Comparison of Phenotypic & Genotypic Methods for Pyrazinamide Susceptibility Testing

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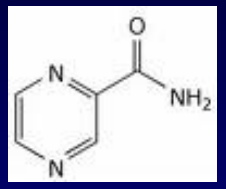
Introduction

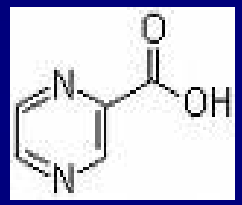
- Pyrazinamide (PZA) - a key primary drug in intensive phase treatment of TB
- Excellent sterilizing effect
- Inhibit semi dormant tubercle bacilli
- shortens the treatment in combination with RIF
- Regimens containing PZA have a lower relapse rate

Mechanism of Action



Pyrazinamide (PZA)

Pyrazinamidase (PZAase), acidic pH



Pyrazinoic acid (POA), inhibits fatty acids synthesis

- PZA resistant strains have lost PZAase activity
- PZAase enzyme is encoded by pncA gene
- pncA gene mutations are responsible for PZA resistance
- Many species of mycobacteria naturally resistant to PZA due to pncA gene alteration eg *M. bovis* 169 CAC→GAC (HIS 57 ASP)

Susceptibility Testing: Drawbacks of current methods

- PZA Susceptibility testing using conventional methods is not always reliable - low pH (5.5) inhibits the growth of mycobacteria
- Other factors – inoculum size, drug concentration etc
- Colorimetric enzymatic assay often difficult to interpret
- Many clinical laboratories do not perform PZA susceptibility testing

- With increasing incidence of MDR & XDR TB rapid & Accurate prediction of PZA resistance is essential

- At our tertiary care centre, we analysed 236 total requests for PZA susceptibility by MGIT 960 TB system during a period of Jan to May 2007

- PZA Resistant strains = 141 (59%)

Objective

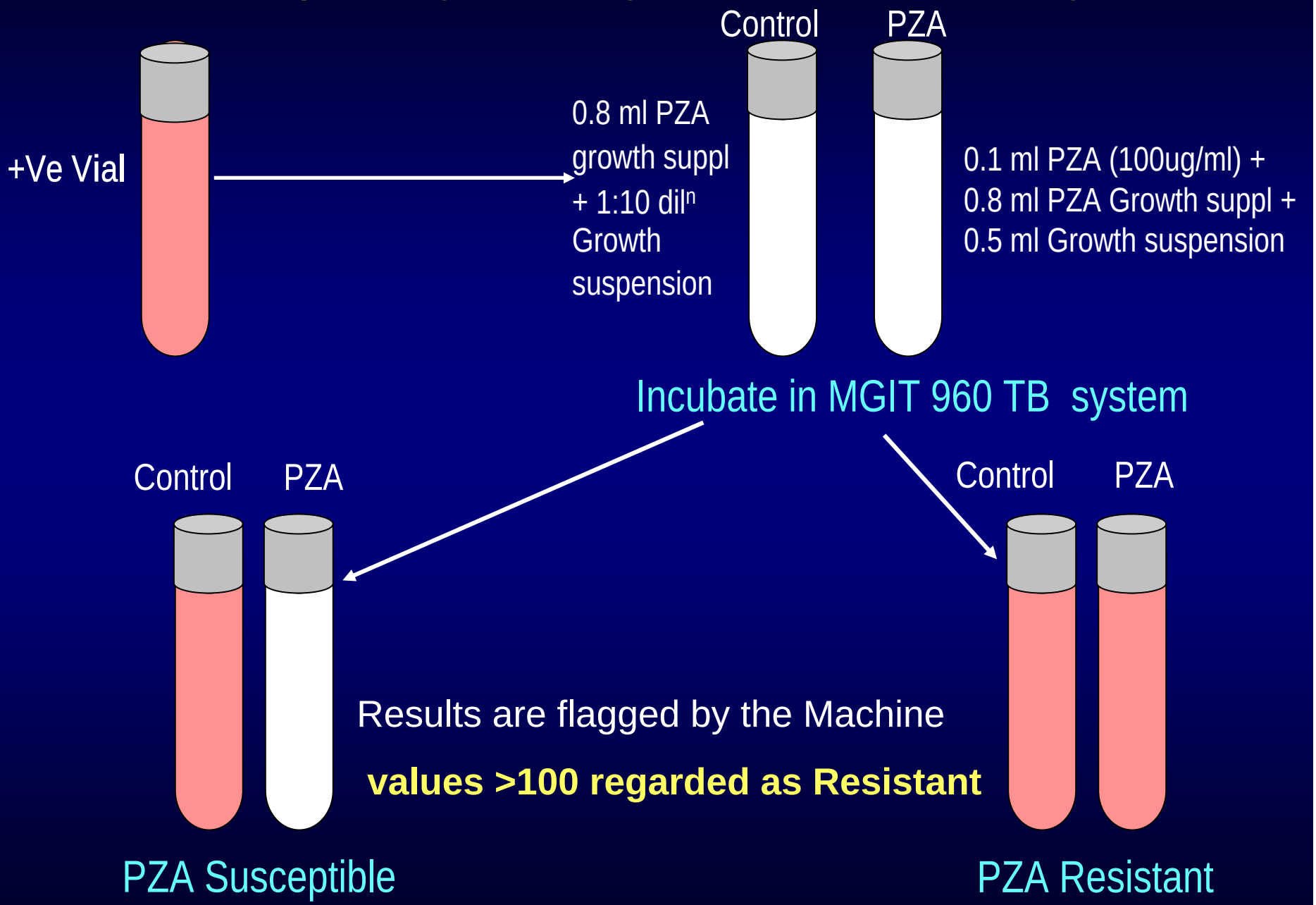
- To compare our PZA susceptibility result using MGIT 960 TB system with
  A. Enzymatic : PZAse (wayne's method)
  B. Genotypic : *pncA* gene sequencing

