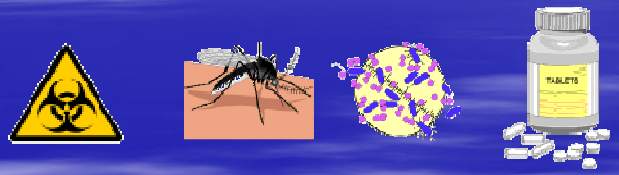
Molecular biology technique for Bacterial diagnosis



Assist. Prof. Dr. Nuvee Prapasarakul
Depart of Vet Micro, Chulalonkorn Univ.

Microbial identification

Microorganisms are identified to the genus level or

to sensitivity test ????

- **Direct techniques such as staining (104 – 105 organisms)**
- **Culture techniques**

  (inoculation onto appropriate media/biochemical tests)
- **Detection of microbial products – (14 CO_2 for M.tuberculosis)**
- **Molecular techniques – DNA probes, PCR, DNA sequencing**

Criteria for Evaluating Typing Systems

- **Typeability** → The proportion of isolates that can be assigned a type
- **Reproducibility** → The ability of the typing system to assign the same type on repeat testing of the same strain
- **Stability** → The stability of clonal expression of markers over time
- **Discriminatory power** → Discrimination index $>_{0.95}$ (5% probability of assigning independent isolates to the same type)
- **Epidemiological Concordance** → The capacity of a typing scheme to classify all epidemiologically related isolates from an outbreak into the same clone

Conventional Phenotypic Typing Systems

- **Antibiogram**
- **Biotype**
- **Phage typing**
- **Bacteriocin typing**
- **Serotyping**
- **Protein profile (PAGE)**
- **Multilocus Enzyme Electrophoresis (MLEE)**

Genotype → Gene expression → **Phenotype**

Antibiogram

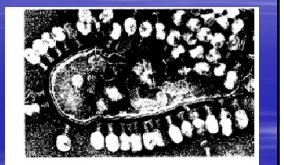
- Clinical isolates are routinely tested for antibiotic resistance/sensitivity
  - Qualitative results – disk diffusion; breakpoint assay
  - Quantitative results – MIC determination; E-test strip
- Discrimination dependent upon
  - the diversity and prevalence of resistance
  - the antibiotics tested
- Limitations of the method
  - Variable phenotypic expression of low level or inducible resistance

Biotyping

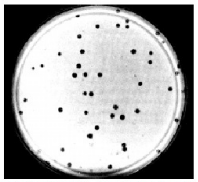
- Based on subspecies diversity of
  - Colony morphology
  - Metabolic activity
  - Toxin production
- Commercial systems now in use that generate "biotype codes" automatically
- Limitations of the method
  - Better for identification than typing
  - Show poor discrimination and repeatability

Phage Typing

- Bacteriophage are viruses that infect bacteria
- Susceptibility to lysis requires presence of phage receptor
- A single phage can replicate 200 fold in 15–20 minutes and then lyses the host cell – releases phage particles
- Plaques of growth inhibition can be seen on an indicator lawn of sensitive cells



Phage binding to an *E.coli* cell



Phage plaques in bacteria lawn

Phage Typing

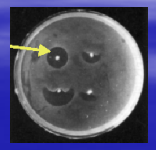
Used in reference laboratories for typing some important pathogens

- Salmonella/Shigella/S. aureus
- Susceptibility to lysis by a defined set of bacteriophages
- One of the most discriminating phenotypic typing systems
- Limitations of the method
  - Incomplete typeability
  - Poor reproducibility
  - Genetic instability of phage typing

Bacteriocin Typing

Protein antibiotics that kill sensitive indicator strains

- Bacteriocin typing is based upon;
  - the ability of test strains producing bacteriocins to lyse a standard set of indicator strains
  - the sensitivity of test strains to bacteriocins produced by a set of standard producing strains
- Originally used in reference laboratories for typing Klebsiella pneumoniae, Pseudomonas aeruginosa
- Limitations of the method
  - Difficult to standardise
  - Poor reproducibility
- **Little used today**

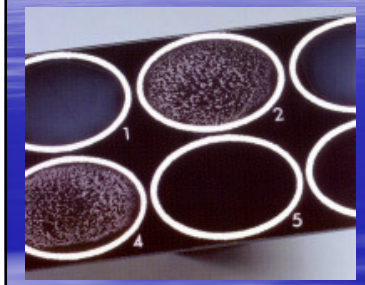


Serotyping

Antigens expressed on the microbial cell surface can be characterised by species-specific antibodies

