TEHRAN UNIVERSITY
OF
MEDICAL SCIENCES

MDR tuberculosis, results of bacteriology and molecular diagnosis

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Drug resistance in Tuberculosis

- A major threat to worldwide control of tuberculosis (TB)
- 3.3% of new cases and 20% of previously treated cases are MDR-TB
- Accurate and rapid detection of resistant strains is critical to provide appropriate treatment and to intercept the transmission of drug-resistant TB

Estimated number of MDR TB in the World, EMR and Iran

- **World:** 580,000
- **EMR:** 39,000
- **Pakistan:** 26, 000
- **Afghanistan:** 3000
- **Turkey:** 710
- **Iran:** 250

Resistance determination to first-/second-line anti-TB drugs in *Mycobacterium tuberculosis* strains using WGS and MIC testing in compare with LPA

Conventional Drug Susceptibility Testing (DST)

- ✓DST: solid & recently in liquid media
- ✓Detection and identification: 8 weeks
- ✓DST for first line drugs: may take an additional 3-6 weeks

Rapid diagnosis

- ✓Accurate and rapid detection: Appropriate treatment, stopping the transmission of resistant strains
- ✓Lack of access to quality laboratory diagnostics: jeopardize efforts to control the worldwide transmission of TB

Molecular DST

- ✓An improved understanding of the molecular basis of drug resistance has resulted in the ability to determine susceptibility in less than 24 hours

Molecular DST

Drug	Gene	Gene product	Mutations
RIFAMPIN	*rpoB*	RNA pol (β subunit)	hot-spot region (98%) cod. 508 to 535; N-term region
RIFABUTIN	*rpoB*	RNA pol (β subunit)	cod. 144, 146, 148, 505, 512, 526, 531
INH	*katG*	Catalase-peroxidase	cod. 315 (60-80%) Polymorphism Leu463Arg
	inhA	NADH-dep enoyl-ACP red	promoter region (ribosome binding site) pos. -8; -15 (15%); coding region (example: Ser94Ala)
	ndh	NADH dehydrogenase	coding region
	ahpC	small subunit of alkylhydroperoxide reductase	promoter region (mutations relatively rare)
	OxyR	regulon (controls expression of katG and several other genes including ahpC)	(mutations relatively rare)
ETHIONAMID	*inhA*	NADH-dep enoyl-ACP red	Ribosome binding site pos. -8; -15 coding region: cod. 16, 21, 47, 78, 94, 95
	ethA	monooxygenase	coding region
	ethR	putative monooxygenase repressor	coding region
	ndh	NADH DH	coding region
PZA	*pncA*	Pyrazinamidase	coding region (72-98%)
ETHAMBUTOL	*embB*	Arabinosyl transferase	ERDR (70%) cod. 306; 239, 240, 247, 282, 285, 299, 311, 330, 368, 397, 466, 469, 471, 630
	embC	Arabinosyl transferase	cod. 251, 254, 270
	embR	(regulator) - ?	
FLUOROQUINOLONES	*gyrA*	DNA gyrase (sub. A)	QRDR (nt. 220 to 339) cod. 74, 88, 90, 91, 94 (70%) Polymorphism at cod. 95
	gyrB	DNA gyrase (sub. B)	QRDR (nt. 1414 to 1530) cod. 495, 516, 533
	mfpA	protein that mimic DNA	?
STREPTOMYCIN	*rpsL*	12S ribiosomal protein	cod. Lys43Arg, Lys88Gln/Arg, Arg9His, Val93Met (60%)
	Rrs	16S rRNA	reg. 530: C to T 491, 512, 516; A to C/T 513 (8%); reg. 915: C to A/G 903; A to G 904; C to T 798; G to A 877; A to C 906
CAPREOMYCIN	*rrs*	16S rRNA	A to G 1401
	tlyA	rRNA methyltransferase	coding region
VIOMYCIN	*rrs*	16S rRNA	coding region
KANAMYCIN	*rrs*	16S rRNA	C to T 1402; 1401, 1483, (>) 1400 (67%)
AMIKACIN	*rrs*	16S rRNA	1400

Molecular DST

- ✓Processing of a large number of specimens at the same time
- ✓Detection of mutations associated with lower levels of phenotypic resistance
- ✓Detection of specific mutations causing drug resistance to an entire family of some classes of drugs

Approved Methods

- Gene Xper TB (Automated Real Time PCR)
- Line Probe Assay
- WGS

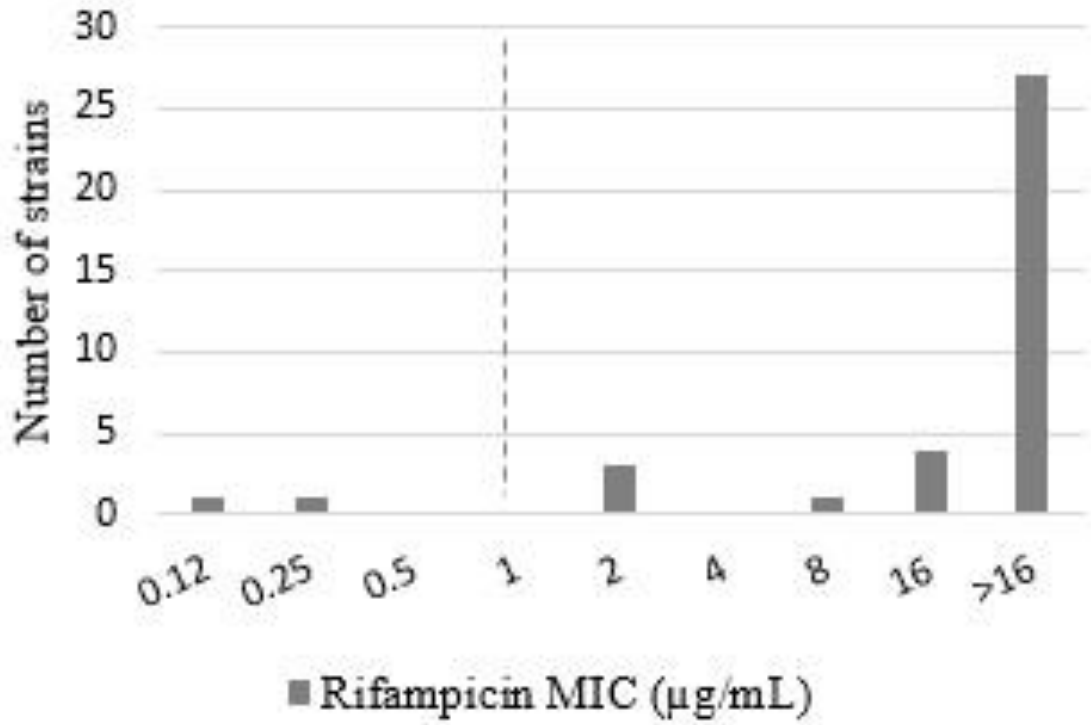
MTB strains used in this study (2014-2018)

- MTB was primarily cultured from patients on LJ medium at Tehran Regional Reference Laboratory for Tuberculosis
- Initial identification was based in biochemical
- Under supervision of TB Supranational Reference Laboratory Network in Stockholm, Sweden (WHO)
- RR strains of *M. tuberculosis* strains (n=37) were selected
- WGS was done by TB Supranational Reference Laboratory (SRL-Milan)

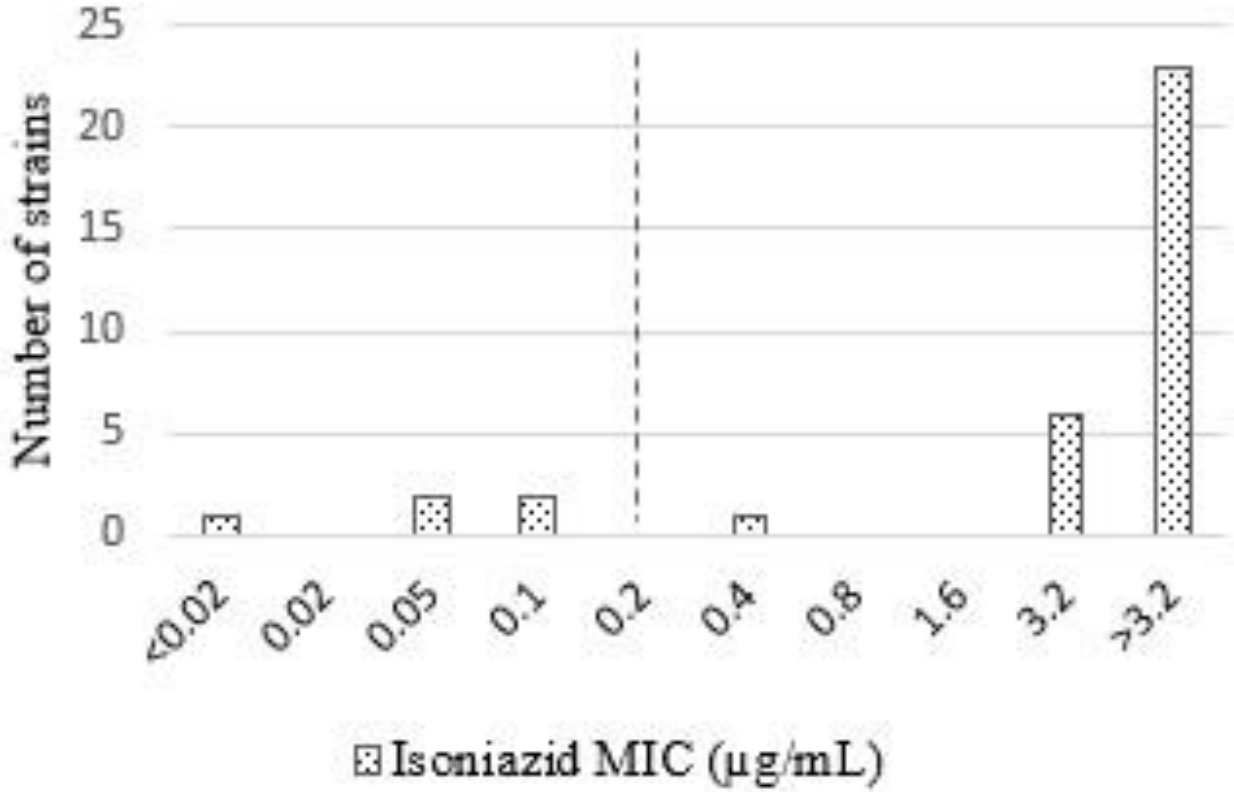
Phenotypic DST

- Resistance to the first-line drugs was assessed using proportional method
- The MICs of first- and second-line drugs were determined by a broth microdilution method (Middlebrook 7H9 medium supplemented with OADC
- 96-well microplates used for: Rifampicin, Isoniazid, Ethambutol, Levofloxacin, Moxifloxacin, Amikacin , Kanamycin , Capreomycin, Prothionamide, D-Cycloserine, Clofazimine, Linezolid, Bedaquiline, and Delamanid
- This experiment was done in duplicate
- *M. tuberculosis* H37Rv was used as a standard strain

