

*Drug susceptibility testing to 1st
& 2nd line drugs in the diagnosis
of MDR & XDR TB*

Dr Camilla Rodrigues MD
Consultant Microbiologist

TB - constantly on the back burner

Tuberculosis –
*a disease which medicine never cured, wealth
warded, or poverty could boast exemption from;
which sometimes moves in giant strides &
sometimes at a tardy pace
but
slow or quick is ever sure & certain*

Nicholas Nickleby – Charles Dickens



Drug Resistant TB – “ Ebola with wings ”

Primary Resistance : no past history of TB drugs

Initial Resistance : no reliable record of TB drugs

Sec / Acq Resistance : past h/o TB drugs

MDR : Resistant to INH + RIF

XDR : MDR plus

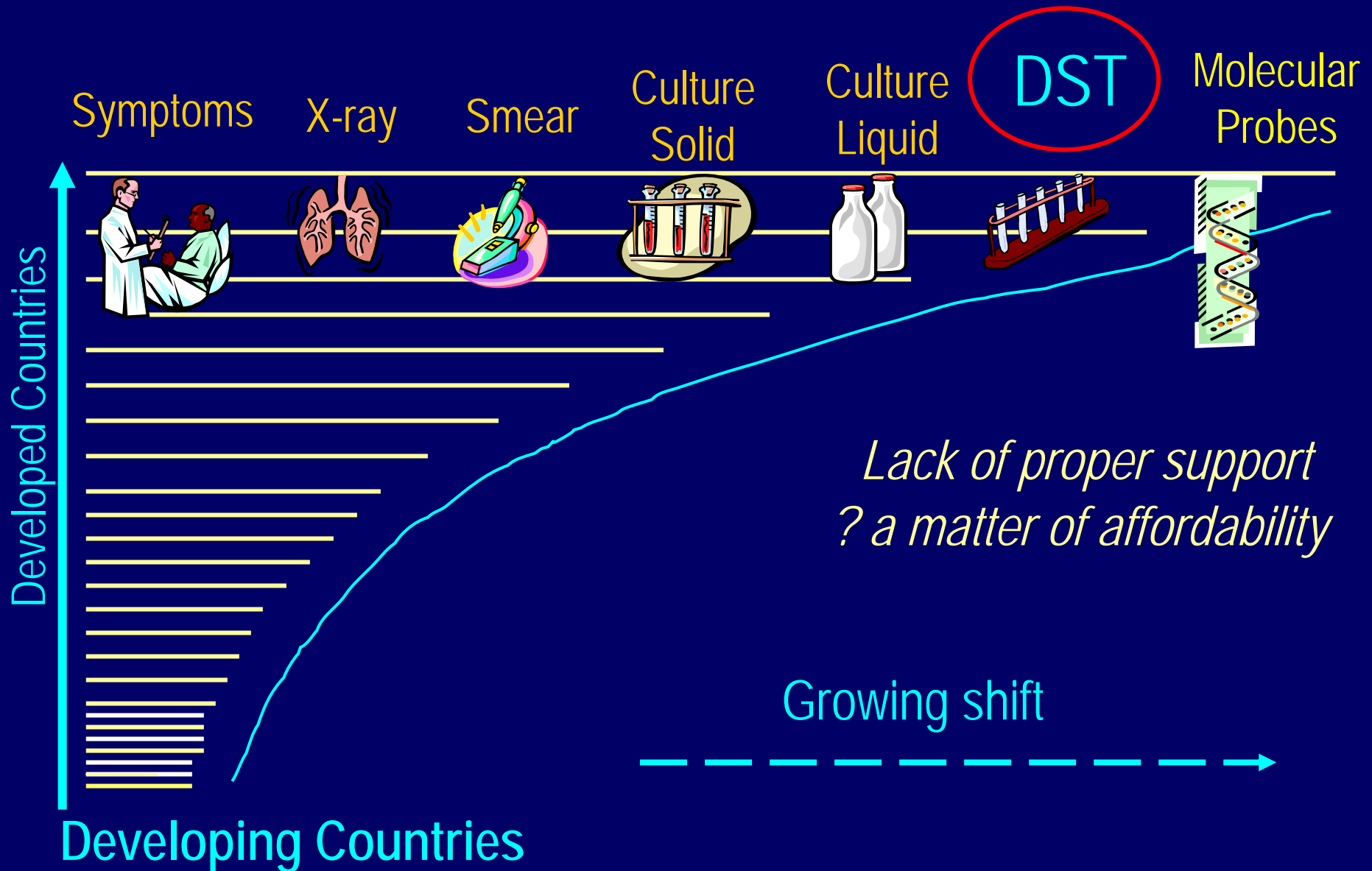
R to one of three injectables Ak Km Cs
& R to any fluoroquinolones

The resumption of consumption

Patients with resistant TB are

- difficult to treat
- remain infectious for longer
- pose a public health hazard - transmit to 12 persons / year
- more likely to die
- HIV further compounds the problem

TB - Diagnostic Test Coverage



MDR-TB SPREADING WORLDWIDE



- Many national TB programs do not have the resources to screen all TB cases for resistance
- Underestimation of the extent of the progressing epidemic of **primary resistance**
- Mono resistance to INH or R contributes to MDR in the continuation phase

DST in *M.tuberculosis* - a neglected problem

- Culture
 - confirmation of species
 - DST a **must** for
 - Relapse or re treatment
 - Change of regimen (pts continuing to be +)
 - Suspected primary resistance
- may be used** as
- a guide in initial treatment
 - to obtain baseline epidemiological data

Priority rank list for DST

Rifampicin

Isoniazid

Ethambutol

Pyrazinamide

Streptomycin

1st line

Aminoglycoside : kanamycin or amikacin

Polypeptide : capreomycin

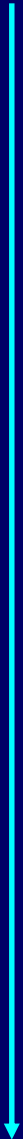
Fluoroquinolone : ciprofloxacin or ofloxacin

Thioamide : ethionamide or prothionamide

PAS

Serine analogue : cycloserine or terizidone

2nd line



Newer second line drugs

- Moxifloxacin
- Rifabutin
- Linezolid
- Clarithromycin
- Clofazimine

Drug Susceptibility Testing (DST)- *M.tuberculosis*

1. Growth observation

Macroscopic observation

Proportion method

Absolute Conc

Resistance Ratio

E test

Nitrate Reduction Assay

Microscopic observation

MODS

2. Detection of Metabolic activity or products

BACTEC / MGIT / MB/Bact /ESP Myco

MABA - microplate alamar blue assay / REMA / MTT

3. Newer methods as Phage based technologies

4. Molecular methods : detection of genetic mutations - Genotypic

Phenotypic

Requirements for Drug Susceptibility Tests

- High intra & inter lab reproducibility
- Shortest TAT
- Distinguish between high & low levels of R
- Practical lab application
- Minimal investment & consummable costs
- Minimal labor time
- Applicability to 1st & 2nd line drugs

Problems in DST for *M tuberculosis* still a bottleneck

- Inoculum standardisation (number / dispersion / viability)
- Stability of drugs
- Alteration of drugs in different media
(inactivation / protein binding / deterioration / inspissation / pH /
antagonistic substances / incomplete dissolution in solvents /
inaccurate dilution)
- Incubation temp / time
- Criteria of resistance
- Type of test performed

Problems in Drug Susceptibility Testing

False Resistance

High inoculum
Contamination mixed
not homogenised
low drug conc

False Susceptibility

Low inoculum / low viability
suboptimal growth
inoculum dilution
incorrect drug conc

Low level resistance

- inconsistent results when tested repeatedly
- discordant when compared to other systems

DST – *M tuberculosis*

1. Growth observation

Macroscopic observation

Proportion Method

Absolute Concentration Method

Resistance Ratio

E test

Nitrate Reduction Assay

Microscopic observation

MODS

Indirect

1. Growth observation - Indirect methods

1. Proportion method

Ratio of colonies on the drug containing medium to those on drug-free medium (set at 1%)

If $>1\%$ is R, the drug will not be useful in therapy

Qualitative as well as precise estimate

2. Absolute concentration method or MIC

Growth is taken as the end point (>20 colonies)

Affected by inoculum size & viability

3. Resistance ratio method

Determines the resistance ratio between the MIC of the test strain and MIC of reference strain ($RR >8$)

Affected by variations in the std strains

DST- Growth observation

- Macroscopic

Absolute conc (>20)

Resistance Ratio (RR > 8)

Proportion Method (set at 1%)

