

**Molecular Diagnostics  
of Bacterial Infections:  
Current Status and Future Trends**

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**Houston, Texas**



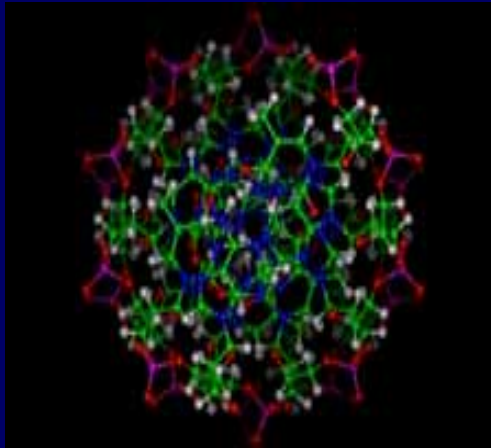
# TEXAS CHILDREN'S HOSPITAL

## Houston, Texas



# Molecular Diagnostics

## DNA and RNA Detection



**Transverse**

**Base Complementarity  
Yields Specificity**



**Longitudinal**

# Molecular Diagnostics

## Why?

- Detection and Diagnosis
  - unculturable or difficult to culture
  - need for rapid answer
  - inadequacy of phenotypic methods (biochemical)
- Prognosis and management
  - need for quantitative information (viral load)
  - susceptibility testing (drug resistance) without culture
    - Molecular resistance testing

# Molecular Microbiology

- Qualitative Testing - detection of pathogen  
- presence or absence
- Quantitative Testing - “viral load” - for  
management of infected individuals
- Drug Resistance Testing - detection of  
mutations associated with resistance
- Molecular Epidemiology - molecular strain  
identification to examine disease  
outbreaks

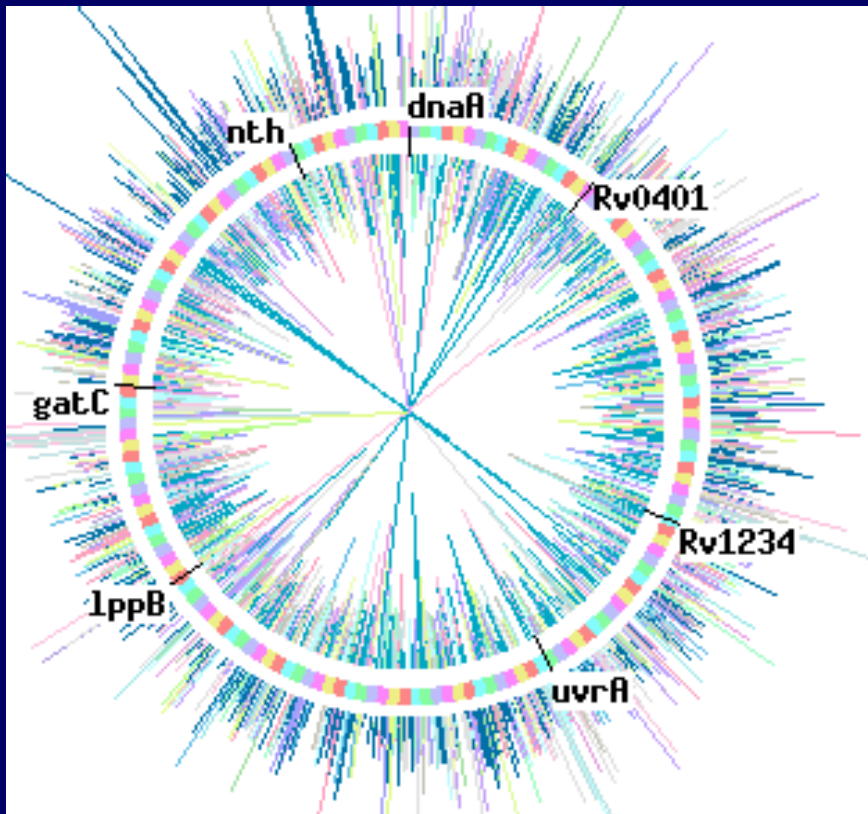
# Bacterial Pathogens

- *Bordetella pertussis* (respiratory)
- *Chlamydia trachomatis/Neisseria gonorrhoeae*
  - detection in cervical specimens or urine
- *Enterococcus* sp. (vancomycin resistance)
- *Helicobacter pylori* (gastric and stool)
- *M. pneumoniae* (respiratory and CNS)
- *M. tuberculosis* (resistance and extrapulmon)
- *Neisseria meningitidis* (meningitis and blood)
- *Staphylococcus* (bloodstream infections)

# Bacterial Targets

- *Mycobacterium tuberculosis*
- Methicillin-Resistant *Staphylococcus*
- *Helicobacter pylori*

