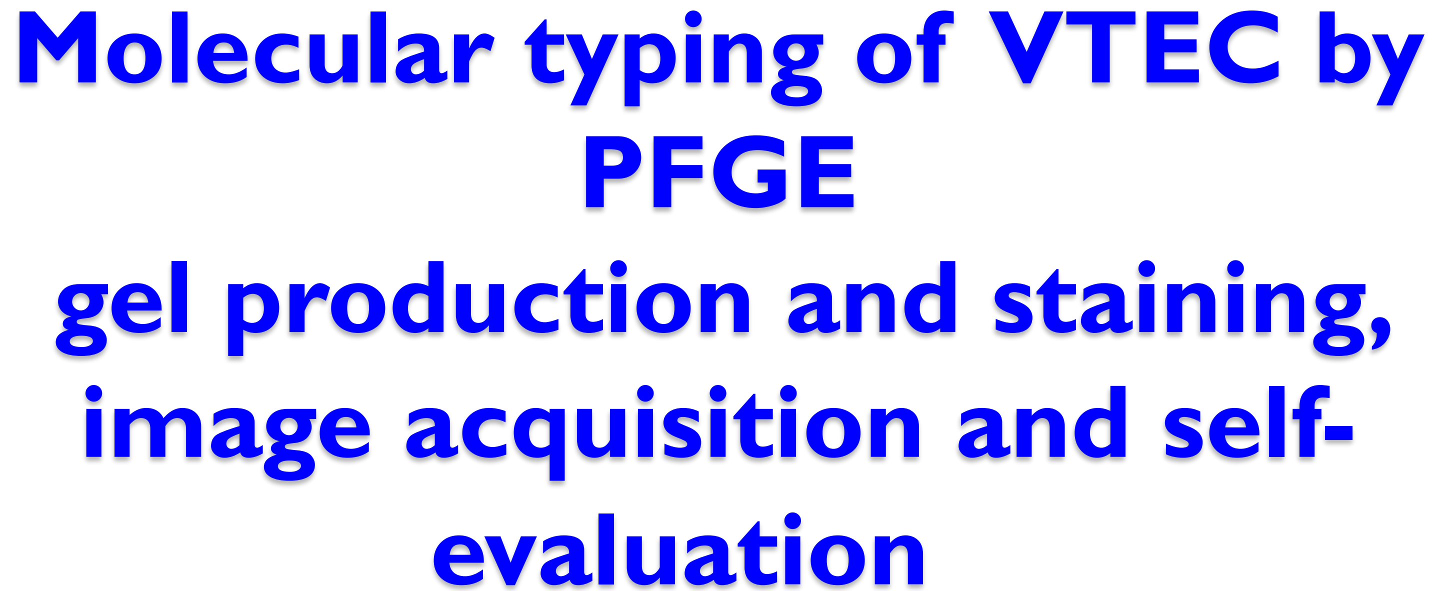


Basic Course on the use of BioNumerics, EU-RL VTEC, Rome12-13 June 2014

Pulsed Field Gel Electrophoresis

Set up 30 years ago

Technique to separate long strands of DNA through an agarose gel by applying a pulsed electric field

PFGE resolves extremely large DNA, raising the upper size limit of DNA separation in agarose from 30-50 kb to well over 10 Mb (10,000 kb)

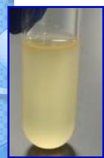
the most commonly used typing method for outbreak identification, surveillance and investigation for a number of import ant pathogens, in particular Salmonella, STEC and Listeria

PulseNet International a network dedicated to tracking foodborne infections world-wide- many efforts for methods standardization, including PFGE

PFGE SOPs produced by EURL VTEC is based on the PulseNet protocol

PFGE trainings at EURL

PFGE analysis at a glance…



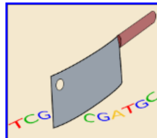
Bacterial cell suspension

Mix with agarose

Plugs preparation

Lysis and washing

Bacterial DNA in plugs



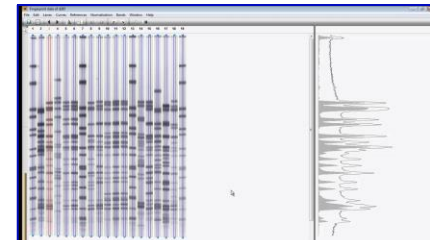
DNA restriction

plugs are loaded onto agarose gel

PFGE run

Adequate gel staning/de-staining

Gel image aquisition → Image Analysis



Bacterial DNA is embedded in agarose plugs to avoid shearing and restricted with rare-cutting Endonuclease

