AO AL Azienda Ospedaliera di ALESSANDRIA Santi Antonio e Biagio e Cesare Arrigo

Dipartimento Attività Integrate Ricerca e Innovazione AO AL - ASL AL

IL SEQUENZIAMENTO NGS NEL LABORATORIO DI MICROBIOLOGIA: VALIDAZIONE, IMPLEMENTAZIONE ED UTILITÀ CLINICA

11 NOVEMBRE *2023*

ore **9.00**

Salone di Rappresentanza
AON SS. Antonio e Biagio e Cesare Arrigo
Via Venezia 16, Alessandria

Diagnosi NGS per TB: lo stato dell'arte

Daniela M. Cirillo

IRCCS Ospedale San Raffaele

OUTLINE

- WGS stato dell'arte
- Possibilità attuali
- Raccomandazioni di ECDC/WHO per sorveglianza e diagnostica
- Strategie attuabile
- Controllo di qualità
- Difficoltà interpretative
- Refertazione
- Possibilità di dismettere il pDST
- Conclusioni

DIAGNOSTIC METHODS USED IN THE ANALYSIS OF M.TB SAMPLES

PHENOTYPIC DST

Reference standard for some drugs, not all

- significant delays in diagnosis (requires a minimum of 2 weeks)
- force suboptimal and empirical treatment

Nimmo et al., 2017 ; Cabibbe & Cirillo, 2016; Outhred et al., 2015

GENOTYPIC DST

Rapid assessment of drug resistance mutations against key first-line anti-TB drugs

- only identify common drug resistance mutations in few gene targets
- Only limited number of mutations evaluated

Soundararajan et al.,2020; Nimmo et al., 2017; Outhred et al., 2015

NEXT GENERATION SEQUENCING

(NGS) WORKFLOW

WHOLE GENOME SEQUENCING (WGS)

CLINICAL SPECIMEN (SPUTUM)

FROM CULTURE

MGIT culture

Culture inactivation and DNA extraction

Library preparation

Sequencing

DIRECTLY FROM SPECIMEN

Specimen inactivation and DNA extraction

qPCR

MTB enrichment (biotinylated RNA baits / Myco Cap)

Library preparation

Sequencing

TARGETED NGS (tNGS)