- To find the prevalence of *M. bovis* in PZA resistant cases by spoligotyping

Material & Methods

- Consecutive 30 PZA susceptible & 33 PZA resistant strains reported for PZA susceptibility testing by MGIT 960 TB system (1% proportion method) were further screened by
  - Enzymatic Wayne's Assay
  - Sequencing
- Fingerprinting was performed by Spoligotyping

PZA susceptibility testing MGIT 960 TB system



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 4 days | 37^0C, 4 days

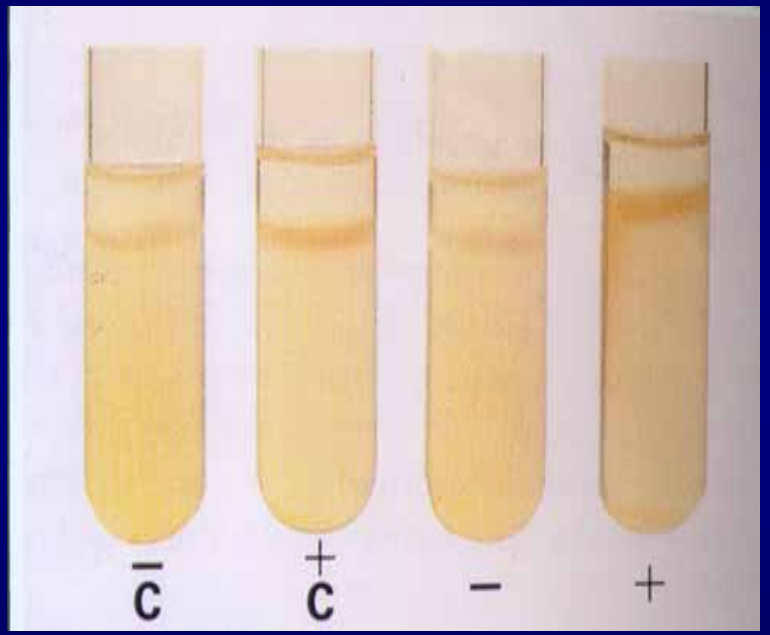
Add 1 ml freshly prepared 1% ferrous ammonium sulphate

↓ RT, at least 30 mins

Examine for a pink band in the agar medium independently by 2 observers

↓

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



Positive: Pink band (Susceptible)

Negative: No pink band (Resistant)

