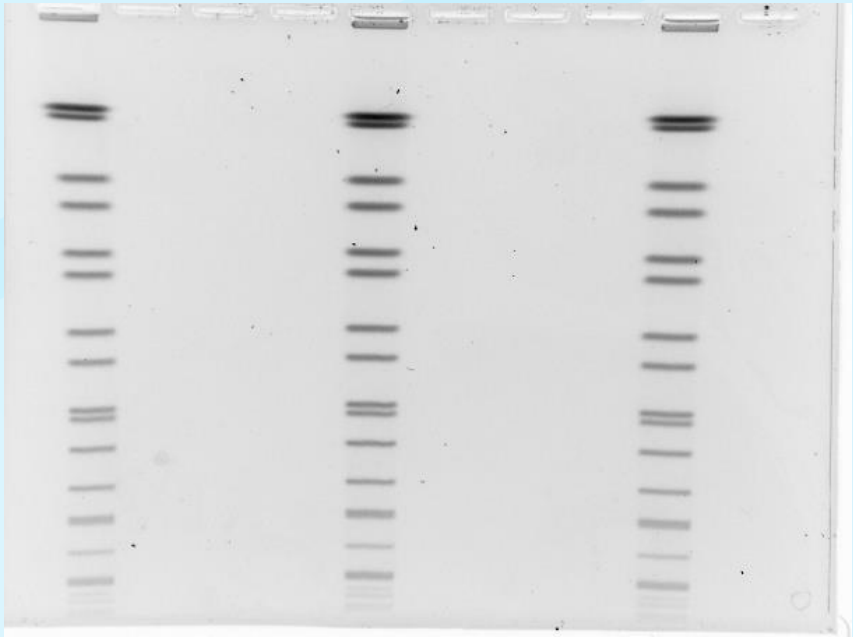
Example of contaminated mapper

- **Contamination in the mapper (fungal, bacterial)**
  - Smearing throughout the lane (usually)
  - Smearing in isolates and controls (usually)

Contaminated mapper



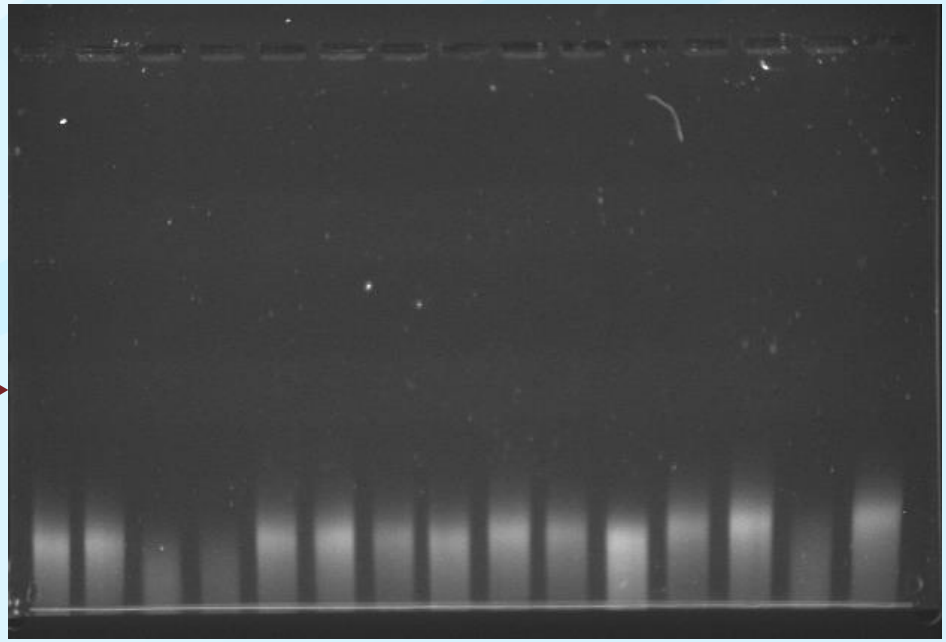
Same mapper after being decontaminated



Example of Contaminated Mapper

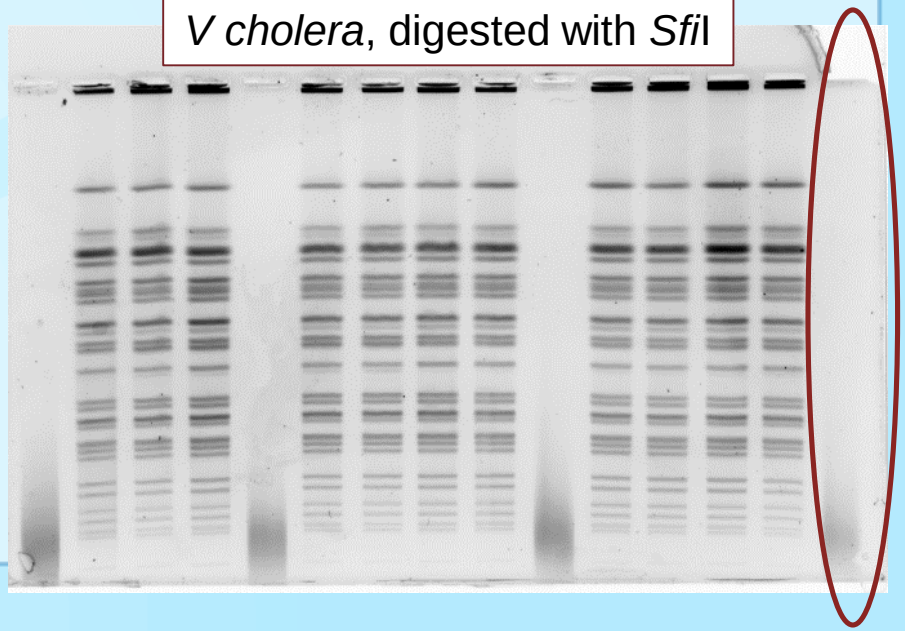
- **Solution**
  - Drain 0.5X TBE / water from mappers / lines after **each use**
  - Once every 3 months (or as needed)
    - circulate ~2 L 5% bleach 20 – 30 minutes, drain
    - circulate ~2 L dH_2O 20 – 30 minutes, drain, repeat at least 3X
    - leave chiller OFF