Clinical sample

Inactivation and DNA extraction

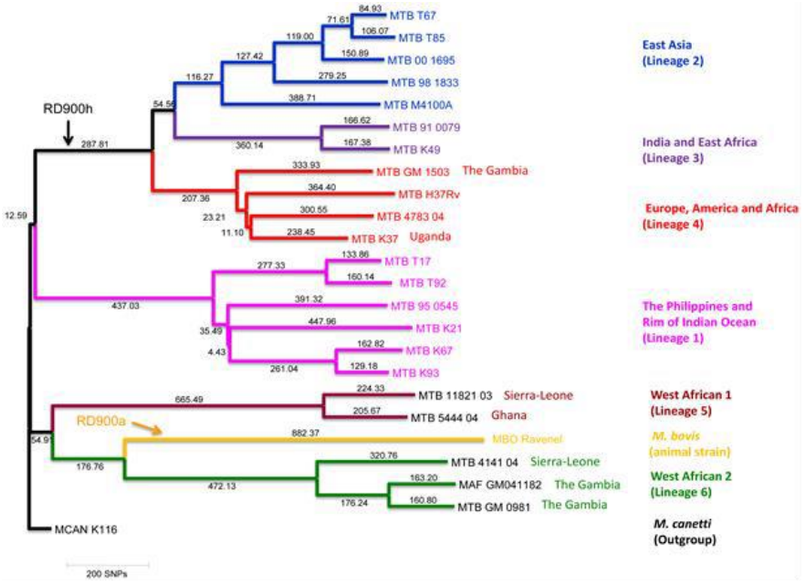
PCR amplification of targeted regions

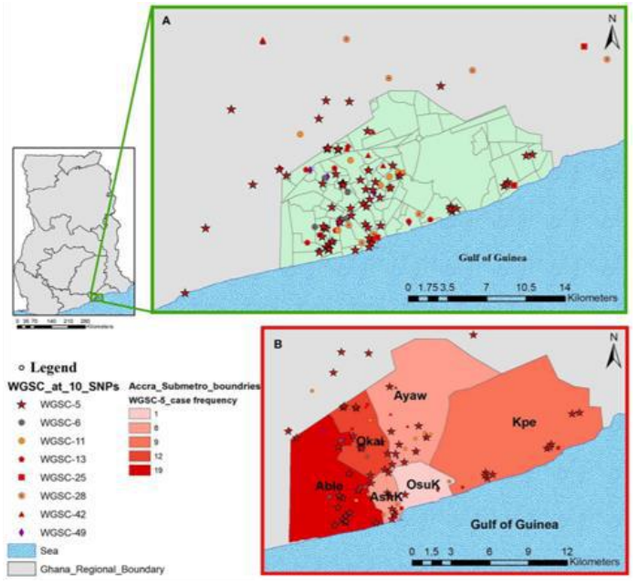
Library preparation

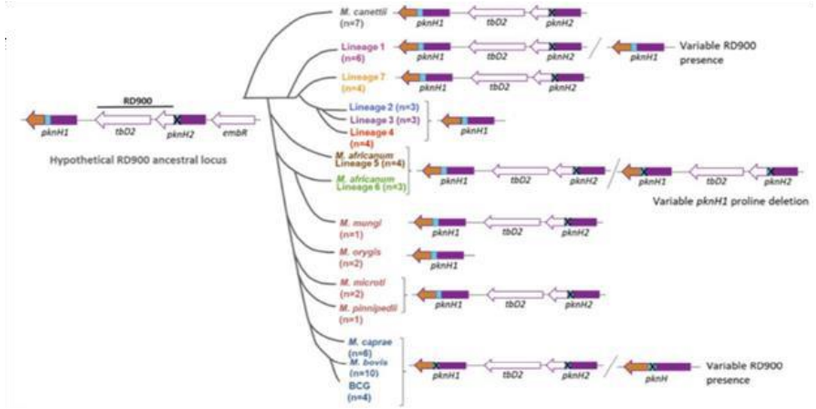
Sequencing

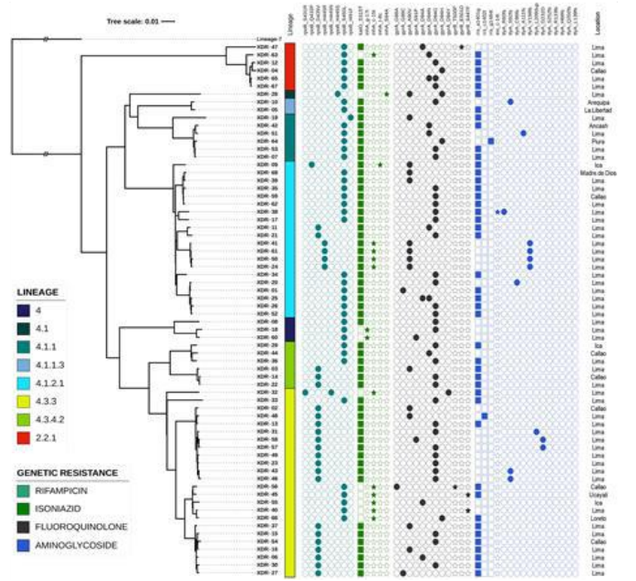
Cabibbe et al. 2016 Macedo et al., 2023; Soundararajan et al.,2020