E - test

Nitrate Reductase Assay

- Microscopic

MODS

} Direct

1. Growth observation : Direct Assays

Nitrate Reduction Assay



Fully Susceptible

R to all 4 drugs
Color change > Control

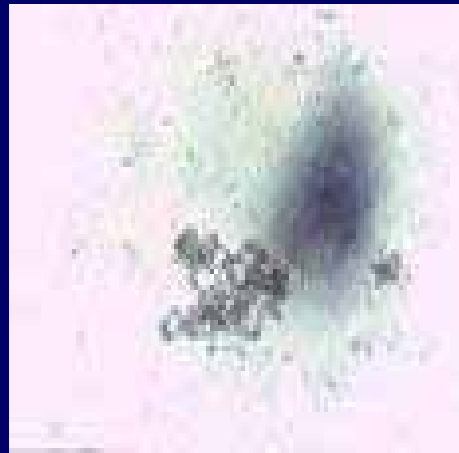
| | | | | |
|---------|-----|----|-----|-----|
| Control | INH | RF | ST | ETH |
| 1:10 | 0.2 | 40 | 4.0 | 2.0 |

J Clin Microbiol 2002;40:553

1. Growth observation

Direct assays for resource limited settings

Microscopic Observation Drug Susceptibility Assay – MODS
accurate detection & simultaneous identification of MDR



Characteristic tangles of *M.tb* seen under inverted light microscope

N Engl J Med 2006;355:1539

Drug Susceptibility Testing (DST): *M tuberculosis*

1. Growth observation

- Proportion method
- Absolute Conc
- Resistance Ratio
- E test
- Nitrate Reduction Assay
- MODS

2. Detection of Metabolic activity or products

BACTEC / MGIT / MB Bact / ESP Myco

MABA / REMA / MTT

3. Phage based technologies

4. Molecular methods : detection of genetic mutations

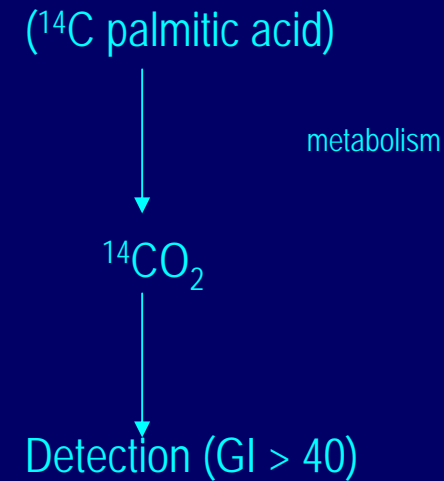
BACTEC 460 TB



- Modified proportion method
- 7H12 does not absorb / inactivate drugs
- Growth monitored radiometrically
- R is determined by comparing rate of growth in control & in drug containing vial
- Critical prop for R is taken as 1% for all drugs

BACTEC 460 TB System

- ❑ Shorter TAT for 1^o isolation
- ❑ Allows for completion in 1 week
DST on solid media (3 - 4 wks)
vs liquid (6 -12 days)
- ❑ Approved for indirect DST
for 1st line drugs



Culture Inoculum – Procedural Flow Chart 460 TB

Test Culture



Subculture on 12B

Incubate at 37 degrees



When GI >500 in 12B



0.1 ml in each drug vial

0.1 ml + 9.9 ml Diluting Fluid
Mix 0.1 ml in Control (w/o Drug)



GI 30 in the control, Interpret results



If growth in drug cont vial progresses to a higher level than the 1:100 diluted control, then >1% R

Critical concentrations

CDC & CLSI recommend testing critical conc .Other conc optional

- Lowest conc of drugs that inhibits “wild” strains (S) while not inhibiting strains isolated from pts not responding to treatment (R)
- *For H & R, the gap bet highest MIC for susceptible & lowest MIC for resistant is substantial . Problem in interpreting cut off values close to critical conc*
- R is growth of $>1\%$ of an inoculum in presence of the critical conc of the drug

Critical concentrations for 1st line drugs ($\mu\text{g/ml}$)

| Drug | LJ | 7H10 | 460 TB | MGIT 960 |
|--------------|-----|-------|--------|----------|
| Isoniazid | 0.2 | 0.2/1 | 0.1 | 0.1 |
| Rifampicin | 40 | 1.0 | 2.0 | 1.0 |
| Ethambutol | 2 | 5.0 | 2.5 | 5.0 |
| Streptomycin | 4 | 2.0 | 2.0 | 1.0 |
| Pyrazinamide | 100 | 100 | 100 | 100 |

CLSI & CDC recommend testing critical concentrations
Discrepancies with Emb /Sm

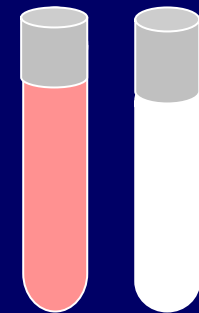
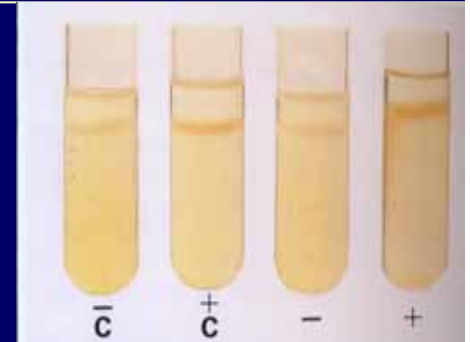
Re testing

- Indeterminate results
- Mono resistance esp rifam, pyrazina, emb
- New case that shows resistance
- Quality Control

Pyrazinamide susceptibility

Issues: low pH inhibits growth

- Enzymatic PZAse (Wayne)
- 460 TB / MGIT 960 TB system
- *pncA* gene sequencing



Increasing demand for 2nd line drugs - when to test & how ?

- MDR (in the absence of drug R, 1st line drugs are highly effective)
- Failure to respond despite long history of treatment
- Follow up in the DOTS Plus - without bacteriological confirmation, utilisation of 2nd line not justified
- Variation in testing systems & methods

Recommended critical concentrations

2nd line drugs - LJ

| Drugs | Drug conc $\mu\text{g/ml}$ | |
|--------------|----------------------------|-------------------|
| | 1% critical prop | 10% critical prop |
| Kanamycin | 30 | 20 |
| Capreomycin | 40 | 20 |
| Ofloxacin | 2 | - |
| PAS | 0.5 | 0.25 |
| Thiacetazone | - | 2 |
| Cycloserine | 40 | 30 |



Critical Concentrations of 2nd line drugs liquid vs solid



| Drug | 460 TB | 7H10 |
|-------------|---|------|
| Capreomycin | 1.25 | 10.0 |
| Ethionamide | 1.25 | 5.0 |
| Kanamycin | 5.0 | 5.0 |
| Amikacin | 1.0 | 1.0 |
| Clofazimine | 0.5 | 1.0 |
| Ofloxacin | 2.0 | 2.0 |
| Rifabutin | 0.5 | 1.0 |
| Cycloserine | inconsistent & unreliable (difficulty in CC in 7H12) | |

J Clin Microbiol 1999;37:3179

DST- M.tuberculosis

- Treatment of MDR should be based on DST
- Considerable difference in critical concentrations with lack of correlation for 2nd line drugs
- DST for 1st & 2nd line drugs established on 460 TB
- Increasing concern about use of radio active methods
- Inadequate worldwide studies for 2nd line on MGIT 960

MGIT 960 TB



Detects O₂ consumption
in the presence or absence of drug



MGIT 960 TB

Validation of MGIT 960 for DST to 2nd line drugs

J Clin Microbiol 2006 ;44:668

J Clin Microbiol 2006;44 :811

Susceptibility testing of 2nd line drugs on MGIT 960

To establish BACTEC MGIT 960 test procedures & concentrations for 2nd line drugs used to treat R pts

Amikacin

Kanamycin

Capreomycin

Ofloxacin

Moxifloxacin

Ethionamide

PAS



DST calibration – study phases

- Phase 1 : Susceptible culture isolates from never treated pts – Including a standard strain, H37Rv (11) - **Probably S**
- Phase 2 : Resistant culture isolates from patients failing 2nd line treatment (20)– **Probably R**
- Phase 3 : Fresh & stock clinical resistant isolates (73)

Study design

Phase 1 : Establish a basic test procedure & determine range of test conc

Phase 2 : Test conc adjusted based on results of Phase 1 (working critical conc in MGIT 960 that would yield results equivalent to 460 TB)

Phase 3 : Final testing of optimal drug conc
" work in the field "

Critical conc considered the conc with the least amt of discrepant results between the 2 systems