# *Mycobacterium tuberculosis*



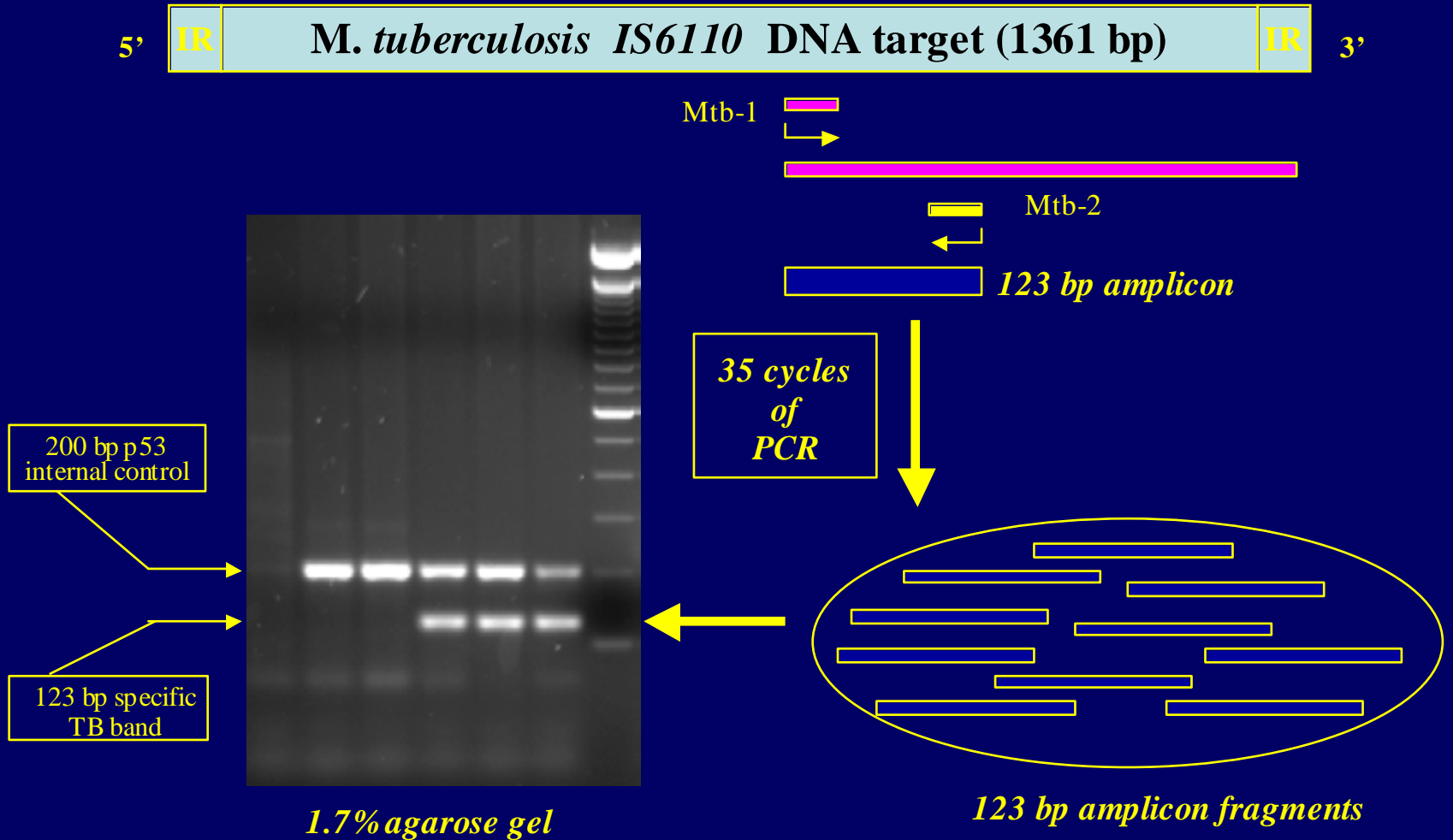
- Translation, ribosomal structure and biogenesis
- Transcription
- DNA replication, recombination and repair
- Cell division and chromosome partitioning
- Posttranslational modification, protein turnover
- Cell envelope biogenesis, outer membrane
- Cell motility and secretion
- Inorganic ion transport and metabolism
- Signal transduction mechanisms
- Energy production and conversion
- Carbohydrate transport and metabolism
- Amino acid transport and metabolism
- Nucleotide transport and metabolism
- Coenzyme metabolism
- Lipid metabolism
- Secondary metabolites biosynthesis, transport and catabolism
- General function prediction only
- Function unknown
- No COG match

4411529 bp  
genome

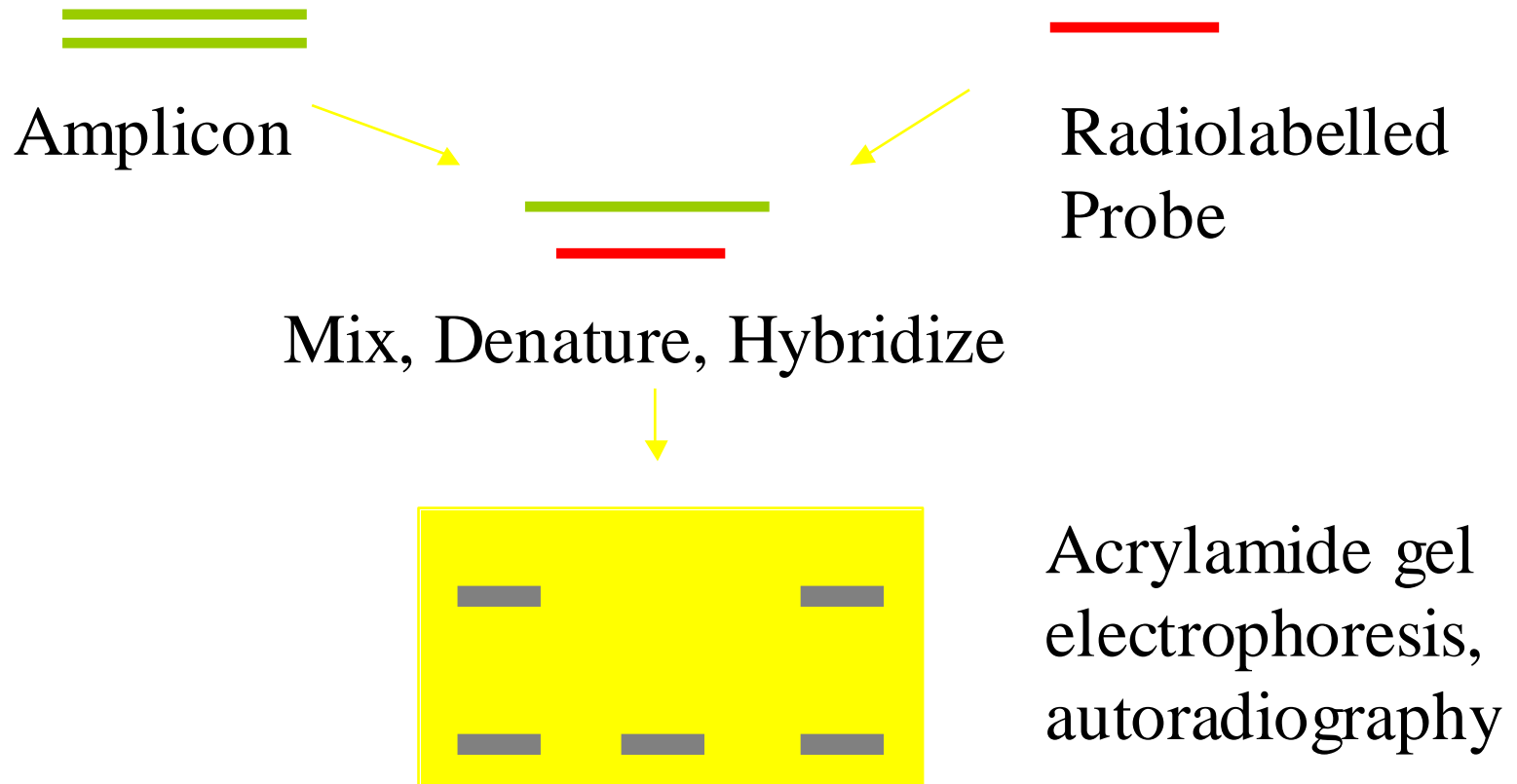
# Significance and Testing

- Major Global Pathogen (“top 3”)
- Diagnosis of Extrapulmonary Tuberculosis
  - Blood – most common isolate in blood cultures in Africa
  - Central nervous system (CSF) –
    - tuberculous meningitis
  - Tissue – Intestinal
- Molecular Resistance Testing