NEXT GENERATION SEQUENCING





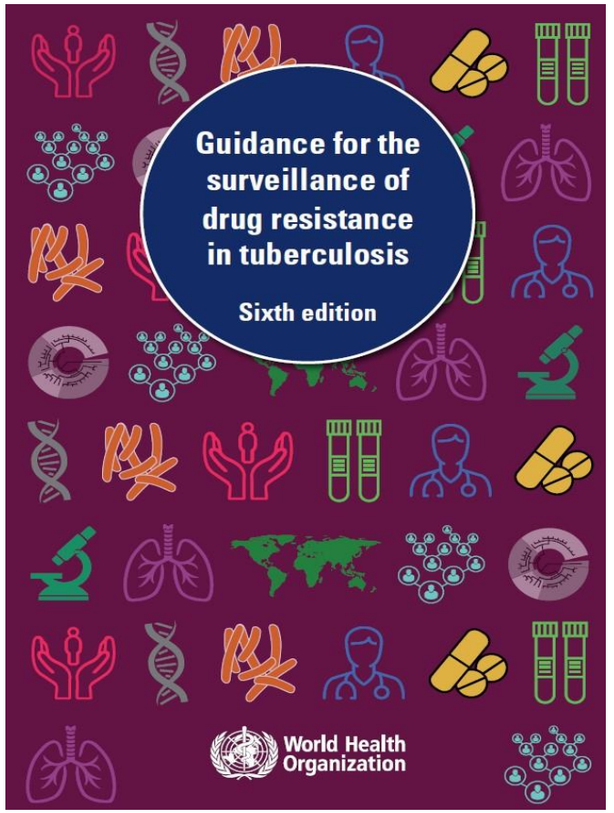




WHOLE GENOME SEQUENCING FROM MGIT CULTURE

- powerful tool for **epidemiological and drug-resistant routine surveillances**
- offers a rapid and simultaneous screening of all the clinically-relevant mutations
- support the conventional contact tracing for epidemiological studies with high discriminatory power
- ensure the required high DNA quality and quantity
- performed on genomic DNA from primary culture samples (2-3 weeks growth period)
- may cause loss of clonal diversity

Next Generation Sequencing-based Algorithms in Anti TB Drug Resistance Surveillance - 2020



Lozano et al., 2021; Soundararajan et al.,2020; Cabibbe & Cirillo, 2016

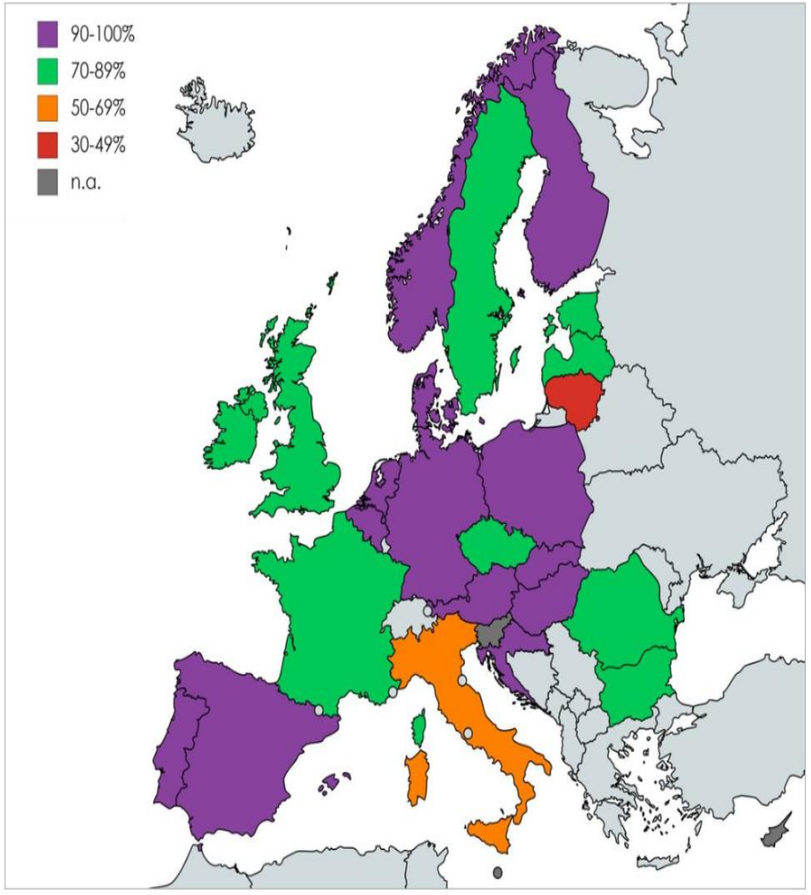
WGS:TOOL FOR CROSS BORDER CLUSTERS EVALUATION