Pseudomonas species isolated from CHEF Mapper unit at CDC



Example of Contaminated Plugs

- **Bad *Xba*I / H buffer?**
  - Unlikely → completely degraded DNA, no ghost bands
- **Solution**
  - Keep plugs in sterile TE at 4°C
  - Keep track of dates – identify and discard "bad" plugs
  - Make a new batch of H9812 every 1 – 3 months, depending on testing volume, storage space
  - Always use previously tested lot of H9812
  - Make H9812 control plug
    - make new H9812 plug same day as unknowns
    - controls for plug making that day

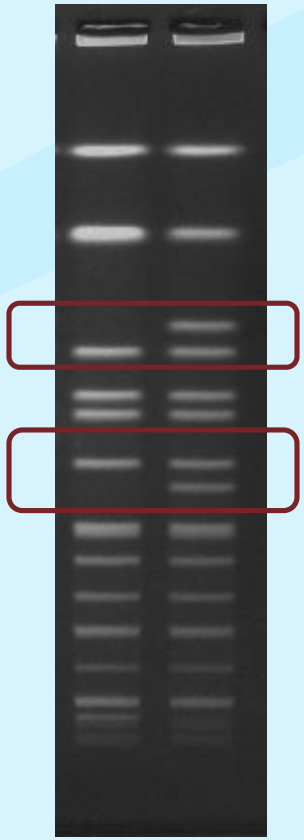


Troubleshooting missing bands

- **H9812 is the universal standard used with all organisms**
  - chosen due to optimal number and distribution of bands
- **Always test "new" lot of H9812 plugs with "old" lot to confirm:**
  - PFGE pattern is same, including band intensity
  - concentration is similar
  - the new standards produce good quality PFGE pattern
  - if pattern is different, streak out new plate from frozen stock
- **Pre-tested H9812 standard plugs can be a positive control and focus troubleshooting efforts**
  - problem observed in sample lanes but **not** in standards, likely due to sample associated affect (i.e. sample plug preparation)
  - problem observed in both sample **and** standard lanes, likely due to step involving sample and standard plugs (i.e. enzyme digests)

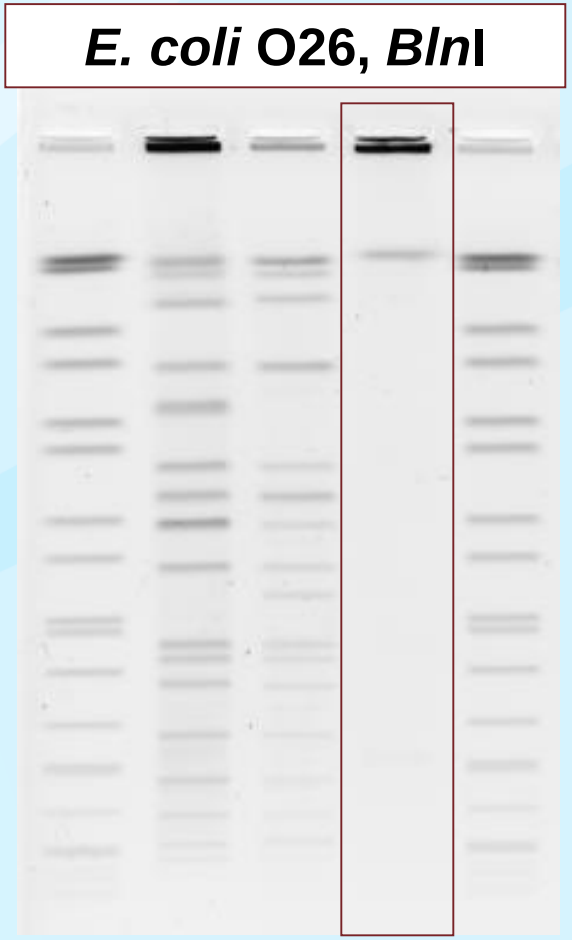
H9812

new old



Trouble shooting no bands – no digestion

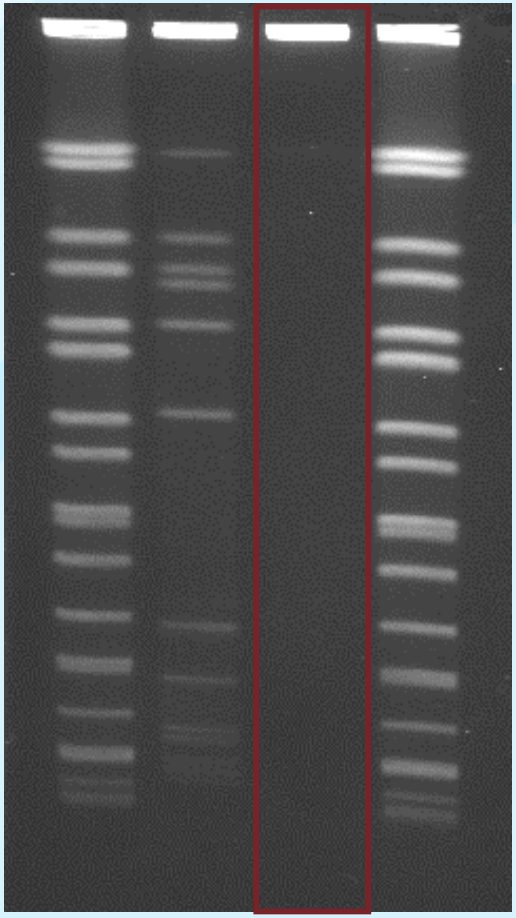
- **Some strains do not restrict with certain enzymes – no site or sites are "hidden"**
- **Appears as a large band at the top of the gel with no DNA smearing in the lane and DNA still left in the well**
- **It's not you – it's the strain (and enzyme)!**
  - *E. coli*, *Salmonella*, *Listeria, Vibrio spp*
  - some serotypes more often than others
  - cannot predict



