MOLECULAR TOOLS IN DIAGNOSIS OF TUBERCULOSIS

DM SEMINAR

08 Apr 05

Scope

- Rapid culture methods
- Serological diagnosis
- Nucleic acid based techniques
- Mycobacteriophage based assays
- Applications
- Future directions

Introduction

- Long generation time of the tubercle bacillus ~18-24 hours
- All microbiology reports are delayed ~4-6 weeks
- Inadequate treatment encourages spread of drug resistant strains adding to disease burden in community
- Molecular tools hold promise for future for early diagnosis and drug resistance testing

Rapid culture methods

- BACTEC system
- Mycobact Growth Indicator Tube(MGIT)
- MB/Bac T system
- Septi-chek
- ESP culture system
- Microscopic observation of broth/slide cultures

BACTEC System

- Radiometric method
- ^{14}C labelled palmitic acid added to liquid 7H12 medium
- Detects MTB by metabolism rather than growth
- $^{14}CO_2$ produced detected by specialized eqpt
- Growth index(GI) measured
- Results available in 7-14days (87-96%)

Ramachandran et al, Ind J TB 2003

MGIT

- Automated system
- Capable of analyzing 960 specimen
- Metabolism of MTB produces O_2
- Fluorescence of dye with oxygen measured
- Results available in 7-14 days
- Cost effective for high load microbio-labs

MB/Bac T system

- Automated
- Colorimetric detection of CO_2
- Slightly longer time (10-15 days)
- Prone to contamination

ESP Myco system

- Changes in gas pressure in a sealed culture broth bottle by gas production/consumption
- Reliable & less labour intensive
- Used in combination with solid medium not stand alone

Microscopic observation of broth culture

- Rapid detection method
- Relatively inexpensive
- As quick
- Equal sensitivity and specificity
- Suitable for endemic countries with high disease burden

Identification of isolates

- Standard biochemical tests
- Radiometric methods
- Enzymatic/colorimetric method
- Lipid analysis- HPLC
- Mycobacteriophage based
- DNA probes
- Ribosomal RNA probes
- Gene amplification – PCR/TMA/SDA

Serologic tests

Serologic tests

- Applied for variety of cases incl smear +ve pulmonary tuberculosis to smear & culture negative EPTB at inaccessible body sites.
- ELISA based methods for the detection of mycobact antigen in body fluids
- Most often for the diagnosis of neurological and pleural tuberculosis

Serologic tests

- Positive test may perhaps “rule in” a diagnosis, but a negative test cannot “rule out” a diagnosis of tuberculosis
- Used as supportive evidence along with conventional tests.
- Some workers have advocated testing for a panel of antigens rather than single antigen.

Limitations - Serologic tests

- Affected by BCG vaccination, previous infection and environmental NTM exposure
- Persistence of antibodies leads to difficulty in distinguishing between infection and disease
- Low sensitivity in smear negative, HIV co-infection, and disease endemic countries

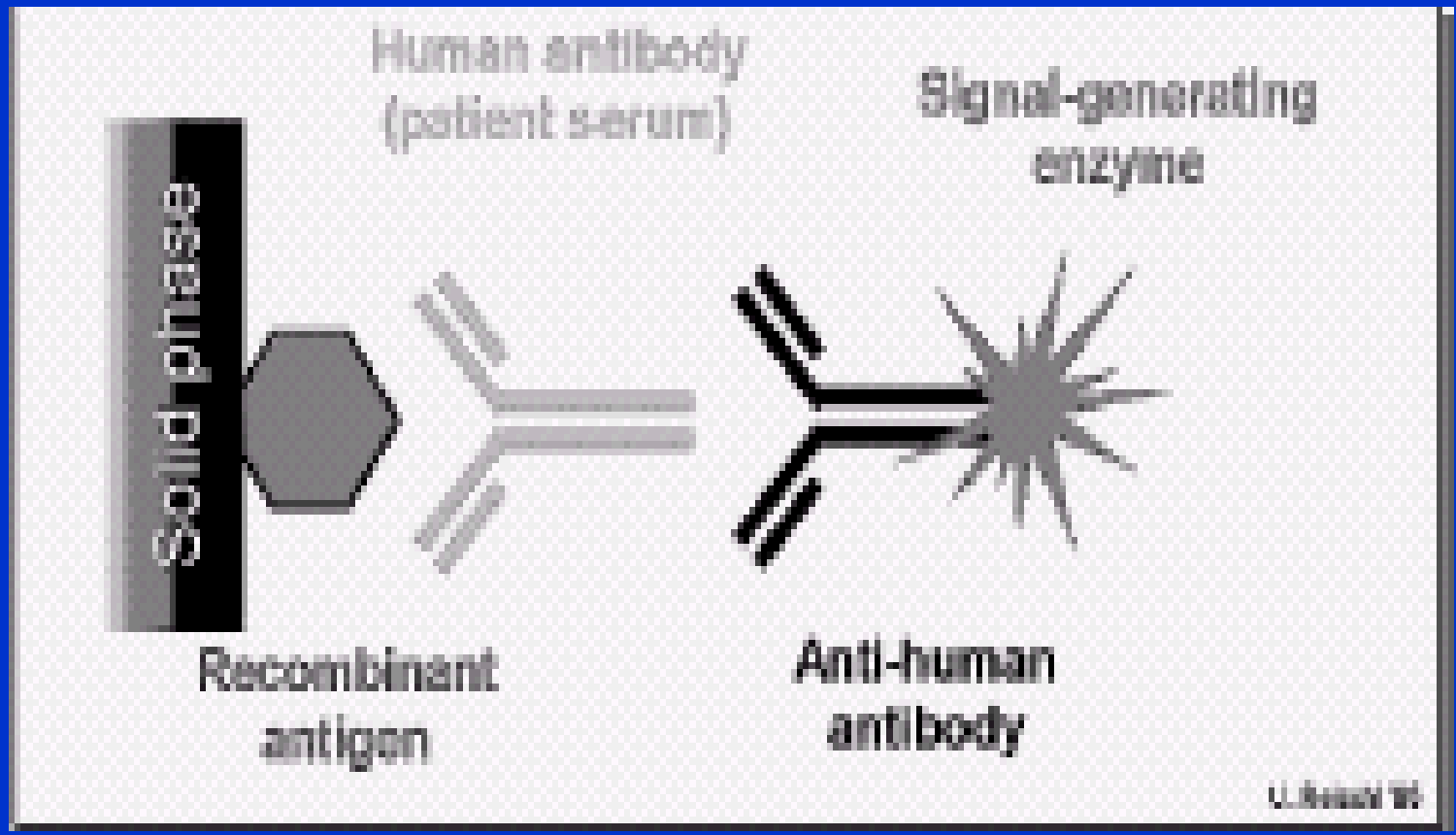
Antigen detection tests

- Lipoarabinomannan
- Extracted glycolipids
- Purified protein derivative
- 38 Kda Ag
- 45/47 Kda Ag
- Cord factor(trehalose dimycolate)