Table 1: Country contribution to cross-border clusters

Country	RR/MDR-TB cases in cluster (N)	Percentage among clustered strains	Percentage of clustered RR/MDR-TB cases among submitted ones (N=2,217)
Austria	9	2.8	36.0
Belgium	5	1.6	26.3
Bulgaria	6	1.9	9.2
Croatia	0	-	-
Czech Republic	6	1.9	24.0
Denmark	0	-	-
Estonia	5	1.6	7.6
Finland	2	0.6	22.2
France	11	3.5	11.1
Germany	40	12.7	16.3
Hungary	2	0.6	9.1
Ireland	3	0.9	13.0
Italy	38	12.0	29.9
Latvia	3	0.9	3.5
Lithuania	43	13.6	24.0
Netherland	5	1.6	20.8
Norway	1	0.3	10
Poland	7	2.2	7.1
Portugal	0	-	-
Romania	101	32.0	11.7
Slovakia	2	0.6	25.0
Slovenia	0	-	-
Spain	9	2.8	13.6
Sweden	8	2.5	32.0
UK	10	3.2	12.3
Total	**316**	**100**	**14.3**

[a] A cross-border cluster is defined as two or more RR/MDR-MTBC isolates having a SNP difference ≤ 5, and isolated in at least two different countries.

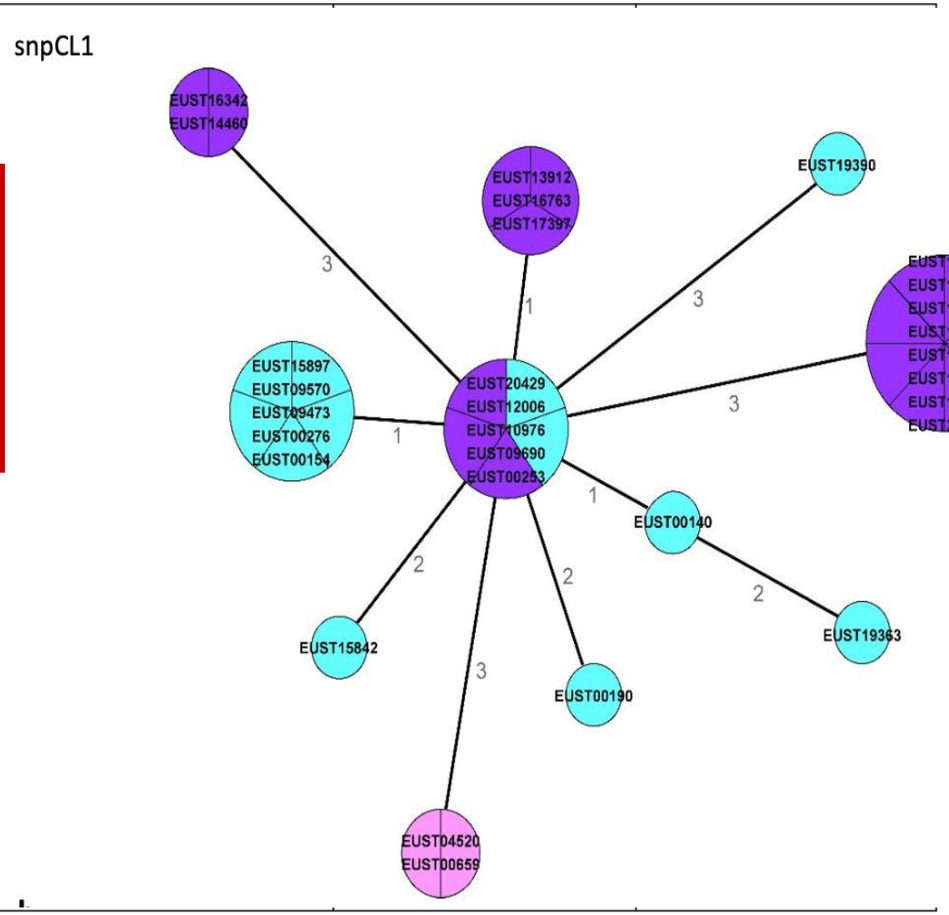
Figure 1: Whole genome sequencing coverage of reported RR/MDR-TB cases in European Union/European economic area, 2018