Trouble shooting no bands – no digestion

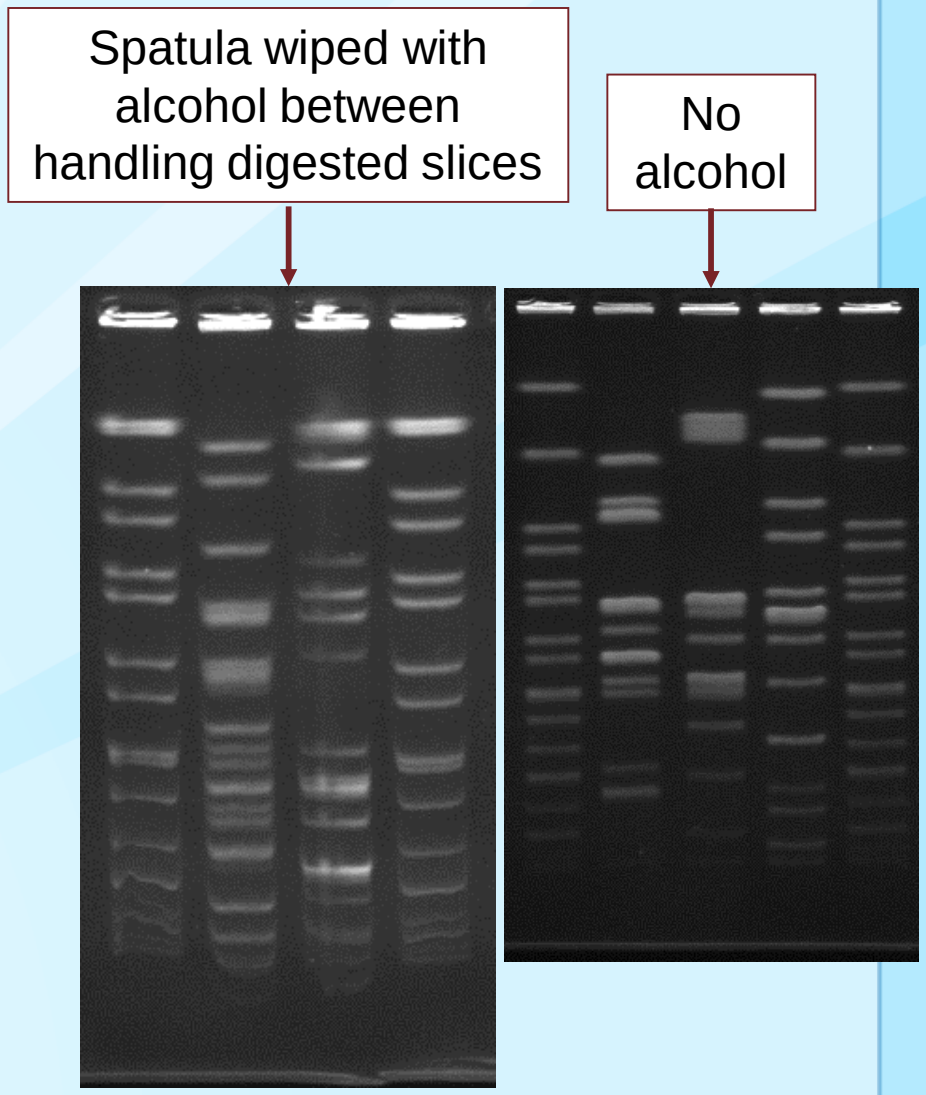
- **Other slices digested with master mix are a positive control for enzyme working**
- **Valid pattern for that strain and can be uploaded**
  - not the same as untypeable!
- **Solution → digest with other enzymes (secondary +/or tertiary) for that organism**

***Listeria*, *Apa*I**



Troubleshooting wavy bands

- **Possible causes**
  - rusty, nicked, damaged razor or scalpel
  - damaged plug slices, debris in gel and/or plug
- **Possible solutions**
  - use new razor or scalpel
  - make new plugs
  - completely melt plug agarose and mix completely with cell suspension
  - completely melt gel agarose, check for cell debris
  - **do not** use alcohol wipes between plug slices