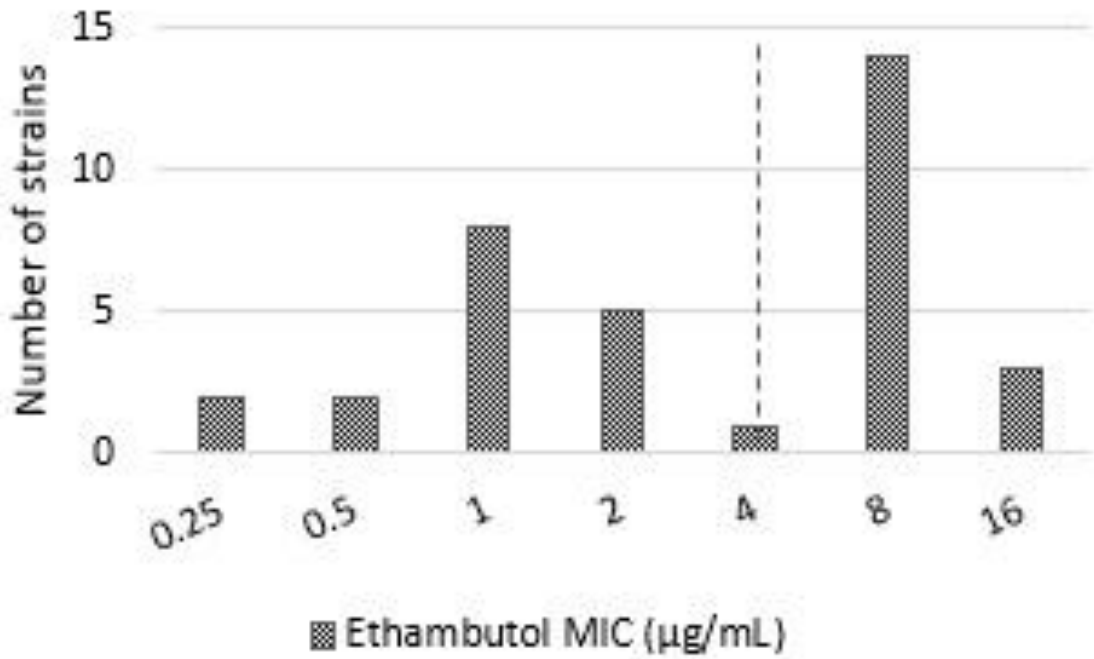
A

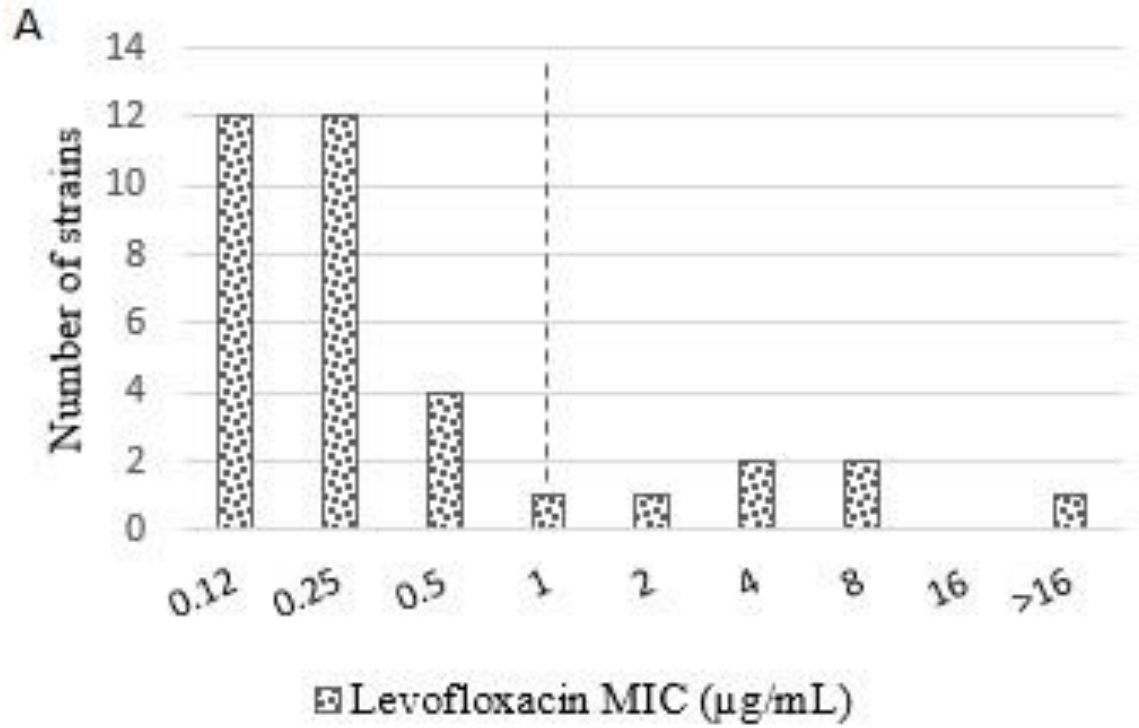

30
25
20
15
10
5
0
Number of strains
0.12
0.25
0.5
1
2
4
8
16
>16
Rifampicin MIC (µg/mL)

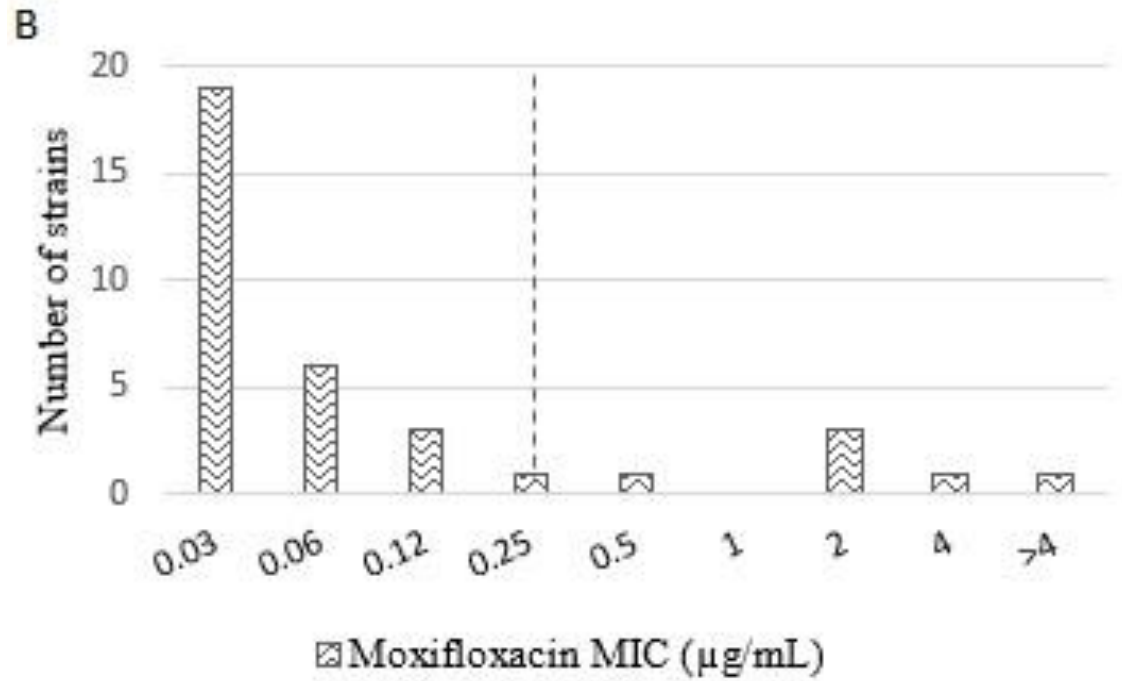
B


25
20
15
10
5
0
Number of strains
<0.02
0.02
0.05
0.1
0.2
0.4
0.8
1.6
3.2
>3.2
Isoniazid MIC (µg/mL)