Tagliani et al. ERJ 2019

EUSeqMyTB: cross border clusters

Cluster name	Number of strains in cluster	Lineage classification	WGS-based drug resistance profile (gene; mutation; number of strains with mutation)
snpCL1	30	4.8	R-R (*rpoB;* S450L; n=30); H-R (*katG;* S315T; n=30; *inhA* prom; c-15t; n=30); E-R (*embB;* M306I; n=30); Z-R (*pncA;* A146V; n=30); FQ-R (*gyrA*; D94Y; n=11; *gyrB*; A504V; n=3); BDQ-R (Rv0678; large deletion; 5/30)
snpCL3	16	4.6.2	R-R (*rpoB;* S450L; n=16); H-R (*katG;* S315T; n=16); E-R (*embB;* M306I; n=16); Z-R (*pncA;* W68C; n=16); CAP-R (*tlyA*; N236K; n=16);
snpCL8	12	4.2.2	R-R (*rpoB;* S450L; n=12); H-R (*katG;* S315T; n=12); E-R (*embB;* G406A; n=12); Z-R (*pncA;* T76P; n=12);



R: rifampicin; H: isoniazid; E: ethambutol; Z: pyrazinamide; FQ: fluoroquinolones; BDQ: bedaquiline; CAP: capreomycin; R: resistance; n: numbers.
All strains were isolated between 2017 and 2019.