Methods

- All 7 antimicrobial drugs were tested by BACTEC 460 TB & MGIT 960 in parallel
- All drugs obtained in a chemically pure form
- Three concentrations were tested for each drug by both methods
- Stock solutions of drugs aliquoted & stored at -70° C
- *M. tb* H37Rv ATCC 27294 used for QC



Concentrations $\mu\text{g} / \text{ml}$

| | MGIT 960 | 460 TB |
|--------------|----------------|----------------|
| Amikacin | 0.5, 1.0, 2.0 | 0.5, 1.0, 2.0 |
| Kanamycin | 1.25, 2.5, 5.0 | 1.25, 2.5, 5.0 |
| Capreomycin | 1.25, 2.5, 5 | 1.25, 2.5, 5 |
| Ofloxacin | 1.0, 2.0, 4.0 | 1.0, 2.0, 4.0 |
| Moxifloxacin | 1.0, 2.0, 4.0 | 1.0, 2.0, 4.0 |
| Ethionamide | 1.25, 2.5, 5 | 1.25, 2.5, 5 |
| PAS | 2.0, 4.0, 8.0 | 2.0, 4.0, 8.0 |

Discrepant results based on critical concentrations established in Phase 3

| | | |
|-------------------|---------------------------|-------------------------|
| Total 73 isolates | False Susceptible by MGIT | False Resistant by MGIT |
| Percent | 0.78% | 0.39% |

False Susceptible regarded as a Very Major Error

False Resistant regarded as a Major Error

MGIT critical concentrations -2nd line drugs



| Drug $\mu\text{g/ml}$ | MGIT 960 | 460 TB |
|-----------------------|------------|-------------|
| Amikacin | 1.0 | 1.0 |
| Kanamycin | 2.5 | 2.5 |
| <i>Capreomycin</i> | <i>2.5</i> | <i>1.25</i> |
| Ofloxacin | 2.0 | 2.0 |
| Moxifloxacin | 1.0 | 1.0 |
| <i>Ethionamide</i> | <i>5.0</i> | <i>2.5</i> |
| PAS | 4.0 | 4.0 |

Drug Susceptibility Testing (DST) : *M.tuberculosis*

1. Growth observation

Proportion method /Absolute Conc /Resistance Ratio

Nitrate Reduction Assay

MODS

2. Detection of Metabolic activity or products

BACTEC / MGIT / MB/Bact /ESP Myco

MABA - Microplate Alamar Blue Assay

REMA - REsazurin Microtitre Assay

MTT - Methyl Thiazol diphenyl Tetrazolium bromide

*Rapid, low cost
show good agreement
for H & R
? Biosafety & contam*

3. Phage based technologies

4. Molecular methods : detection of genetic mutations

Drug Susceptibility Tests (DST) – *M.tuberculosis*

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Macroscopic observation in drug free & drug containing media

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Nitrate Reductase Assay

Microscopic observation

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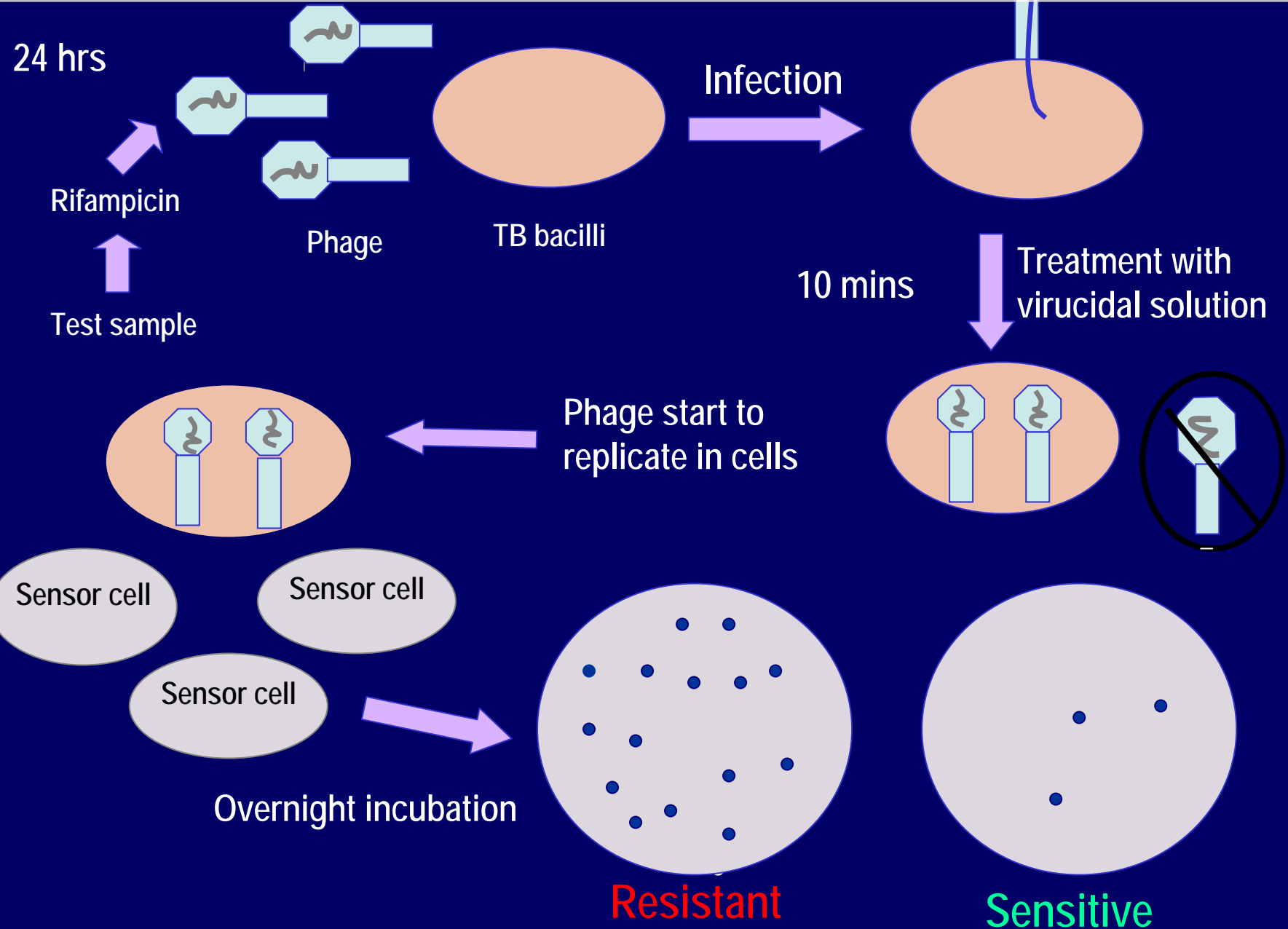
BACTEC / MGIT / MB/Bact