# TB PCR & Detection



# Amplicon Detection Probe Hybridization in Solution



# *Mycobacterium tuberculosis*

## Molecular Resistance Testing

- **Rifampin (RIF)**
  - Binds to  $\beta$  subunit of RNA polymerase (*rpoB*)
  - 96% of resistant Mtb isolates have mutations in 81-bp region – well-studied
  - Four (4) mutations – 75% of resistant clinical isolates
- **Isoniazid (INH) – two genes**
  - *katG* and *inhA* – 75-85%
- **Pyrazinamide – *pncA* – 70%**
- **Streptomycin – *rpsL* – 65-75%**
- **Ethambutol – *embB* – 70%**

# Molecular Methods - Resistance

- Reverse hybridization
  - Line probe assays
- RNase Cleavage
- Diagnostic Sequencing (Genotyping)



Looking for mutations

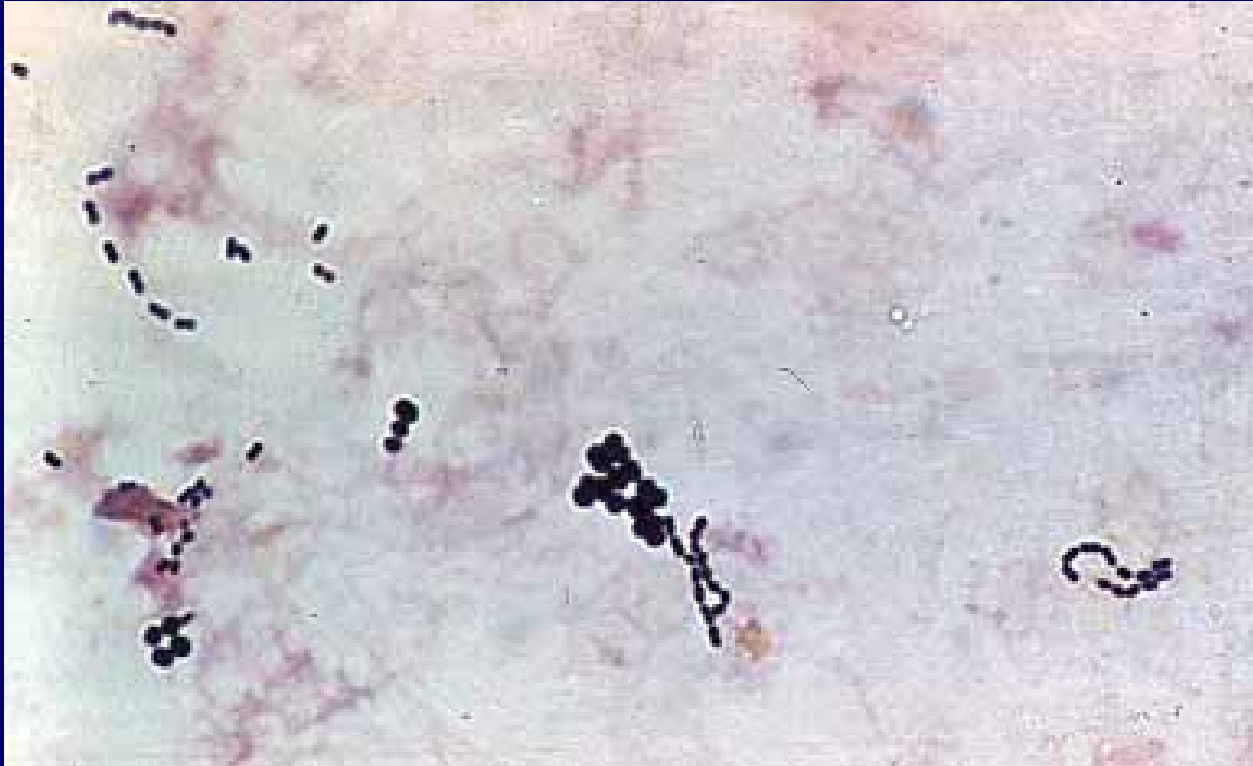
# RNase Cleavage

- Watterson et al., J Clin Microbiol (1998)
- Mutation scanning – excellent correlation with LiPA
- Nested PCR of wild type and patient
- Inner promoter primers – SP6 and T7
- In vitro transcription of each RNA strand
- RNA:RNA heteroduplexes
  - RNA proficiency required
- Cleave with RNase 1 and T1 (not RNase A)
- Gel detection; could include isotope incorporation
- NIRCA (Ambion) - \$555 for 120 tests

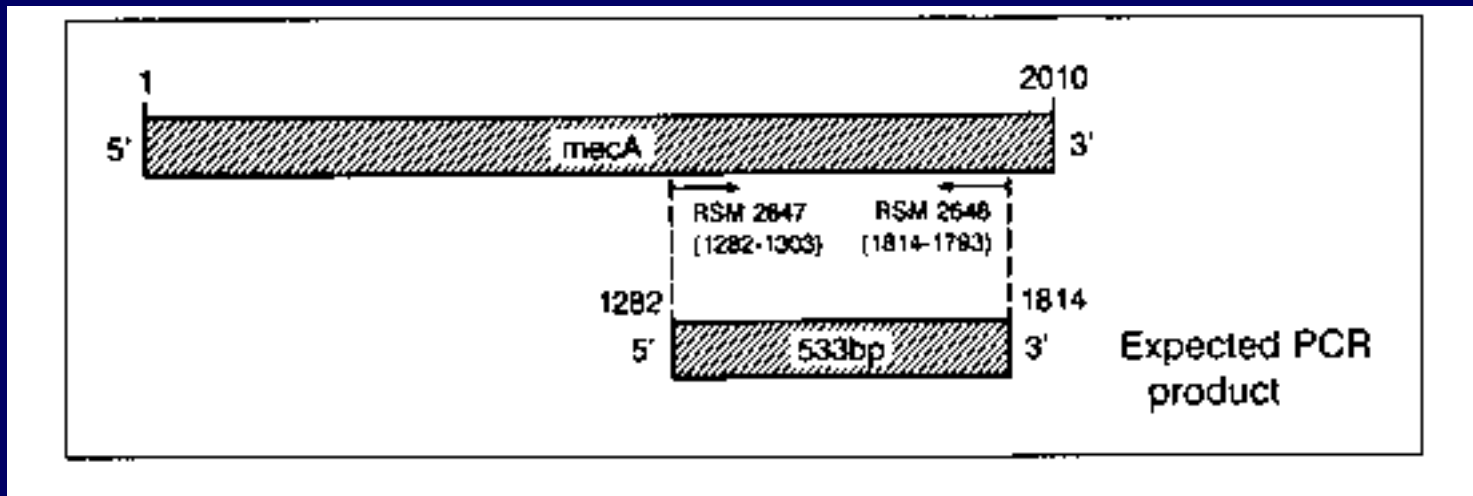
# Methicillin Resistance in *Staphylococcus*