Tagliani et al. ERJ 2019

XDR Cluster

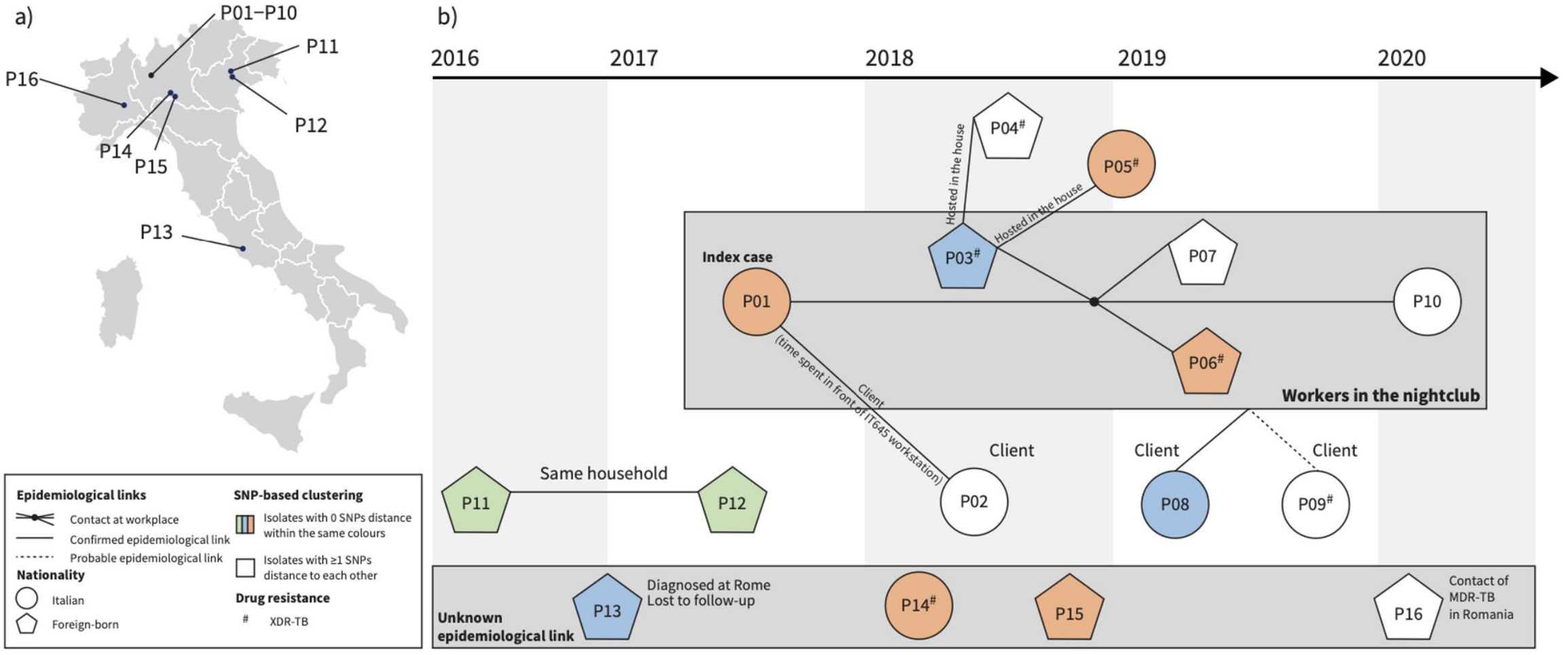


FIGURE 1 a) Spatial and b) temporal distribution and description of epidemiological links of clustered tuberculosis (TB) cases. MDR: multidrug resistant; XDR: extensively drug resistant; SNP: single nucleotide polymorphism.

WGS data submission

- ECDC upload app
- Bionumerics plugin
- ENA/SRA identifiers

Options for WGS data upload

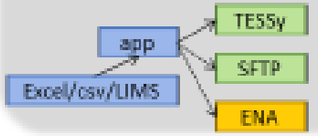
ECDC WGS upload application

How to use

- Download and install the application, see link below
- Read the manual and configure the data upload for each pathogen, personal support is available through the ECDC FWD mailbox (fwd@ecdc.europa.eu)
- Configure data import/mapping from Excel/csv, your LIMS system, or enter data directly
- Start uploading in a few clicks according to the reporting protocol
- The descriptive data are submitted to TESSy only, the WGS reads/assembly is submitted to the selected system(s)

Features

- Can be configured to import data from databases or local files (MySQL, SQL Server, SQLite, Excel, csv)
- Configure only once, single click upload
- Can upload assemblies to TESSy and SFTP, raw reads to SFTP and ENA (configurable)
- Data sharing through SFTP and ENA



ECDC Bionumerics client plugin

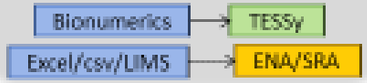
How to use

- Download and install the plugin, see link below
- Read the manual and configure data mapping from your database to TESSy, personal support is available through the ECDC FWD mailbox
- If raw reads are going to be submitted through ENA/SRA, set up ENA/SRA data submission and make the identifiers available in your bionumerics database. Support available from ECDC and from EBI/NCBI.
- Start uploading data according to the reporting protocol

Features

- Requires Bionumerics and that either ENA/SRA run accession or assemblies are stored in the Bionumerics database
- Simple upload process
- Can upload assemblies and ENA/SRA identifiers to TESSy

- Can not share raw reads through SFTP
- Can not submit data to ENA/SRA



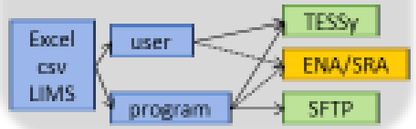
Direct TESSy submission – manual or machine-to-machine

How to use

- TESSy data, including ENA/SRA identifiers and Base64-encoded gzip-compressed FASTA if available are put into a csv file (manual upload) or an TESSy XML-file (machine-to-machine)
- The batch is uploaded and approved manually through TESSy (csv) or through the TESSy application interface (XML)
- Personal support and a Java library for machine-to-machine implementation is available through the ECDC FWD mailbox (fwd@ecdc.europa.eu). For machine-to-machine solutions, SFTP upload can also be implemented.