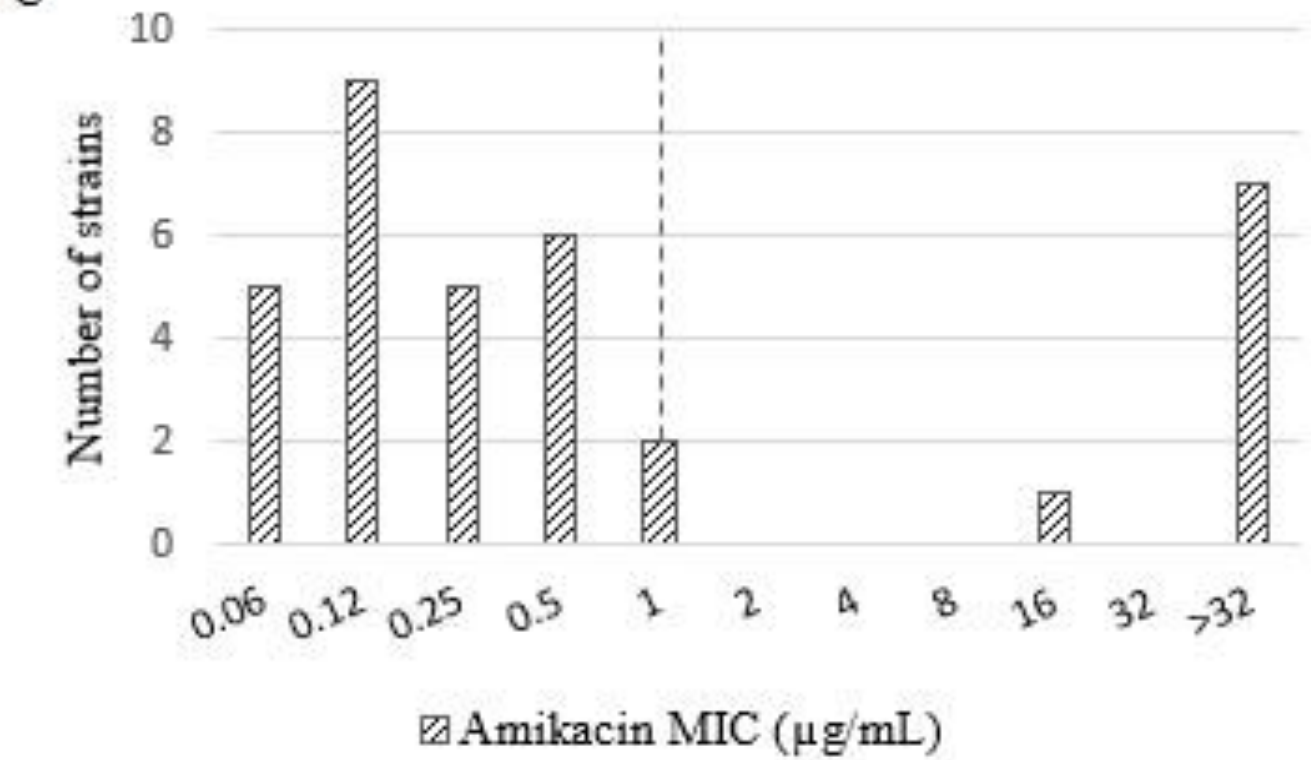
C



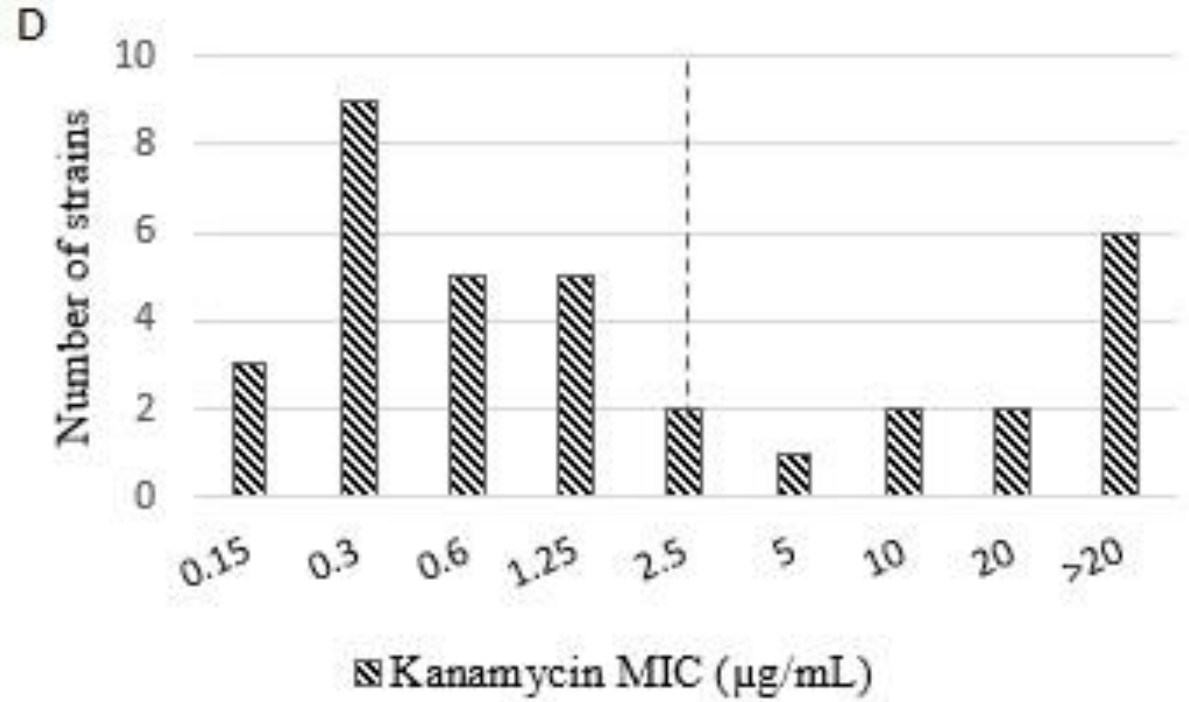

A
14
12
10
8
6
4
2
0
Number of strains
0.12
0.25
0.5
1
2
4
8
16
>16
Levofloxacin MIC (μg/mL)


B
20
15
10
5
0
Number of strains
0.03
0.06
0.12
0.25
0.5
1
2
4
>4
Moxifloxacin MIC (µg/mL)

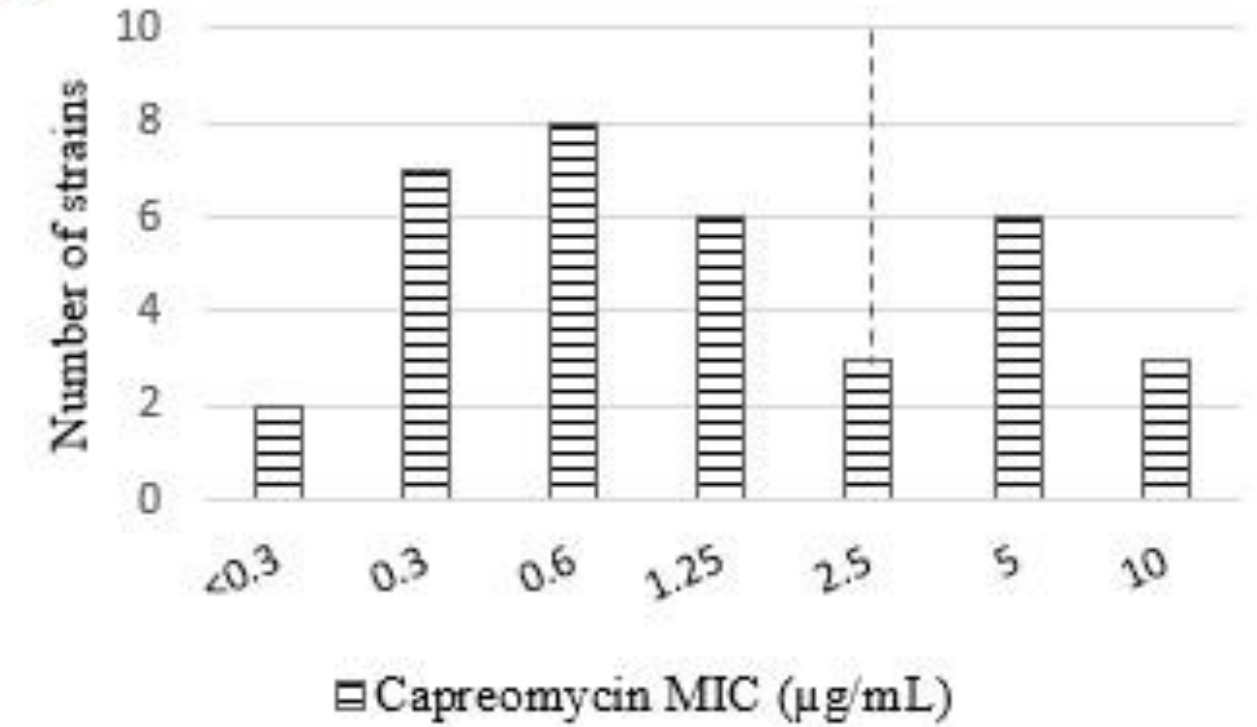
C


10
8
6
4
2
0
Number of strains
0.06
0.12
0.25
0.5
1
2
4
8
16
32
>32
Amikacin MIC (µg/mL)

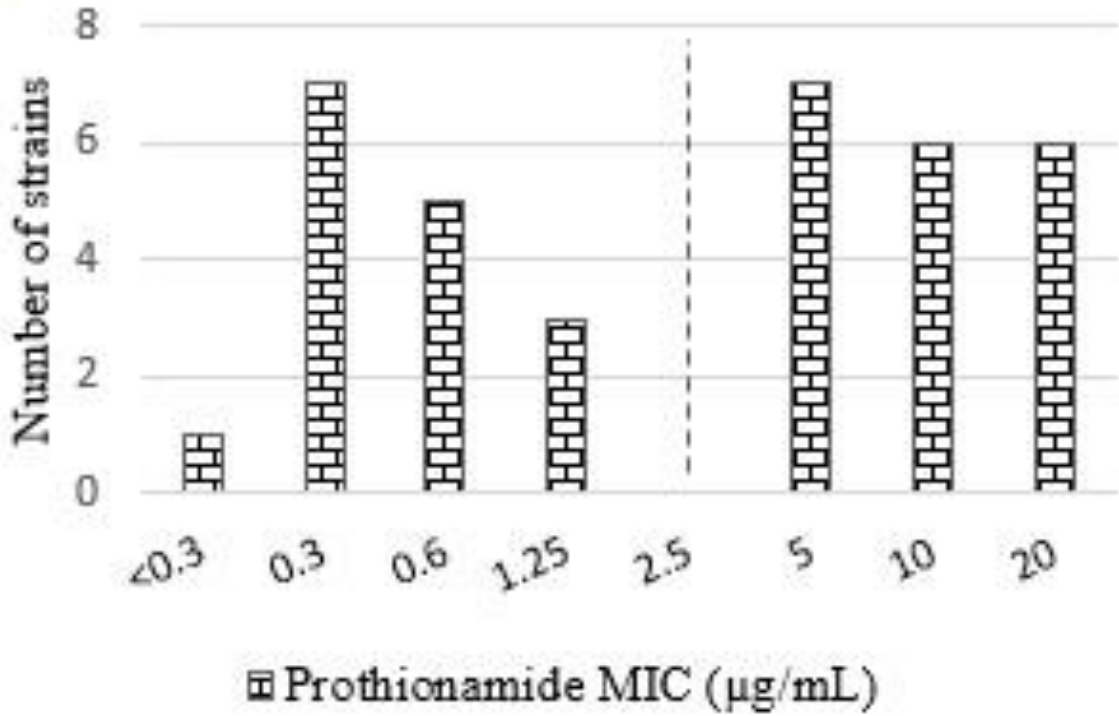
D


10
8
6
4
2
0
Number of strains
0.15
0.3
0.6
1.25
2.5
5
10
20
>20
Kanamycin MIC (μg/mL)