- gene detection
- *mecA* gene encodes PBP 2' (2a)
- low affinity PBP conferring resistance
- integrated approach - phenotype / genotype
- revised NCCLS guidelines
  - distinguish *S. aureus* and CoNS
  - oxacillin disc, oxacillin-salt agar (*S. aureus*)

## ***Staphylococcus* and *Streptococcus* in Blood Cultures**

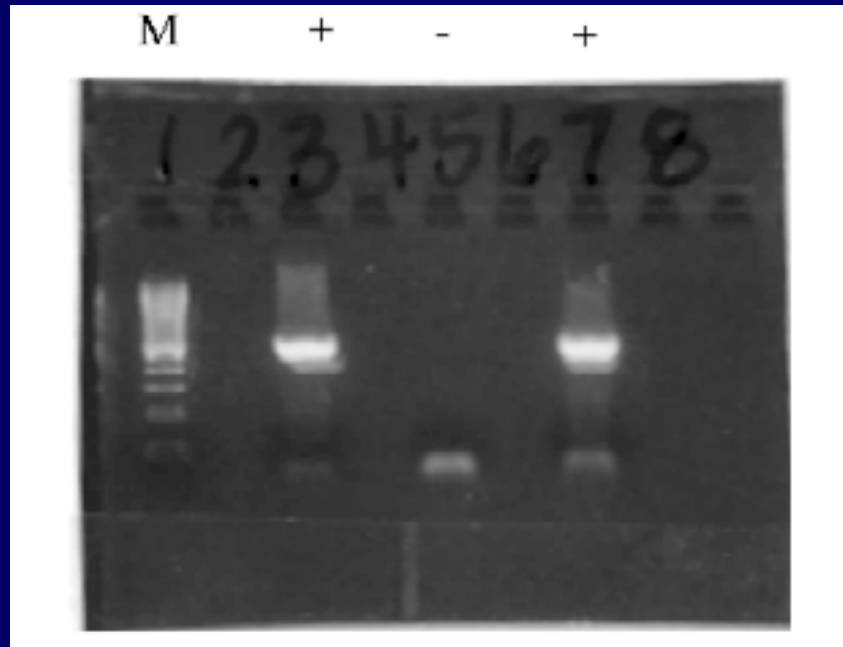


# *mecA* Gene Detection in *Staphylococcus* sp.



Murakami K and Minamide W. PCR Identification of Methicillin-Resistant *Staphylococcus aureus*. Sec 6.4  
Persing, D, T. Smith, F Tenover, and T White. *Diagnostic Molecular Microbiology: Principles and Applications*. Washington, D.C.: American Society for Microbiology, 1993.

# *mecA* Gene Detection in *Staphylococcus aureus*



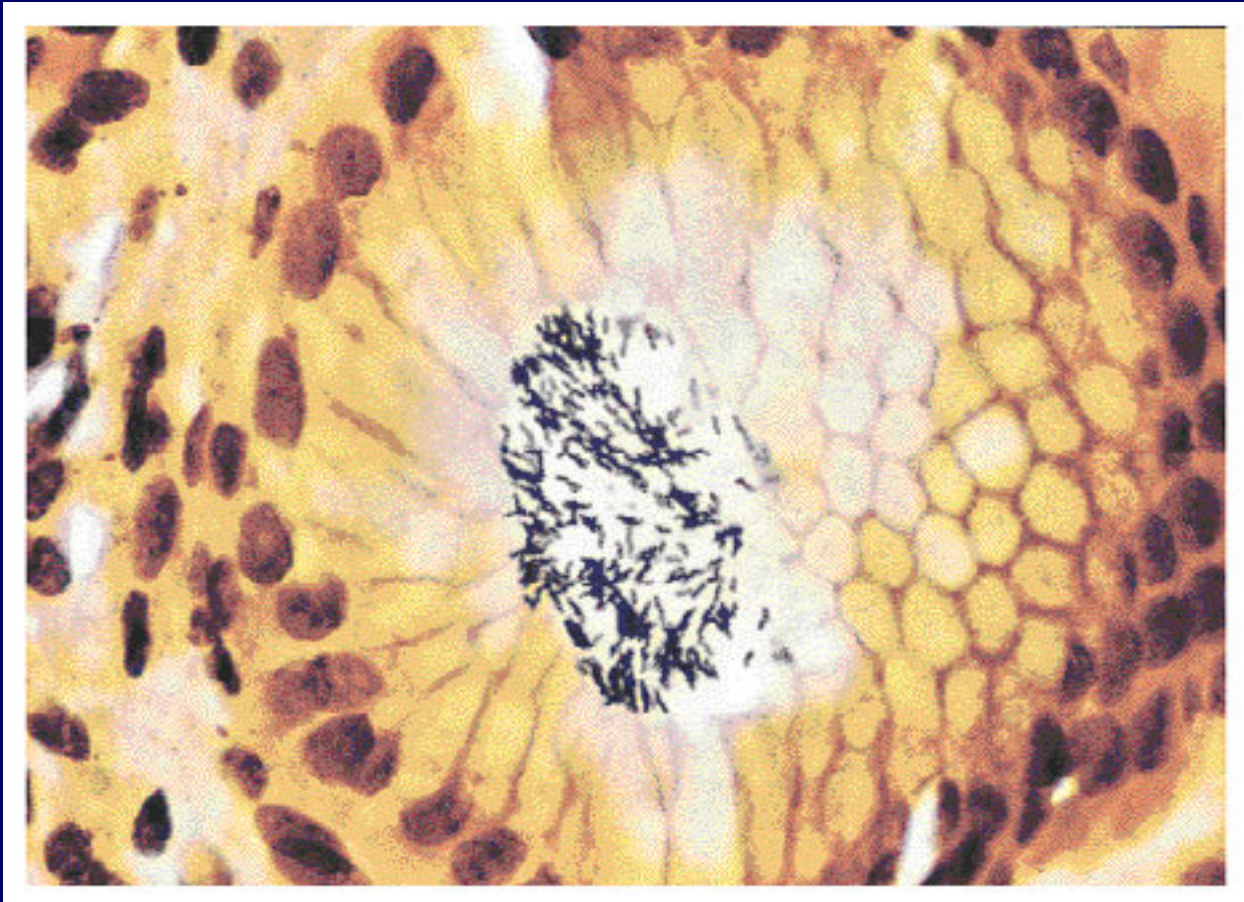
# *Helicobacter pylori* – A Major Global Pathogen



Modified Gram Stain

# *Helicobacter pylori* in Gastric Pit

(Genta R and Graham DY NEJM (1996))



# Amplification of *glmM* from *Helicobacter* sp.



## Lane:

- 1 - *H. bilis*
- 2 - *H. canis*
- 3 - *H. cholecystus*
- 4 - *H. cinaedi*
- 5 - *H. hepaticus*
- 6 - *H. muridarum*
- 7 - *H. mustelae*
- 8 - *H. pullorum*
- 9 - *H. pylori* (Sydney)
- 10 - *H. rodentium*
- 11 - *Lacto. murinus*
- 12 - *E. coli* MC4100
- 13 - negative control
- 14 - 100 bp MW