MABA - microplate alamar blue assay / REMA / MTT

3. Phage based technology : LRP, PhaB, Fast Plaque

4. Molecular methods : detection of genetic mutations

Lysis with mycobacteriophages



Lysis with mycobacteriophages

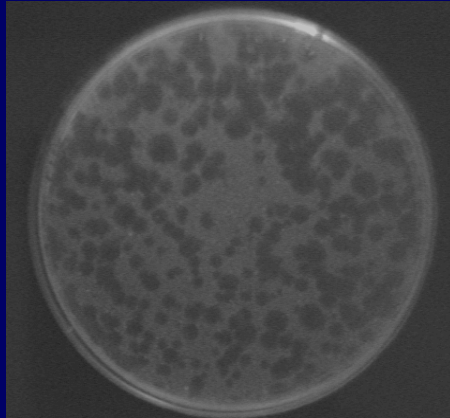


Plate without
Rifampicin

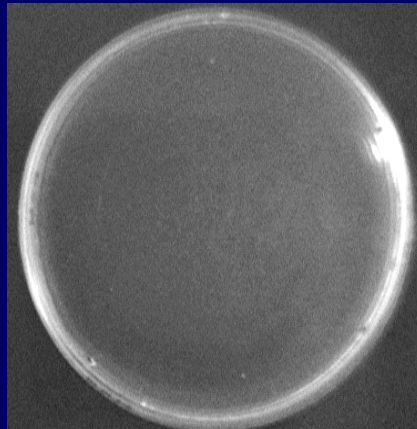


Plate with
Rifampicin

Rifampicin Sensitive

Rifampicin Resistant

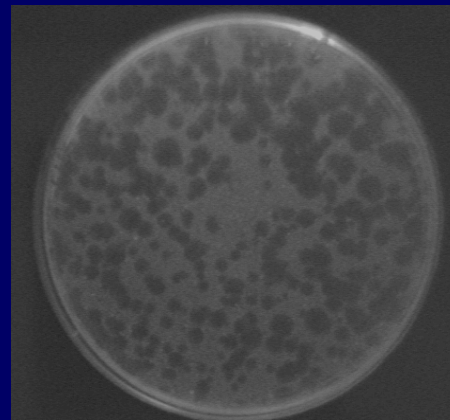


Plate with
Rifampicin

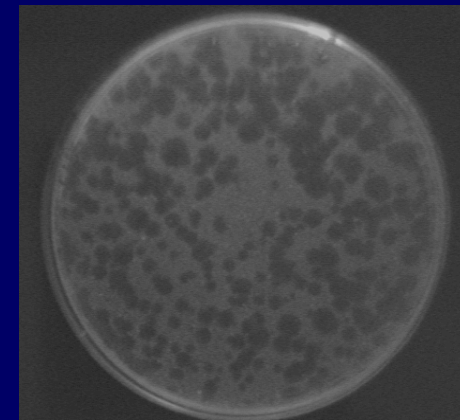


Plate without
Rifampicin

Phage assay

| Test | Ref. Std | Specimen | Results |
|----------------|----------------------------------|-----------------------|-------------------------------------|
| RIF Resistance | BACTEC 460 L.J Proportion mtd | Culture Isolates | Sensitivity 96% Specificity 100% |
| INH Resistance | BACTEC 460 L.J Proportion mtd | Culture Isolates | Sensitivity 97% Specificity 100% |
| RIF Resistant | BACTEC 460 | Smear + Resp. Spec | Sensitivity 93% Specificity 87% |

Limitations

- Analytical sensitivity : 100-300 bacilli/ml
- Not useful in smear negative, paucibacillary specimens
- Pts receiving anti-TB treatment

Ajay K et al. IJMM 2002;20:211-14



Drug Susceptibility Testing (DST): *M.tuberculosis*

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Macroscopic observation in drug free & drug containing media

Proportion Method

Absolute Conc

Resistance Ratio

E-test

Nitrate Reductase Assay

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2. Detection of Metabolic activity or products

BACTEC / MGIT / MB/Bact /ESP Myco

MABA / REMA / MTT

3. Phage based technology

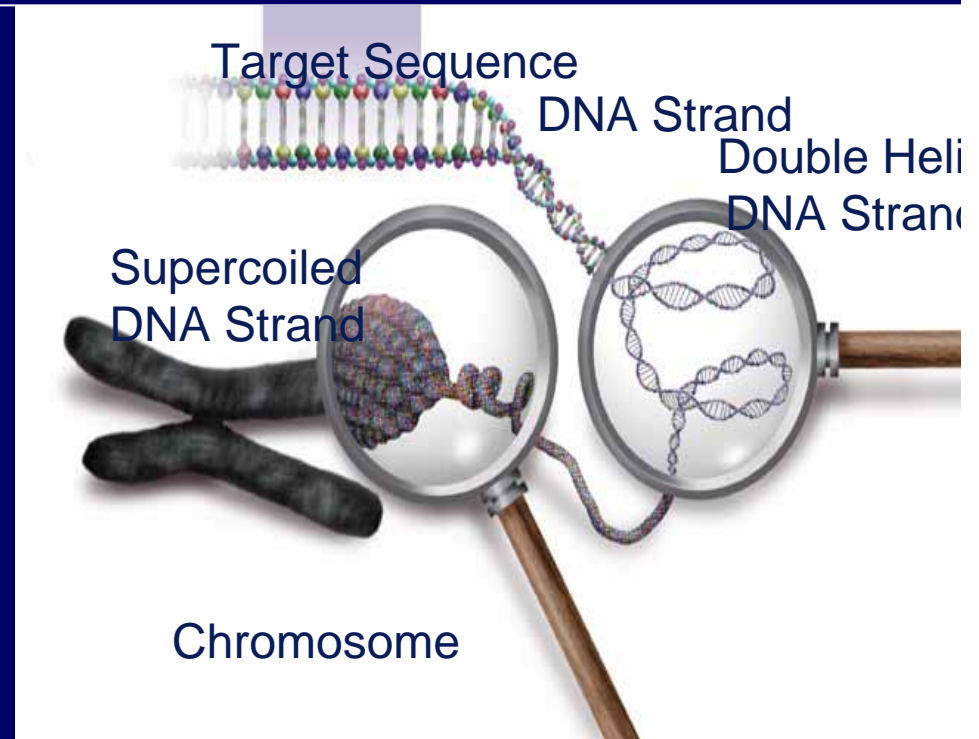
Bioluminescence /Flow cytometry

4. Molecular methods : detection of genetic mutations advances in genotypic hardware

DST – Genotypic molecular methods

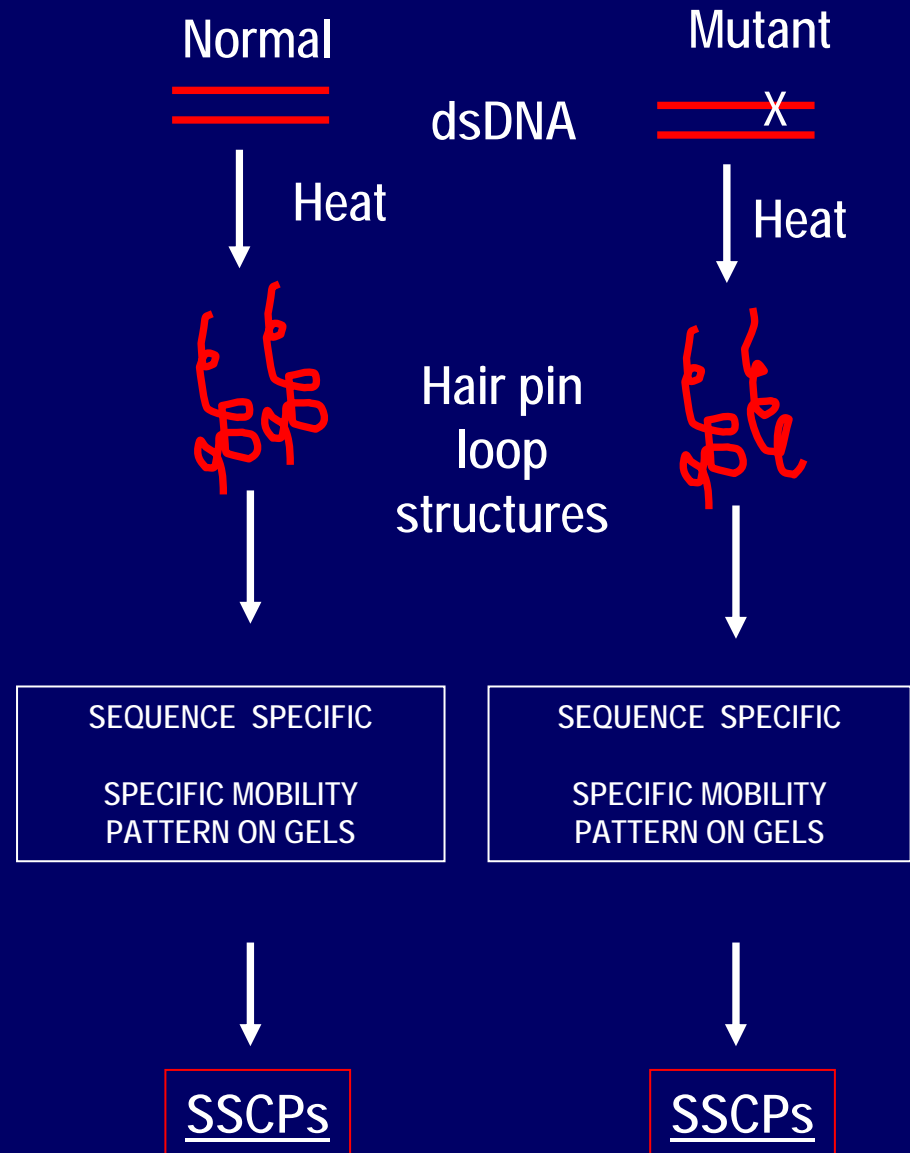
“ Leap frogging technology ”

- DNA sequencing
- PCR – SSCP
- PCR – HDX
- RFLP
- Line probe assays
- Molecular beacons
- **Micro Arrays / microfluidic tech**



Single Strand Conformational Polymorphism (SSCP)