Parameters affecting the gel run: buffer, agarose, angle of the field, voltage, time switch, temperature, time

The production of a good TIFF depends on all the steps mentioned

DNA preparation

Proper DNA restriction

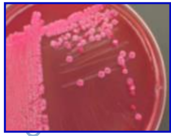
PFGE run

Proper Gel Staining/De-staining

Good Gel Image documentation

quality assessment of PFGE images

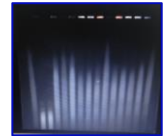
Quality of DNA



Start with pure, fresh bacterial cultures

If cultures are not pure, it is not possible to obtain a single pattern

If cultures are not fresh, bacteria may produce endonucleases resulting in DNA degradation



Standardize the bacterial suspensions

If different concentrations of bacterial cells are used each lane will have darker and lighter bands - difficult analysis

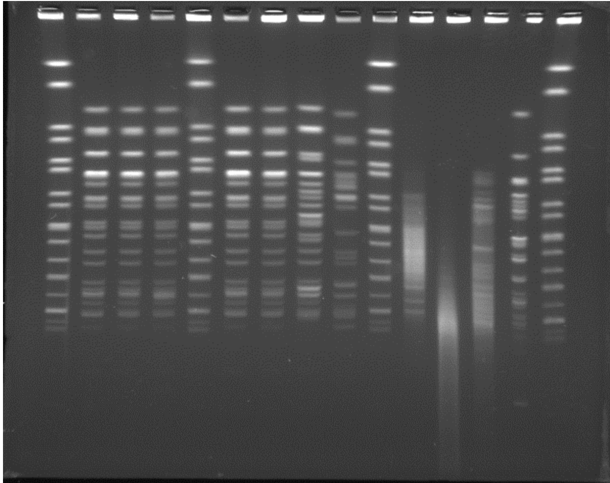
Use sterile, freshly prepared solutions

The concentration may vary in old solutions, nucleases may be present affecting the DNA integrity

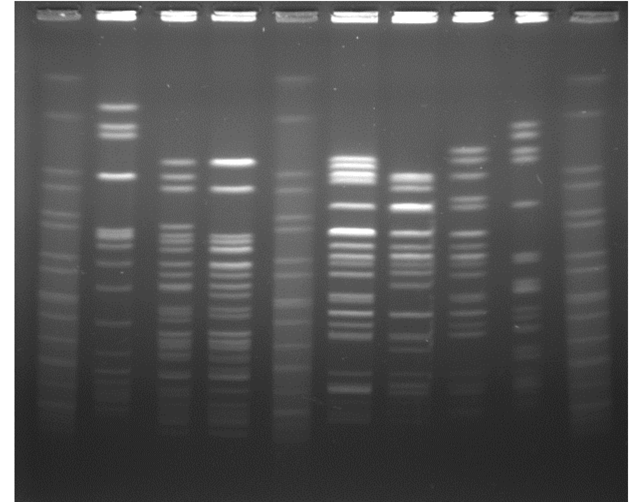
Wash thoroughly the plugs after lysis

Proteinase K must be completely removed - it could inhibit restriction

Some examples….

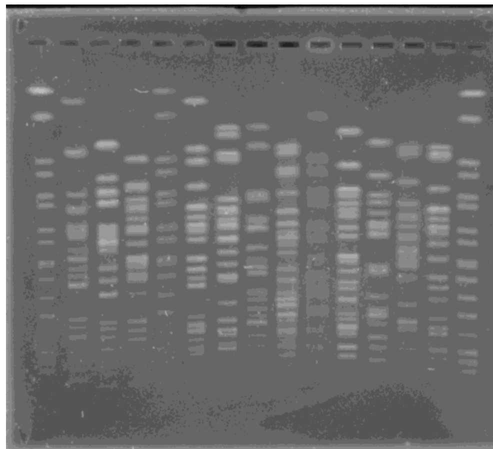


Degradation of DNA



Different intensity of DNA in each lane

Partial Degradation of DNA



Different intensity of DNA in each lane

DNA restriction

Use appropriate sized-plugs

When using 15-well DNA comb cut the plugs

Pre-restrict the plugs

Letting the plugs equilibrate with the restriction buffer will improve digestion

Use BSA

BSA is a stabilizer of restriction endonucleases, so its use enhances the enzymes activity

