Clinical Utility of target amplicon sequencing for rapid diagnosis of drug resistant *Mycobacterium tuberculosis*

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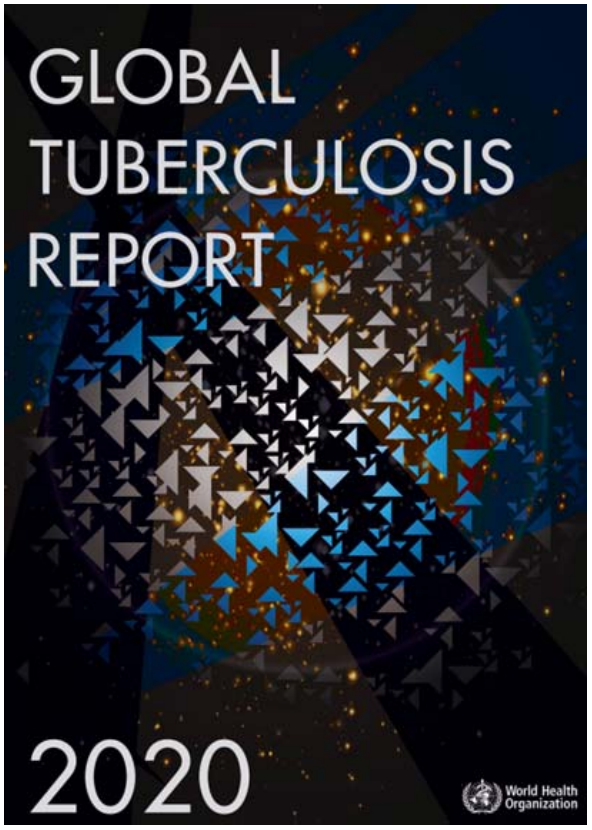
Tuberculosis (TB)

Mycobacterium tuberculosis (Mtb)

- Obligate aerobic acid-fast bacilli (AFB)
- Spread from person to person by aerosols
- Re-emerging problem in industrialized countries
- Infections among immuno-compromised patients
- Multi-drug resistant strains (MDR-TB)
- Pulmonary tuberculosis

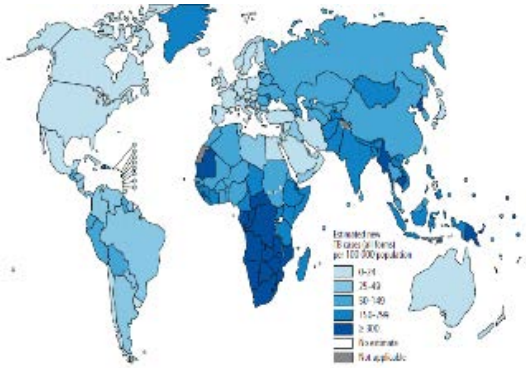
The Global Tuberculosis (結核) Situation

WHO Tuberculois Report 2020



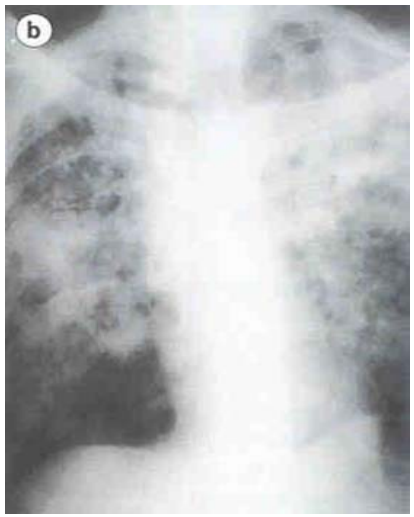
Summary

- Estimated 10.4 million new cases of TB
- 1.4 million people died from TB
- Hong Kong ~ 3,600 new cases / year
- Top five high TB burden countries:
  - India
  - China
  - Nigeria
  - Indonesia
  - Pakistan

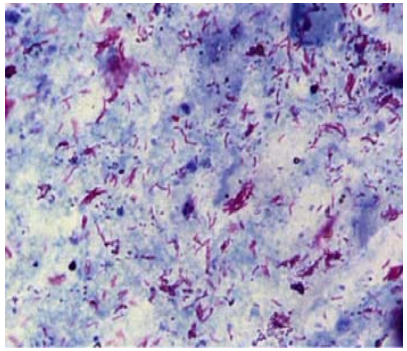


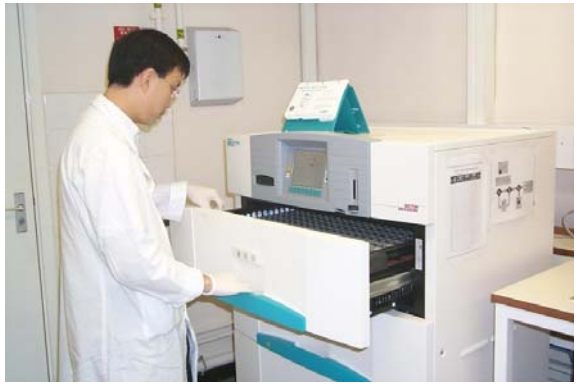
Diagnosis of Pulmonary Tuberculosis

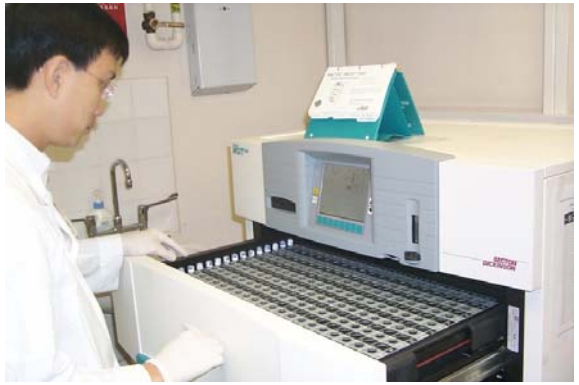
- Chest X-ray
- Direct smear for AFB in sputum
  - Turnaround Time < 2hr
  - Low sensitivity (<50%)
- Sputum culture for *M. tuberculosis* (very slow)
  - 2~4 week (Solid LJ medium); 1-3 week (Liquid MGIT broth)
  - 1~2 week (identification)
  - High sensitivity (gold standard)