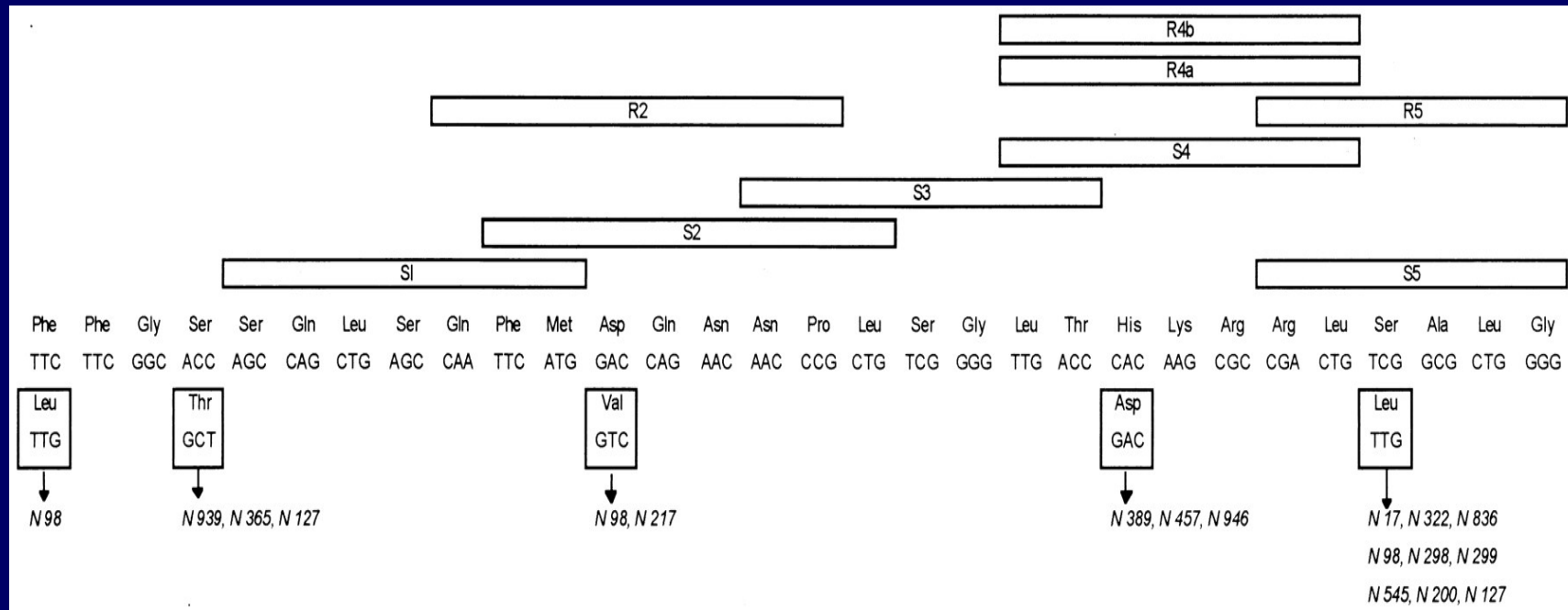
- SSCP analysis was done on 12% poly AA gel with
- Gels are run overnight at Room temp. and 4°C
- Stained with EtBr and observed under UV light



Drugs & genes involved in their resistance

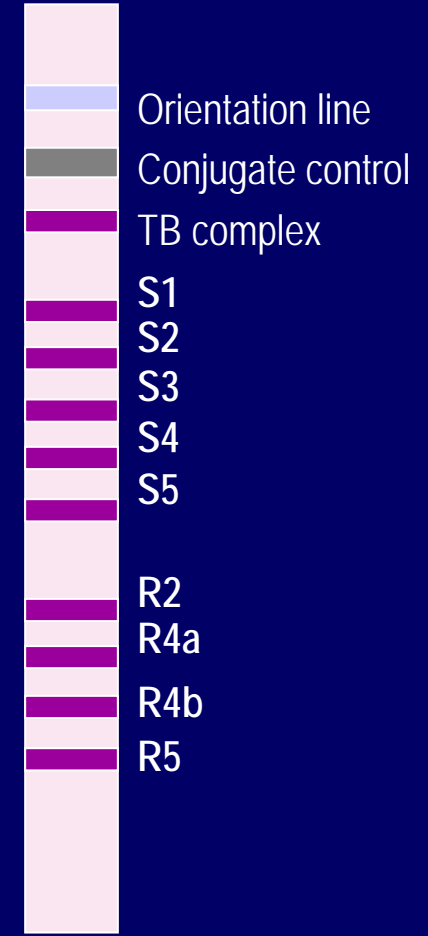
| Drug | Gene | Mutation frequency |
|--------------|--|--------------------|
| Rifampicin | RNA polymerase subunit B (<i>rpoβ</i>) | 96% |
| Isoniazid | Enoyl acp reductase (<i>inhA</i>) | 10-20% |
| | Catalase–peroxidase (<i>katG</i>) | 30-60% |
| | Alkyl hydroxyperoxide <i>oxy</i> reductase (<i>ahpC</i>) | 2-8% |
| Ethambutol | Arabinosyl transferase (<i>embC,A,B</i>) | 80% |
| Streptomycin | Ribosomal protein subunit 12 (<i>rpsL</i>) | 52-59% |
| | 16 S ribosomal RNA (<i>rrs</i>) | 8-21% |
| Pyrazinamide | Pyrazinamidase-nicotinamidase (<i>pncA</i>) | 72-97% |
| Quinolones | DNA gyrase subunit A (<i>gyr A</i>) | 75-94% |

Probes used in Line probe assays



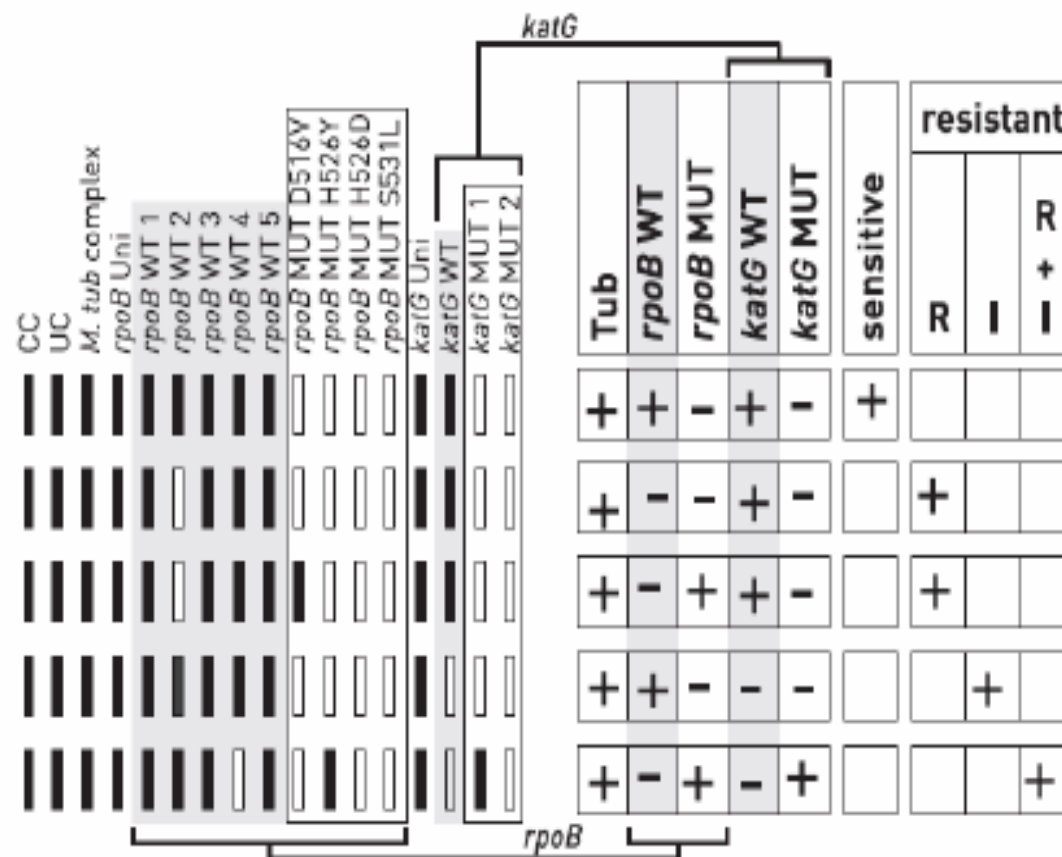
Rifampicin resistance determining region (RRDR) -81 base-pair region encoding 27 amino acid and involving codon 507-533