- Agglutination; immunofluorescence

- Technically simple, reproducible, low cost method
- Limitations of the method
  - Limited by availability of specific antisera
  - Errors in typing caused by antigen variation within a clone
  - Evolutionary convergence of unrelated clones to expression of identical epitopes



Technique Serotyping

Strain typing	+
Taxonomy	-
Population genetics	-

Notes: antigenic variation often not correlated with genetic variation

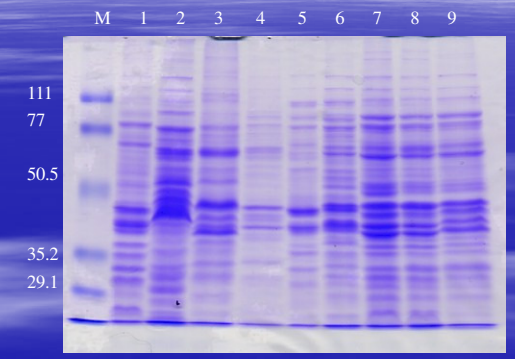
Protein Profile

- Analyse variation in whole cell proteins using polyacrylamide gel electrophoresis (PAGE)
- Can combine with immunoblotting to give more discrimination
- Can be applied to a number of pathogens
- Relatively cheap to perform

Limitations of the method

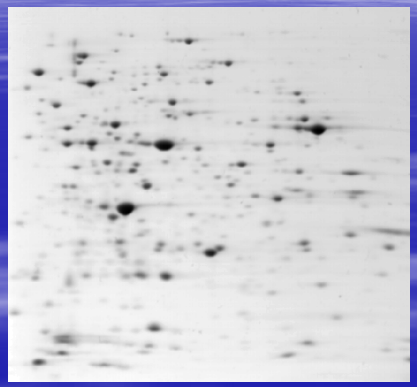
- Difficult to standardise
- Complex protein profiles are difficult to interpret

Comparison of protein profiles of unidentified canine isolates and the reference belonging to genus *Brachyspira*.

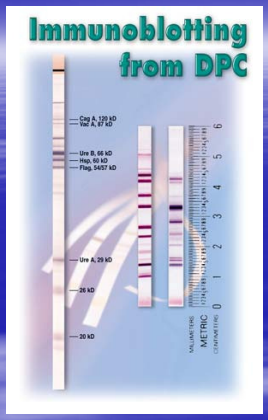


Lanes 1, *B. hyodysenteriae* ATCC27164; 2, *B. pilosicoli* P43/6/78; 3, *B. innocens* ATCC29796; 4, *B.aalborgi* NCTC11492;5, *B. avinipulli* C1; 6, D3a/8/31; 7, D1a/10/25; 8, D2a/10/259, D1d/6/15

2D PAGE ANALYSIS



Immunoblotting from DPC



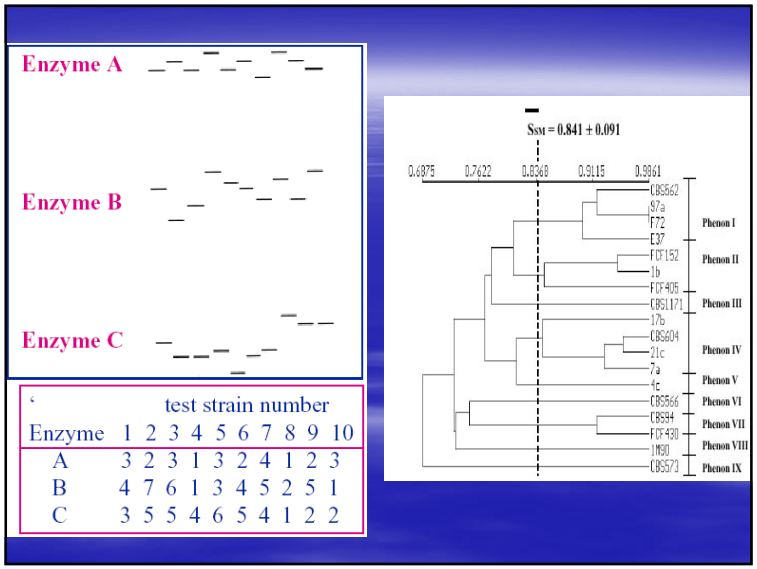
Technique Protein profiling and Western blotting