Conventional Laboratory Diagnosis for Tuberculosis





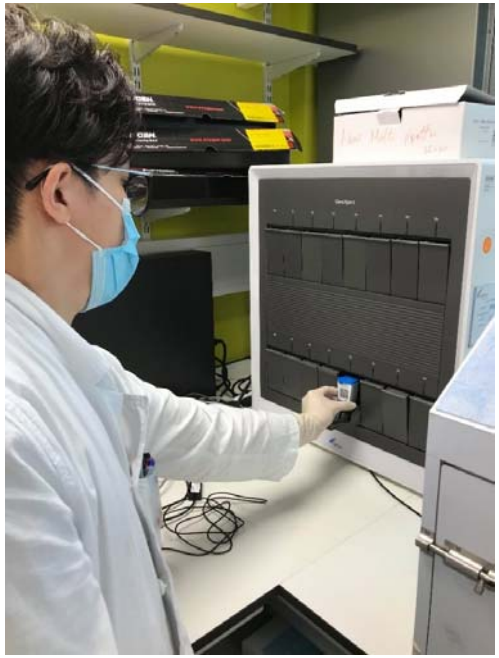


Molecular Diagnosis for Tuberculosis

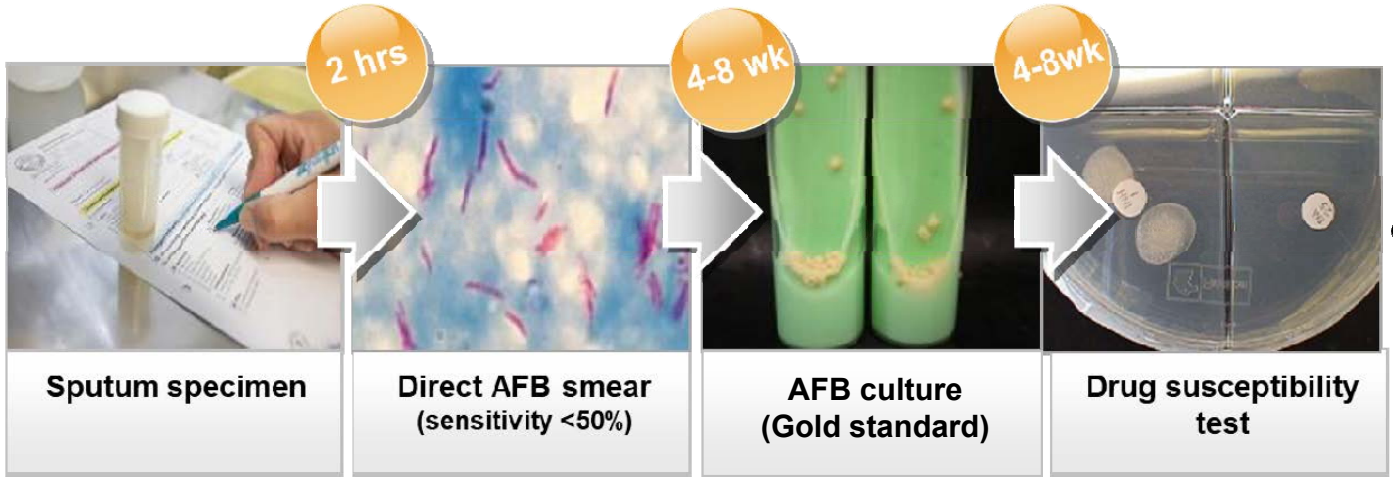
DNA Amplification assay (eg PCR):

- Rapid diagnosis
- High sensitivity
- High specificity
- Simple setup (Commercial systems)

Cepheid (GeneXpert)



Abbott (m2000TB)



Conventional culture-based method (2-3 months)

Full Report

DNA extract → qPCR for Mtb → **3 – 5 hrs** → PCR+ → Mtb DNA detected

Sensitivity: ~91 – 97%
Specificity: ~98 – 99%

Molecular Diagnosis

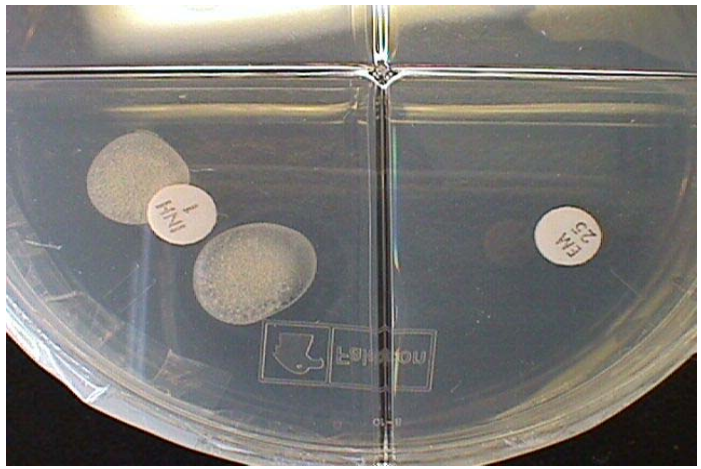
Multidrug Therapy for Tuberculosis

3 - 9 months treatment of 2-3 primary drugs:

- Rifampicin
- Isoniazid (INH)
- Streptomycin
- Ethambutol
- Pyrazinamide

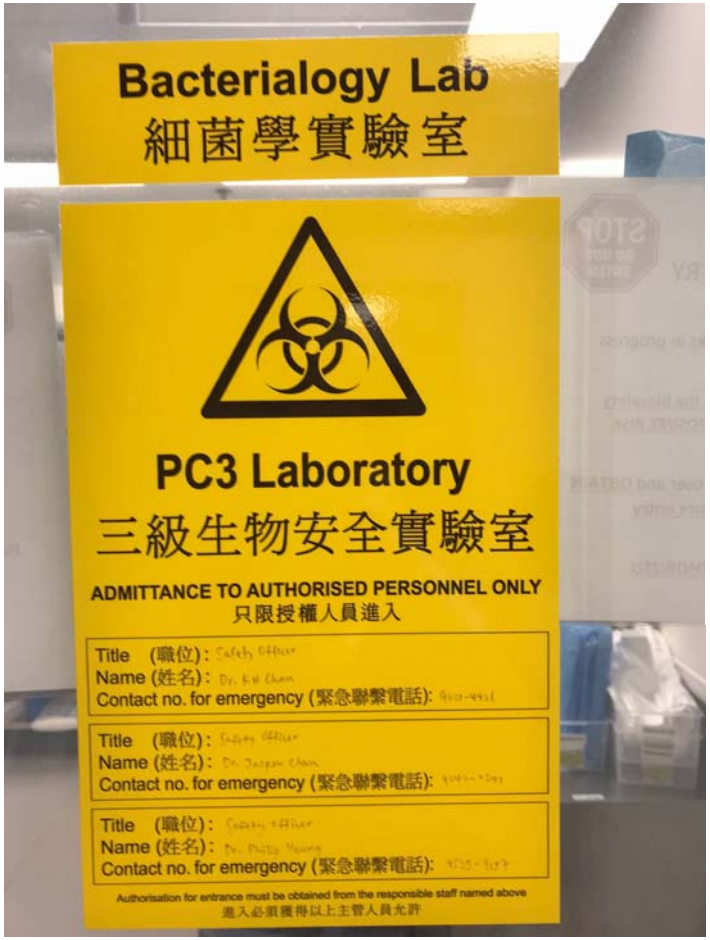
Drug Susceptibility Testing (DST) for *Mycobacterium tuberculosis*