Make sure that the plugs are covered with pre-restriction/restriction buffer

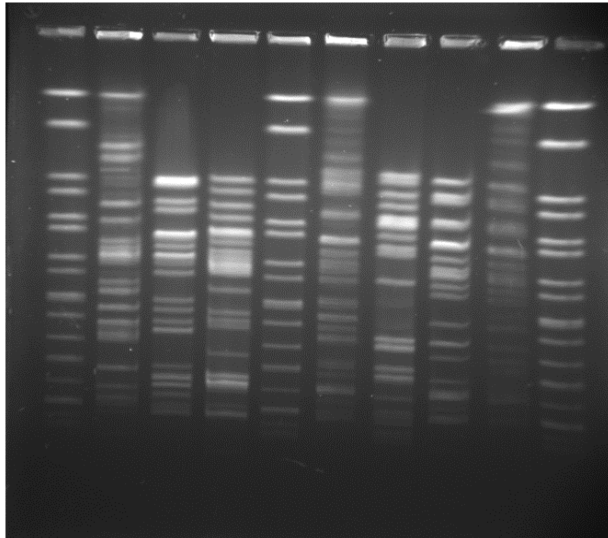
Use the appropriate amount of Enzyme

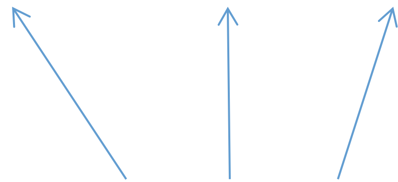
Using less units of restriction endonuclease may result in incomplete digetion

Don’t let restriction go too far

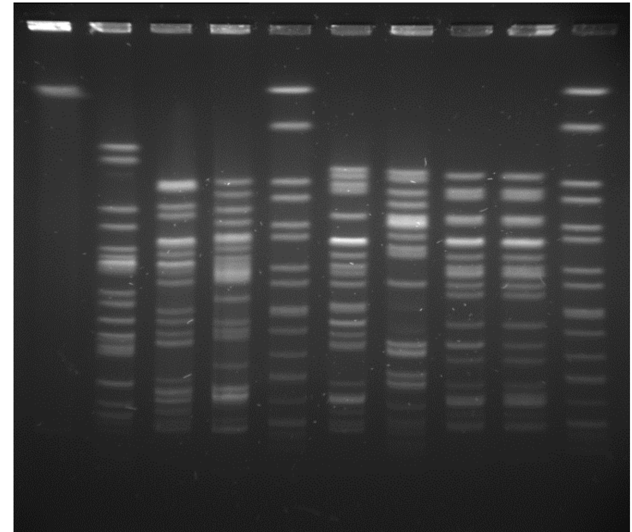
Too long restriction of DNA may result in degradation

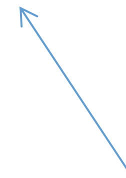
A few examples….





Partial restriction





No restriction!!!!

PFGE run

- **Buffer must be prepared fresh and used only one time**

Let the gel solidify completely before removing the comb

Cover the gel for preventing that dust can go into it

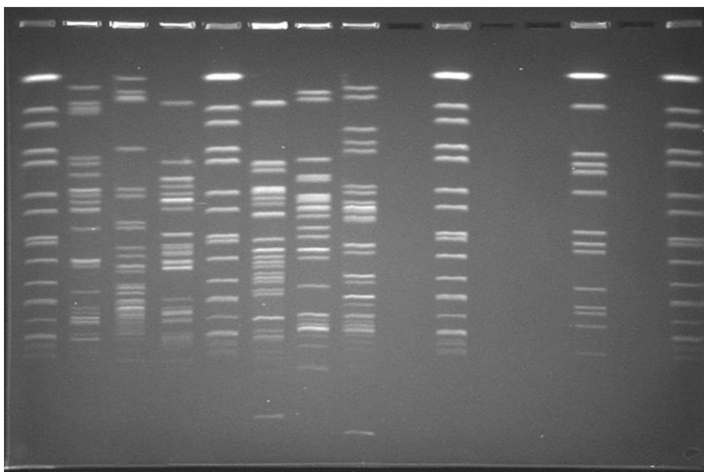
The gel must not be too thick as this would affect the resolution

Carefully load the plugs into the wells and make sure that they won’t move during the run

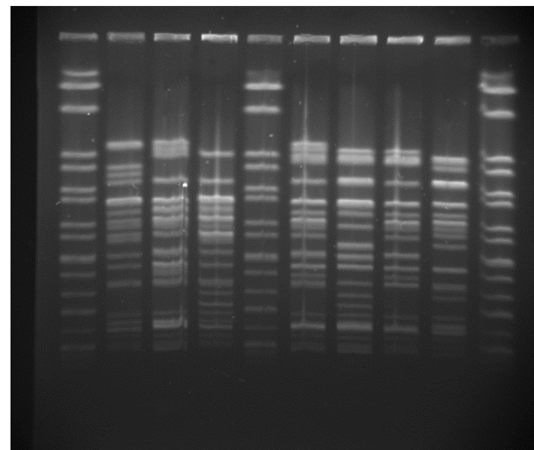
Temperature and running conditions are crucial