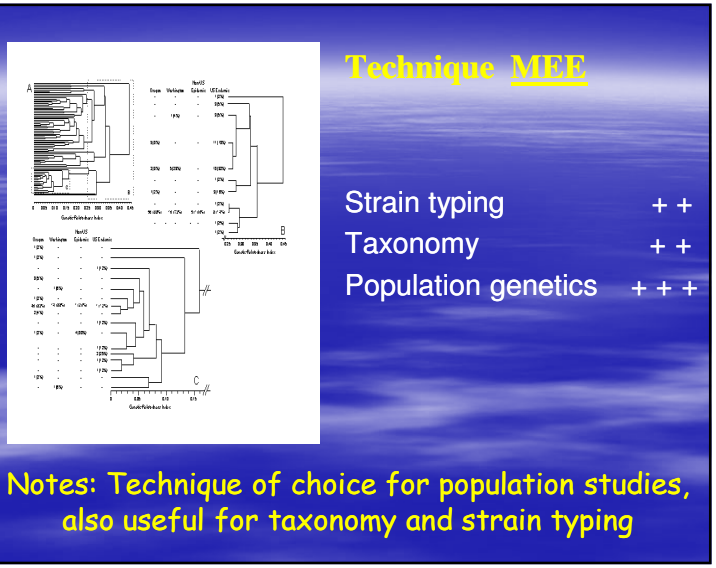
Strain typing	+ +
Taxonomy	+ +
Population genetics	-

Notes: useful adjunctive technique but technique difficult to interpret objectively.

Multilocus Enzyme Electrophoresis

- Search for electrophoretic variants of a set of 10–15 standard "housekeeping enzymes"
- Many strains can be compared on one gel
- Detect the enzyme activity using chromogenic substrates
- The data is then used to assign an electrophoretotype
- Widely used method for a number of pathogenic bacteria



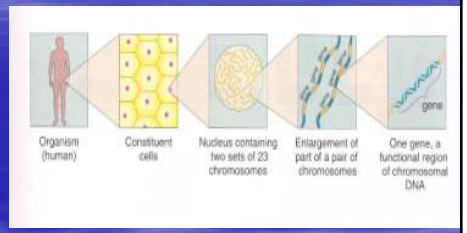


Conventional Phenotypic Typing Systems

System	Typeability	Discrimination	Reproducibility	Stability	E C
Antibiogram	Excellent	Variable	Good	Variable	Good
Biotype	Excellent	Variable	Good	Good	Moderate
Serotype	Good	Moderate	Good	Good	Good
Phage type	Moderate	Good	Moderate	Moderate	Good
Bacteriocin	Moderate	Good	Moderate	Moderate	Moderate
PAGE	Excellent	Moderate	Moderate	Moderate	Moderate
Immuno-blotting	Excellent	Good	Good	Moderate	Good
MLEE	Excellent	Good	Excellent	Excellent	Good

Molecular typing System

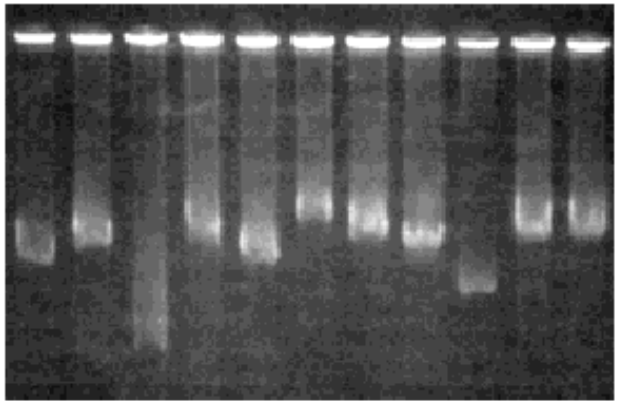
Techniques
- Plasmid analysis
- PCR
- G+C content
- 16S rRNA/DNA
- Gene sequencing
- Hybridization
- RFLP analysis
- REA
- PFGE
- RAPD



Genotype → Gene expression → Phenotype

Plasmid Analysis

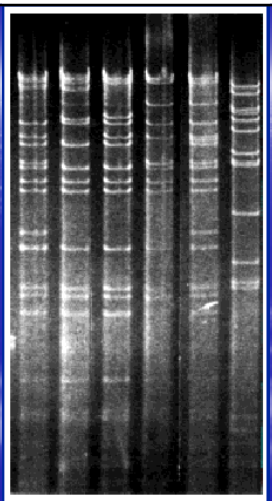
Extrachromosomal genetic elements that replicate independently of the chromosome
- Easily purified and separated by size on an agarose gel
- Encode useful properties to the host cell
- Antibiotic resistance; metabolic activities
- The first DNA-based typing method for epidemiological studies of nosocomial infections (1988)
- Limitations of the method;
- Requires species to harbour multiple, diverse plasmids
- Can be difficult to accurately size the plasmids
- The presence of identical plasmids can be the result of horizontal transfer rather than clonal relatedness