Antigen based tests

- Capture/ Sandwich ELISA
- Monoclonal Ab
- Latex agglutination
- Reverse passive hemagglutnation
- Antigen level – 3-20 ng /ml can be detected
- Sens: 40-50 % spec: 80-95%
- Sputum, pleural ,CSF,urine

Recombinant Antigen



Antibody based tests

Name of the assays	Antigen used
MycoDot (Dot-blot)	Lipo arabino mannan (LAM)
Detect-TB (ELISA)	Recombinant protein Peptide
Pathozyme Myco (ELISA)	38 kDa (recombinant Ag) and LAM
Pathozyme TB(ELISA)	38 kDa (recombinant)
Antigen A60 (ELISA)	Antigen – 60
ICT diagnostics (membrane based)	38 kDa (recombinant)

Ramachandran et al, Ind J TB 2003

Newer tests

- Mycobact Superoxide dismutase Ab: has high PPV(93-94%) useful in low prevalence countries as compared to endemic areas (77-88%)

  Chan et al Tuberc lung disease 2000

- Tests to find antibody associated with active disease than infection are being evaluated
  - Insta test TB
  - TB STAT PAK

Ramachandran et al, Ind J TB 2003

Skin tests

- TB MPB 64 patch test- Specific MTB antigen, becomes positive in 3-4 days and remains for a week
- Sens 98.1% and spec 100%
- Requires further evaluation

Nakamura et al Int J Tuberc lung Dis 1998

Gamma Interferon assay

- QUANTIFERON-TB assay
- Specific MTB Ag ESAT6, CFP are used to stimulate mononuclear cells in vitro and IFN gamma is measured by ELISA
- Useful to differentiate TB disease from NTM infection
- Not affected by BCG vaccination

Streeton et al, Int J Tub Lung dis 1998

T Cell based tests

- ELISA based test compared with TST
- To detect T cells specific for MTB antigens(absent from NTM,M Bovis)
- More sensitive than TST for detecting latent infection among 535 school students and was unaffected by BCG vaccination

Ewer et el Lancet 2003

ELISPOT assay

- Enzyme linked immunospot assay
- Study of 293 S African children with suspected TB were subjected to TST, microbiologic and ELISPOT test
- Had sens ~83%compared to 63 % of TST
- Sens remained high even with HIV co-infection, malnutrition , which affect the tuberculin skin test.

Susan et al Lancet 2004

Phage based assays

Phage amplified biologic assays

- Pha B assay :for protection of phage by mycobacteria in clinical samples
- virucidal solution added to kill extracellular phages
- intracellular phages replicate and lyse the MTB and new phages are released
- Quantified by counting plaque on culture of M smegmatis

Fast plaque Assay

- Commercial assay
- Direct detection of MTB in sputum and urine samples
- Fast assay : reduces the detection time to 1 day from sample collection
- Suitable for use in resource poor countries

Marei AM et al J Med Microbiol 2003

Luciferase reporter phage assay

- LRP are phages carrying firefly Luciferase gene which produce light in presence of luciferin(substrate) and ATP.
- Infect viable MTB and sample releases light detected by luminometer / photograph
- Species identification and drug resistance testing can be carried out with results within 54 hours