Agar Proportion Method



MGIT960

P3 Laboratory in QMH, HKU

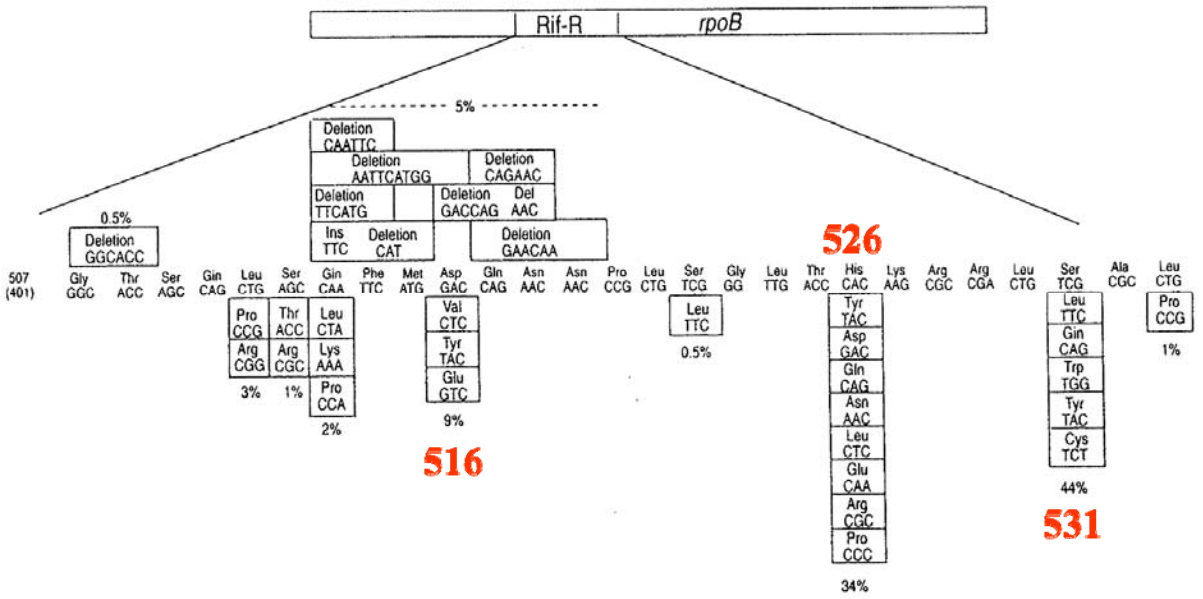


Rifampicin (1)

- Resistance caused by the mutation in *rpoB* gene (Rifampicin resistance-determining region - RRDR)

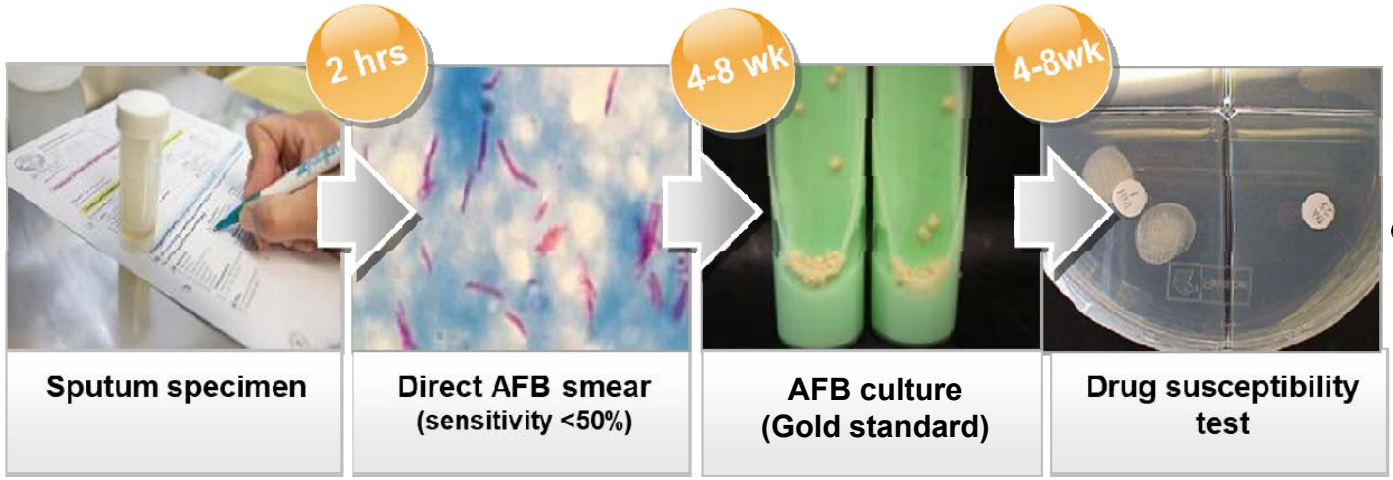
RNA Polymerase β Subunit Gene

157bp (RRDR)



Rifampicin (2)

- An effective anti-tuberculosis agent
- A surrogate marker of Multidrug-resistant tuberculosis (MDR-TB)
- Rapid detection is important for the treatment and control of tuberculosis
- Drug resistance detection by qPCR in commercial systems:
  - GeneXpert - Rifampicin [Molecular Beacons Probes]
  - Abbott m2000TB - Rifampicin and Isoniazid (INH) [Taqman Probes]



Conventional culture-based method (2-3 months)