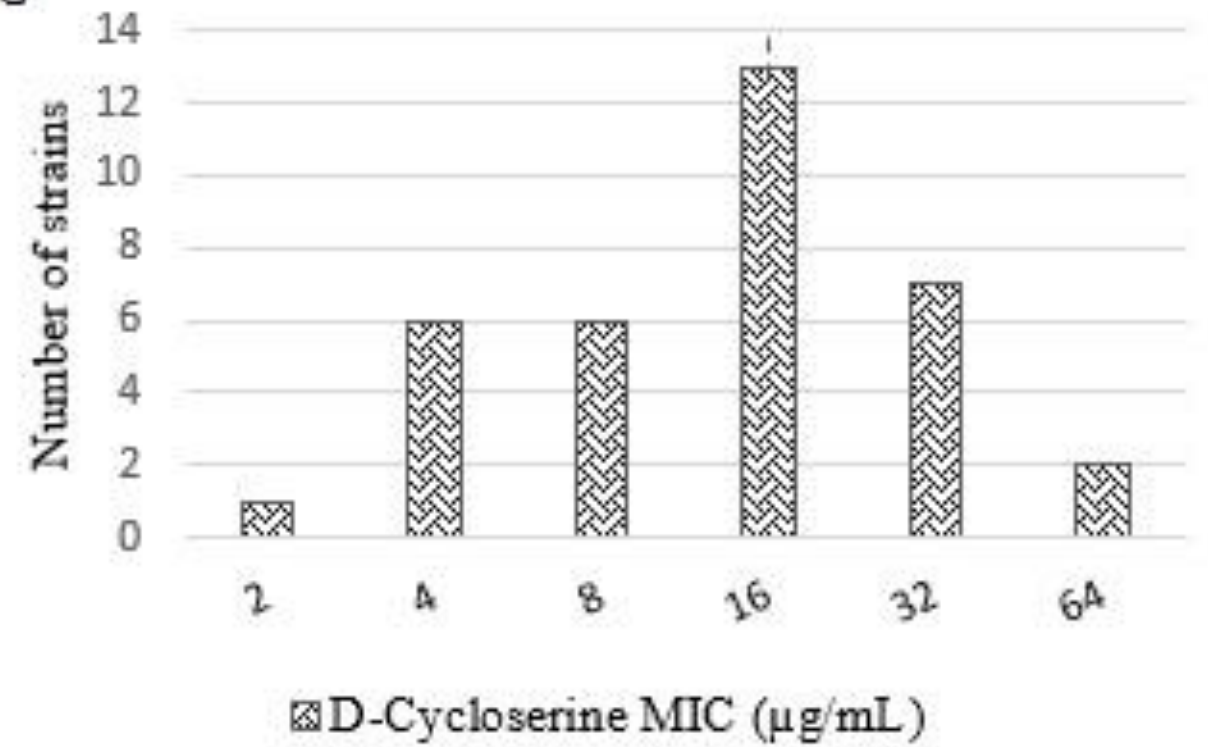
E


10
8
6
4
2
0
Number of strains
≤0.3
0.3
0.6
1.25
2.5
5
10
Capreomycin MIC (μg/mL)

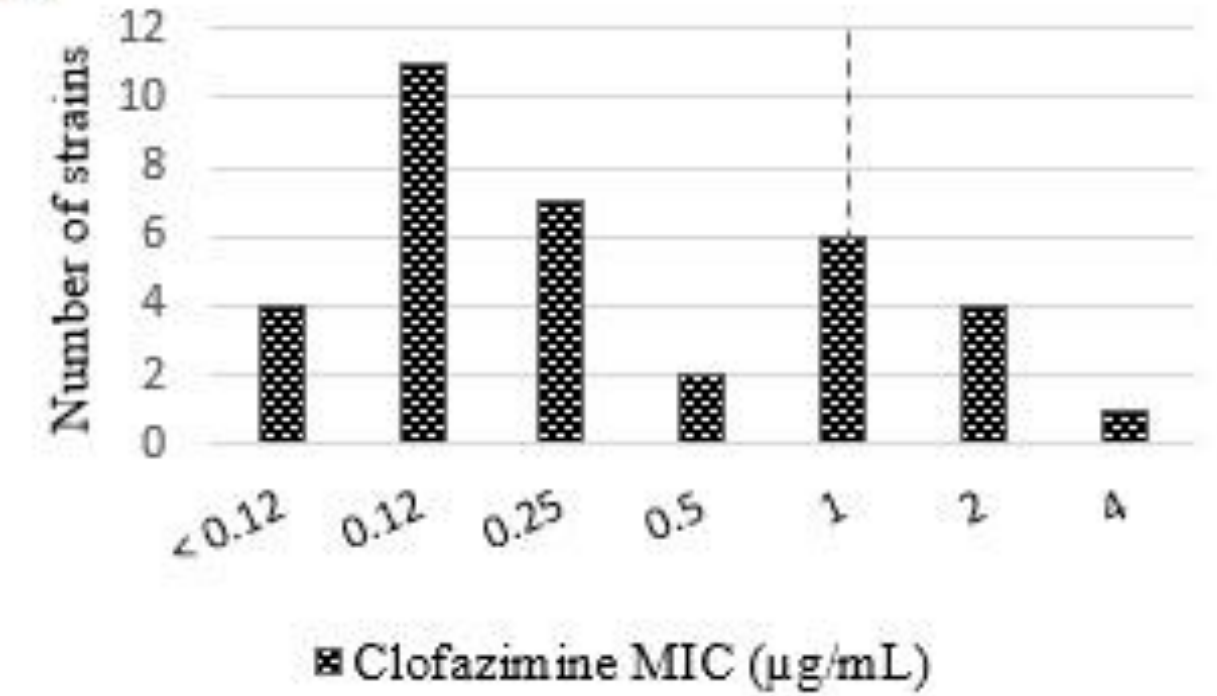
F


Number of strains
8
6
4
2
0
<0.3
0.3
0.6
1.25
2.5
5
10
20
Prothionamide MIC (µg/mL)

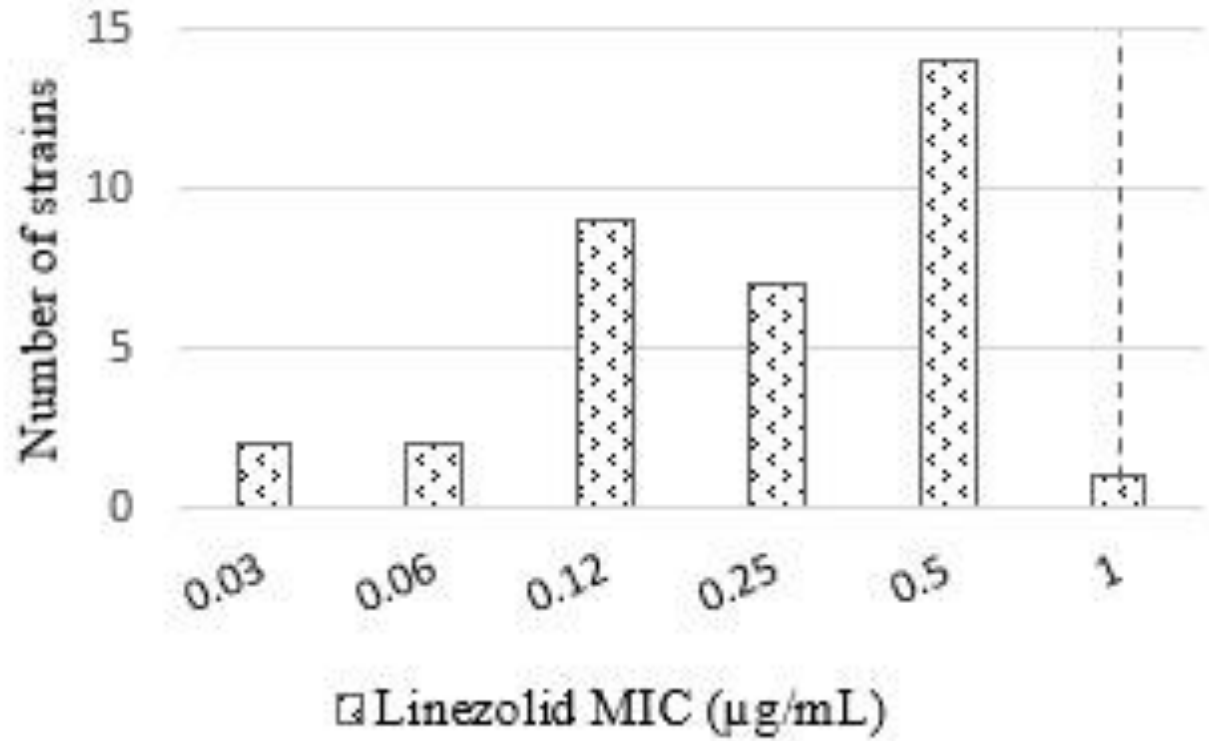
G


14
12
10
8
6
4
2
0
Number of strains
2
4
8
16
32
64
D-Cycloserine MIC (µg/mL)