Nucleic Acid Amplification Techniques

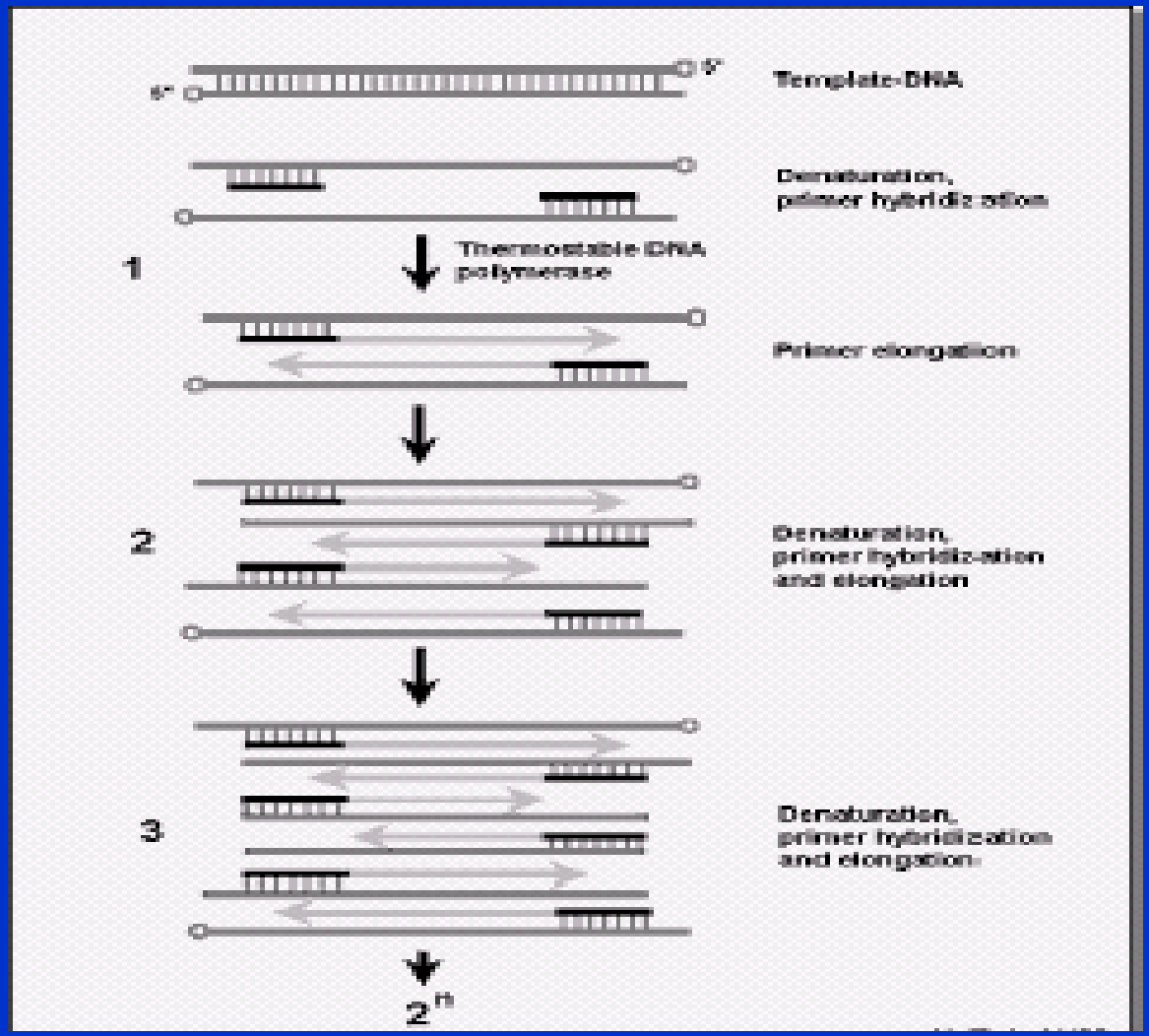
NAT

- Polymerase chain reaction
- Transcription mediated amplification
- Strand displacement amplification
- Nucleic acid sequence based amplification
- Ligase chain reaction
- Q beta replicase amplification
- Branch DNA amplification

PCR

- Synthesis of d s-DNA by hybridization of oligonucleotides to target s s-DNA
- Uses thermal cycler to denature the target DNA
- Thermostable polymerase for DNA amplification
- Repeated cycles by varying temp for primer annealing(70-72 C) and denaturation(94-96 C)
- Amplified product are then detected by southern blotting and fluorescent/radiolabelled probes hybridization

PCR



Denaturation

Primer annealing

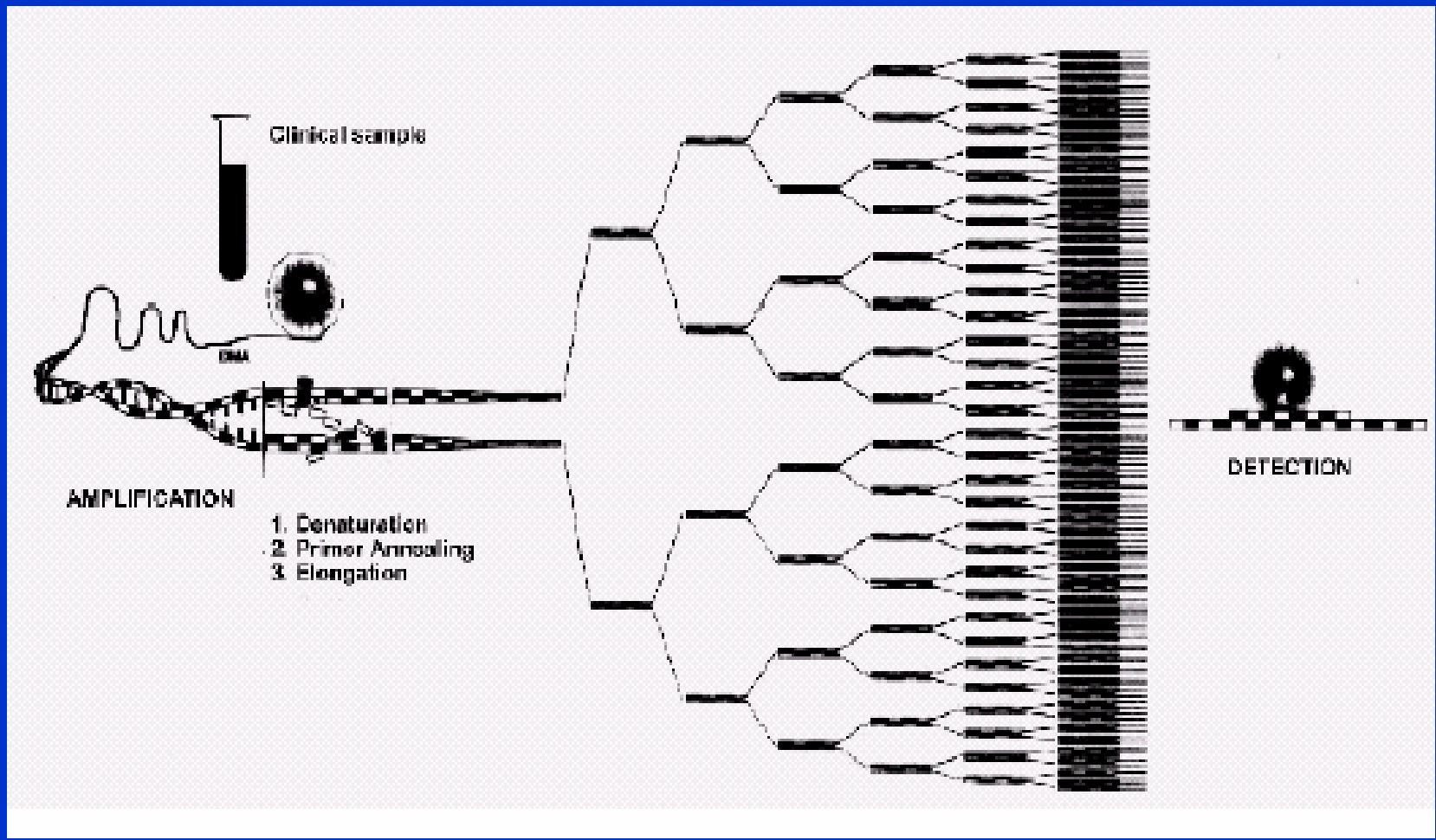
↓

Polymerization

↓

Repeat 25-30 cycles in Thermal cycler

Exponential amplification



PCR

- PCR is capable of detecting even 1-10 organism in clinical specimen(ideal condtn)
- Amplification leads to rise in nucleic acid to 10^{6-7} copies in few hours(25-30 cycles)
- Results are available within hours rather than days

Targets for PCR

- IS6110
- IS1081(nested PCR developed by CDFD)
- 65 kDa protein gene
- 16S r DNA gene
- MPB64 gene
- 35 kDa protein gene
- TRC 4

Types of PCR

- DNA PCR
- RT PCR
- NESTED
- INVERSE
- IN SITU

COMMERCIAL
OR
IN HOUSE

Indian PCR tests

- CDRI, Lucknow
- AIIMS,New Delhi

  These assays have been found to be of acceptable sensitivity and specificity for detection of MTB in sputum

  Katoch VM , Ind J Med Res 2004
- CDFD, Hyderabad –modified IS1081 nested PCR has shown promising results.