Full Report

DNA extract → qPCR for Mtb → **3 – 5 hrs** → PCR+

Sensitivity: ~91 – 97%
Specificity: ~98 – 99%

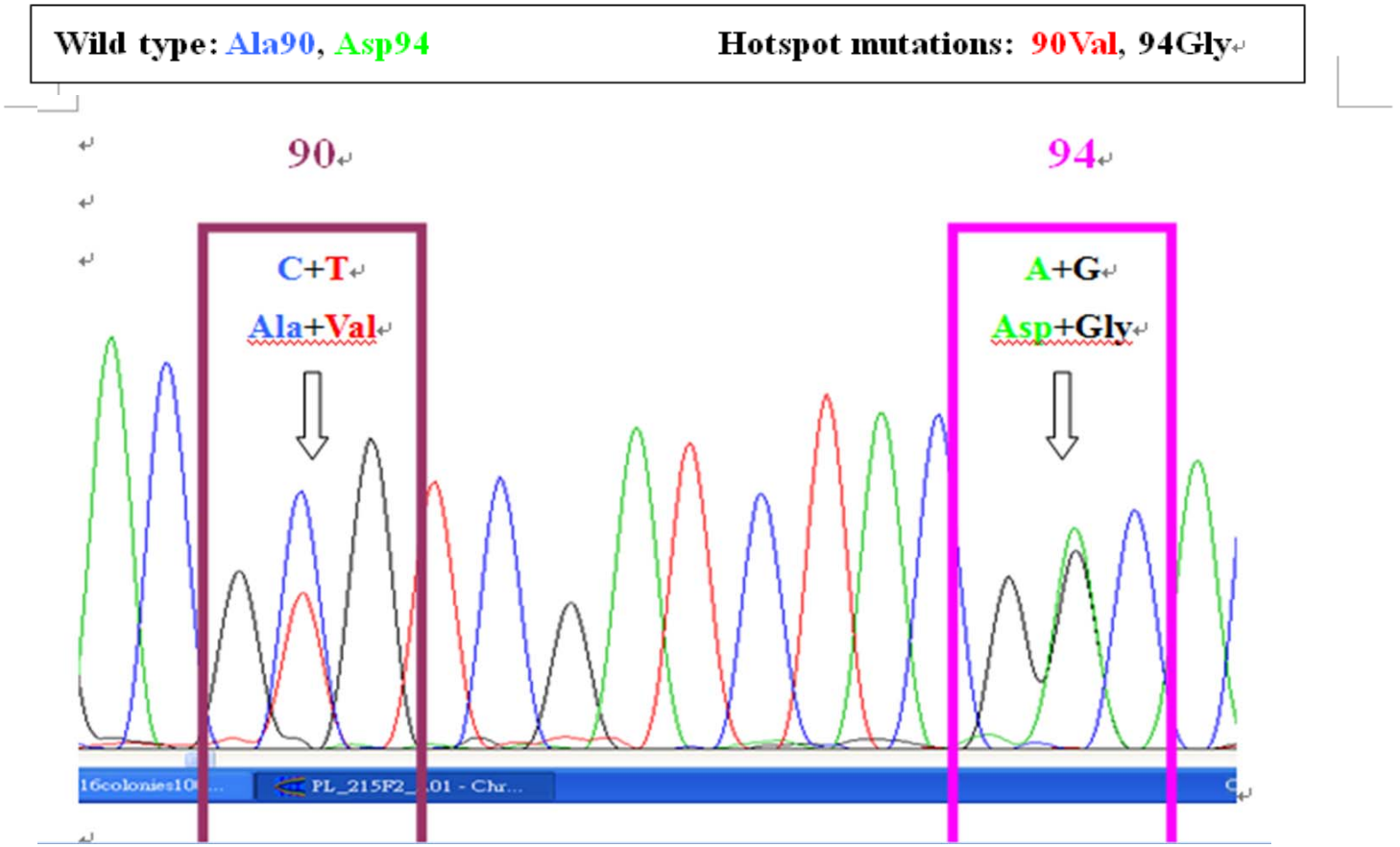
Mtb DNA detected:
- Rifampicin / INH resistance mutations detected **OR** not detected * *

* * Retrospective Analysis of False-positive and Disputed Rifampin Resistance Xpert MTB/RIF Assay Results in Clinical Samples from a Referral Hospital in Hunan, China. *J Clin Microbiol. 2019 Apr; 57(4)*

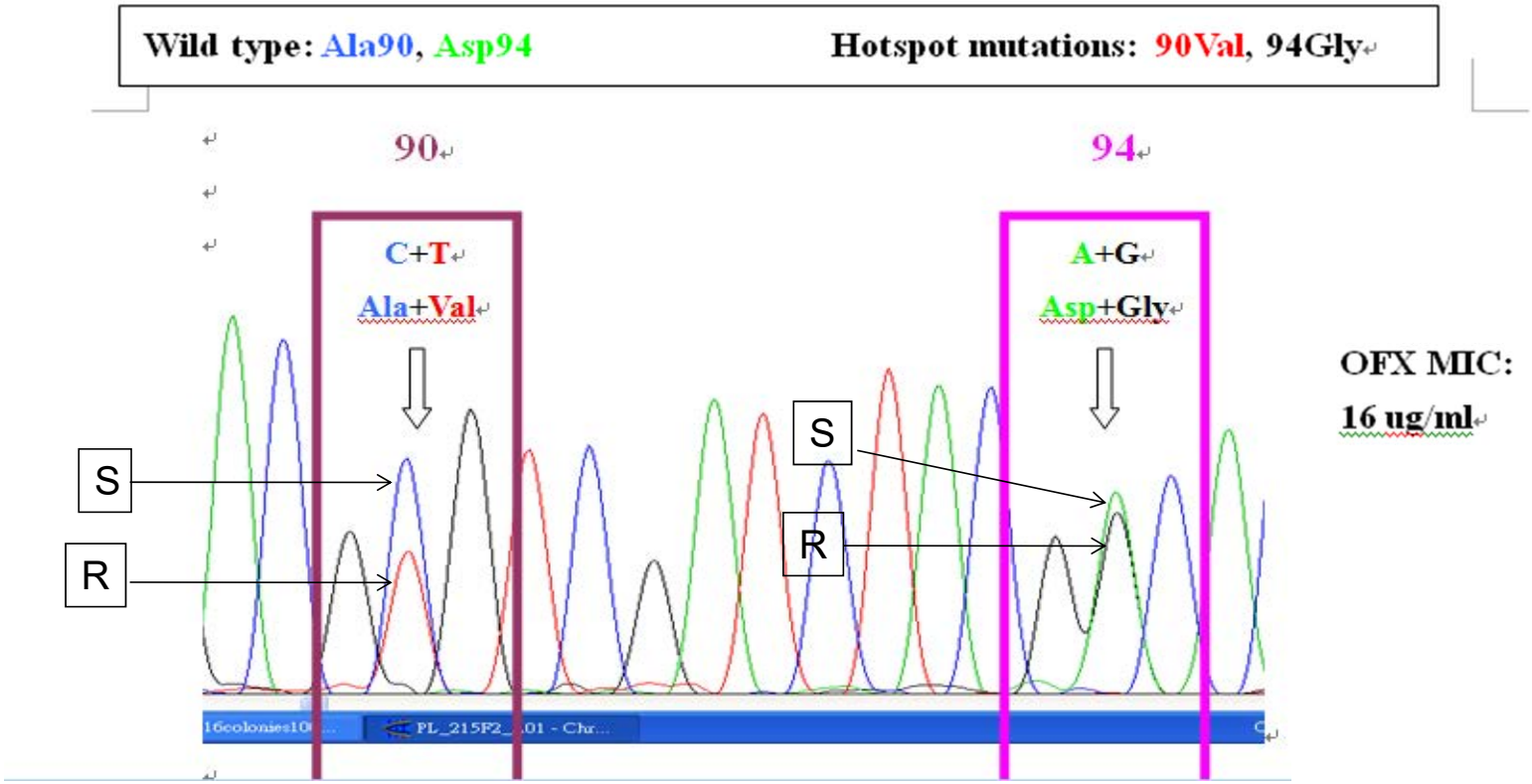
Fluoroquinolones

- Resistance caused by the mutation in *gyr* gene
- DNA gyrase (Quinolone resistance-determining region - QRDR) - Ofloxacin
- Most gyrase A missense mutations were found at positions 90, 91, and 94 that were located within QRDR.
  - Significant increase in MIC (>4.8μg/ml).