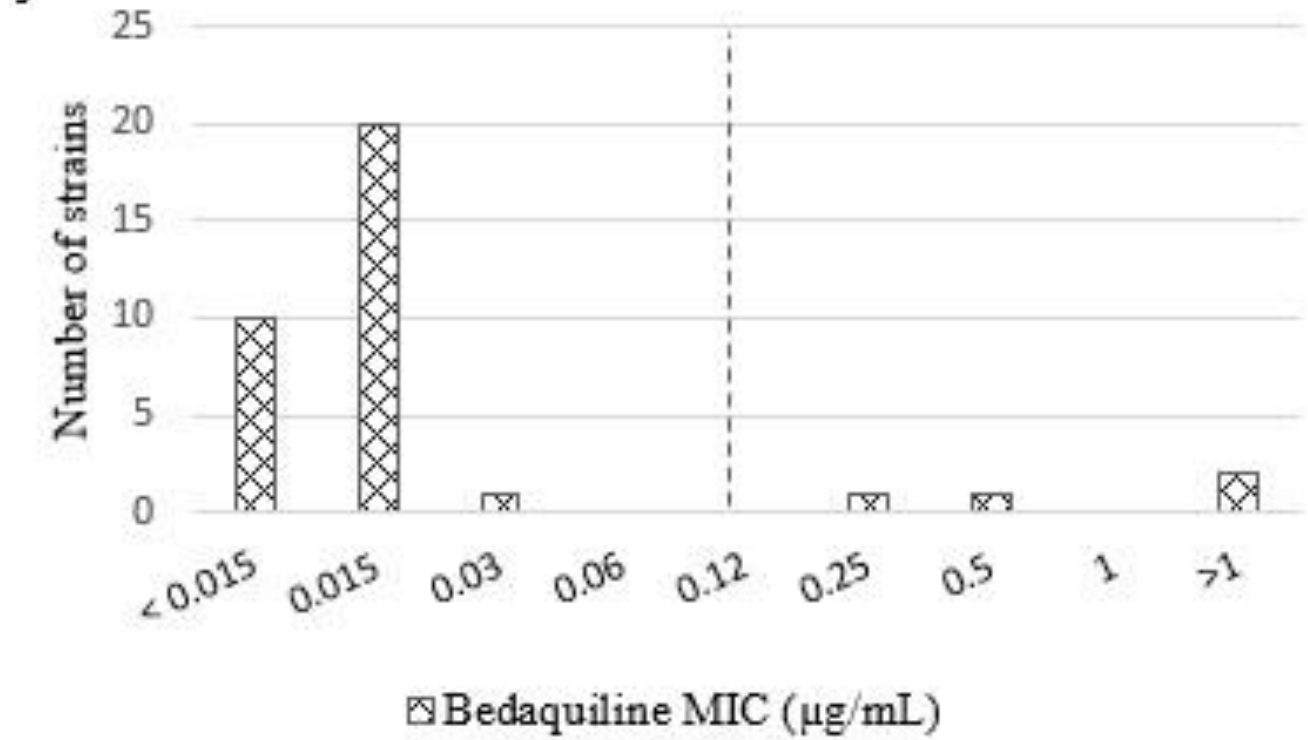
H

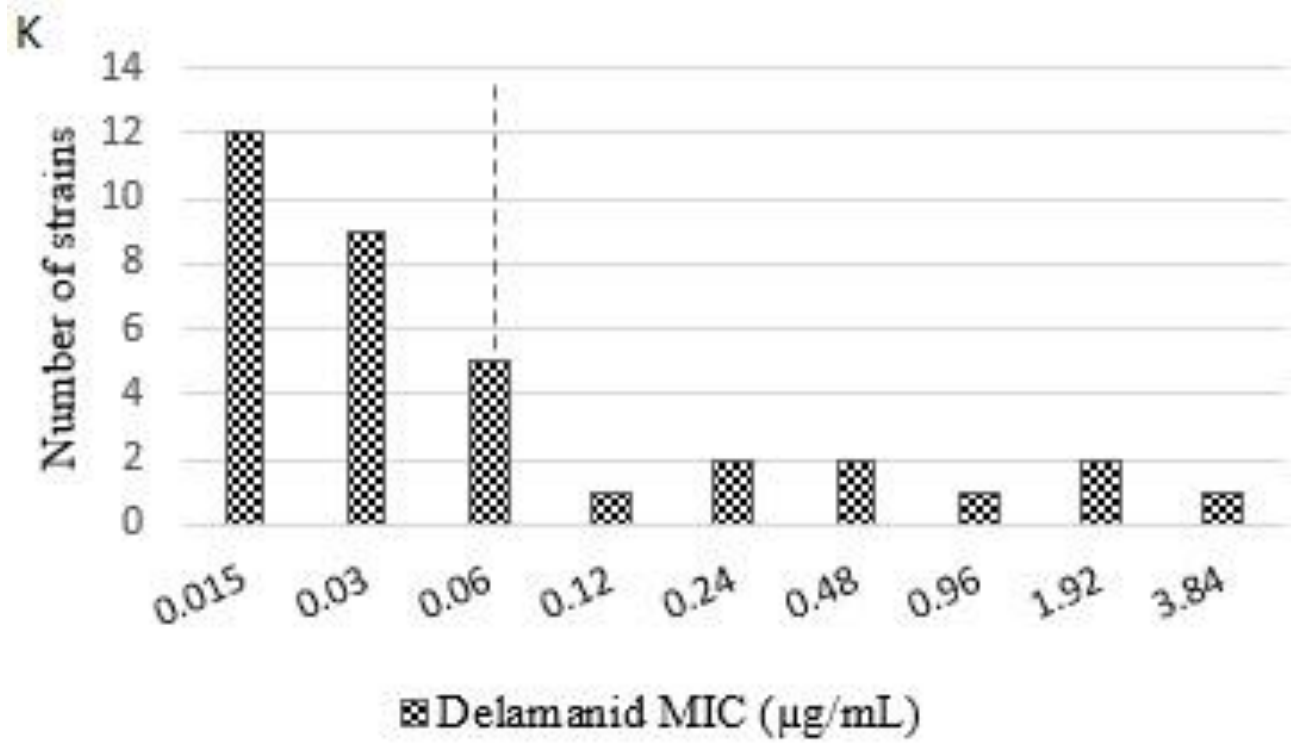

Number of strains
12
10
8
6
4
2
0
< 0.12
0.12
0.25
0.5
1
2
4
Clofazimine MIC (μg/mL)

I


15
10
5
0
Number of strains
0.03
0.06
0.12
0.25
0.5
1
Linezolid MIC (μg/mL)

J


25
20
15
10
5
0
Number of strains
< 0.015
0.015
0.03
0.06
0.12
0.25
0.5
1
>1
Bedaquiline MIC (µg/mL)


K
14
12
10
8
6
4
2
0
Number of strains
0.015
0.03
0.06
0.12
0.24
0.48
0.96
1.92
3.84
Delamanid MIC (µg/mL)

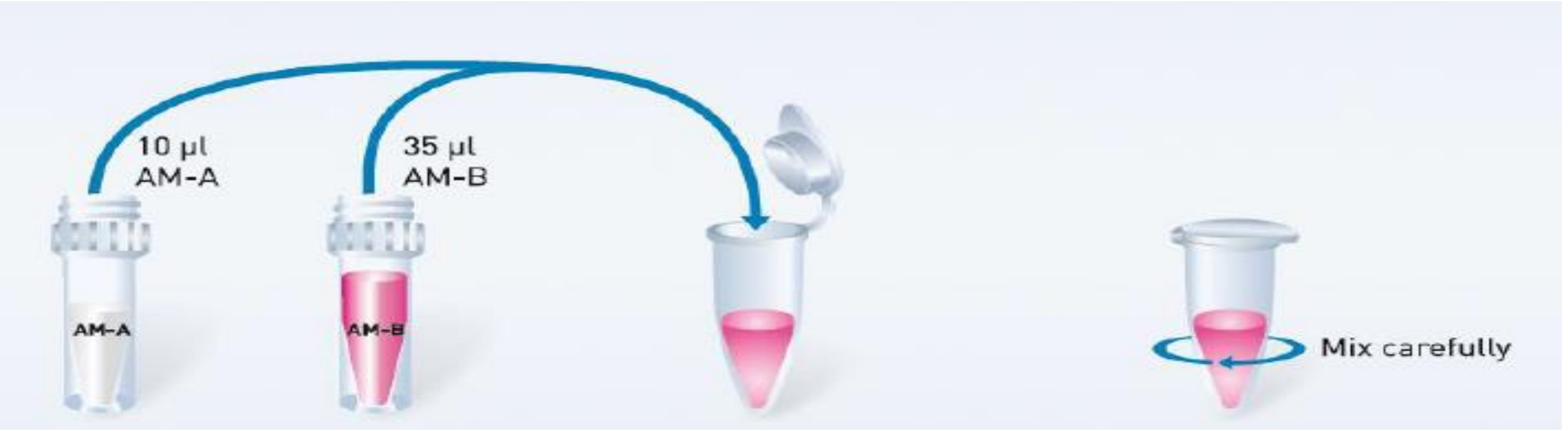
Application of LPA on MTB strains from Iran

- ✓Identification of *M. tuberculosis* complex
- ✓Resistance to Rifampicin
- ✓Resistance to Isoniazid
- ✓Resistance to Fluorquinolones
- ✓Resistance to injectable antibiotics
- ✓Pulmonary smear-positive and smear-negative specimens or cultivated samples