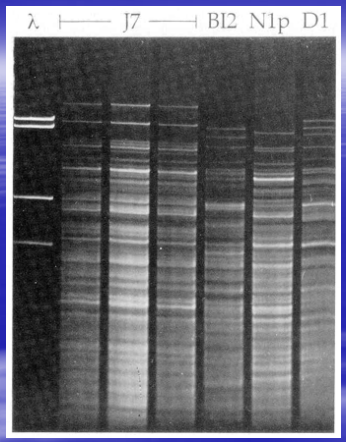
Plasmid profile of *E.coli* strains

Plasmid Restriction Enzyme Analysis

Helps distinguish two dissimilar plasmids of identical size
- Restriction endonucleases cut the plasmid DNA at defined sequences and generate linear fragments
- i.e. EcoRi cuts at GAATTC ds sequences
- The size of fragments can be compared directly on the agarose gel (sieving effect)
- Image capture and computer analysis of the bands is possible
- Limitations of the method;
- Only works if plasmids are present
- Are plasmids missing a few bands identical?



*Eco*RI Plasmid profile



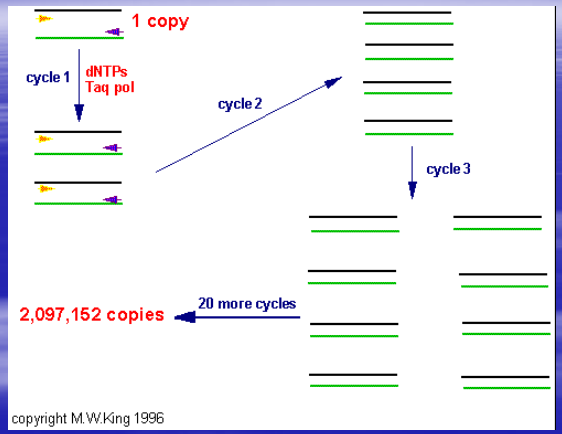
Restriction Endonuclease Analysis (REA)

Strain typing	+ +
Taxonomy	+
Population genetics	-

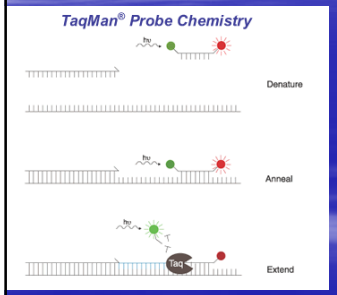
Notes: whole genomal technique, large of band interpretation hard.

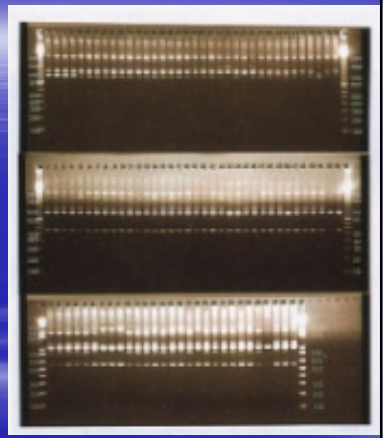
Polymerase Chain Reaction

- The PCR is a powerful technique used to amplify DNA millions of fold, by repeated replication of a template, in a short period of time.
- The process utilizes sets of specific in vitro synthesized oligonucleotides to prime DNA synthesis.
- The technique is carried out through many cycles (usually 20-50) of melting the template at high temperature, allowing the primers to anneal to complimentary sequences within the template and then replicating the template with DNA polymerase.
- The process has been automated with the use of thermostable DNA polymerases isolated from bacteria that grow in thermal vents in the ocean or hot springs.