Line Probe Assays : Inno Lipa Rif TB

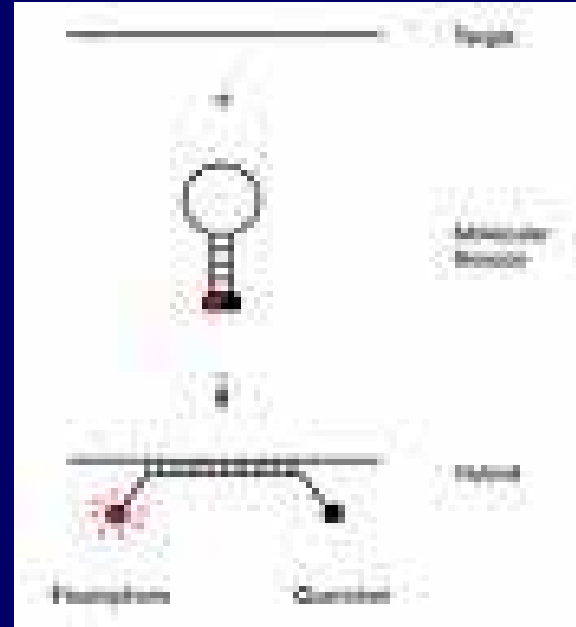
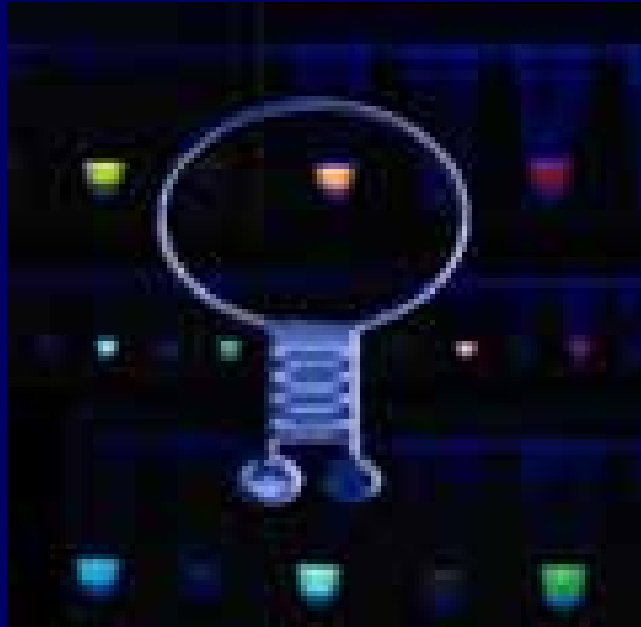


Strip hybridization

- PCR-based strip assay
- Detects *M. tuberculosis* complex 3S-rRna/16S-RNA
- Detection of both H (*catG*) and R (*rpoB*) resistance



Molecular beacons for established mutations

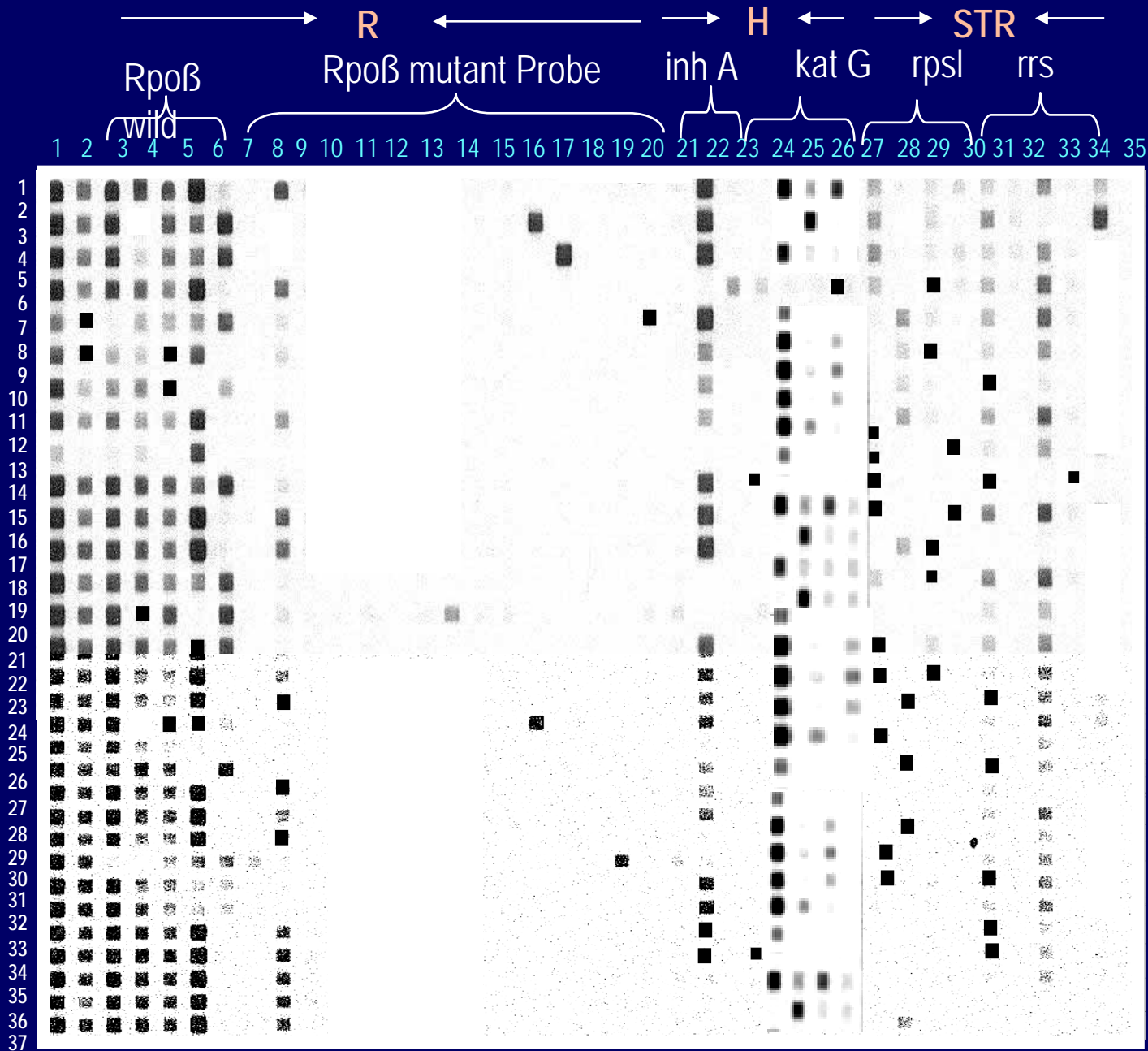


Based on stem & loop structure with the probe in the loop
Fluorescence detected in Real time within 4 hrs w/o post PCR
manipulation

J Clin Microbiol 2004;42 :4204

MTB Drug Resistance Testing

P
A
T
I
E
N
T
S



Probes

1. MYC
2. MTB
3. Rif wt 1
4. Rif wt 2
5. Rif wt 3
6. Rif wt 4
7. Rif wt 5
8. Rif 533 mt
9. Rif 531 TTG
10. Rif 531 TGG
11. Rif 526 TAC
12. Rif 526 GAC
13. Rif 526 CGC
14. Rif 526 TGC
15. Rif 526 AAC
16. Rif 522 TTG
17. Rif 522 TGG
18. Rif 516 TAC
19. Rif 516 GTC
20. Rif 516 GTG
21. Rif 516 GGC
22. Rif 513 CCG
23. Rif 511 CCA
24. Inh A wt
25. Inh A mt
26. KG 315 wt
27. KG 315 mt
28. KG 463 wt
29. KG 463 P
30. RpsL 43 wt
31. RpsL 43 mt
32. RpsL 88 wt
33. RpsL 88 mt
34. RRS 491 wt
35. RRS 491 mt
36. RRS 513 wt
37. RRS 513 mt
38. RRS 516 mt

Wild – Susceptible,
Mutant - Resistant

TAT of genotypic with phenotypic methods

| | Identification Recovery | | Susceptibility testing | | Total Time for Identification & Susceptibility testing |
|--------|----------------------------|---------|------------------------|-----------|--|
| | Rate | Time | Efficiency | Time | |
| L.J | 30-50% | 2-6 wks | 80-99% | 2-4 wks | 4-12 wks |
| BACTEC | 50-75% | 2-3 wks | 92-100% | 8-12 days | 3-5 wks |
| MGIT | 50-75% | 1-2 wks | 98.6-100% | 6-10 days | 2-4 wks |
| RLBH | 95-98% | 4 days | 98-100% | 4 days | 4 days |