Run the gel with the O157-running conditions

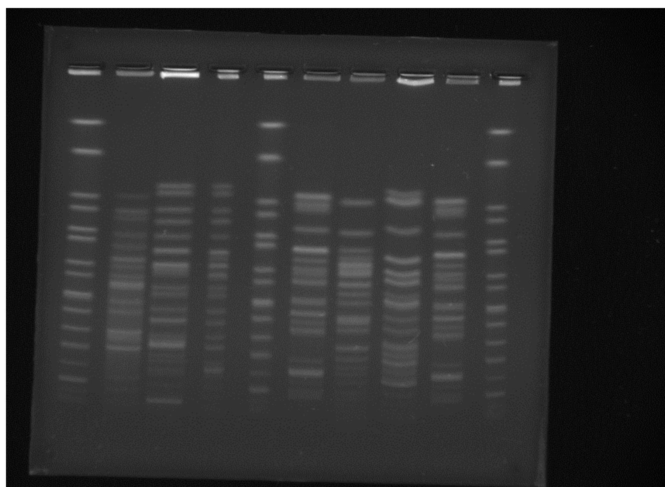
Examples



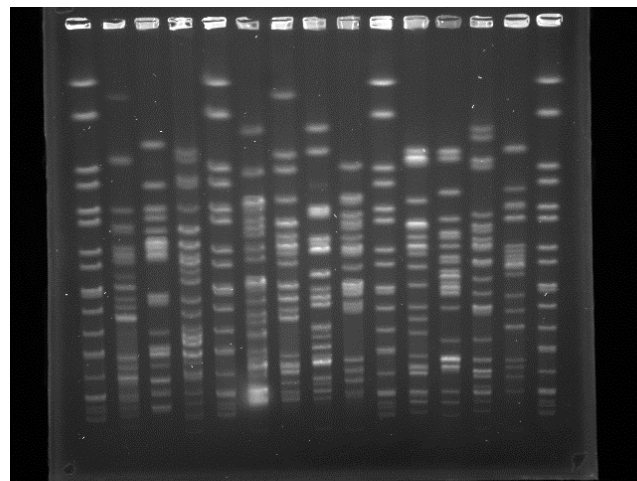
Non-O157 running conditions



Plug damaged



«Smile» effect



The pattern is not sharp in a few lanes

Gel Staining/De-staining

Stain the gel in freshly prepared 1 µg/ml EtBr solution

Do not stain for more than 20 minutes

this would make it difficult to remove background

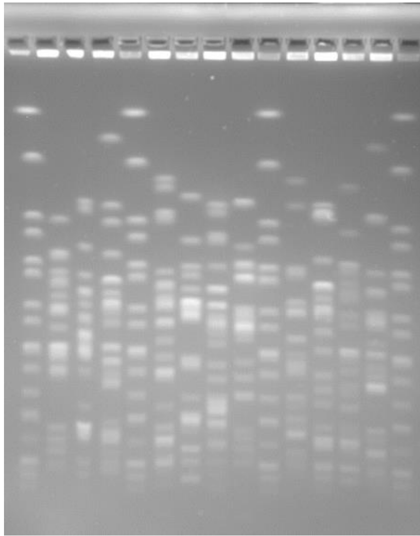
De-stain the gel in pure water

More than one time

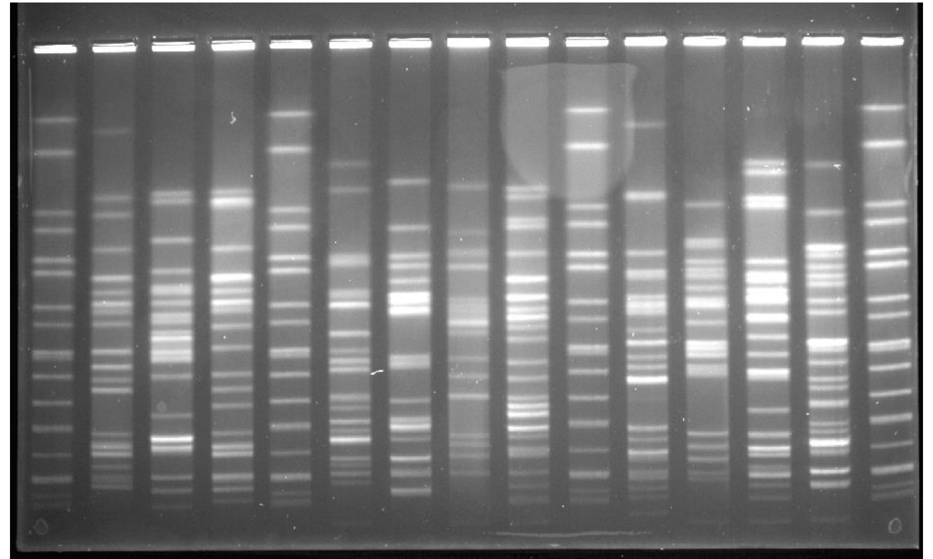
Staining and de-staining shall be done in clean boxes with slow agitation