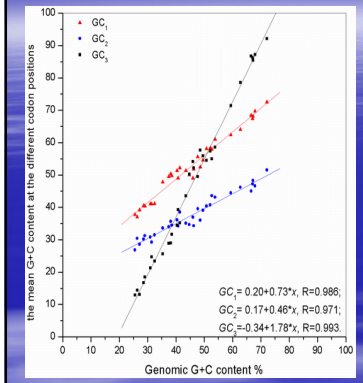


Pcr.swf





Technique G+C content

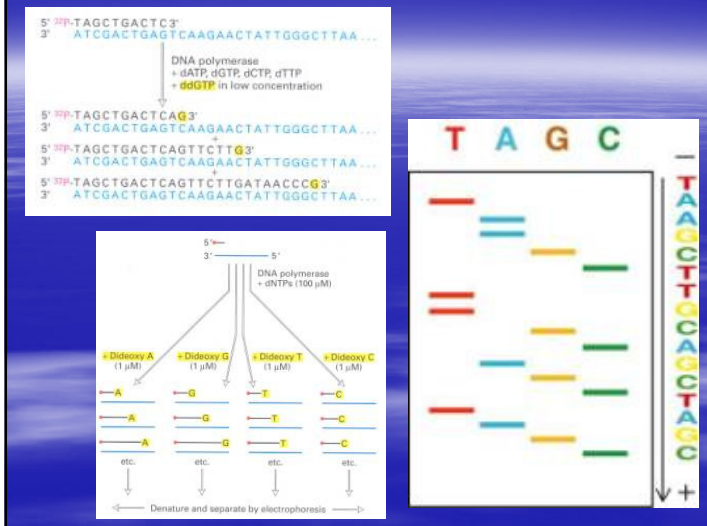


Strain typing	-
Taxonomy	+
Population genetics	-

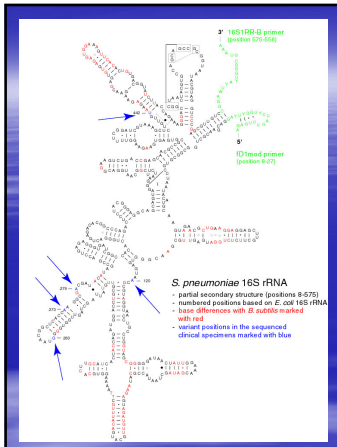
Notes: important taxonomic characteristic but of little discriminatory value.

16S DNA Sequencing

- Since PCR amplifies selective DNA targets, the ultimate resolution would come by sequencing the amplified DNA
- This technology can be used for bacteria, viruses and fungi
- Easy to standardise between laboratories by using standard reference strains to assess the sequencing accuracy
- In bacteria it is usual to sequence a hypervariable region of $_{16}$S RNA using PCR sequencing
- Limitations of the method:
  - Cost of the procedure
  - How many bases can be different before two strains are not in the same typing group ??



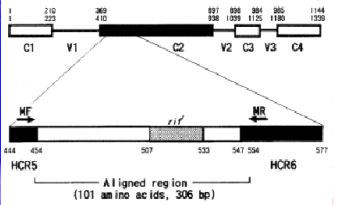
Technique 16S rRNA/DNA



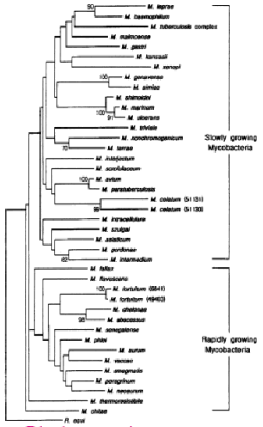
Strain typing	-
Taxonomy	+++
Population genetics	-

Notes: technique of choice for phylogeny and relationships at the genus level.

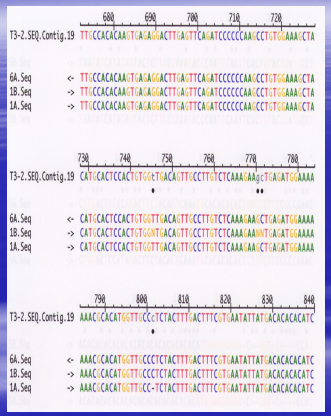
- Kim *et al* report the use of PCR sequencing of the *rpoB* gene of Mycobacterial species