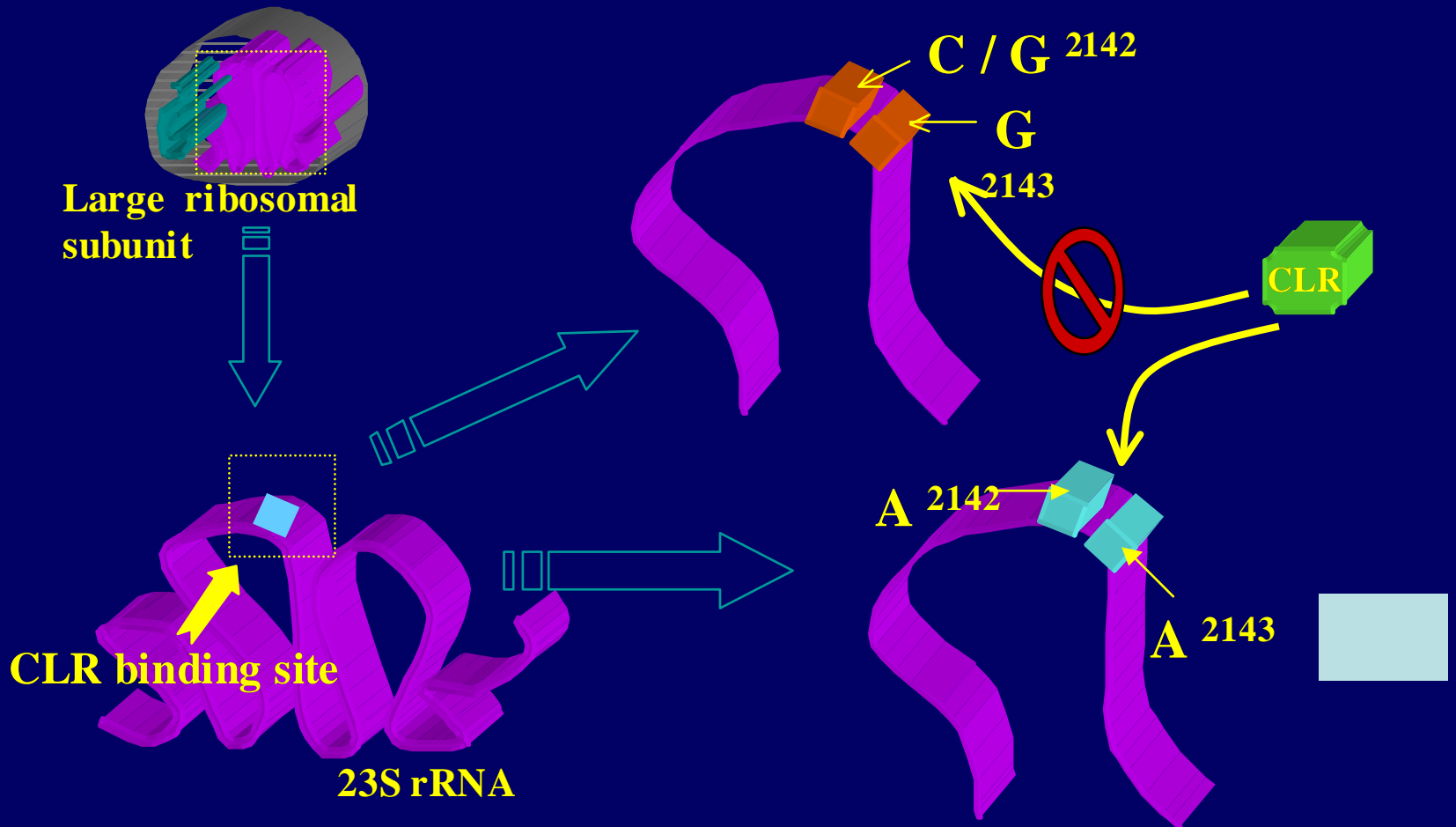
# Antimicrobial Agents and *H. pylori*

- **Clarithromycin or Metronidazole**
- Tetracycline HCl or Amoxicillin
- Bismuth Subsalicylate

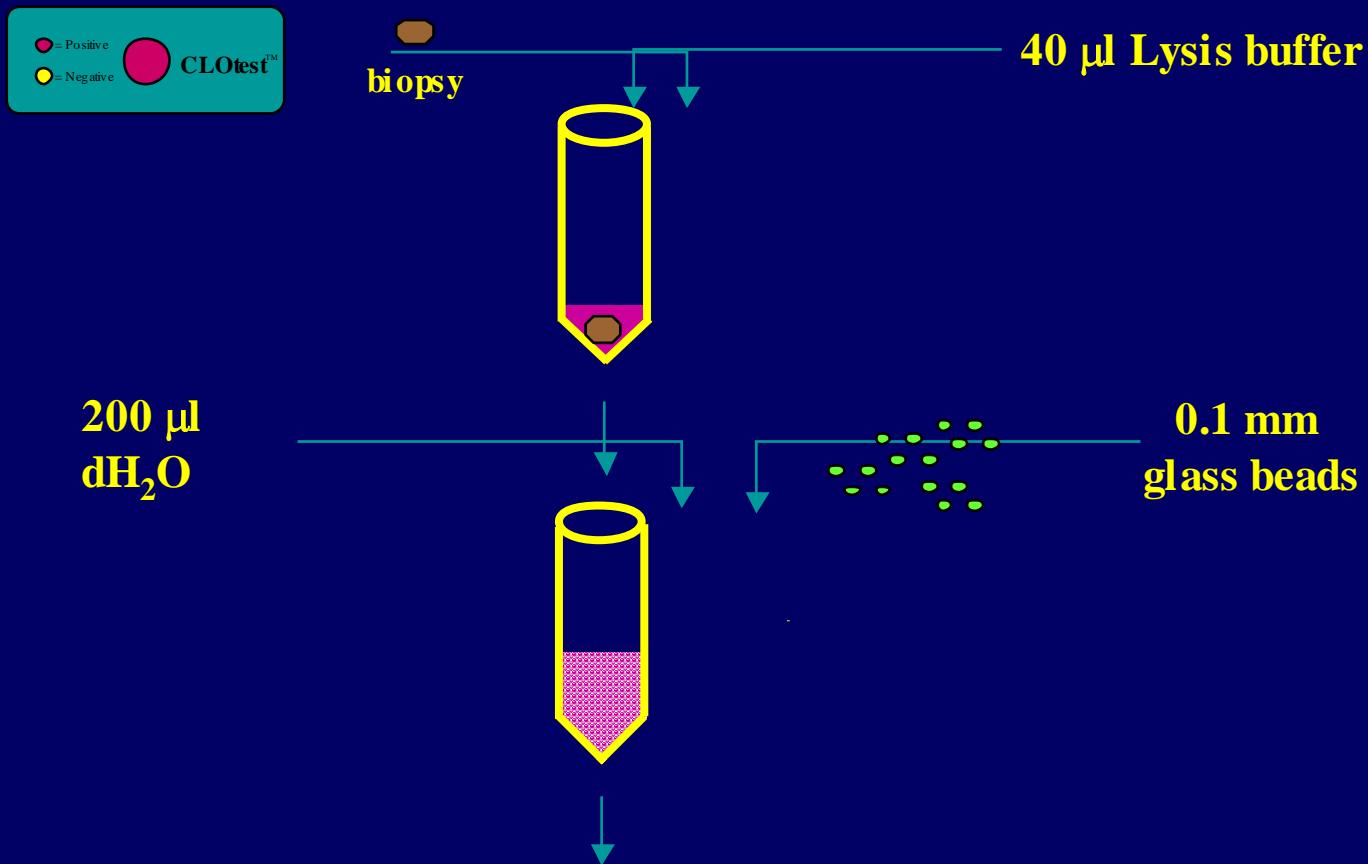
# Macrolide Resistance Predicts Treatment Failure