Sanger Sequencing of *gyrA* gene Mtb in Sputum



Culture confirmation : Mtb - INH^R; Rif^R; OFX^R



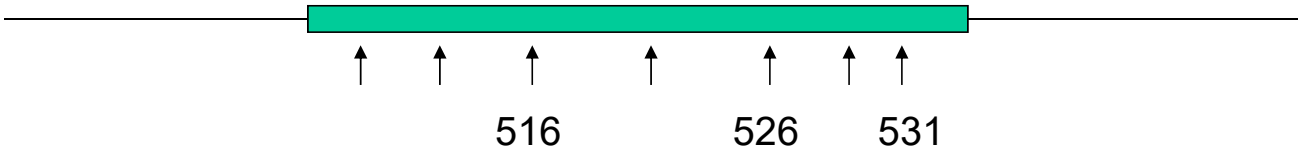
Extensively Drug Resistant Tuberculosis (XDR-TB)

- pre-XDR-TB is: MDR-TB strains which are also resistant to any fluoroquinolone.

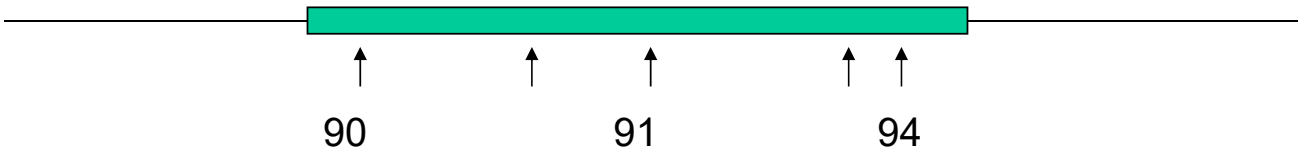
- XDR-TB is: MDR-TB strains which are also resistant to any fluoroquinolone and at least one additional Group A drug (Group A drugs are the most potent group of drugs in the ranking of second-line medicines for the treatment of drug-resistant forms of TB using longer treatment regimens and comprise levofloxacin, moxifloxacin, bedaquiline and linezolid).

Known mutations associated with Rifampin and Ofloxacin resistance

rpoB (hot point mutations)



gyrA (hot point mutations)



Novel mutations associated with Rifampin, Ofloxacin and Pyrazinamide resistance

Rifampin MIC = 16 - 64 ug/ml

rpoB (hot point mutations)

***Gilman Siu** et al J Antimicrob Chemother 2011 66(4)*

Ofloxacin MIC = 8 ug/ml

gyrA (hot point mutations)

***Ricky Lau** et al, Antimicrob Agents Chemother 2011 55(2)*

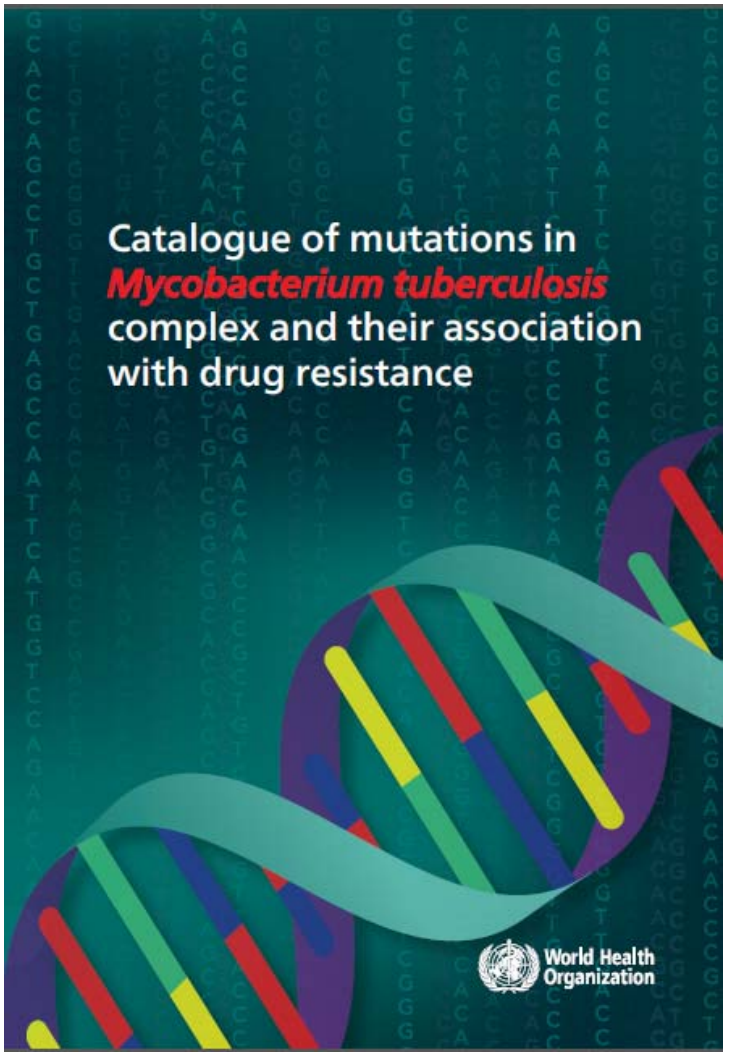
Pyrazinamide

Direct Detection of Pyrazinamide Resistance in Mycobacterium tuberculosis by Use of ***pncA*** PCR Sequencing.

Kingsley Tam et al, *J Clin Microbiol 2019 57.*

Submission of **novel** drug resistance mutations to World Health Organization (WHO)

2021



DNA Sanger Sequencing:

- Provides more reliable results to resolve False Resistant/ Susceptible data of commercial diagnostic kits
- Tedious and time consuming (long turnaround time) unsuitable for routine diagnostic application
- Requires higher bacterial load in the sputum for DNA sequencing most drug resistance mutations of common anti-tuberculosis drugs.

Next Generation Sequencing (NGS) for Infectious Diseases

- Full Genome Sequencing for epidemiological tracing:
  - SARS-CoV-2 causing COVID-19 pandemic
  - EHEC O157:H7 causing food poisoning
- Target Amplicon Sequencing:
  - Detection of gene mutations associated with microbial drug resistance
  - A total 163 drug resistant isolates (HK: 93 + Africa: 70) were tested in parallel with Phenotypic Drug Susceptibility Test (pDST)

Table 1. Gene targets for target amplicon sequencing

Drug	Gene	Amplicon Size (bp)	Major Mutation covered*
Rifampicin	*rpoB*-RRDR[1]	288	*rpoB* RRDR
	rpoB-full[1]	1,311	*rpoB* RRDR, V146F, I572F
Isonizid	*katG*	435	*katG* S315T
	inhA promoter	454	*inhA* C-15T
	inhA structural	922	*inhA* codon 94 and 95
	katG-furA intergenic region	892	*furA* codon 4, -134bp upstream deletion
Ethambutol	*embB*	955	*embB* M306V/I, G406A/D/S
	ubiA	1,119	Compensatory mutation
Pyrazinamide	*pncA*	813	Entire gene
	rpsA	1601	Entire gene
Fluoroquinolones	*gyrA*	751	90-94 QRDR
	gyrB	1054	N538D/E540V
Aminoglycosides	*eis* promoter	593	C-14T,C-12T, G-10A, G-10C
	rpsL	472	K43R/K88Q
	rrs	1,211	C1400,A1401/C1483
Capreomycin	*tylA*	945	R3*; Q22* Lost of tlyA expression
Linezolid	*rplC*	710	G2061T/G2576T
	rrl	1,102	T460C

[1] Two sets of primers were designed for *rpoB* to ensure a better coverage at 81bp RIF resistance determining regions

Target Amplicon Sequencing for *Mycobacterium tuberculosis* isolates

Table 1. Phenotypic drug resistance profiles of 163 *M. tuberculosis* clinical isolates.