Primer design



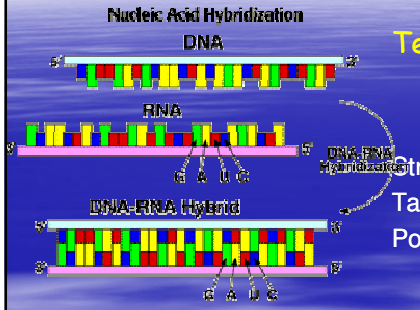
Phylogenetic tree



Technique Gene sequencing

Strain typing	+ +
Taxonomy	+++
Population genetics	+ +

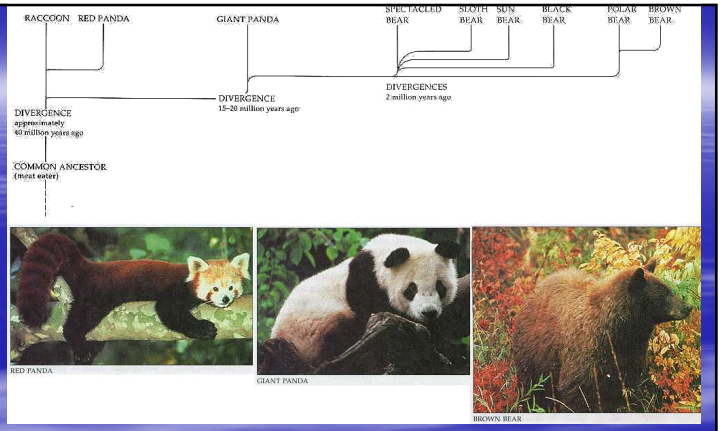
Notes: not routine used at present for certain bacteria.



Technique Hybridization

Strain typing	-
Taxonomy	+++
Population genetics	-

Notes: antigenic variation often not correlated with genetic variation

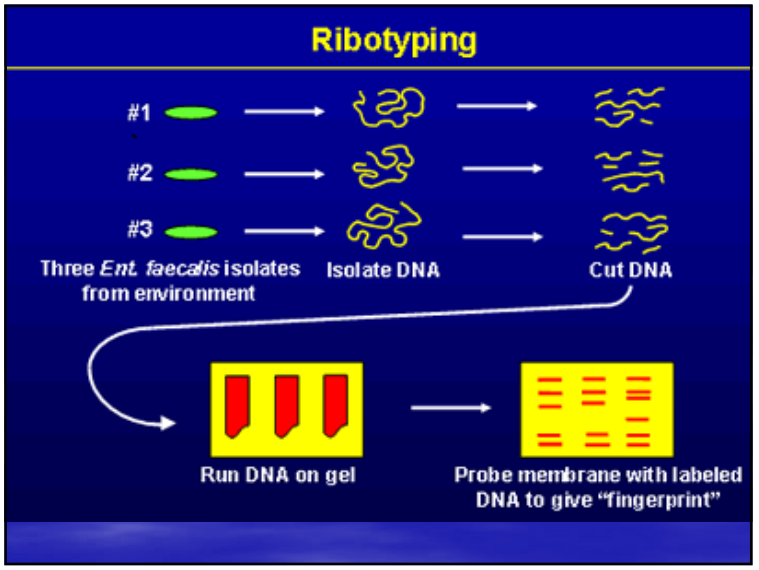


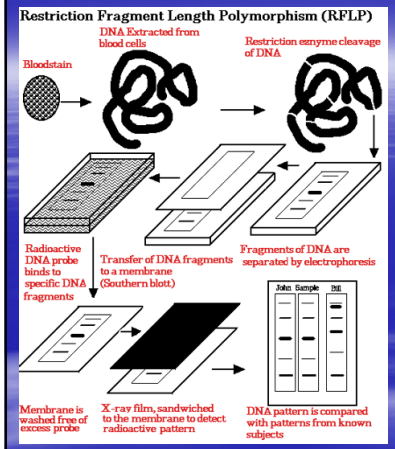
Evolutionary relationships among red pandas, giant pandas, and bears, according to DNA-DNA hybridization studies

Ribotyping

Based upon hybridization of probes with genomic restriction digests

- rRNA probes provide a universal system for all species - ribotyping
  - High conservation of sequences within rRNA genes
  - Multiple copies of rRNA operons
  - Stable sequences
  - Can use rRNA or fragments of genes
- Comparison of the bands can be automated
- Limitations of the method;
  - Discrimination depends upon the restriction enzyme and the probe used.

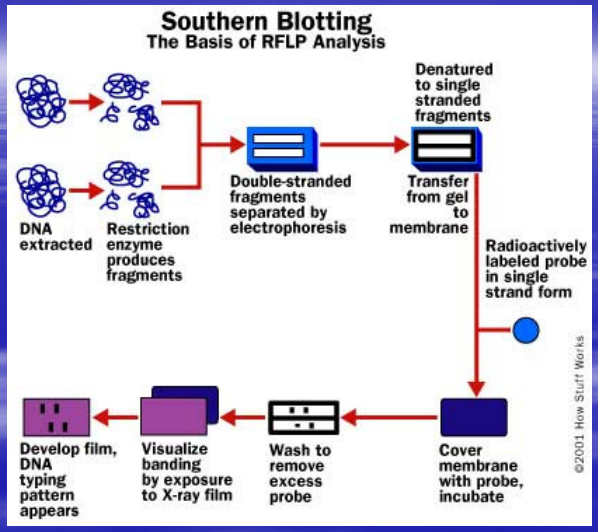




Technique RFLP

Strain typing	+ +
Taxonomy	+ +
Population genetics	+++

Notes: wide application for large populations gene probe-dependent discrimination.