- *Helicobacter pylori*
- treatment efficacy reduced by 55%
- nearly 100% predictive of treatment failure
- 83-98% cured if susceptible
- 25-50% failed if resistant

# Base Substitutions in 23S rRNA Confer Resistance to Macrolides

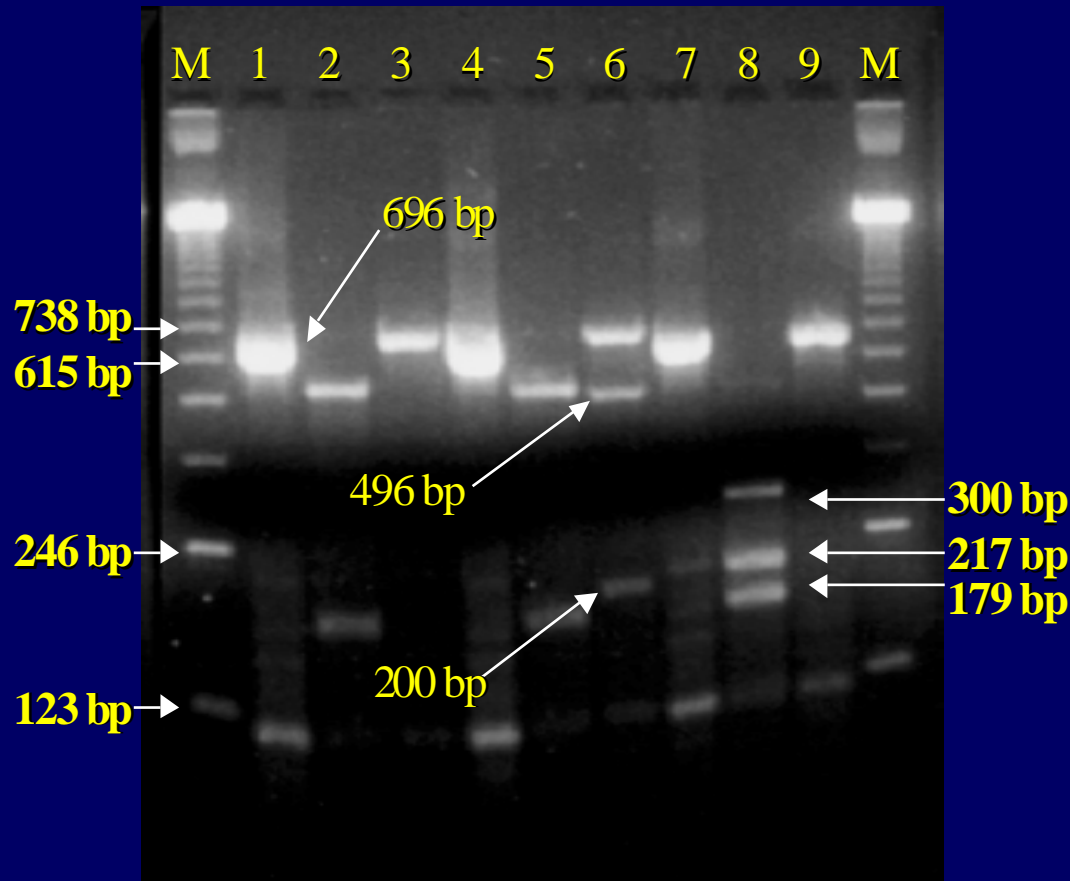


# Differential Lysis of CLOtest™ Biopsy Material



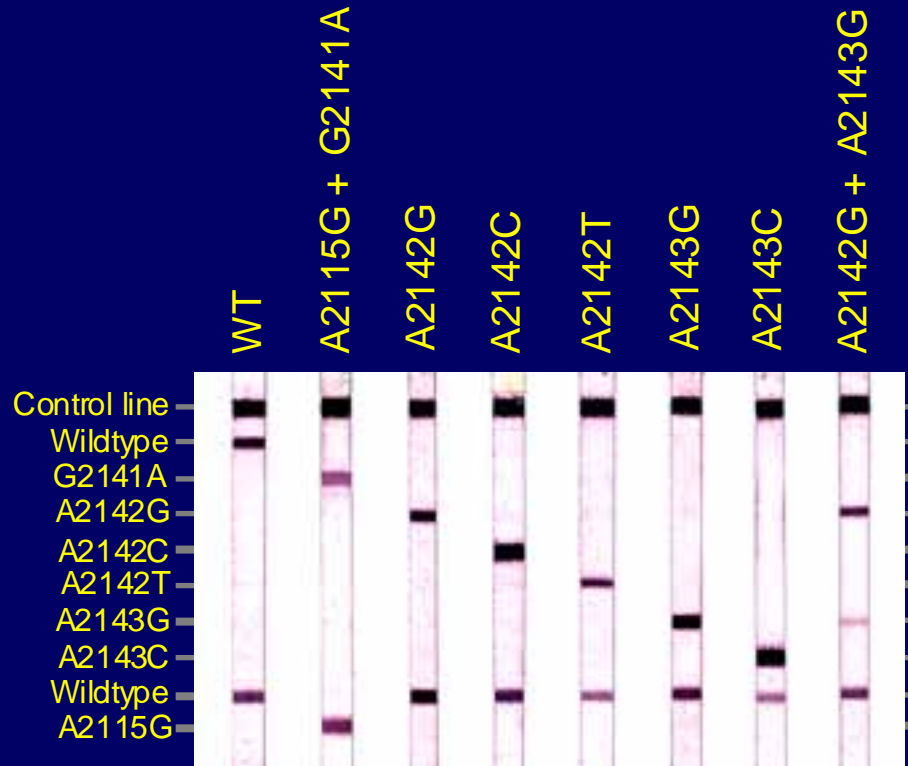
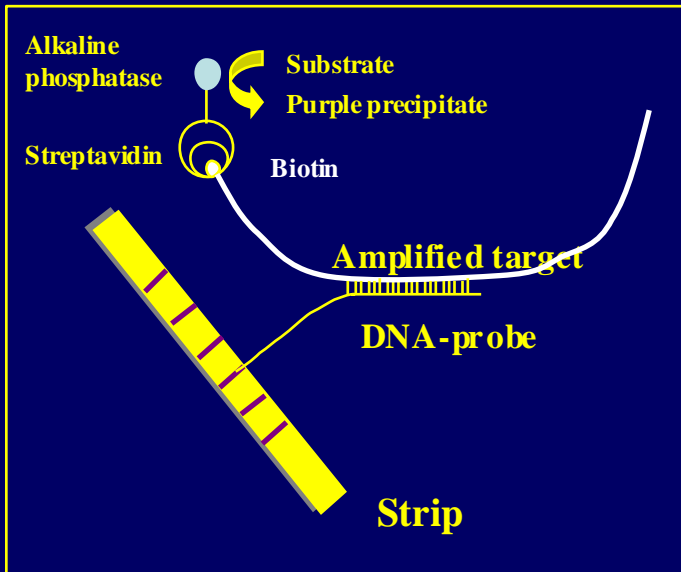
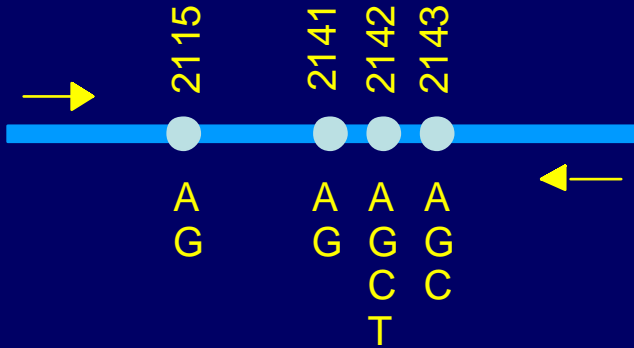
Differential cell lysis by Mini-Beadbeater™ at 5000 rpm for 5 min followed by centrifugation at 12,000 x g for 5 min and sup collection.

# *H. pylori* Molecular Resistance Testing - PCR-RFLP



M - molecular weight markers; Lanes 1-3: WT (undigested, *Bsa*I digest and *Mbo*II digest, respectively); Lanes 4-6: A2142G mutant (undigested, *Bsa*I digest and *Mbo*II digest, respectively); Lanes 7-9: A2143G mutant (undigested, *Bsa*I digest and *Mbo*II digest, respectively).