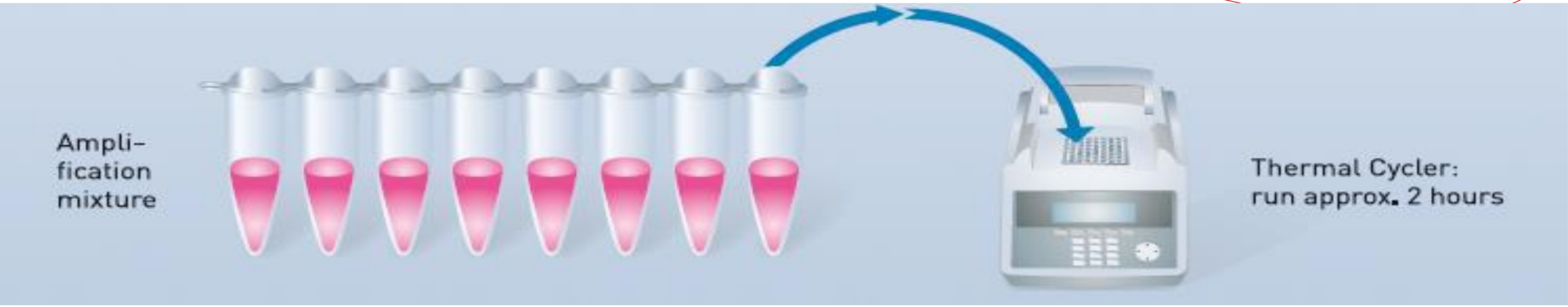
Specimen processing

- ✓Decontamination
- ✓DNA isolation

Master mixes

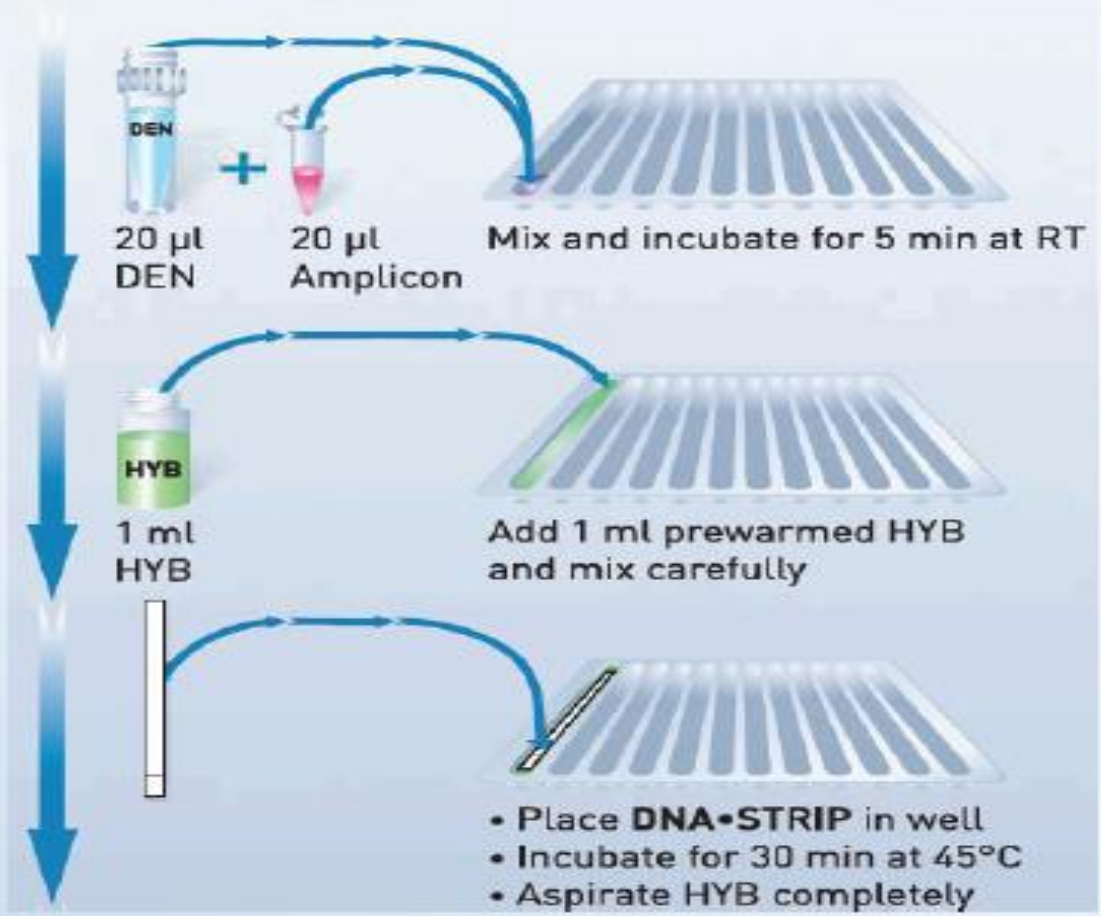


Master mixes



20 cycle	30 cycle	Final step
30 sec 95°C **2 min 65°C**	**25 sec 95°C** **40 sec 50°C** **40 sec 70°C**	8 min 70**°C**

Hybridization and detection



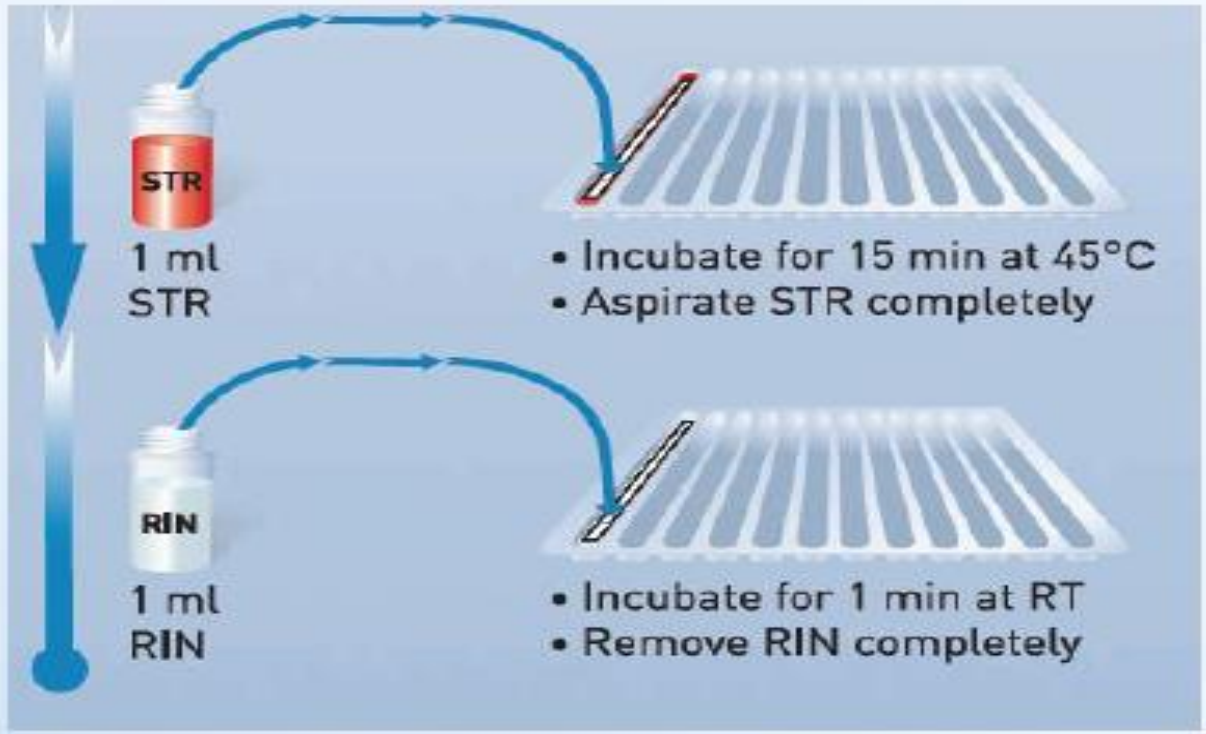
Denaturation DEN

- Separation of amplicons in single strands

Hybridization HYB

- Adding of HYB solution
- Placing of strips in wells and incubation
- Binding of labelled amplicons to probes