Genomic PFGE Analysis

Analysis of genomic DNA avoids requirement for plasmids

- Restriction enzyme used must generate a useful number of visible fragments
- PFGE avoids this problem by using "rarecutters" that generate large fragments (50kb-12Mb)
- These are resolved in a gel run with an alternating electrical field. The change in direction of the DNA leads to retardation of larger fragments

- Limitations of the method;

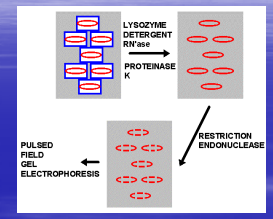
– Specialised apparatus, skill-intensive

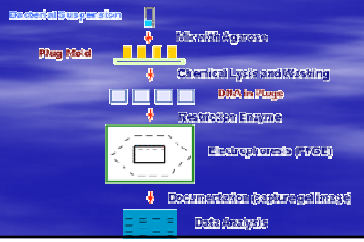
– >24 hours to get a result

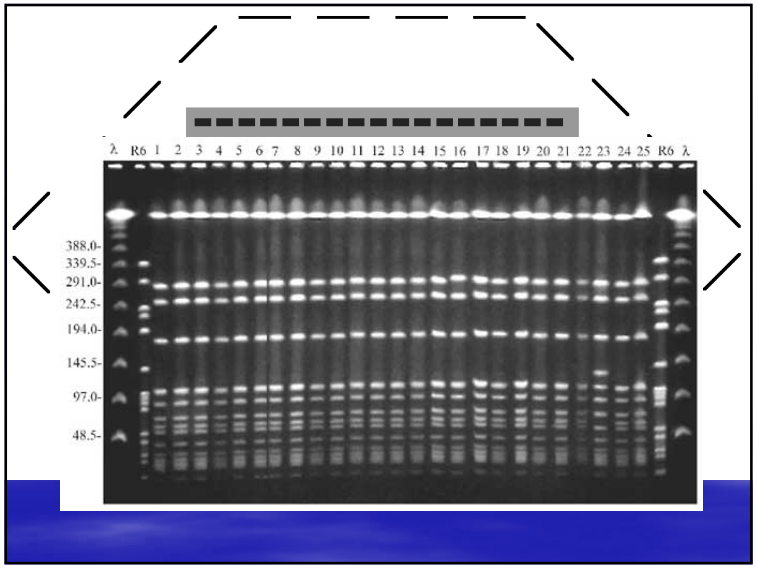
Notes: best technique for epidem. Typing also applicable for population studies

Technique PFGE

Strain typing	+ + +
Taxonomy	-
Population genetics	+ +







PCR-RFLP

Based upon amplification of genome DNA targets

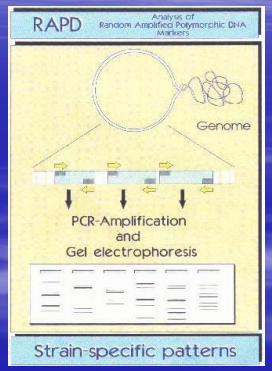
- In PCR-RFLP typing a 1-2kB sequence known to show polymorphism among strains of the species of interest is specifically amplified and then cut with restriction endonucleases
- Quicker than traditional RFLP because no hybridization step is required

- Limitations of the method:

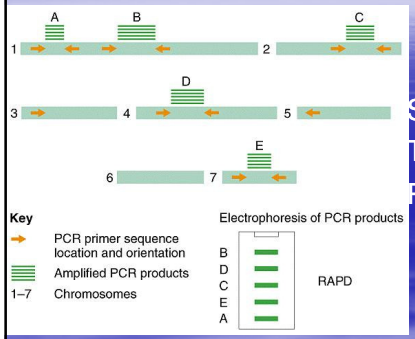
– Knowledge of the polymorphic gene sequences and selection of the restriction enzymes is critical

Arbitrary-Primed PCR (Randomly Amplified Polymorphic DNA)