Drug	Total number of isolates	pDST tested isolates	Number of resistant isolates (%)	Drug resistance pattens (number, %)	
INH	163	163	87 (53.4)	MDR (60, 36.8%)	XDR (10, 10.8%)[c]
RIF	163	163	65 (40.0)		
EMB	163	163	37 (22.7)	MDR + EMB/STR (54, 33.1%)	
STR	163	163	72 (44.2)		
PZA	163	93[a]	44 (47.3)	MDR +PZA (40, 43.0%)[c]	
KAN	163	93[a]	32 (34.4)	MDR +SLIDs (30, 32.3%)[c]	
AMK	163	93[a]	31 (33.3)		
CAP	163	90[a]	28 (31.1)		
OFX	163	93[a]	25 (26.9)	MDR + FQs (20, 21.5%)[c]	
MOX	163	92[a]	23 (25.0)		
BQ	163	0[b]	0		
LZ	163	0[b]	0		

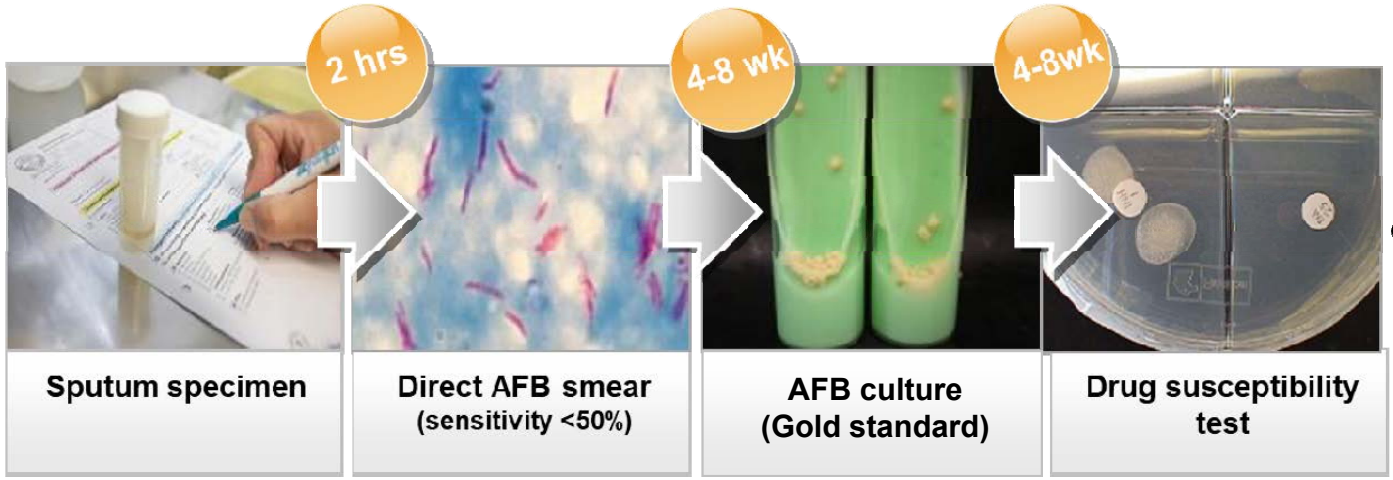
INH, Isoniazid; RIF, Rifampicin; EMB, Ethambutol; PZA, Pyrazinamide; STR, Streptomycin; KAN, Kanamycin; AMK, Amikacin; CAP, Capreomycin; OFX, Ofloxacin; MOX, Moxifloxacin; BD, Bedaquiline; LZ, Linezolid; MDR, multidrug resistance; XDR, extensive drug resistance; FQ, fluoroquinolones; SLIDs, second-line injectable drugs.

[a]Based on routine practice in the Asella Hospital in Ethiopia, pDST for *M. tuberculosis* clinical isolates included only INH, RIF, EMB and STR. Therefore, the pDST results for PZA, KAN, AMK, CAP, OFX, and MOX were not available for *M. tuberculosis* strains isolated from Ethiopia.

[b]BQ and LZ pDST results were not available in this study because of unavailability of the respective drugs in our regions.

[c]The percentages were calculated based on the total number of M. tuberculosis isolates subjected to pDST for PZA, KAN, AMK, CAP, OFX, and MOX.

Gilman Siu et al Clinical Chemistry 66:6 809-820 (2020)

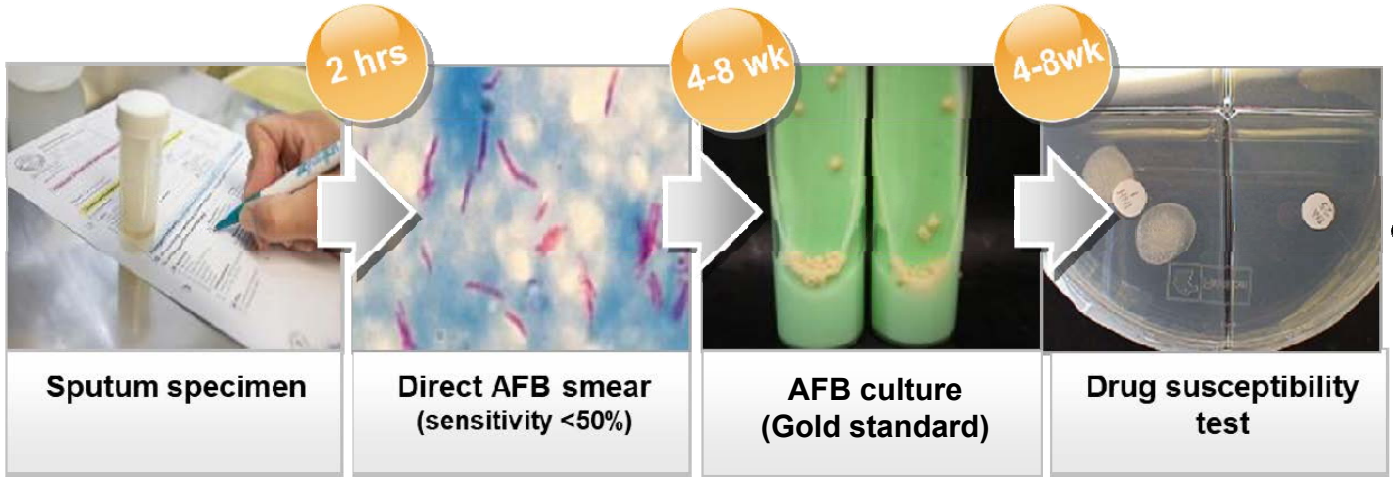


Conventional culture-based method (2-3 months)