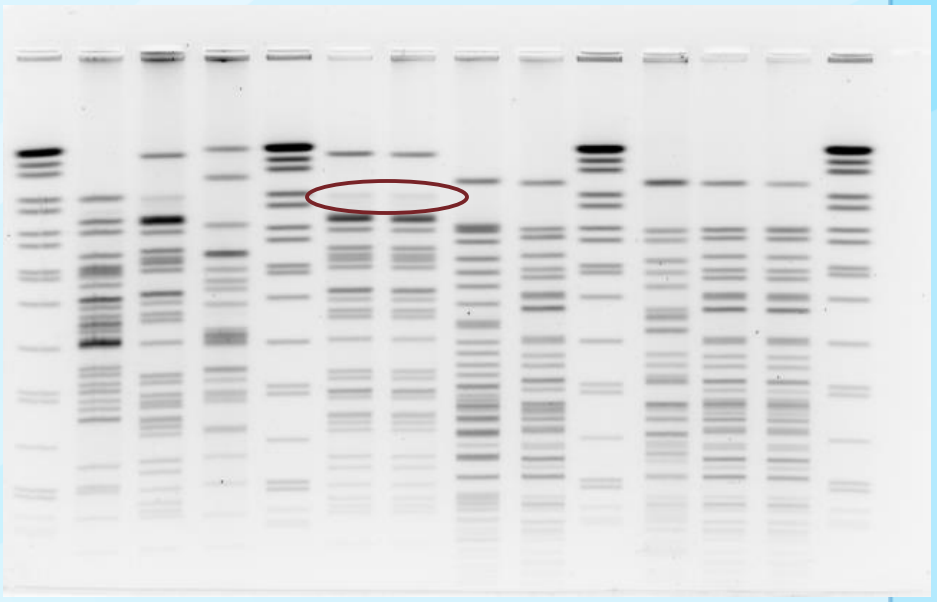


“Ghost” or “Shadow” Bands

- **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 and/or buffer 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

Troubleshooting "ghost" bands

- **Possible solutions**
  - decrease cell suspension concentration
  - wash plugs 2X more with TE buffer
  - include BSA (0.1 mg/ml) in enzyme master mix
    - only use molecular grade BSA
    - make aliquots to reduce freeze/thaw cycles
  - increase units of enzyme
  - use concentrated (40 U/μl vs. 10 U/μl) enzyme to decrease the amount of glycerol in the master mix

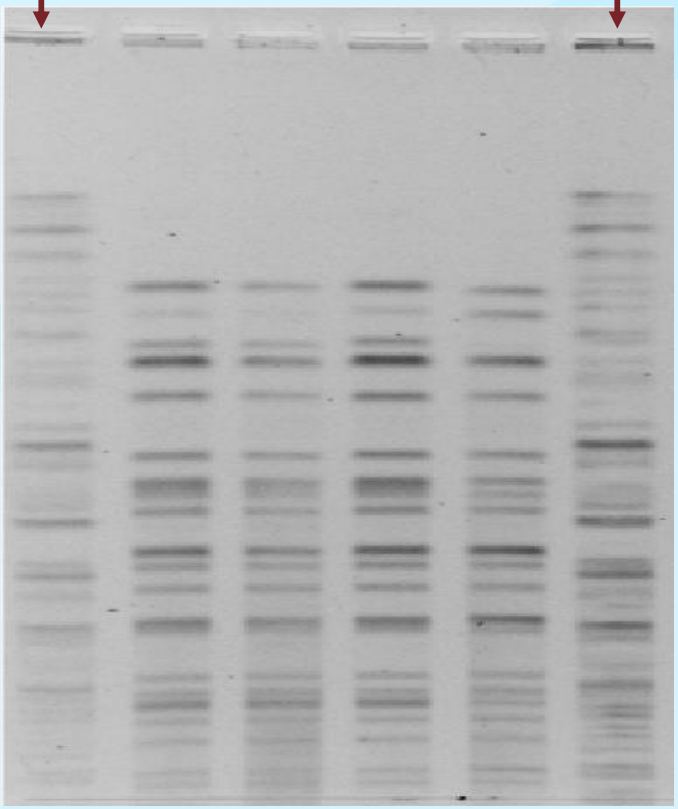


➢ **Do not mark during analysis**

Troubleshooting "ghost" bands

- **Possible solutions, continued**
  - 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
    - use suggested incubation temp
  - new vial and/or lot and/or vendor of enzyme and/or buffer
  - avoid high-fidelity and fast digest enzymes
  - use different spatula for plug slices from each enzyme

H9812 double digest – enzyme not inactivated prior to loading gel



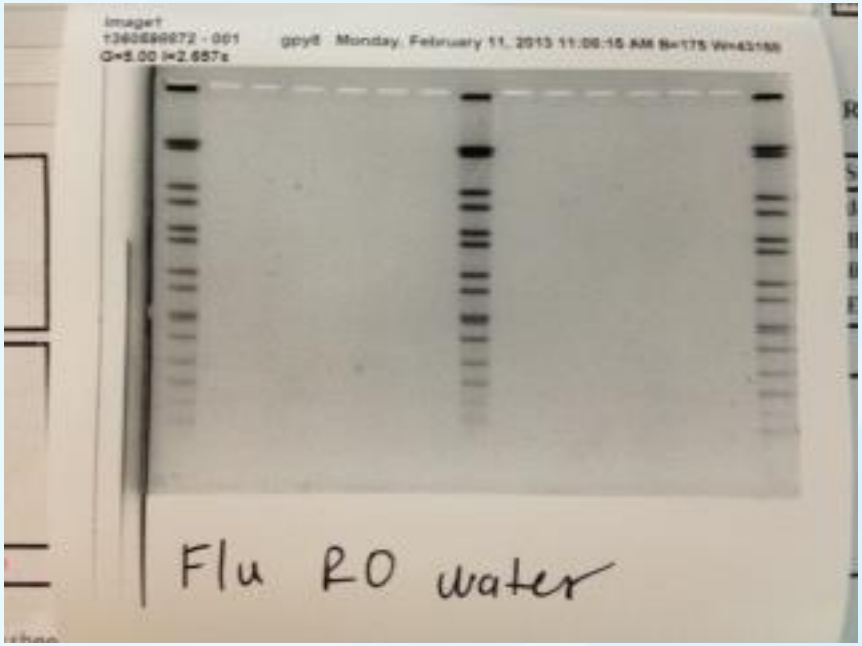
Troubleshooting “ghost” bands – reagent quality