pncA gene Sequencing

DNA extraction by CTAB –NaCl mtd

Amplification

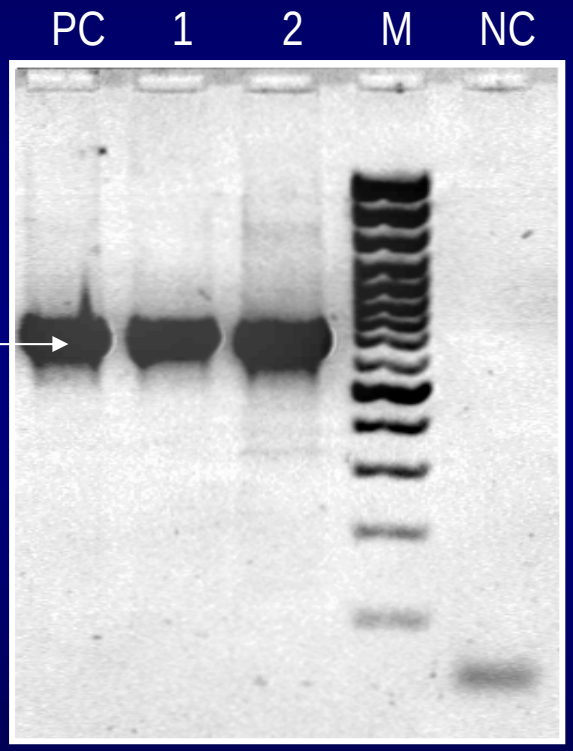
Primers

pncA F 5' GCT GGT CAT GTT CGC GAT CG-3‘

pncA R 5' GCT TTG CGG CGA GCG CTC CA-3‘

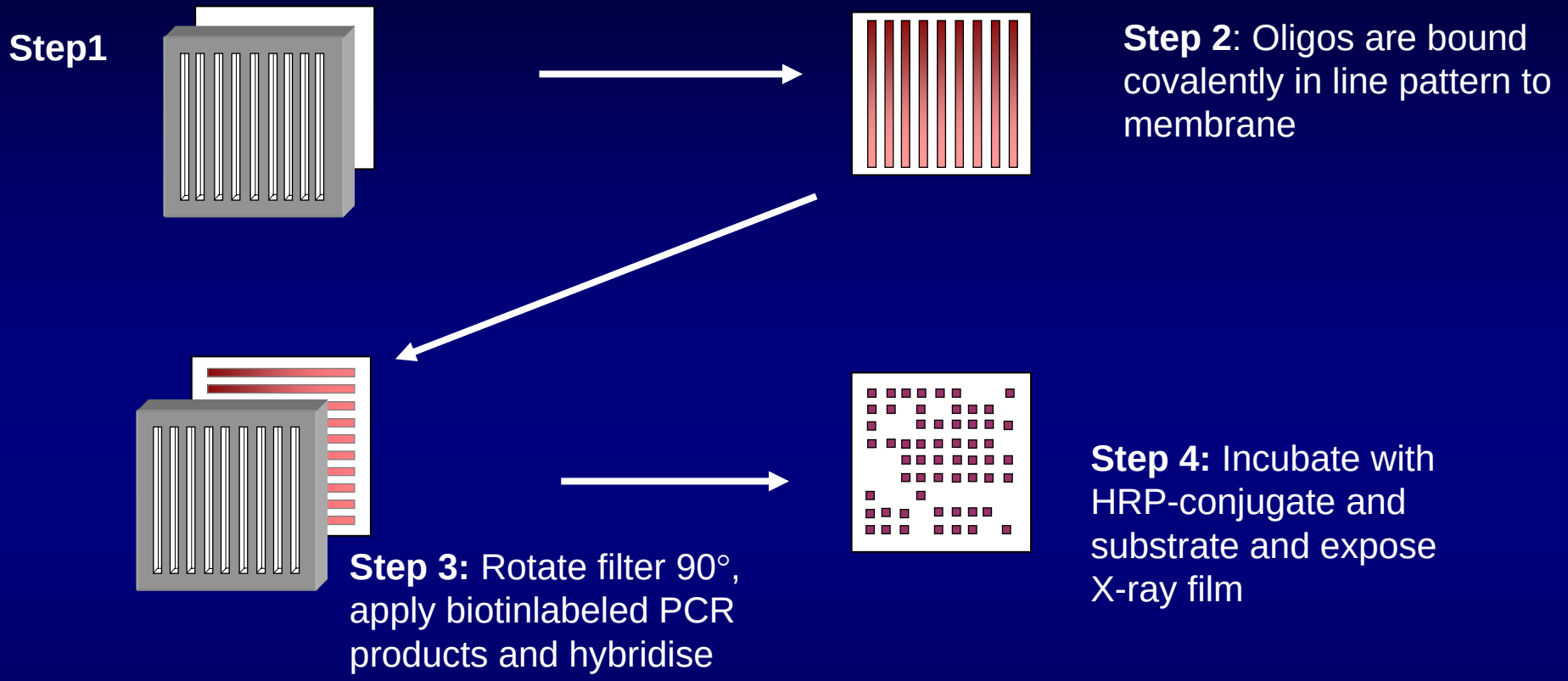
Conditions

95^{0}C 5’, 95^{0}C 1 ‘, 62^{0}C 1 ‘,72^{0}C 1’, 72^{0}C 5’, hold 15^{0}C



Sequencing (ABI sequencer)

Fingerprinting ……Spoligotyping



⇒ The PCR products allowed to hybridize with the 43 spacer sequences immobilized on the membrane