Proper de-staining helps in avoiding high background noise

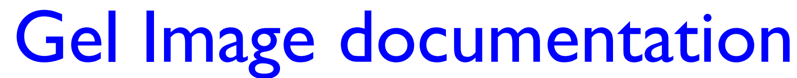
Examples



Very high background



high background, lot of stains



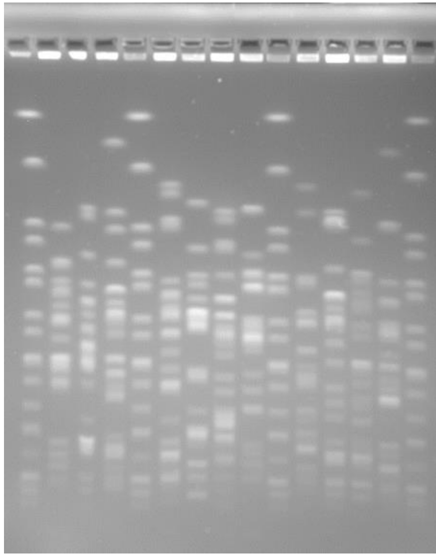
Capture the whole gel in the image (from top to bottom)

The image must be in focus

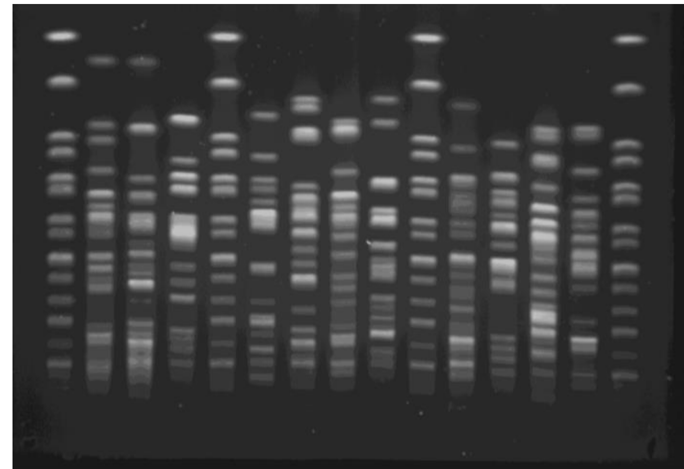
Avoid over-exposure as it would make difficult the following analysis

More than one image aquisitions may be done, but only one must be further used for the analysis

Examples



Overexposure



Out of focus

Self-evaluation of quality assessment of TIFF files

1. The gel image should fill the entire window screen (without cutting off wells or lower bands, the end of the gel must be visible in the TIFF image).
2. The gel image should be in focus with no over-exposure of the bands.
3. The bottom band of the standard must be 1-1.5 cm from the bottom of gel.
4. The resolution of the images must be an 8 bit uncompressed gray-scale TIFF image.
5. The intensity of the bands should be approximately the same in each lane.
6. The bands should be clear and distinct all the way to the bottom of the gel; some band distortion can be accepted, but it should not interfere with the analysis.
7. The gel background should be mostly clear, not affecting the analysis.
8. DNA degradation should be avoided, in order to product clear bands.

The BioNumerics Software: database creation, experiment type, import of TIFF files, and setting up experiments

A SIX STEPS PROCESS:

- **STEP 1-** Create a new database
- **STEP 2-** Create the experiment type (Fingerprint)
- **STEP 3-** Import the TIFF files into the Database
- **STEP 4-** Analyze a TIFF:
  1. Convert a TIFF to Gel Strips
  2. Define Curves
  3. Normalize the gel
  4. Find Gel Bands
- **STEP 5-** Link Lanes to Database Entries
- **STEP 6-** Add information on the Isolates (virulence genes, serogroups etc…)

Coffee break now!

And then we'll excercise on the assessment of quality of gel images