Full Report

(6 - 8 working days)

Next Generation Sequencing



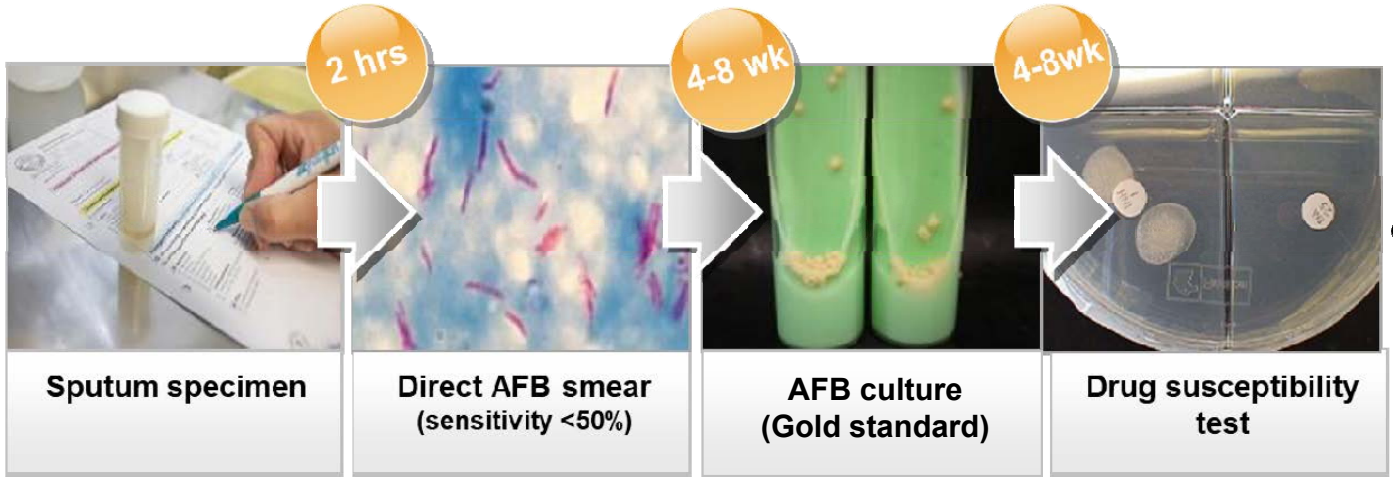
Next Generation Sequencing

Joint Study (>4,000 specimens)

- HKU/QMH/Chest Clinics
- KingMed Diagnostics, Guangzhou, China

NGS setup (CE-IVD)

- Illumina® Miseq
- Bacteriochek™ (Advanced Biological Laboratories S.A., Luxembourg)



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HK / Guangzhou study

DNA extract → qPCR for Mtb → **3 – 5 hrs** → PCR+

4,047 sputum

503 PCR+

~ 6 days

Report

Mtb DNA detected;
Drug resistance mutations to Rif, INH ….etc detected

Phenotypic DST profiles of MTBC culture positive strains from Hong Kong and Guangzhou by phenotypic DST in this study

Specimen type	Resistance pattern defined by TB-NGS	No. of specimens	Phenotypic drug susceptibility patterns									
			culture (+)	RIF	INH	EMB	PZA	STR	FLQ	CAP	AMI	LZD
Genotypic drug resistance defined by MTBDR assay (n=24)	Drug resistant TB (n=14)	2	1	R	S	S	S	S	S	S	S	S
		1	1	R	S	S	S	R	S	S	S	S
		5	2	S	R	S	S	S	S	S	S	S
		1	1	S	R	R	S	S	S	S	S	S
		1	1	S	R	R	S	R	S	S	R	S
		4	3	S	R	S	S	R	S	S	S	S
	MDR-TB (n=4)	2	2	R	R	R	R	S	S	S	S	S
		2	2	R	R	S	S	R	S	S	S	S
	Pre-XDR-TB (n=6)	2	2	R	R	R	S	S	R	S	S	S
		4	4	R	R	R	S	R	R	S	S	S
Drug resistance not detected by MTBDR assay (n=479)	Drug resistant TB (n=2)	2	2	S	S	S	S	R	S	S	S	S
	Minor variant (n=3)[a, b, c]	3	3	S	S	S	S	S	S	S	S	S
	Pan- susceptible TB (n=462)	462	448	S	S	S	S	S	S	S	S	S
	Unsuccessful sequencing (n=12)	9	7	S	S	S	S	S	S	S	S	S

Abbreviation: RIF, rifampicin, INH, isoniazid; EMB, ethambutol, PZA, pyrazinamide; FLQ, fluoroquinolones; STR, streptomycin; AMIs, aminoglycosides; CAP, capreomycin; LZD, linezolid

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		1	1	R	S	S	S	R	S	S	S	S
		5	2	S	R	S	S	S	S	S	S	S
		1	1	S	R	R	S	S	S	S	S	S
		1	1	S	R	R	S	R	S	S	R	S
		4	3	S	R	S	S	R	S	S	S	S
	MDR-TB (n=4)	2	2	R	R	R	R	S	S	S	S	S
		2	2	R	R	S	S	R	S	S	S	S
	Pre-XDR-TB (n=6)	2	2	R	R	R	S	S	R	S	S	S
		4	4	R	R	R	S	R	R	S	S	S
Drug resistance not detected by MTBDR assay (n=479)	Drug resistant TB (n=2)	2	2	S	S	S	S	R	S	S	S	S
	Minor variant (n=3)[a, b, c]	3	3	S	S	S	S	S	S	S	S	S
	Pan- susceptible TB (n=462)	462	448	S	S	S	S	S	S	S	S	S
	Unsuccessful sequencing (n=12)	9	7	S	S	S	S	S	S	S	S	S