- **Intermittent issues with ghost bands, incomplete digestion**
- **10X TBE made in-house**
- **Change in production of EDTA from supplier**
  - same order number but no longer labeled as “*For Molecular Use*”
- **Took several months to identify the issue**
- **Keep track of date, lots, etc… of all reagents**

Effect of Water Quality

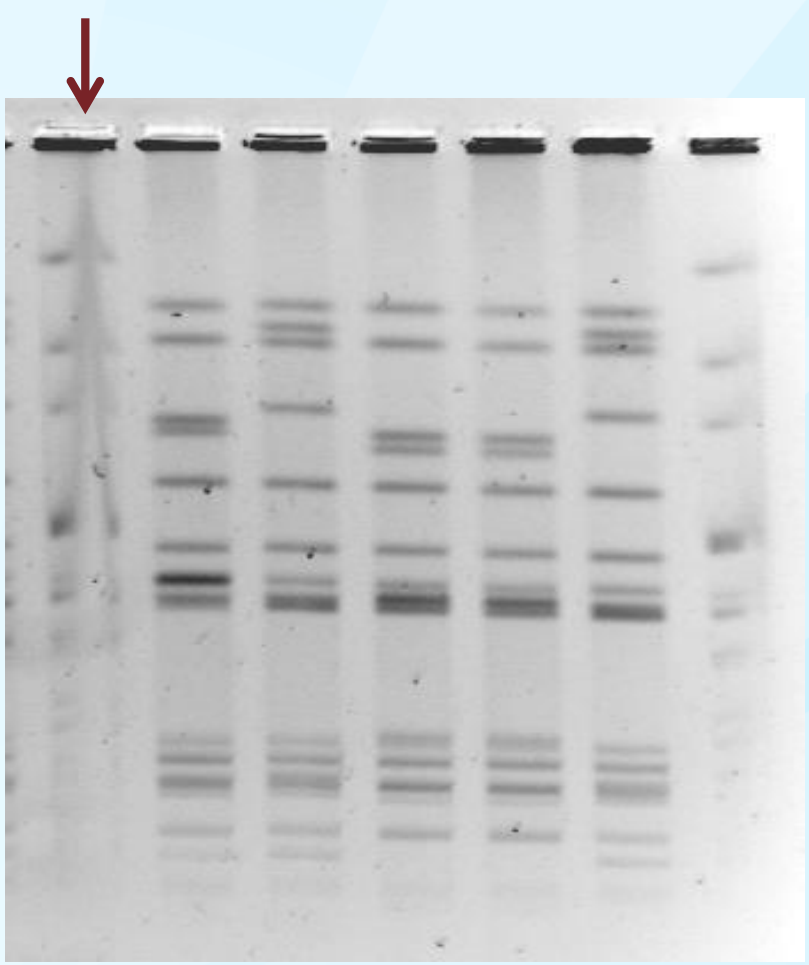
- **Difficult to replicate issues due to water quality**
- **Make sure the quality of water used for reagents is the highest possible quality**
  - change filters in water system on regular basis
  - **avoid** storing water in plastic carboys
- **Use sterile ultrapure (Type I or Reagent Grade) water for all master mixes**
- **Non-sterile ultrapure water can be used for:**
  - buffer used to make gels
  - electrophoresis running buffer
  - de-staining gels
- **Never use tap water**