⇒ Hybridized products visualized by ECL detection(Amersham)

Results
N = 63

Wayne's pyrazinamidase method

Susceptible	Resistant
26	37

MGIT 960 TB system

Susceptible	Resistant
30	33

Sequencing

Wild types	Silent Mutations	Mutations
22	9	32

Genotypic analysis of PZA susceptible strains N = 30

No. of Isolates	Resistance by MGIT	PZAase activity	Nucleotide Change	Change
19	S-SHREZ	S	Wild type	No change
1	S-SHREZ	R	Wild type	No change
6	S-SHREZ	S	195 C → T	Silent, 68 Trp → Arg
2	R-SHRE,S-Z	S	Wild type	No change
2	R-H,S-SREZ	R	195 C → T	Silent, 68 Trp → Arg

9 MTB isolates showed a silent mutation 195 C → T which probably represents the natural polymorphism of the gene

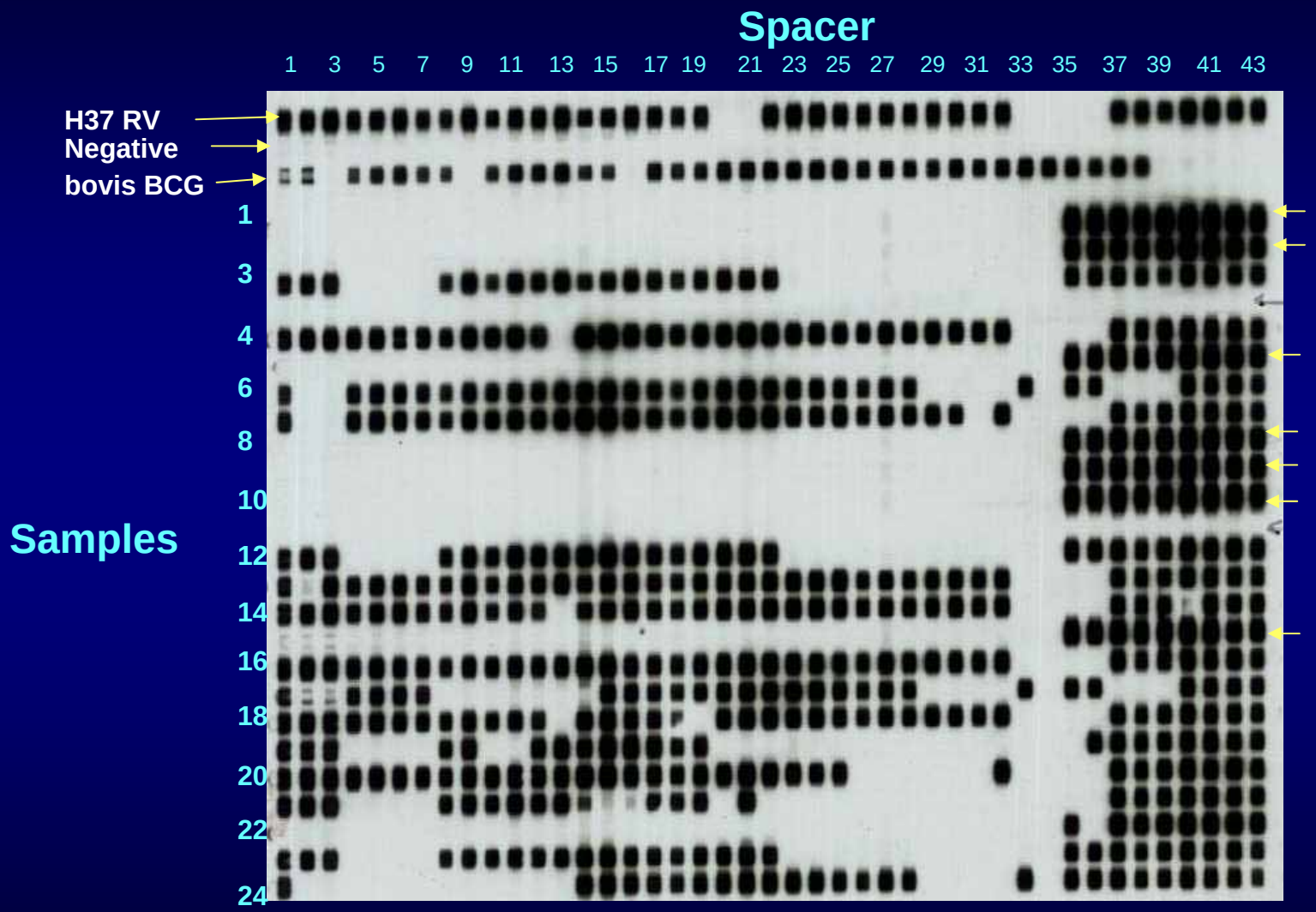
Genotypic analysis of PZA Resistant Strains (N=33)