MDR TB : in urban & rural settings

A study done at our centre comparing sputum samples (pulmonary TB) from urban & rural areas

| | HNH | Rural |
|------------|-----|-------|
| MDR-TB | 51% | 2% |
| S, H, E, R | 25% | 0.6% |
| S, H, R | 15% | Nil |

- Tertiary care centre with a referral bias towards non responding cases*

Clin Infect Dis 2003;36:152-4





Fingerprinting of MDR :

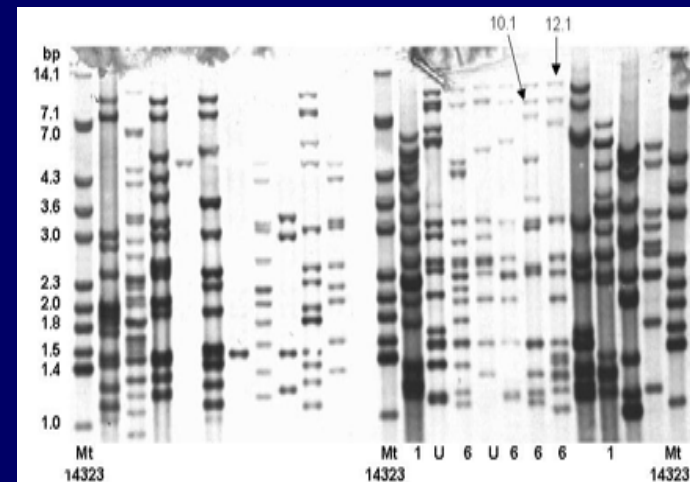
? Outbreaks / ongoing community spread

- At HNH, Mumbai , Beijing genotype most predominant (35%) among MDR isolates

Second largest prevalent group (31%) found to belong to Delhi genotype

RFLP showed Beijing genotypes to be closely related

Clin Infect Dis 2005;40:881-6

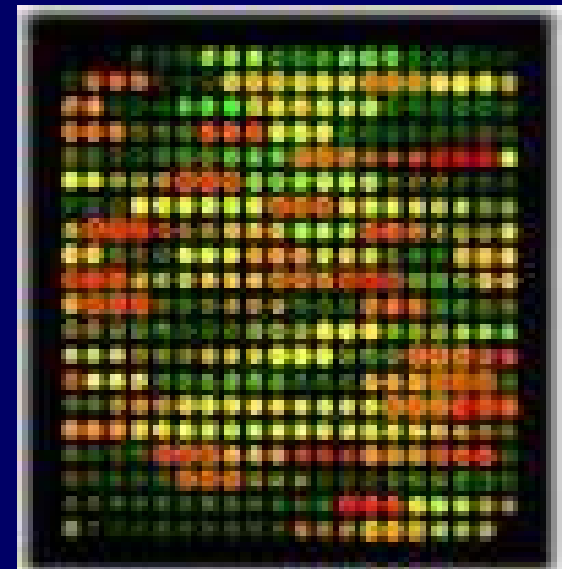


In the near future..... Lab on a chip

- Several thousand probes on a chip
- Automated data management systems with paperless reporting

.....and these tests will provide

- Identification of pathogens
- drug susceptibilities
- Nature of host response



The men who gave us the cure for tuberculosis



..... losing curable status ?

Ending neglect.....

Central role of the laboratory in the
early diagnosis & management of TB

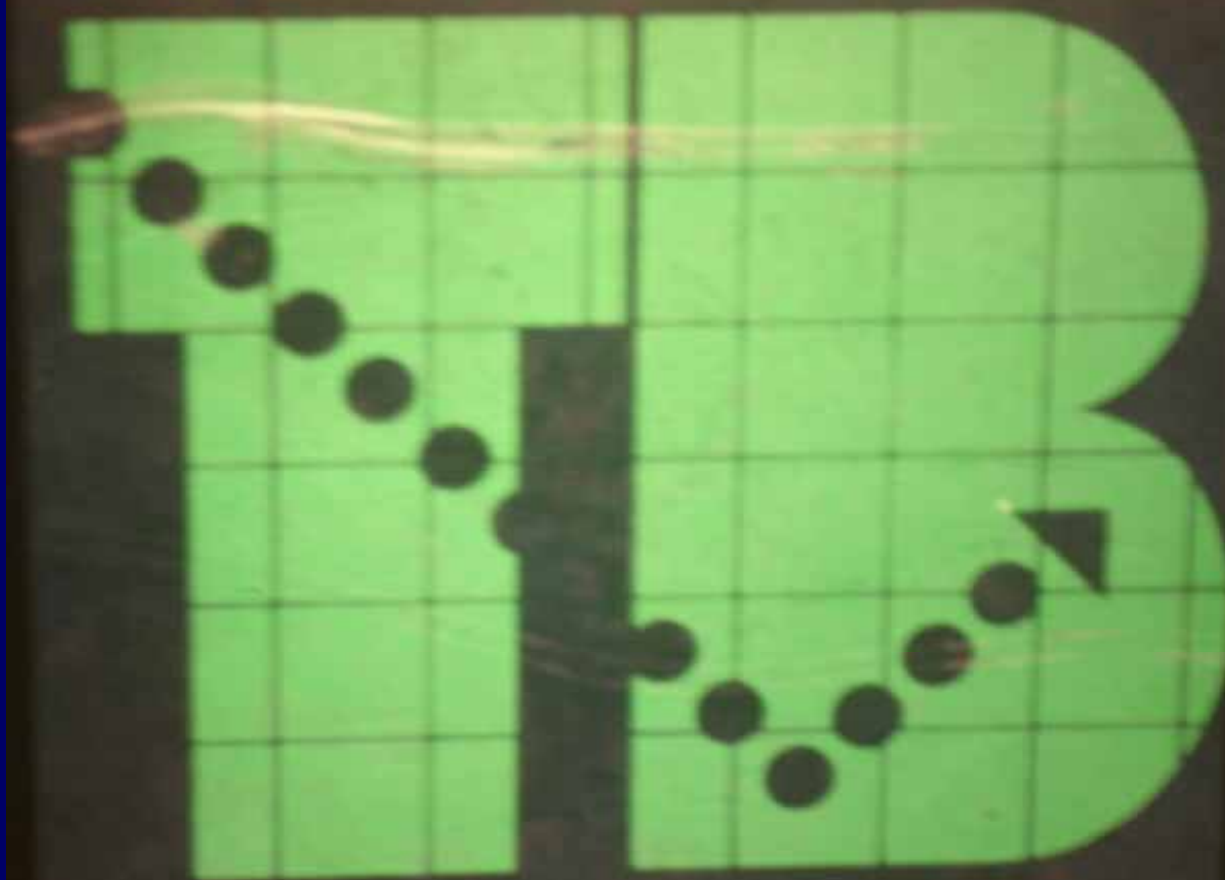
Knowing is not enoughwe must apply

Willing is not enough we must do

Goethe

Thank you

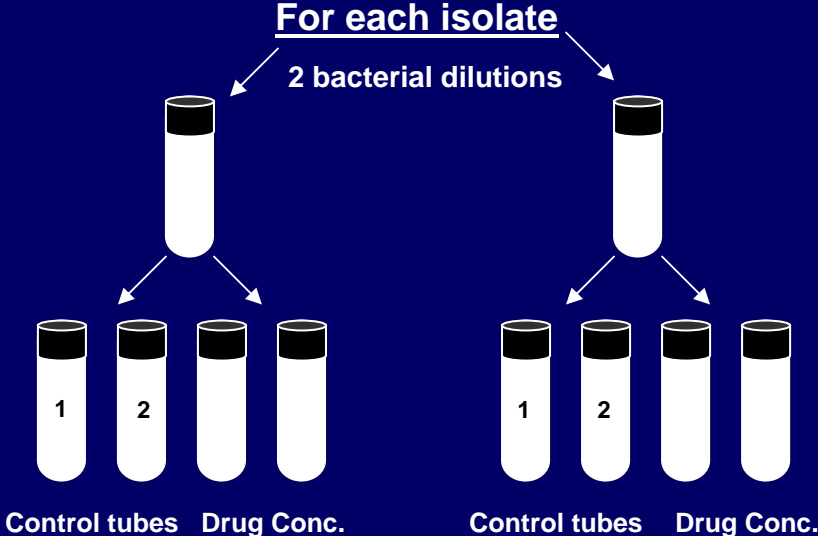
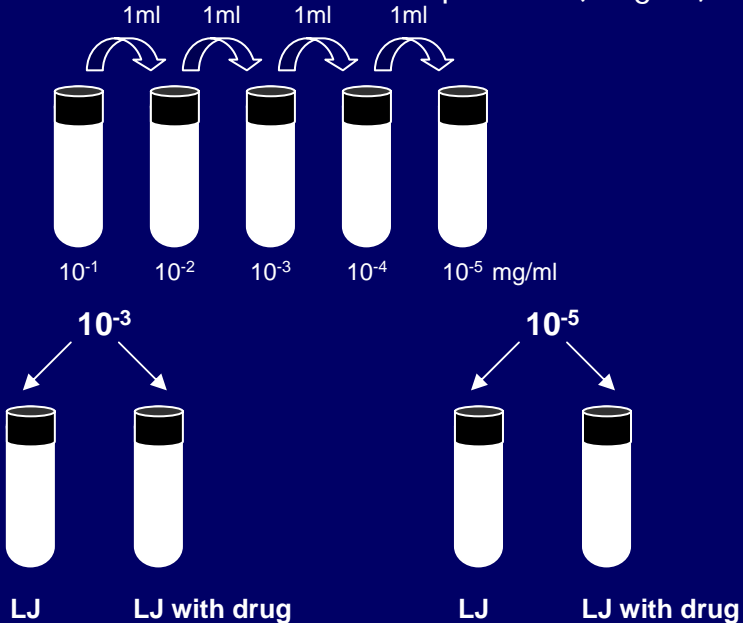
TUBERCULOSIS