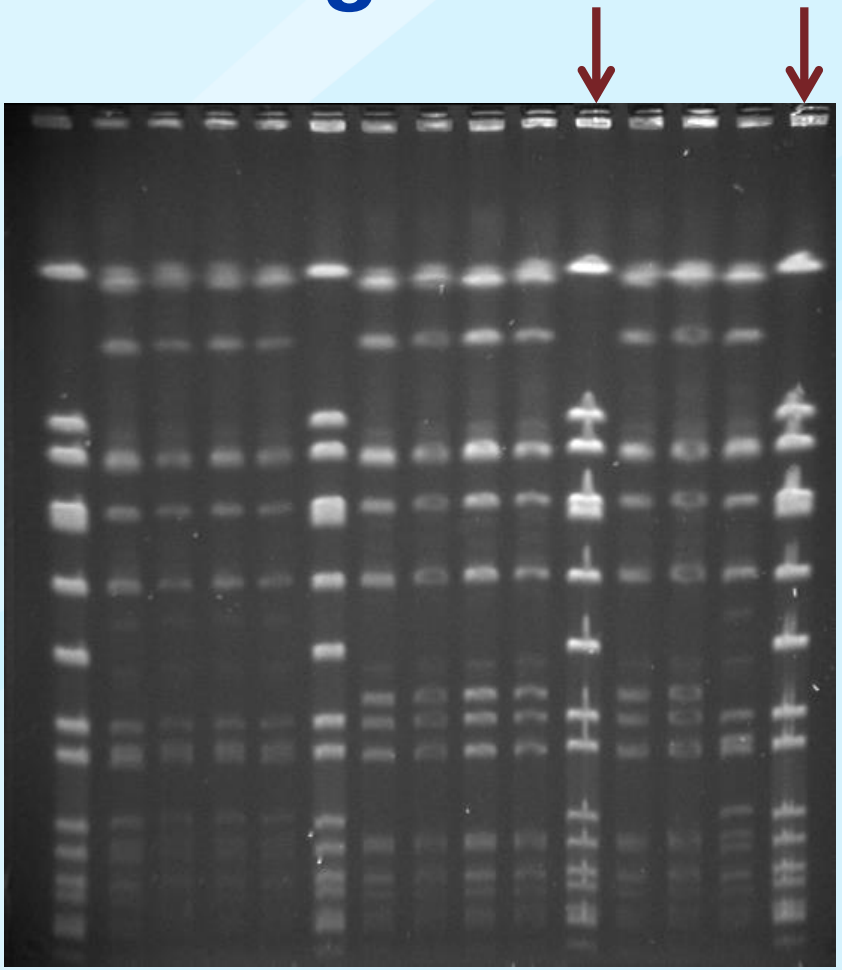
Effect of Water Quality



- **Sudden, dramatic, but intermittent failures; would seem to resolve and then reappear**
- **Tested and eliminated all possibilities – organism, enzyme, buffers, etc…**
- **Water system was cleaned, replaced and gels have looked beautiful since then**

Foreign Particles or Bubbles Embedded in Plugs





Common Questions

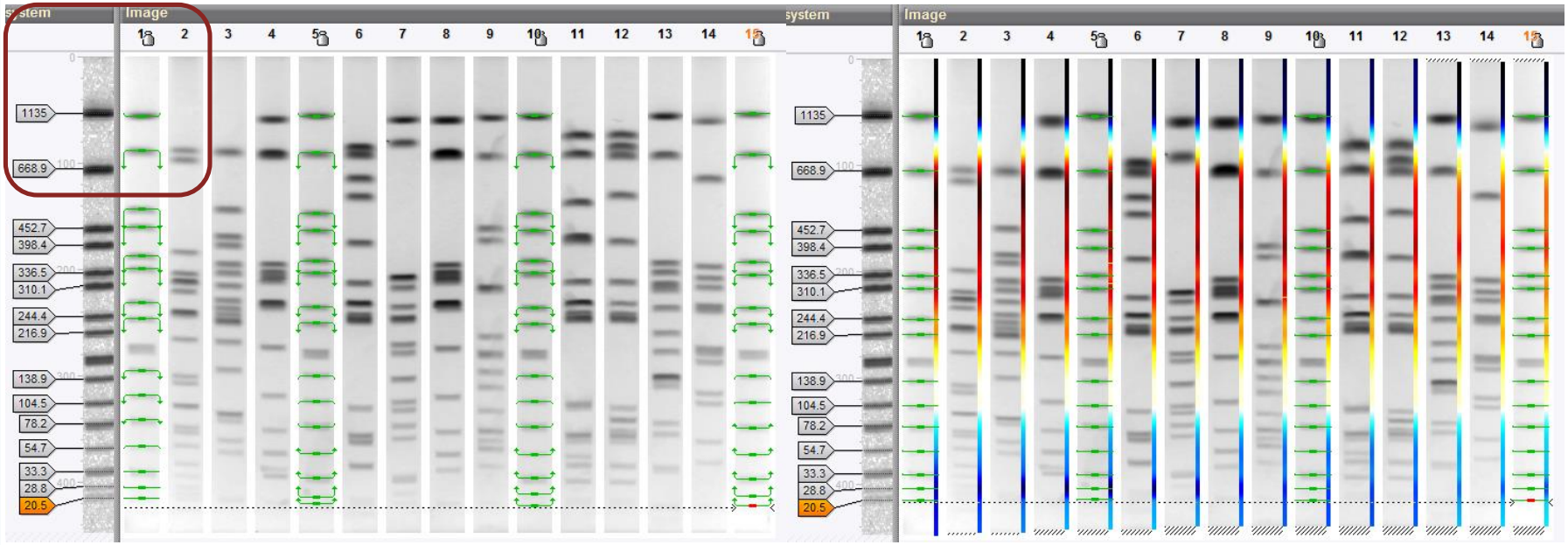
- **Slanted gels, run length, normalization**
  - Power supply – building, dedicated line
  - Pump flow rate – check rate annually (at least), ~70 ≈ 1L / minute, if less then adjust setting, reverse flow to flush out agarose pieces, air bubbles or “kinks” in tubing
  - Chiller – temperature accuracy (probe can go bad)
  - TBE – variations between vendors and in-house, volume ~2.2L
  - Water – use the highest grade possible, not sterile unless indicated
    - Milli-Q is great, DI or “polished” water may be OK if good quality going in
    - quality may change over time – new building, aging filter, storage in plastic carboys may leach chemicals, etc…
  - Humidity, air temp, ventilation, neighboring equipment
  - Agarose – different lot, different kelp, dirty glassware

Normalization

- ✓ **Always check normalization – if bad, do not upload**
- **Programming error**
  - Accidentally programmed 30kb – 600kb, instead of 700kb, etc…
  - *Salmonella* isolates run with *E. coli* conditions and vice versa cannot be analyzed – must re-run with correct conditions
- **Wrong agarose**
  - "Megabase (Bio-Rad lot# 45100031) does **not** separate large molecular weight bands correctly and **cannot** be used
  - **Only** SeaKem Gold (Lonza) can be used for gel running agarose
- **Instrument malfunction**
  - Electrophoresis box or "brain" may go bad
  - Swap out boxes and brains to isolate the problem
  - Contact Bio-Rad to have equipment repaired