Studies :Sputum PCR

Study	No. of Specimens	Prevalence %	Sensitivity %		Specificity %		PPV %	
			C	R	C	R	C	R
Abe[3]	135	28	81.3	84.2	94.2	300	81.3	84.0
Beige[4]	103	47	98.0		70.0		75.0	
Clarridge[5]	>5000	4.4	83.6	86.1	98.7	100	94.2	98.4
Miller[6]	750	21	78.2	92,3				100.0
Nolle[7]	3J3	40	91.0		100.0		100.0	
Shawar[8]	384	18	74.0	80.0	95.0	97	77.0	86.0
Yuen[9]	519	8	96.0		85.0	100		

Rattan A Ind J TB, 2000

Studies :Sputum PCR

Study	PCR Sensitivity (%) in different studies		
	Overall	Smear+ Culture +	Smear- Culture +
Abe[5]	84	96	50
Clarridge[5]	86	94	62
Miller[6]	92	98	78
Nolte[7]	91	95	57
Yuen[9]	96	100	92

Rattan A Ind J TB, 2000

Limitations of PCR

- False positive: due to contamination ,carry over from previous test
  - strict discipline among lab personnel
  - proper technique
  - use of In Situ PCR
- False Negative:
  - proper sample collection/ preparation
  - presence of inhibitors to Taq polymerase

Amplicor^{R} MTT

- Roche Amplicor^{R} –DNA PCR based test
- Specimen preparation,DNA PCR amplification, hybridization, detection
- Approved by FDA for detection of MTB in smear + resp samples(not recd ATT for > 7 days or within 12months)
- Results are available in 6.5 hours
- Sens and specificity of 91.9/99.8% compared to 95.3/ 100% for culture

-ATS work shop, AJRCCM 1997

Nucleic acid Amplification

Isothermal procedures –work without thermal cycler

- Strand displacement amplification (SDA)
- Transcription mediated amplification(TMA)
- Q beta replicase amplification(QBR)

TMA

- Transcription mediated amplification
- Sample preparation- releases r-RNA
- Reverse transcriptase copies the RNA target
- RNA polymerase mediated amplification- RNA amplicon
- Hybridization protection assay detects RNA amplicon.

Amplified MTD

- Geneprobe^{R} Amplified mycobacterium tuberculosis direct test –based on TMA
- Approved by FDA for detection of MTB in smear + resp samples(not recd ATT for > 7 days or within 12 months)
- No statistically significant difference in sens and spec in resp (86.6/96.4%) and non resp samples (93.1/97.7%)

ATS work shop, AJRCCM 1997

SDA

- Isothermal synthesis of ss- and dsDNA.
- Sample containing target DNA treated with restriction enzyme *HincII*
- Primer annealing extension & displacement of strands by E coli DNA polymerase I.
- Sense and antisense strands act as template for further amplification

Gene Amplification Methods

Table I. Comparative sensitivity of DNA, rRNA targeting probes and gene amplification assays for *Mycobacterium tuberculosis*

Technique	Sensitivity	Application
DNA targeting probe[14,15,17]	10,000-00,000 copies	Identification of isolates
rRNA targeting probe[2,14,15,17]	100-1000 copies	Identification of probes; also limited application on clinical specimens
Gene amplification assays[27-29]	1-10 copies	Direct application on clinical specimens; also used to identify isolates by PCR-RFLP/sequencing

Katoch VM , Ind J Med Res 2004

Commercial available NAT

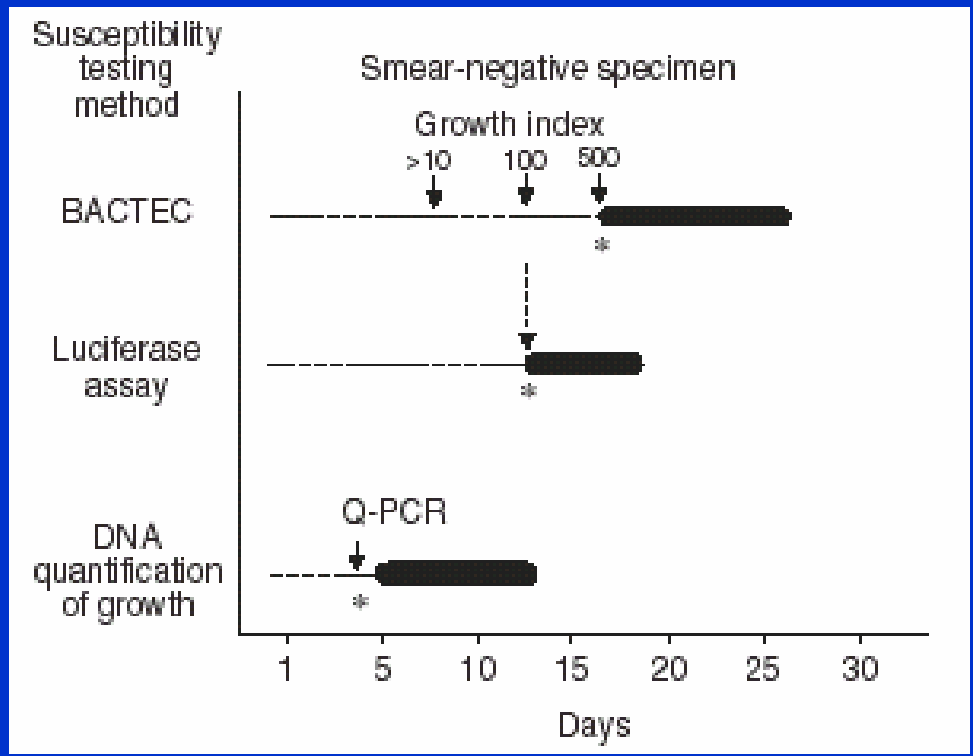
Sl.No.	Method	Target
1	PCR	IS 6110 65 kDa
2	TMA	16 S r RNA
3	SDA	IS 6110
4	NASBA	16 S r RNA
5	b DNA	As for PCR
6	LiPA	As for PCR