BACK TO THE FUTURE

Media Inoculation

1 ml of standard suspension (1mg/ml) + 9ml of sterile distilled water



DST: Reliability of results

Therapeutic index (diff bet in vitro MIC & serum drug levels)

High for INH & Rif

Low for 2nd line drugs as cycloserine / ETA / PAS

In general, Susceptibility results are predictable

If Resistance prevalence is <10%, resistance results show a low PV

In vitro criteria should be performed with strains from pts.that

1. Never been treated
2. Failed treatment
3. DST then standardised to obtain reproducible results

Wayne's Pyrazinamidase Assay

Detects active Pyrazinamidase enzyme by hydrolysis of PZA to Pyrazinoic acid as evidence by a colour change.

Inoculation of actively growing culture into 2 Dubos Agar butts containing 100mg/Litre of PZA

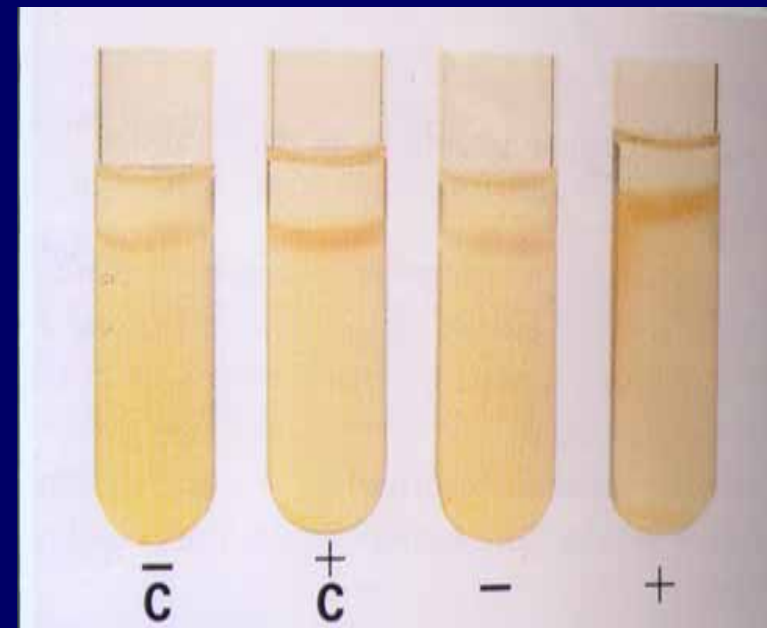
Incubation 37°C, 4 days

Add 1 ml freshly prepared 1% ferrous ammonium sulphate

RT, 30 mins

Examine for a pink band in the agar medium

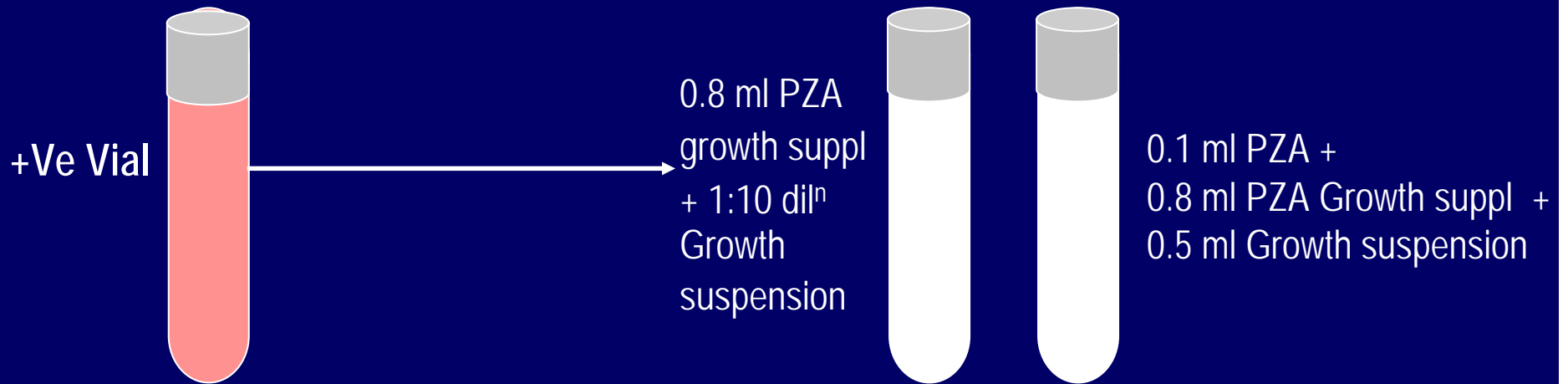
If 4 day tube is negative or doubtful, repeat the test at 7th day using the second tube



Positive: Pink band (Susceptible)

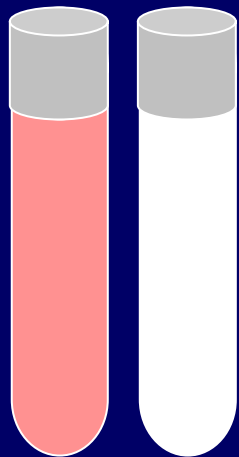
Negative: No pink band (Resistant)

MGIT-PZA



Incubate in MGIT 960 TB system

Control PZA

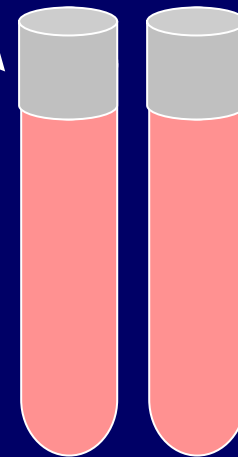


PZA Susceptible

Results will be flagged by the Machine

Cut off value is 100

Control PZA



PZA Resistant

Multidrug resistance in new TB cases in India

| Place | Study period | Sample size | MDR % |
|----------------|--------------|-------------|-------|
| Gujarat | 1983-89 | 570 | 0 |
| Pondicherry | 1985-91 | 2127 | 0.7 |
| Bangalore | 1980-86 | 1024 | 1.3 |
| Kolar | 1987-89 | 292 | 3.4 |
| Rajasthan | 1988-91 | 1009 | 0.9 |
| Tamil Nadu | 1985-2000 | 6771 | 2.0 |
| Delhi | 1990-91 | 324 | 0.6 |
| Madhya Pradesh | 2001-02 | 273 | 1.0 |
| Maharashtra | 1982-1989 | 1108 | 2.0 |
| West Bengal | 2000-01 | 350 | 3.0 |
| Orissa | 2000-02 | 343 | 0.7 |

Multidrug Resistance in previously treated TB cases in India

| Place | Study period | Sample size | MDR% |
|----------------|--------------|-------------|------|
| Tamil Nadu | 1986-2000 | 1408 | 24.8 |
| Karnataka | 1988-1989 | 111 | 17.1 |
| Maharashtra | 1998-99 | 90 | 67.0 |
| Gujarat | 1983-2001 | 2089 | 30.6 |
| Delhi | 1990-91 | 81 | 33.3 |
| Haryana | 1991-95 | 196 | 49.0 |
| Madhya Pradesh | 1998-99 | 1426 | 8.1 |