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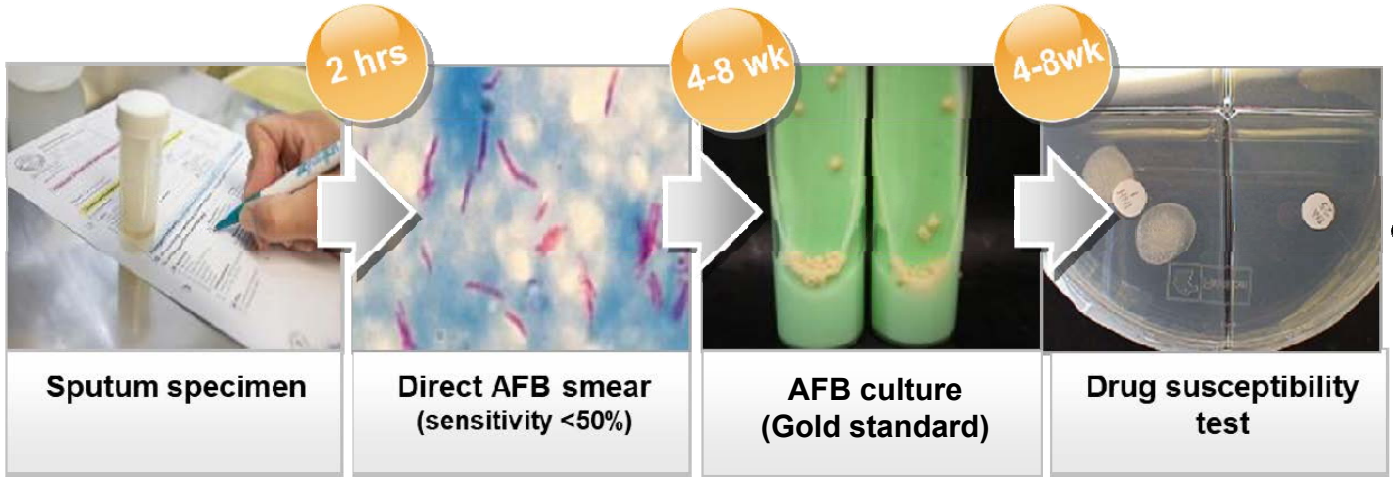

Conventional culture-based method (2-3 months)

Full Report

HK / Guangzhou study

4,047 sputum → DNA extract → qPCR for Mtb → **3 – 5 hrs** → **503 PCR+** → Rif / INH resistance → **24** → ~ 6 days

Report

Mtb DNA detected;
Drug resistance mutations to Rif, INH ….etc detected

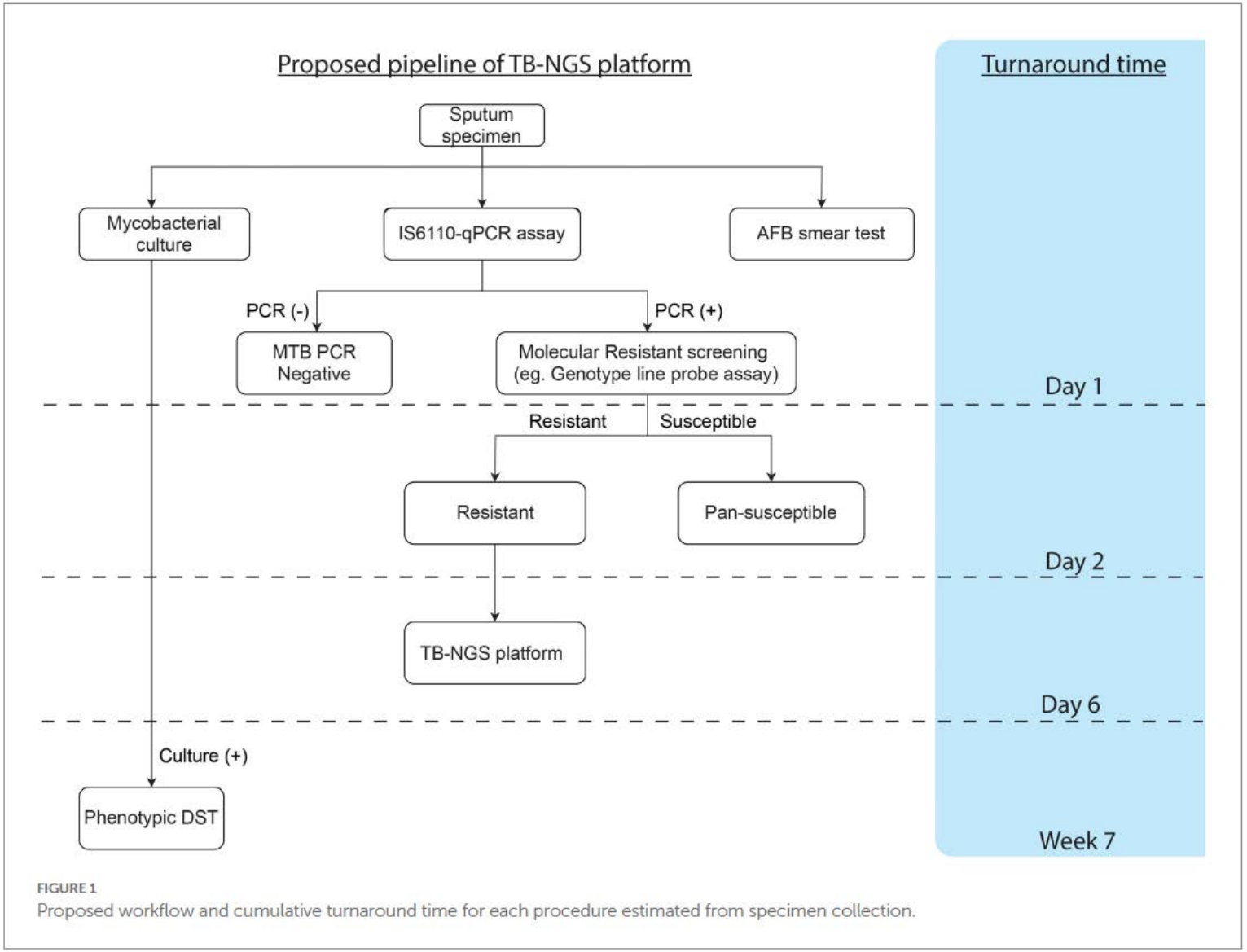

FIGURE 1
Proposed workflow and cumulative turnaround time for each procedure estimated from specimen collection.

Kenneth Leung et al. Front Microbiol. 2022 Sep :13

Conclusions

- PCR provides rapid molecular diagnosis of *M. tuberculosis*
- As Next Generation Sequencing (NGS) is expensive and technically complicated, a good diagnostic algorithm and workflow would provide a cost-effective routine service with an average turnaround time of 6 working days.
- Molecular diagnosis cannot replace conventional laboratory practice:
  - Conventional culture of *M. tuberculosis* isolates is required for epidemiological surveillance
  - Anti-mycobacterial susceptibility testing (DST)

Acknowledgements

The University of HK:
Dr. Kenneth Leung
Dr. Kingsley Tam

TB & Chest Service,
Dept of Health, Hong Kong:
Dr. CC Leung
Dr. CK Chan
Dr. KC Chang

HK Polytechnic University:
Dr. Gilman Siu

Johns Hopkins University:
Prof Y Zhang

KingMed Diagnostics, Guangzhou, China.

Funding:

- The Science and Technology Planning Project of Guangdong Province, China (Grant Number: 2019B121205010)