Applications

MTB resistance detection

- Detection of point mutation ,early detection of resistant strains from cultured isolates/ clinical specimen
- Results are available within hours to days as compared to weeks with conventional tests
- Helps clinician in initiating appropriate drug therapy and prevents further transmission of resistant strains

Drug susceptibility testing



Roth A et al E Resp J,1997

Drug resistant MTB

Drug	Mutant gene	Frequency
Rifampicin	rpo B	~96%
INH	kat G/ inhA	75-85%
Sreptomycin	rpsL	65-75%
PZI	pnc A	~70%
Ethambutol	emb B	~ 70%

Drug Resistant MTB

- METHODS
  - DNA Sequencing
  - Line probe assay
  - PCR based assay
  - Single strand conformation polymorphism(SSCP)
  - Mycobacteriophage (LRP)assay

Sputum negative PTB

- Sensitivity of NAT is much more in smear +ve cases(95-96%) than smear –ve cases(48-53%)
- FDA panel has approved use of NAT in respir specimen both smear +ve and –ve cases from patients with suspected TB
- Clinically Intermediate /high suspicion of TB sensitivity 75-88% and spec was 100%

ATS statement ,AJRCCM 2000

Extrapulmonary TB

- Paucibacillary tuberculosis- conventional methods are insensitive
- Histopathology tissue may be unaccessible or non representative and nonspecific
- Potential for molecular tools is tremendous for diagnosis of EPTB

Katoch VM , Ind J Med Res 2004

Conventional methods :EPTB

Variable	Pleural fluid	Pericardial fluid	Cerebrospinal fluid
Smear microscopy	< 10%	< 1%	5-37%
Mycobacterial culture	12-70%	25-60%	40-80%

Sharma SK et al I J TB 2004

Extra-pulmonary TB

- CNS TB:PCR has 50-70% positivity compare to CSF AFB /culture which has 5-20%
- Ocular :confirm diagnosis in 50- 70%(aqueous /vitreous)
- Cutaneous :positivity 50-60% compared to culture

Katoch VM , Ind J Med Res 2004

Extra-pulmonary TB

- Lymph node TB:positivity rates varying from 40-90%
- Bone/Genitourinary TB:useful in confirming diagnosis

Katoch VM , Ind J Med Res 2004

Amplicor^{R} in TBM

- Compared with direct ZN stain of smears, radiometric culture for MTB and clinical and CSF findings.
- More sensitive(60%) than the combination of ZN stain smears and radiometric culture for MTB
- Rapid and highly specific(100%) diagnostic test for TBM.

Bonington et al, J Clin Microbiol 1998

Serologic & Mol tools-EPTB

Diagnostic method	Pleural fluid	Cerebrospinal fluid
ELISA:		
Detection of antibody in the fluid		
Sensitivity	0.22 - 0.68[a]	0.60 - 0.90[b]
Specificity	0.90 -1.00[a]	0.58 - 1.00[b]
Detection of antigen in the fluid		
Sensitivity	0.48 - 1.00[c]	0.61 - 0.79[d]
Specificity	0.98 - 1.00[c]	1.00[d]
Molecular methods :		
Polymerase chain reaction		
Sensitivity	0.22 - 0.81[e]	0.50 - 0.90[f]
Specificity	0.77-1.00[e]	1.00[f]

Sharma SK et al I J TB 2004

RFLP

- Restriction fragment length polymorphism
- Restriction endonucleases will cut the ds- DNA at specific recognition sites so fragments of different lengths result
- Gel electrophoresis followed by southern blotting to produce patterns which are Genomic or DNA Fingerprints

Barnes P, N Engl J Med 2003

IS6110

- Insertion sequence(IS) are 1355 base pairs in size and are randomly distributed throughout the genome of MTB
- They are present in varying numbers from 0-30 copies
- RFLP analysis of the distribution of the insertion sequence IS6110 in different strains
- Large data bases of IS6110-based genotypes are available (DNA Fingerprints)

Barnes P, N Engl J Med 2003

IS6110 RFLP

- Strains with fewer than 6 IS6110 insertion sites have limited polymorphism and require other methods of genotyping
- Requires sufficient quantity of DNA and are done on cultured isolates after several weeks

Barnes P, N Engl J Med 2003

MIRU

- Mycobacterial interspersed repeat units
- MTB genome has repeat units some identical some variable
- MIRU genotyping characterizes the type of repeats into 12 independent MIRUs
- Performed directly on culture(no DNA purification)
- Detected by PCR followed by electrophoresis