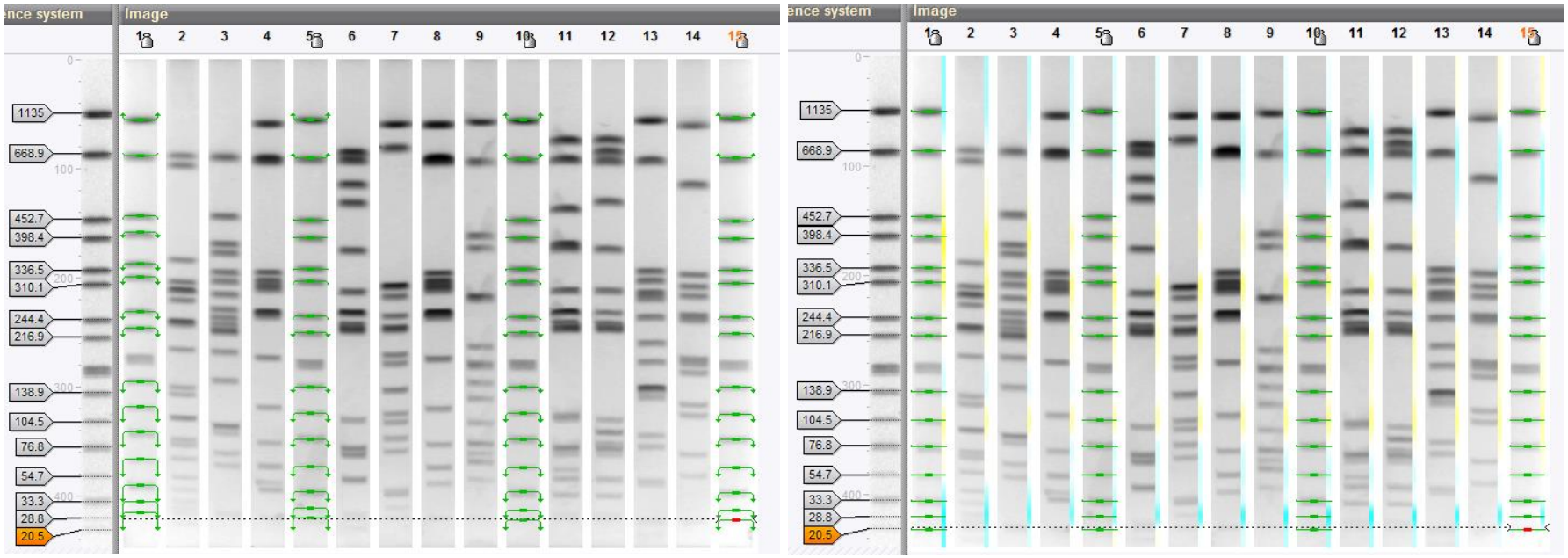
Bad Normalization – wrong conditions

- **Gel was run with "*Salmonella*" conditions and analyzed in *Salmonella* database**
  - Dark distortion bars indicate poor normalization
  - Re-run with 2.16 s – 63.8 s (30 kb – 700 kb)



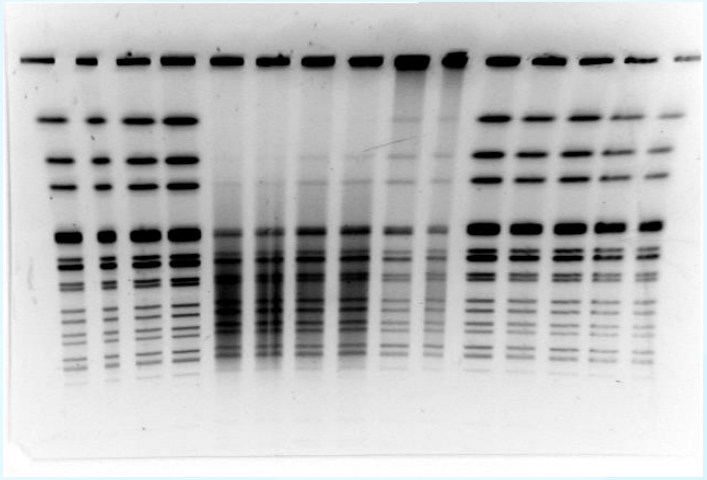
Bad Normalization – wrong conditions

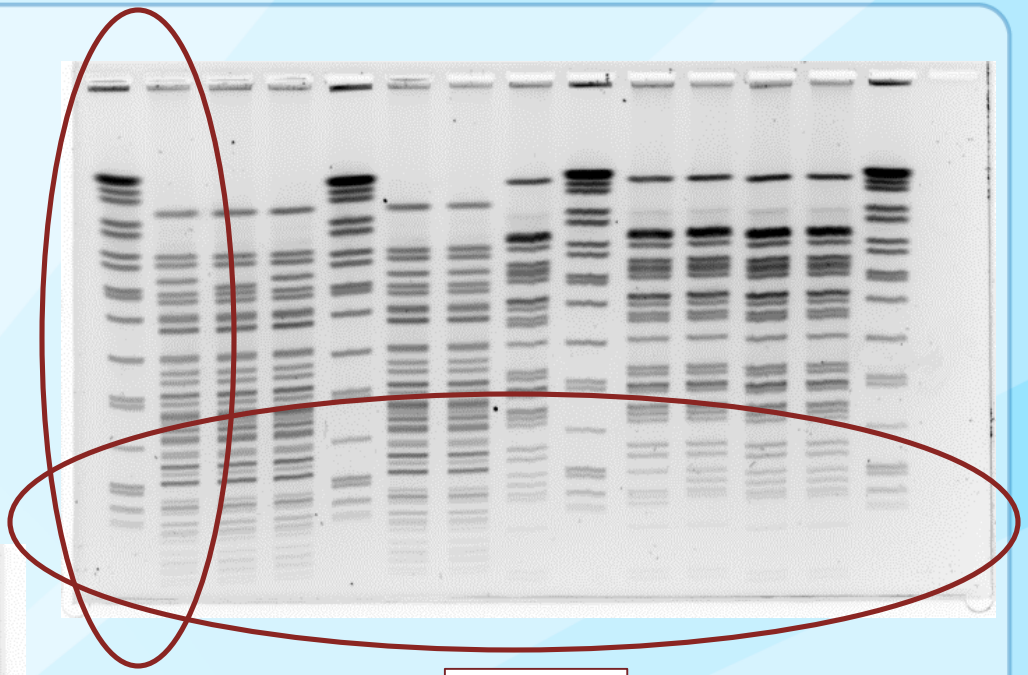
- **Gel was run with "*Salmonella*" conditions and analyzed in *E. coli* database**
  - Light distortion bars indicate proper normalization
  - Strongly suggests 2.16 s – 54.17 s (*E. coli* O157) conditions