- Identity strain-species variation in DNA
- Arbitrarily-chosen primers pairs to amplify
- DNA fingerprint



Technique RAPD

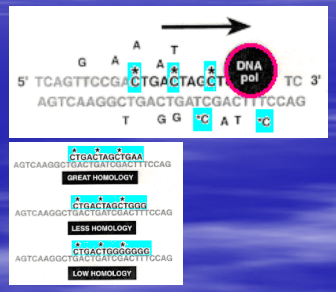


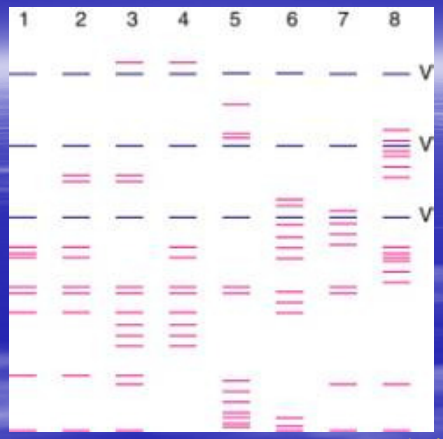
Strain typing	+ +
Taxonomy	+ +
Population genetics	+ +

Notes: Good for within and between species reproducibility not guaranteed.

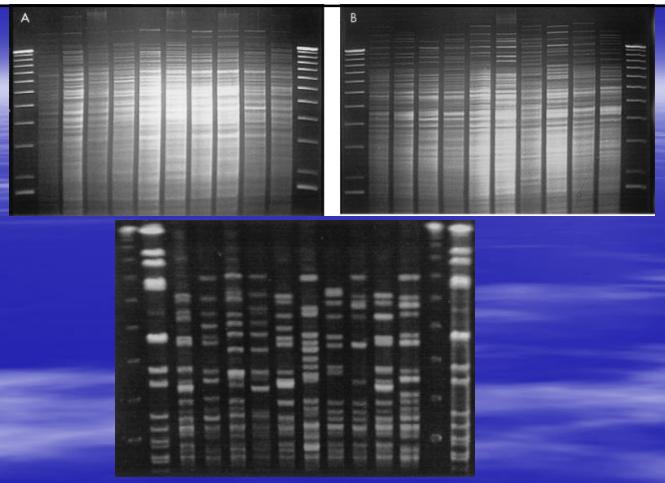
DNA Fingerprint

1. RPLF
2. PCR
3. RAPD
4. VNTRs





ถ้าหมายเลข 1 คือ DNA ของนายเฉลิม หมายเลขใดคือ DNA ของนายวันเฉลิม



Technique ? Any Explanation?

Comparison of Genomic Methods for Differentiating Strains of *Enterococcus faecium*: Assessment Using Clinical Epidemiologic Data

TABLE 2. Description of isolate sources and genomic typing results from the second potential outbreak with eight VRE strains

Isolate code	Date isolated (mo/day/yr)	Nursing unit source[a]	Specimen source	Type		
				REA		PFGE
				*Hin*dIII	*Hae*III	
EF20	3/21/93	8E	Urine	C_1	D_0	D
EF21	3/15/93	8W	Urine	B_2	B_2	B_6
EF22	4/7/93	SICU	Rectum[b]	B_2	B_2	B_7
EF23	4/7/93	SICU	Rectum	B_5	E_0	E
EF24	4/10/93	8E	Urine	D_0	F_0	F
EF26	4/22/93	SICU	Blood	B_2	B_2	B_1
EF27	4/21/93	14E	Rectum	B_6	G_0	G
EF28	4/21/93	14W	Rectum	B_7	G_1	H

Molecular Typing Systems

System	Typeability	Discrimination	Reproducibility	Stability	E C
Plasmid	Variable	Variable	Moderate	Moderate	Good
Plasmid REA	Variable	Variable	Good	Moderate	Good
Genome REA	Excellent	Good	Moderate	Good	Good
Ribotyping	Excellent	Moderate	Excellent	Excellent	Excellent
RFLP	Excellent	Variable	Excellent	Excellent	Excellent
PFGE	Excellent	Excellent	Good	Good	Excellent
PCR-RFLP	Excellent	Variable	Excellent	Excellent	Excellent
DNA sequence	Excellent	Excellent	Excellent	Good	Excellent

Selecting Molecular Typing Systems

Currently

- Antimicrobial sensitivity testing
- Serotyping
- PCR
- PFGE for confirmatory genotypic analysis

• In the future

- Automated DNA sequence analysis both for identification and typing systems