No. of Isolates	Resistance by MGIT	PZAase activity	Nucleotide Change	Change
1	R-HREO	R	Wild type	No change
2	R-SHR	R	-11 A → G	Promoter mutation
1	R-SHREKTHO	R	-11 A → G, 483 G → A	Promoter mutation
1	R-SHRE	R	-11 A → G, 535 Deletion A	Promoter + Frameshift mutation
1	R-HRE	R	-11 A → G, 195 C → T	Promoter + Silent mutations
2	R-SHRE	R	14 T → G	Iso 5 Ser
1	R-SHR,S-E	R	29 A → G, 195 C → T	Gly 10 Arg + Silent mutation
1	R-SHRE	R	42 C → A, 535 Deletion A	Cys 14 stop codon + Frame shift mutation
1	R-Z	R	Deletion of 9 NA 382-389	Frameshift mutation
1	R-SHREO	R	152 A → G	His 51 Arg
1	R-SHRE	R	156 C → A, 546 G → T	Iso 92 Iso*, Leu 182 Phe
1	R-SHR	R	195 C → T, 406 G → A	Silent mutation + Asp 136 Asn
1	R-SHR	S	195 C → T, 515 T → C	Silent mutation + Leu 172 Pro
1	R-S	R	195 C → T, 535 Deletion A	Silent + Frame shift mutation
1	R-SIHRE	R	195 C → T, 314 G → A, 535 Deletion A	Silent + Gly 105 Glu + Frameshift mutation
1	R-HRE	R	195 C → T, 328 G → T, 535 Deletion A	Silent + Asp 110 Tyr +Frameshift mutation
1	R-SHRE	R	Insertion of 6 NA at 200	Frameshift mutation
1	R-HRE	S	226 A → C	Thr 76 Pro
1	R-HRE	R	226 A → C	Thr 76 Pro
1	R-HRE	R	286 A → G	Lys 96 Glu
1	R-SHR	R	289 G → T	Gly 97Cys
1	R-HRE	R	347 T → G	Leu 116 Arg
1	R-SHR	R	359 T → G	Leu 120 Arg
1	R-SHRE	R	389 Insertion of GG	Frameshift mutation
1	R-HRE	R	389 Insertion of G	Frameshift mutation
1	R-SHRTHO	R	395 G → C	Gly 132 Ala
1	R- SHRO	R	488 T → C	Val 163 Ala
1	R-HRE	R	538 G → T	Val 180 Phe
1	R-SHRPO	R	559 T → G	Pro 186 Pro*
1	R-SHREO	R	80 T → C	Leu 27 Pro
1	RHRTHO	R	418 C → T, Del T 412	Arg 140 Cys, Frameshift mutation

Comparison of Phenotypic results vs. Genotypic Results

N =63	MGIT		Wayne's Assay	
	Susceptible (30)	Resistant (33)	Susceptible (26)	Resistant (37)
Sequencing Wild type/ Silent mutation (31)	21/9	1	19/5	3/4
Sequencing Mutants (32)	00	32	2	30

Fingerprinting…..Spoligotyping



Of the 33 PZA resistant cases 31 were MDR of these 15 belonged to Beijing genotypes

Not a single isolate of *M. bovis* was detected among PZA resistant isolates

Discussions & Conclusions

- PZA resistance is associated with MDR TB
- PZA susceptibility by MGIT 960 showed better correlation with sequencing than PZAase assay
- Cost & Feasibility remains the main constraint

- PZAase test can not replace the modified proportion method as

- Can not perform directly from liquid culture broth as heavy inoculum from L.J is required

- Subjectivity in result interpretation

- PZA resistant isolates are not always PZAase negative *(Butler WR Antimicrob Agens Chemother 1983, Miller M et al JCM 1995)*

- 32 / 33 PZA culture resistant strains had nucleotide changes like point mutations insertion & deletions, scattered along the pncA gene, 1 PZA resistant isolates did not have any pncA gene mutation suggesting that another mechanism might involved in conferring PZA resistance in this isolate

- 14 different mutation found in this study had not been described earlier

- Sequencing is rapid & accurate however difficult to perform in Mycobacteriology lab routinely

- Unique mutation at 169 (CAC→GAC) defined originally in *M. bovis* was not observed

- Among PZA resistant strains no *M. bovis* was detected by spoligotyping


THANK TOU !