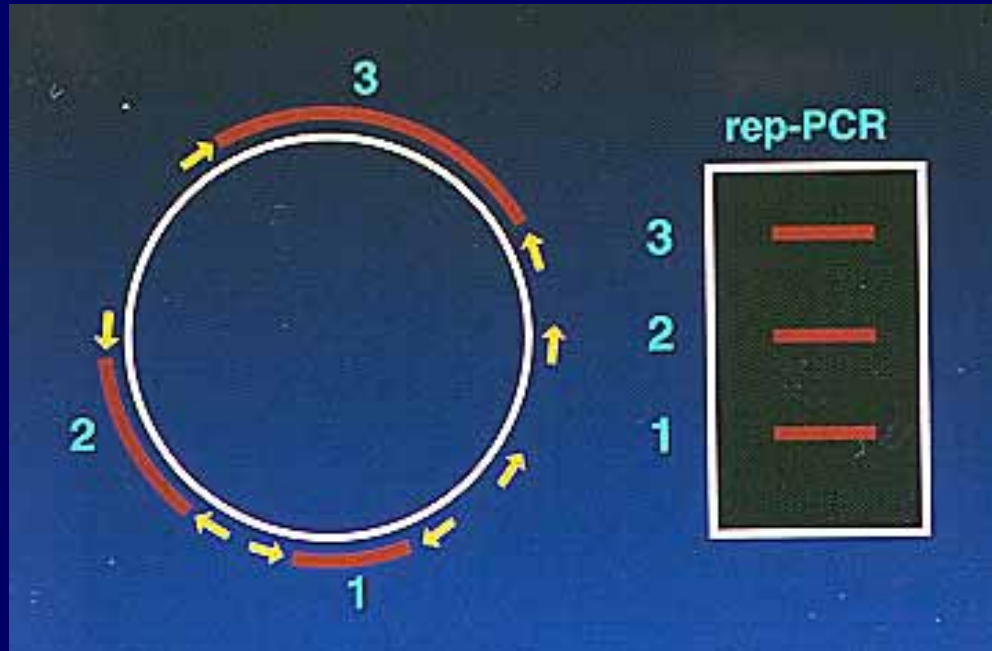
# The 23S rDNA LiPA



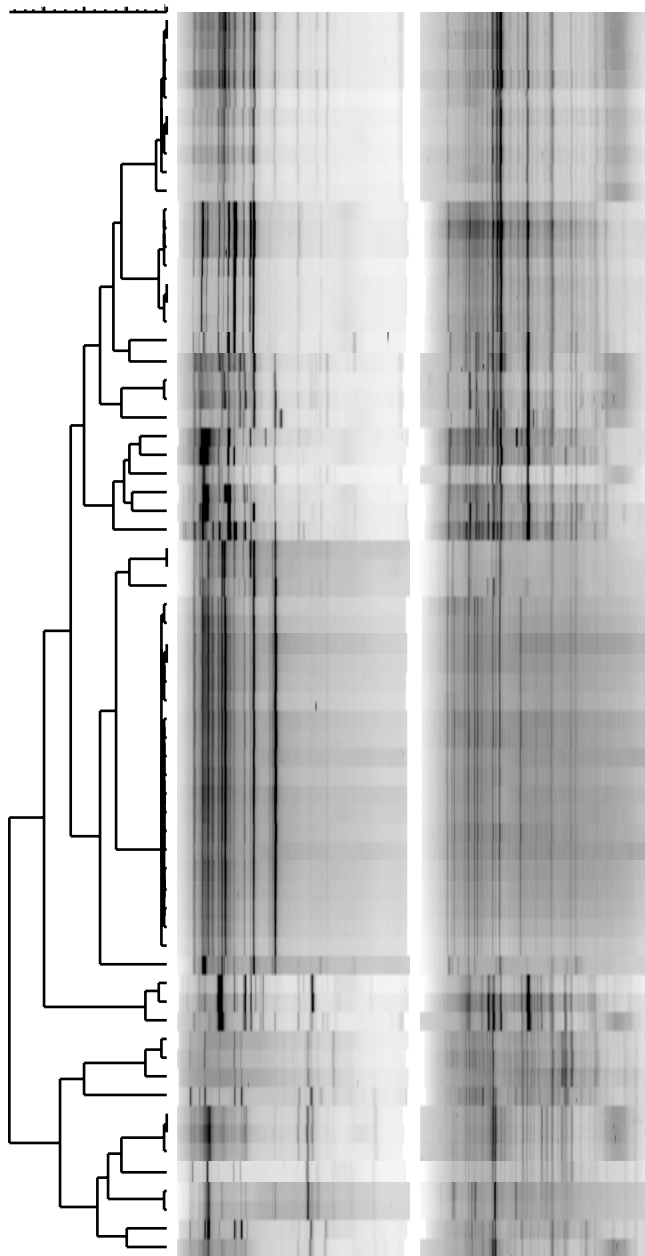
# Molecular Epidemiology

- DNA FINGERPRINTING of microbial pathogens for infectious disease outbreak investigations
- Hybridization - ribotyping
- Pulsed-field gel electrophoresis (PFGE)
- PCR-based typing - repetitive sequence-based PCR (rep-PCR)
- Hybridization and PCR-based typing

# Repetitive DNA sequence –based PCR and Bacterial Strain Typing (Molecular Epidemiology)



**rep-PCR**



- Esc herichia col 7 70
- Esc herichia col 7 69
- Esc herichia col 7 72
- Esc herichia col 1 603N
- Esc herichia col 6 80
- Esc herichia col 1 664
- Esc herichia col 1 691
- Esc herichia col 1 658
- Esc herichia col 7 56
- Esc herichia col 1 656
- Esc herichia col 2 00N
- Esc herichia col 4 36
- Esc herichia col 4 31
- Esc herichia col 7 15
- Esc herichia col 5 51
- Esc herichia col 5 55
- Esc herichia col 5 58
- Esc herichia col 6 79
- Esc herichia col 7 73
- Esc herichia col 1 657
- Esc herichia col 7 73N
- Esc herichia col 1 678
- Esc herichia col 3 53
- Esc herichia col 6 77
- Esc herichia col 1 738
- Esc herichia col 1 82
- Esc herichia col 4 61
- Esc herichia col 2 27
- Esc herichia col 0 1-11 24
- Esc herichia col 0 1-11 06N
- Esc herichia col 0 1-11 06
- Esc herichia col IL-10 KO 5JIM-1
- Esc herichia col IL-10 KO 4JIM-2
- Esc herichia col IL-10 KO 18
- Esc herichia col IL-10 KO 1-CO L-2
- Esc herichia col IL-10 KO 3-CO L-1
- Esc herichia col IL-10 KO 5-CO L-1
- Esc herichia col IL-10 KO 19
- Esc herichia col IL-10 KO 21
- Esc herichia col IL-10 KO 16
- Esc herichia col IL-10 KO 1-JIM-2
- Esc herichia col IL-10 KO 12
- Esc herichia col IL-10 KO 11
- Esc herichia col IL-10 KO 20
- Esc herichia col IL-10 KO 17
- Esc herichia col IL-10 KO 10
- Esc herichia col IL-10 KO 9
- Esc herichia col IL-10 KO 6
- Esc herichia col IL-10 KO 5
- Esc herichia col IL-10 KO 2
- Esc herichia col 0 1-11 01
- Esc herichia col 1 64N
- Esc herichia col 3 57N
- Esc herichia col 1 655
- Esc herichia col 2 26-1
- Esc herichia col 6 78
- Esc herichia col 2 26-2
- Esc herichia col 4 60-B
- Esc herichia col 7 62L
- Esc herichia col 7 62N
- Esc herichia col 7 62
- Esc herichia col 7 61N
- Esc herichia col 0 1-10 60
- Esc herichia col 0 1-10 75
- Esc herichia col 4 36N
- Esc herichia col 7 68

# rep-PCR DNA Fingerprinting - *Escherichia coli* of the Mouse Intestine

← **IL-10<sup>-/-</sup> mice**

# IAEA RAS 6/034



**Promoting Laboratory Quality for Diagnosis of  
Tuberculosis and Viral Hepatitis**

# Quality Assurance

- competency of personnel
- laboratory environment
- verification and validation of tests
- content of procedure manuals
- test methods and procedures