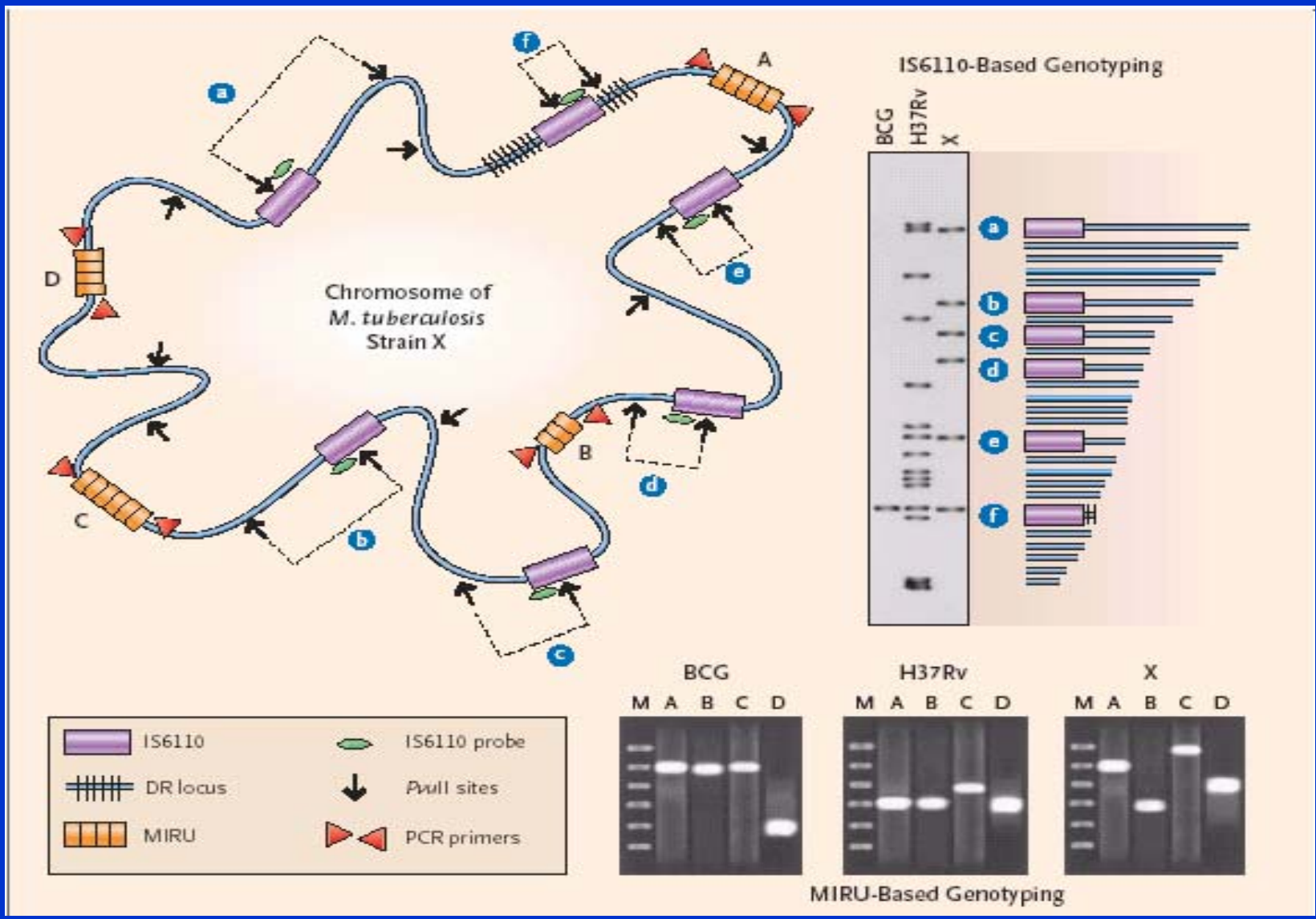
Barnes P, N Engl J Med 2003

MIRU

- Discriminatory power like IS 6110
- Automated analysis
- Digital results which can be catalogued on computer database
- Website has been created for updating the patterns worldwide

Barnes P, N Engl J Med 2003

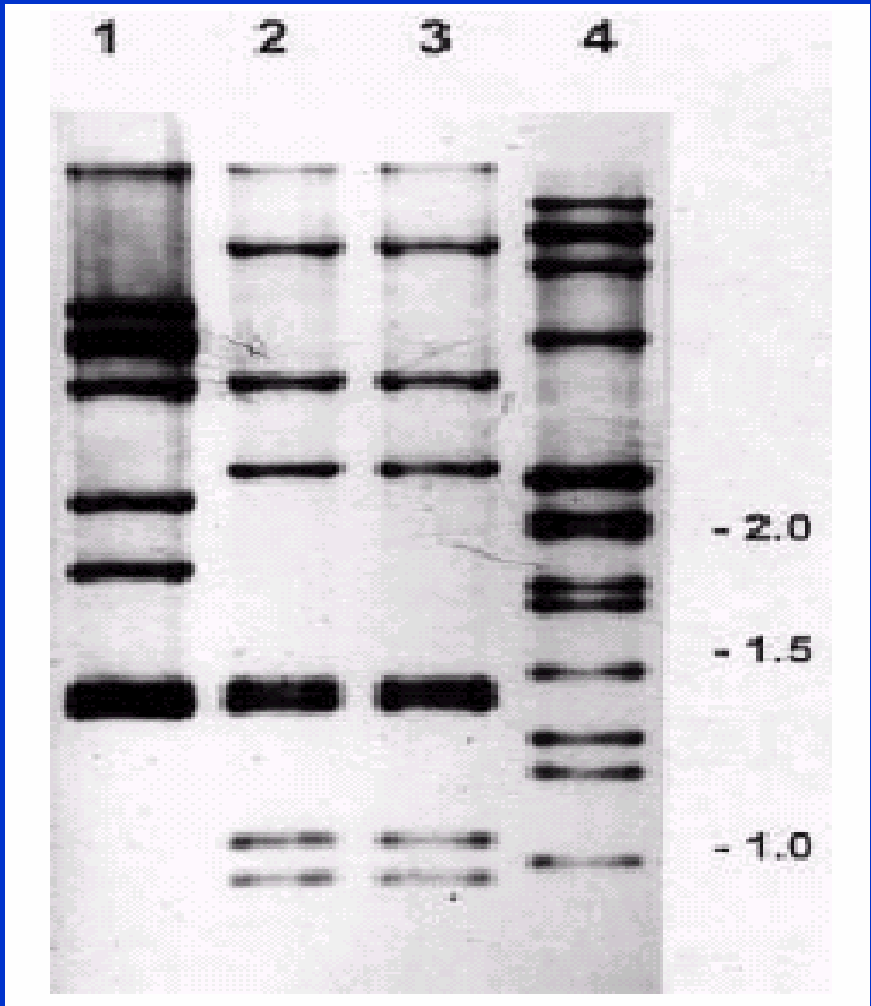
IS6110 RFLP


Chromosome of
M. tuberculosis
Strain X
A
B
C
D
a
b
c
d
e
f
IS6110-Based Genotyping
BCG
H37Rv
X
IS6110
DR locus
MIRU
IS6110 probe
PvuII sites
PCR primers
BCG
H37Rv
X
M A B C D
MIRU-Based Genotyping

Epidemiologic tool

- Out break of TB (Clustering)
- DR Strains M .Tuberculosis
- Recent infection vs reactivation
- Lab cross contamination

Lab Cross Contamination



Roth A et al Euro Resp J 1997

Spoligotyping

- MTB genome has direct-repeat locus which

  contains 10 to 50 copies of 36-bp direct repeat
- Separated from one another by spacers that have different sequences.
- Spacer sequences between any two specific direct repeats are conserved among strains.