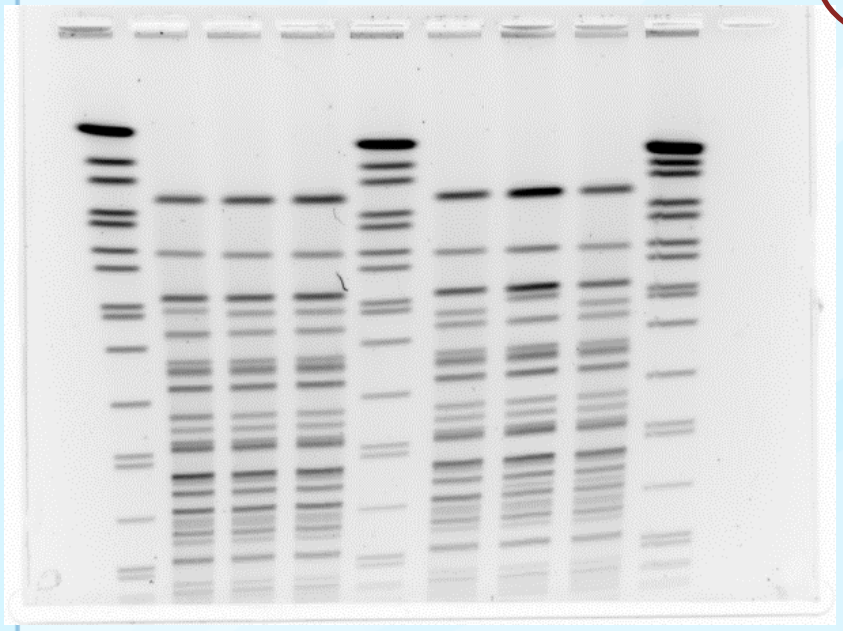
Factors Causing Lane Curvature

- Gel not level when poured
- Electrophoresis chamber was not level
- Gel was not flush against black platform
- Buffer not flowing evenly or not enough buffer in electrophoresis chamber
  - CHEF Mapper requires ~2.2 L in chamber, older models require ~2.0 L
  - clean and check for agarose particles
- Equipment putting off heat near lines
- Temperature of buffer fluctuated during run, more than 14°C ± 2° C
- Broken electrode(s)
- Mapper “personality”
- Electrophoresis chamber needs to be serviced

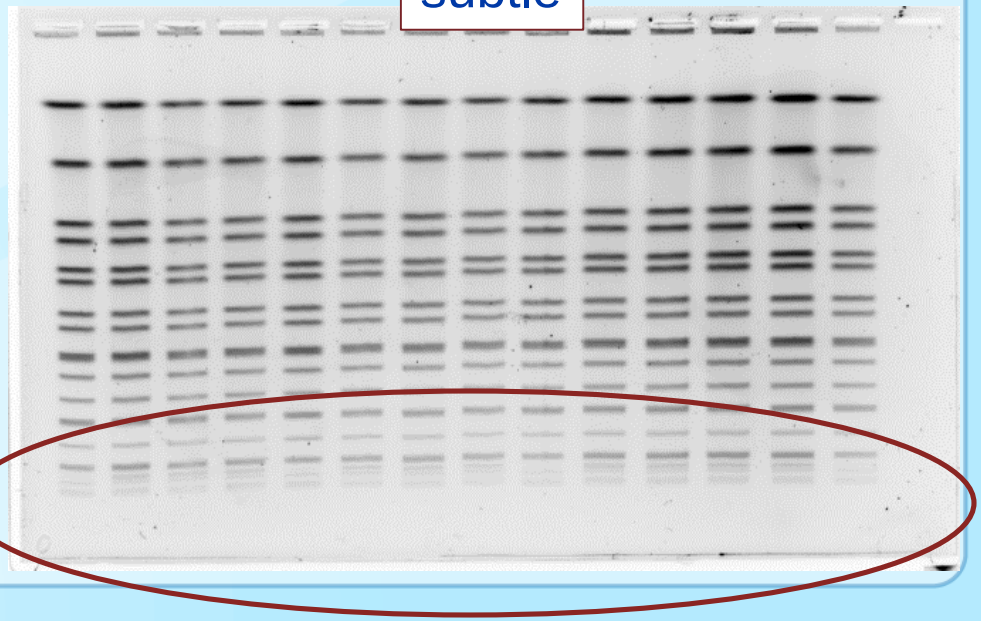




subtle



dramatic



Unique, sporadic, or chronic issue?

What to consider → electrical supply, power interruption, dedicated line, power surge, programming mistake, faulty instrument

Send Your Troubleshooting Questions via e-mail to:

pfge@cdc.gov

Write "**Troubleshooting**" in the subject line of e-mail message