- content and storage of records and reports
- EQAS - proficiency testing

# Technical Competency Assessment

- **Initial Training**
  - Details – Who, When (Date), What
  - Techniques Mastered incl. QC
- **Acquisition of New Skills**
  - Details – Who, When (Date), What
- **Annual Competency**
  - Abbreviated check by assay
  - Assess by technical review –
    - may include performance of proficiency testing

# Test Validation

- establish assay with control specimens
- clinical validation
  - actual clinical specimens
- analytic versus clinical sensitivity
- in-house assays - each lab must perform its own validation and not rely on literature
- novel pathogens may require extensive correlation/validation

# Pre-Analytic – Before Testing

- Specimen acceptability criteria
- Specimen transport and storage
- Specimen processing in laboratory
  - Immediate – blood to serum
  - Time of testing – nucleic acid isolation
- Internal controls to assess adequacy of processing (TB)

# Analytic – PCR Methods

- PCR or nested PCR
- Single tube or two tube
  - RT-PCR and nested PCR
  - If no UNG/isopsoralen, single tube PCR
- Enzymes – recommend specific enzymes – need list
  - Reverse transcriptase
  - DNA polymerase
  - Nucleotide Kinase

# Detection QC – Reverse Hybridization

- Known positive and negative controls (may be internal) to assess detection system with each assay
- Sensitivity controls to assess limits of detection with each assay
- If multiple probes are used, are all probes assessed internally each time?

# Post-Analytic – After Testing

- Data Interpretation and Review
- Correlation of Results with Quality Control
- Reporting
- Timeliness of Reporting
- Who receives the final report and in what form?

# External Quality Assessment (EQAS)

- proficiency testing programs (“surveys”)
  - Frequency and quantity of challenges
  - Nature of challenges
  - Who performs PT – is this part of competency assessment?
  - Methodological
    - DNA/RNA preparation
    - PCR performance
    - agarose gel electrophoresis
  - Application-based

# EQAS – RAS 6/034

- EQAS Program to be launched in February 2003
- Eight (8) laboratories from 8 different countries are participating initially
- Hepatitis B and C, Tuberculosis
- Minimum 10 challenges per year
- “dry” and “wet” challenges
- Corporate support (Ambion) for provision of materials

# IAEA RAS 6/034



# Role of IAEA

- Radioisotope-based Methods are Important in Molecular Diagnostics of Infectious Diseases
- Applications for Diagnosis and Patient Management (Treatment)
- Technology is Here Today and Serves as Foundation for Laboratories
- Tools for Establishment of Quality Programs