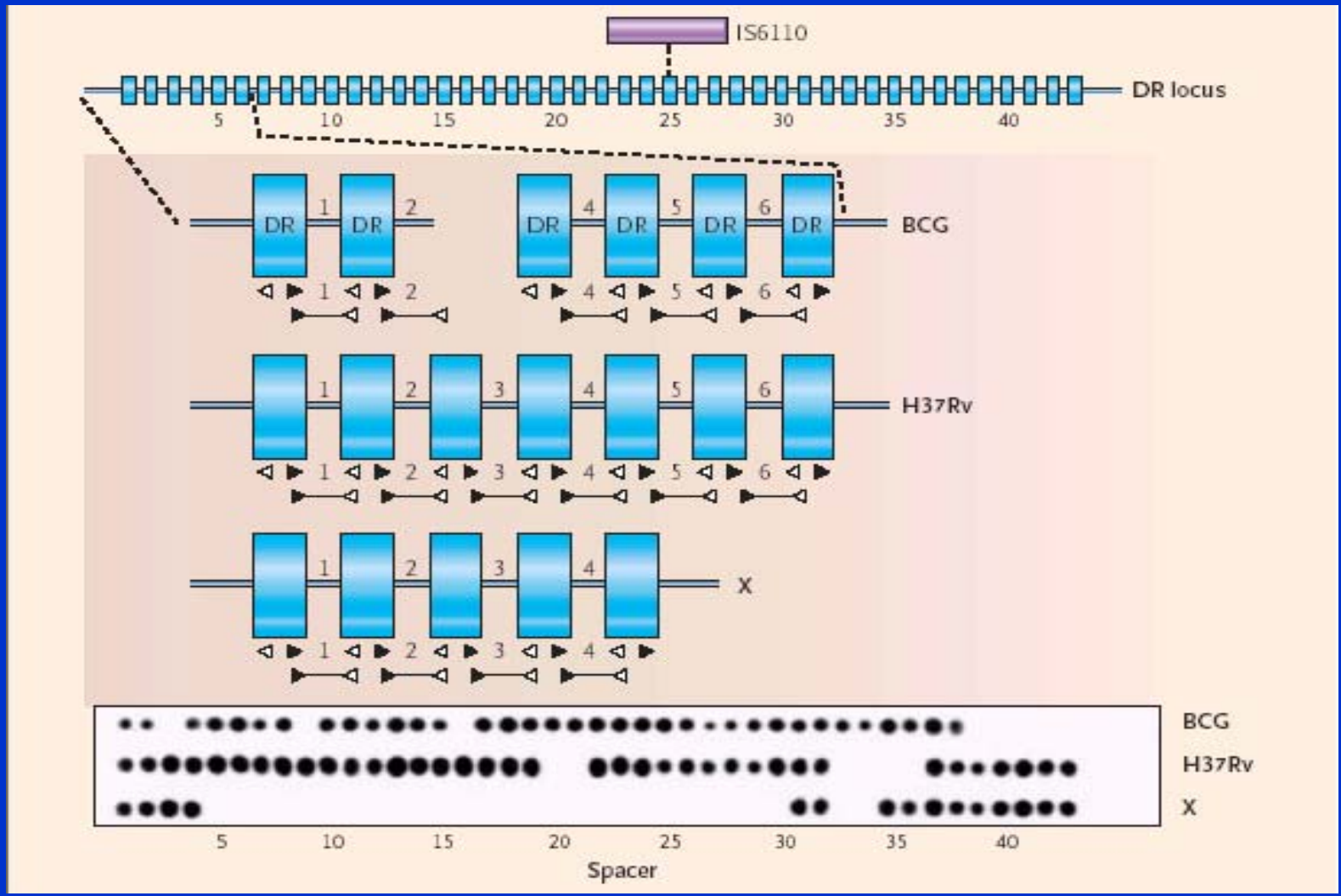
Spoligotyping

- Strains differ in terms of the presence or absence of specific spacers, the pattern of spacers in a strain can be used for genotyping (spacer oligonucleotide typing)
- Advantages:

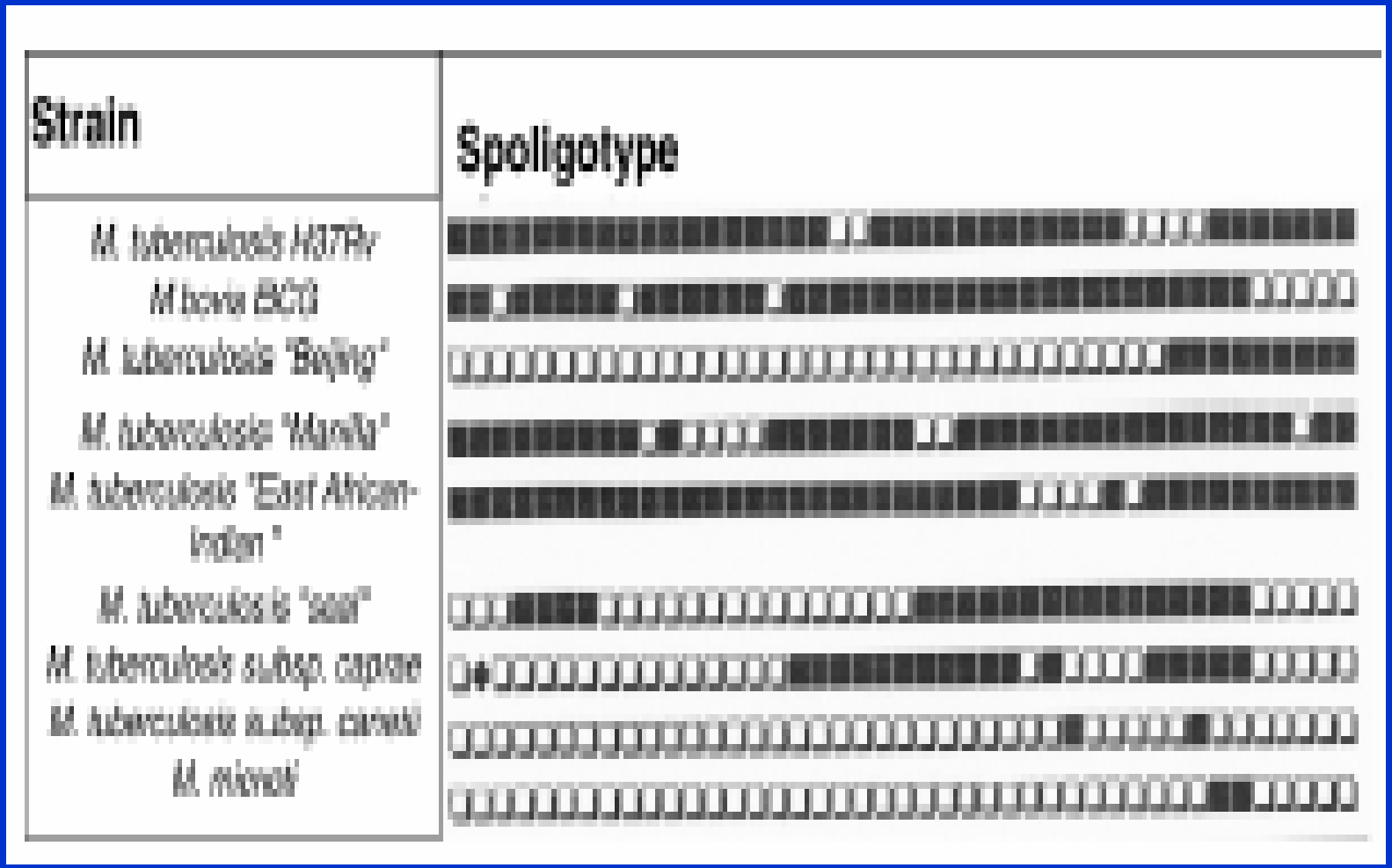
  -Small amounts of DNA are required

  -performed on clinical samples or on strains of *M. tuberculosis* shortly after their inoculation

Spoligotyping



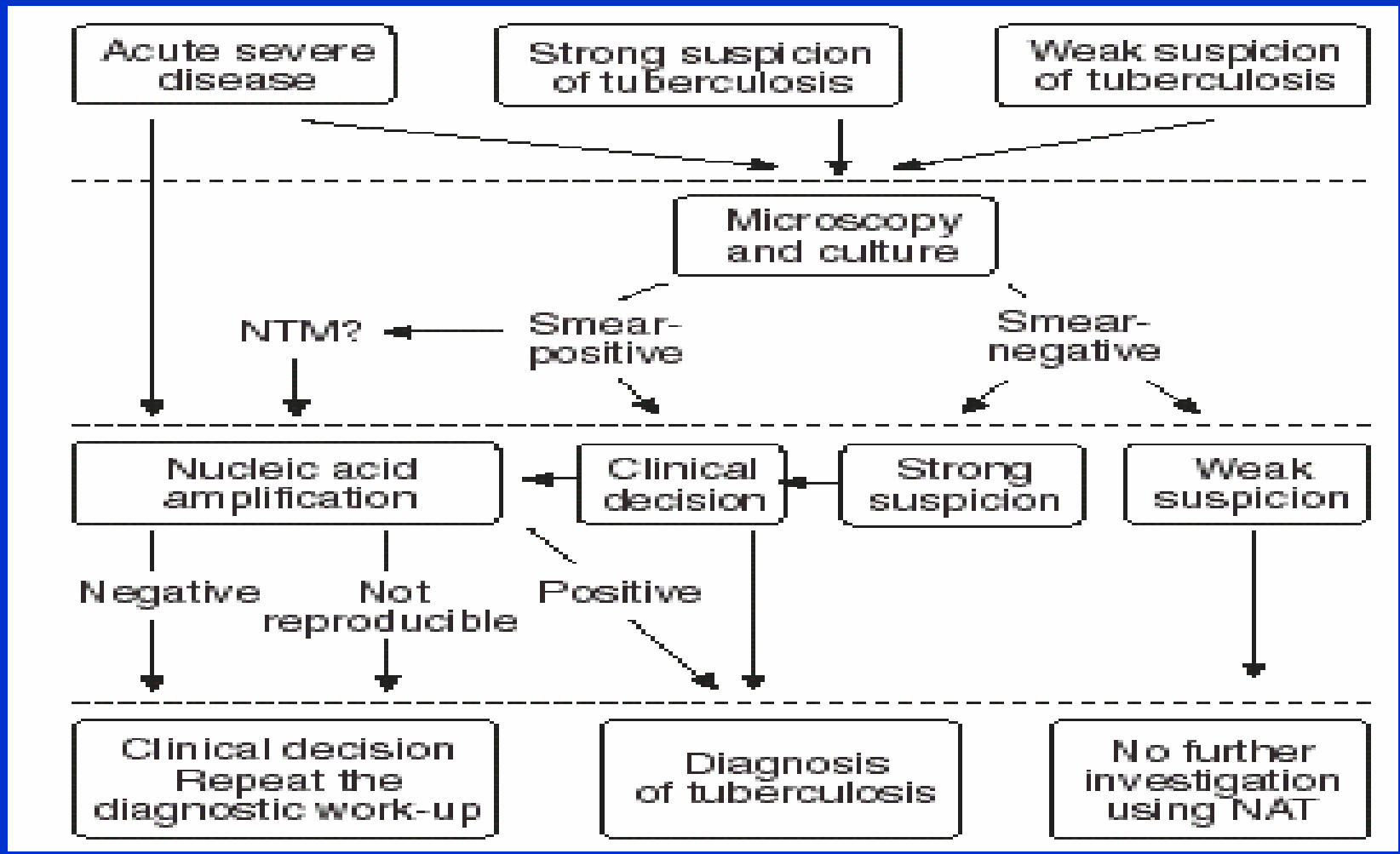
Spoligotyping



Future of NAT

- Molecular beacons
- DNA microarray/DNA chips
- FRET probes(fluorescence resonance energy transfer)

Clinical Decision Algorithm



Roth A et al Euro Resp J 1997

Conclusions

- Molecular methods have a role in early diagnosis of Tuberculosis
- Rapid identification & typing of MTB from cultured isolates and directly from clinical specimen
- Diagnosis of drug resistant mutants
- Tests should be used in conjunction with conventional tests and interpreted with overall clinical picture