Hybridization and detection



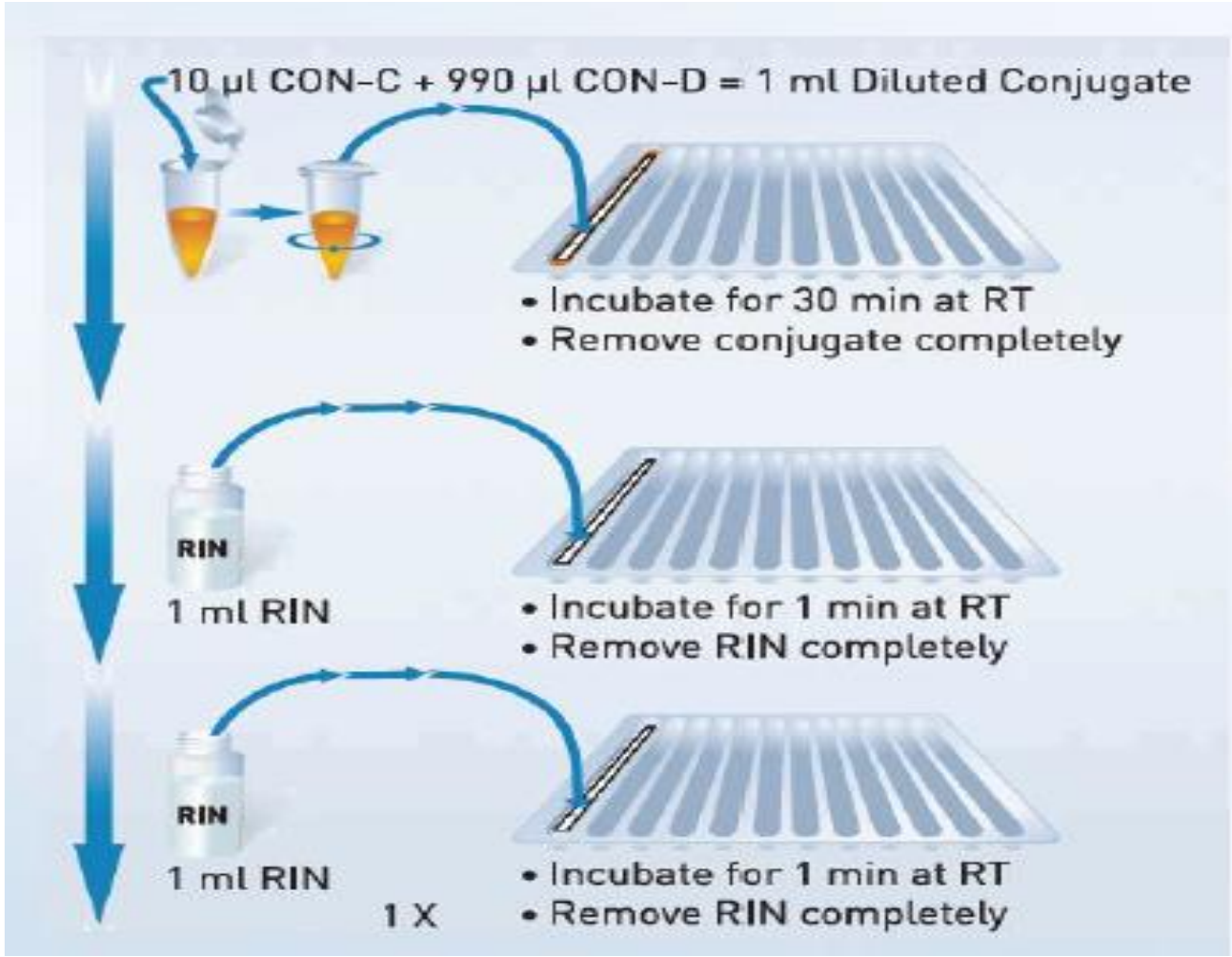
Stringent Wash STR

- Removal of unspecifically bound DNA from probes

Rinse #1 RIN

- Washing step

Hybridization and detection



Conjugate CON

- Binding of an enzyme-conjugated protein

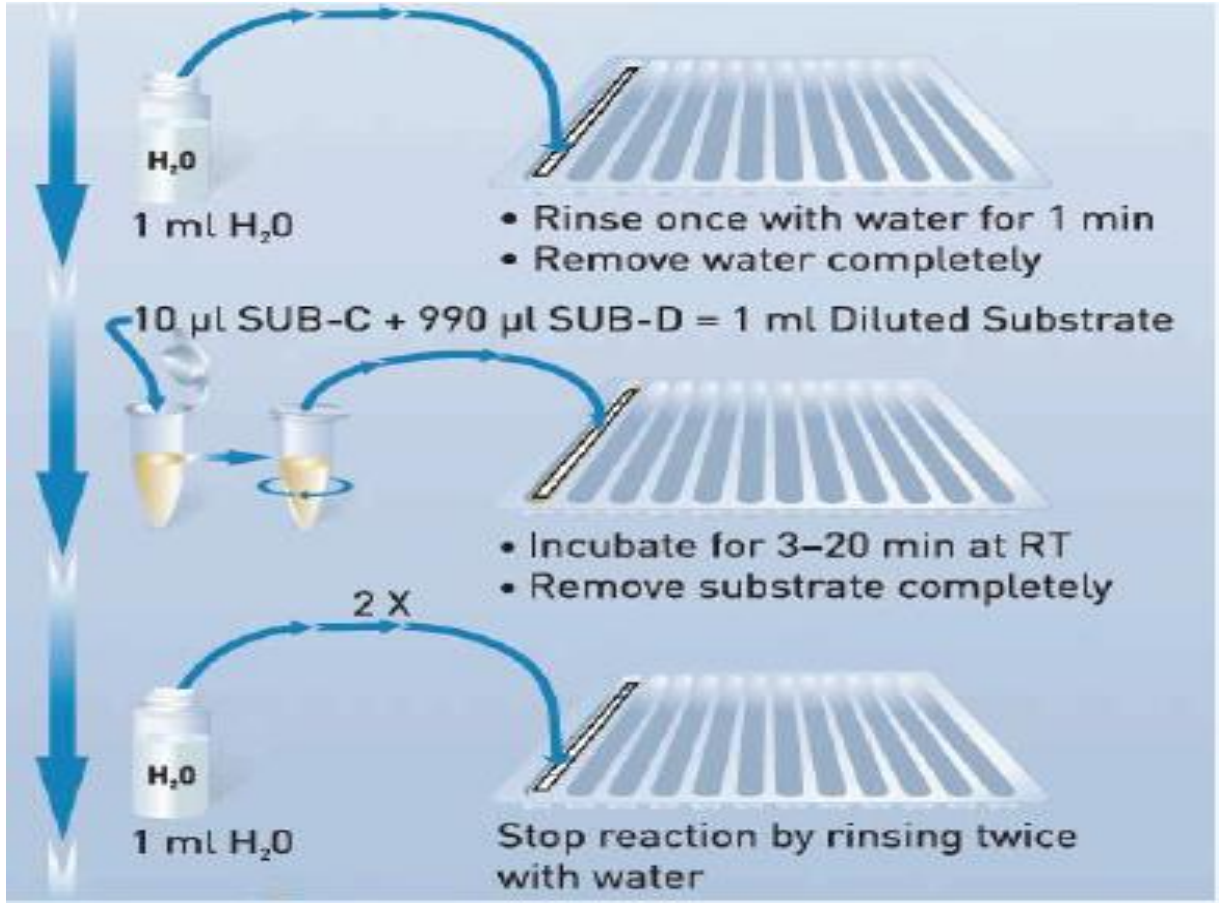
Rinse #2 RIN

- Washing step

Rinse #3 RIN

- Washing step

Hybridization and detection



Water #1 H_2O

- Washing step

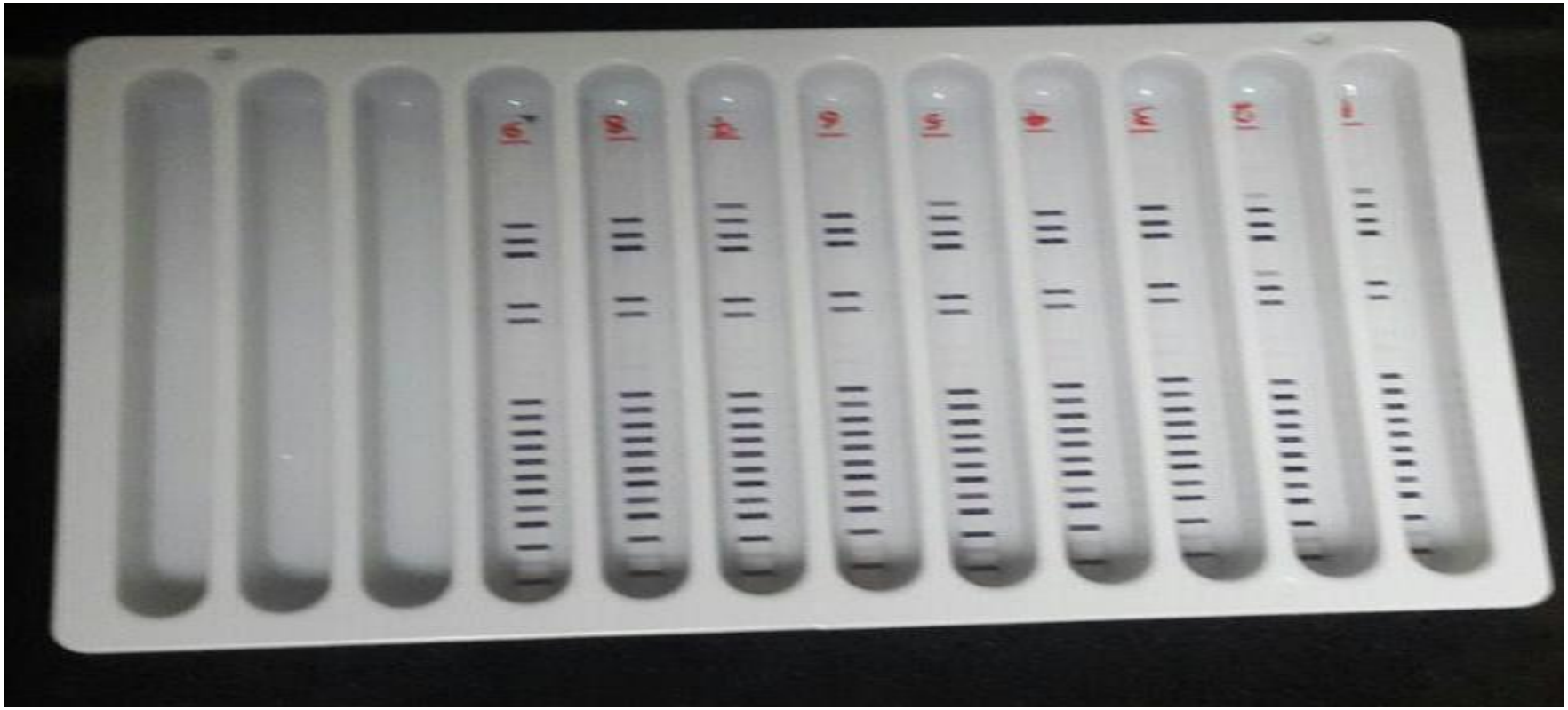
Substrate SUB

- Enzymatic conversion of dye

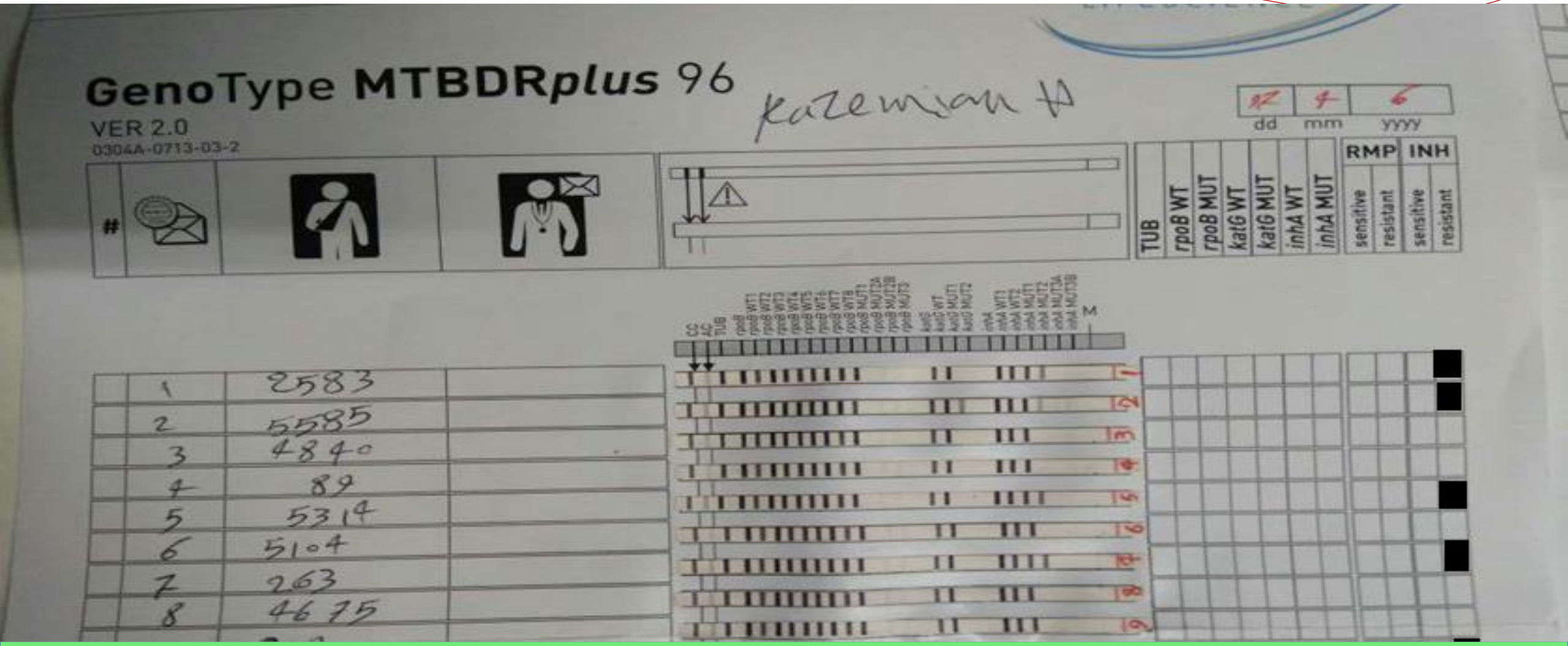
Water #2+3 H_2O

- Washing step

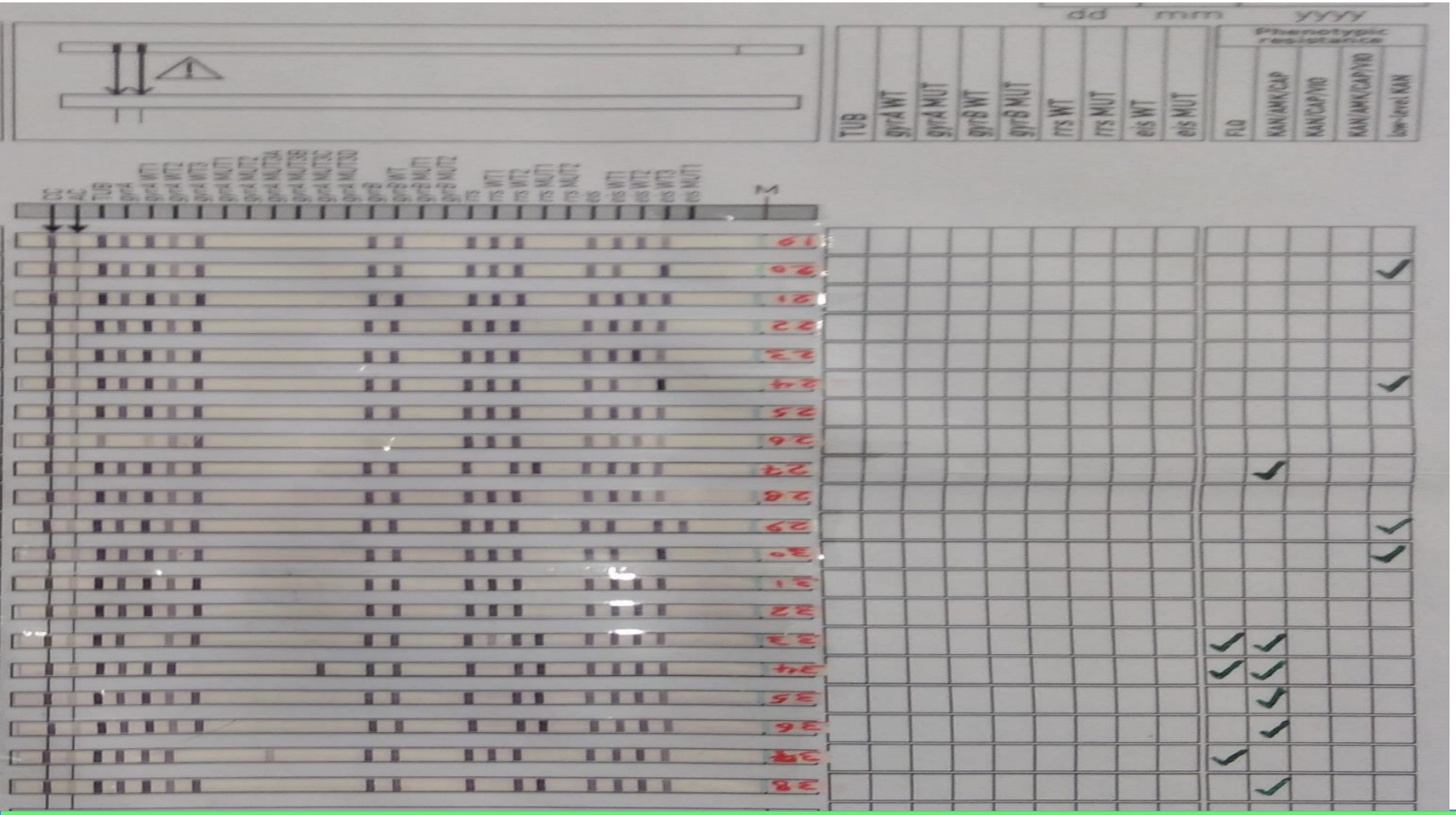
Results



Results


GenoType MTBDRplus 96
VER 2.0
0304A-0713-03-2
dd
mm
yyyy
TUB
rpoB WT
rpoB MUT
katG WT
katG MUT
inhA WT
inhA MUT
RMP
INH
sensitive
resistant
sensitive
resistant
1 2583
2 5585
3 4840
4 89
5 5314
6 5104
7 263
8 4675

Results



Low level Kanamycin resistant

Pre-XDR (Resistant to KAN, AMK, CAP)

XDR (Resistant to KAN, AMK, CAP and FLQ)

Resistant to FLQ

LPA results in Iran

- ✓Highly sensitive (>=97%) and specific (>=99%) for the detection of rifampicin resistance, alone or in combination with isoniazid (sensitivity >=90%; specificity >=99%)

Results

- ✓40 MDR isolates
  - ✓Two pre-XDR strain
  - ✓Three XDR strain
  - ✓Four low level kanamycin resistant

Advantages of LPA

- ✓Quick diagnosis
- ✓Standardized testing
- ✓Potential for high throughput
- ✓Fewer requirements for laboratory biosafety
- ✓Accurate results in compare with culture/DST/sequencing

LPA efficiency

- ✓Simultaneous identification and the detection of drug resistance
- ✓Simultaneous RIF & INH resistance detection (Superiority to GeneXpert)
- ✓Isoniazid mono-resistance or low level INH resistance detection
- ✓Occult MDR-TB detection among Isoniazid mono-resistant isolates by LPA

Conclusion

- ✓Direct testing on sputum specimens allows for the earlier initiation of appropriate treatment
- ✓For patients with confirmed rifampin-resistant TB or MDR-TB, Second Line-LPA may be used as the initial test, instead of phenotypic culture-based DST, to detect resistance to fluoroquinolones & second-line injectable drugs

WGS and Bioinformatic analysis

- Libraries were prepared from extracted genomic DNA using a modified Nextera XT kit and run on the Illumina NextSeq 500 next generation sequencing
- Sequenced reads were submitted to the NCBI sequence read archive.
- Data analysis, SNPs calling and lineage/sub-lineage classification were performed using the MTBseq pipeline, BWA, SAMtools, PICARD-tools (https://broadinstitute.github.io/picard/), and the Genome Analysis Toolkit.
- 27 genes that are known to cause resistance to anti TB drugs

Detection of Resistant Mutants by WGS

- 85.7% of RR-MTB strains were also resistant to isoniazid (MDR)
- Resistance to ethambutol (EMB) was detected in 48.6% RR/MDR strains

- Targeted genes

	Genes
Rifampicin (RIF)	*rpoB*
Isoniazid (INH)	*katG*, *inhA*, *ahpC*, *fabG1*, *furA* and their upstream regions
EMB	*embA*, *embB*

Resistance to rifampicin

- Genetic resistance to rifampicin (RIF) were found in 97.1% of resistant phenotypes
- The *rpoB* Ser450leu (Ser531leu) was the most prevalent mutation (65.7%)
- Mutations in the 81-bp rifampicin resistant determinant region (RRDR) were found in 94.4% of RR-MTB strains

- WGS
  - Sensitivity: 97.1%
  - Specificity: 100%
  - Accuracy: 97.3%

Resistance to isoniazid

- Genetic resistance to INH were found in 96.6% of resistant phenotypes
- The *katG* Ser315Thr and C-15T at *fabG1-inhA* regulatory region were dominant mutations found in 60% and 33.3% of the strains respectively

- WGS
  - Sensitivity: 96.7%
  - Specificity: 100%
  - Accuracy: 97.1%

Resistance to ethambutol

- Genetic resistance to EMB were found in 100% of resistant phenotypes
- The majority of these strains had *embB* Met306Val mutation
- However, 6 out of 18 EMB-susceptible strains showed *embAB* mutations

- WGS
  - Sensitivity: 100%
  - Specificity: 66.7%
  - Accuracy: 82.9%

Resistance to second-line drugs

- We found 22.9%, 31.4% and 25.7% rates of resistance to amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) respectively

- 17.7% fluoroquinolone (FLQ) resistance
- 54.3% prothionamide (PTO) resistance
- 11.4% bedaquiline (BDQ) resistance
- Resistance to linezolid (LZD) was not detected

14.3% clofazimine (CFZ) resistance

25.7% D-cycloserine (DCS) resistance

25.7% delamanid (DLM) resistance

Resistance to second-line drugs

- Targeted genes

	Genes
FLQ	*gyrA, gyrB*
AMK	*rrs*
KAN	*rrs, eis promotor*
CAP	*rrs, tlyA*
PTO	*ethA, inhA, fabG1, ndh*
DCS	*ald, ddlA, alr, cycA*
CFZ	*rv0678, rv1979c, pepQ*
LZD	*rrl, rplC*
BDQ	*rv0678, atpE*
DLM	*ddn, fbiA, fbiB, fbiC, fgd1,*

Resistance to fluoroquinolones

- Genetic resistance to levofloxacin (LFX)/moxifloxacin (MFX) were found in 83.3% of resistant phenotypes
- An LFX/MFX susceptible strain showed *gyrA* Asp94Ala mutation
- Quinolone-resistant determining region of the *gyrB* gene was wild type in all strains
- WGS
  - Sensitivity: 83.3%
  - Specificity: 96.5%
  - Accuracy: 94.3%

Resistance to second-line injectable drugs

- Genetic resistance to AMK, KAN, and CAP were found in 87.5%, 90.0%, 88.9%, of resistant phenotypes
- The A1401G was the frequent mutation found in the *rrs* gene
- Mutations in the *eis* promoter was correctly linked with the low-level KAN resistance (except 1 strain having C-14A substitution)

- WGS

	AMK	KAN	CAP
Sensitivity:	87.5%	90.9%	88.9%
Specificity:	100%	95.8%	92.3%
Accuracy:	97.1%	95.3%	91.4%

Resistance to prothionamide

- Genetic resistance to PTO were found in 100% of resistant phenotypes
- Five susceptible strains had different mutations in *ethA* gene
- WGS
  - Sensitivity: 100%
  - Specificity: 68.8%
  - Accuracy: 85.7%

Resistance to D-cycloserine

- Genetic resistance to DCS were found in 11.1% of resistant phenotypes
- The *cycA* Pro188Ala was the only mutation found among DCS-resistant strains
- 10 DCS-susceptible strains showed different mutations in targeted genes!

- WGS
  - Sensitivity: 11.1%
  - Specificity: 61.5%
  - Accuracy: 48.6%

Resistance to clofazimine

- Genetic resistance to CFZ were found in 20% of resistant phenotypes
- All the CFZ resistance related genes were wild type except *Rv1979c* (found in 1 strains)
- MICs of 3 strains with various substitutions were on breakpoint
- WGS
  - Sensitivity: 20%
  - Specificity: 90%
  - Accuracy: 80%

Resistance to linezolid

- All the strains were susceptible to linezolid
- Two strains having *rrl* mutations (C1537T and C1331T) and two strains with *rplC* Ala72Thr mutation were also susceptible to linezolid
- WGS
  - Sensitivity: -
  - Specificity: 88.6%
  - Accuracy: 88.6%

Extensively drug resistant tuberculosis

- Three strains identified as extensively drug resistant (XDR)
- We found 11 pre-XDR strains:
  - 3 FLQ-resistant
  - 5 AMK/KAN/CAP resistant
  - 2 KAN mono-resistant
  - 1 KAN/CAP resistant
- All the pre-XDR/XDR strains were susceptible to LZD, BDQ, and DLM except three pre-XDR strains

Conclusion

- Precise detection of resistance level using the broth microdilution compared to the proportion method
- 8.6% XDR and 31.4% pre-XDR among the RR/MDR strains
- 11.4% and 25.7% of strains were resistnt to BDQ and DLM without exposure to these drugs

Conclusion

- Mutations in drug resistance related genes were mostly linked to the MICs results especially for key anti-TB drug

- The RR/MDR/preXDR/XDR strains can be identified with high confidence using WGS

Discussion

- The lowest sensitivity of WGS for detecting resistance to BDQ (0.0%), DCS (11.1%) and CFZ (20.0%) followed by DLM (44.4%)
- Detecting resistance using known genes for DCS, CFZ, BDQ, LZD, DLM are most controversial
- New candidate genes for drug resistance (especially newer agents) and role of other resistance mechanisms as efflux pumps

Thanks

For

your attention