Features

- Can upload assemblies and ENA/SRA identifiers to TESSy
- Manual upload is easy to set up but involves recurring manual work
- Machine-to-machine upload requires development but enables high levels of automation



TESSy:
Stores descriptive data, WGS assemblies, links to ENA/SRA or to files submitted through SFTP. Uses Bionumerics for analysis

ECDC SFTP:
Temporarily stores read files, using anonymized identifiers.
Can also be used for data sharing

ENA/SRA:
Stores reads and minimal metadata indefinitely.
Data have to eventually be made public (immediately if reads are not also shared through SFTP)

Support:
fwd@ecdc.europa.eu
Tools download link:
https://tessy.ecdc.europa.eu/TessyHelp/index.aspx?navigation=TechnicalGuidelines

Flusso ceppi

- Continua la raccolta di MDR-TB da inviare alla attenzione di
  - E. Borroni o DM CIRILLO Ospedale San Raffaele
  - borroni.emanuele@hsr.it
- Dopo sequenziamento I Fastq saranno disponibili per chi li richiede
- Saranno depositati mensilmente su EPIPULSE
- Nel caso Fastq siano già disponibili contattare
  - Spitaleri.andrea@hsr.it

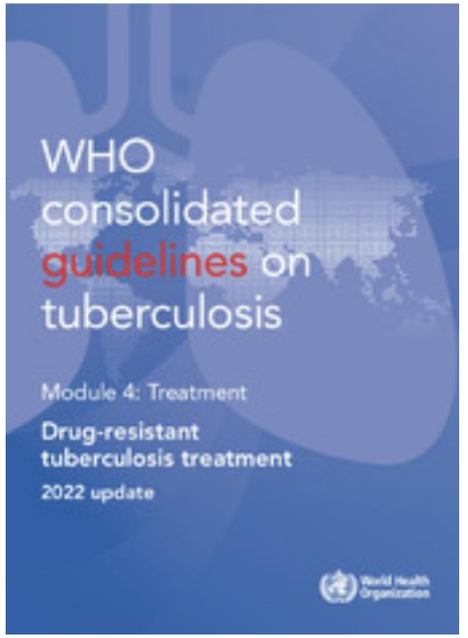
  OSR trasferirà I fastq insieme a quelli sequenziati da OSR dopo controllo di qualità

DR/MDR-TB regimens and definition of XDR-TB

Drug-resistant

Groups and steps	Medicine	Abbreviation
Group A: Include all three medicines	Levofloxacin *or* moxifloxacin	Lfx Mfx
	Bedaquiline[b,c]	Bdq
	Linezolid[d]	Lzd
Group B: Add one or both medicines	Clofazimine	Cfz
	Cycloserine *or* terizidone	Cs Trd
Group C: Add to complete the regimen and when medicines from Groups A and B cannot be used	Ethambutol	E
	Delamanid[e]	Dlm
	Pyrazinamide[f]	Z
	Imipenem–cilastatin *or* meropenem[g]	Ipm–Cln Mpm
	Amikacin (*or* streptomycin)[h]	Am (S)
	Ethionamide *or* prothionamide[i]	Eto Pto
	P-aminosalicylic acid[j]	PAS

XDR 2021



Section 1. The 6-month bedaquiline, pretomanid, linezolid and moxifloxacin (BPaLM) regimen for MDR/RR-TB (NEW)

1.1 Recommendation

NEW RECOMMENDATION

No.	Recommendation
1.1	WHO suggests the use of a 6-month treatment regimen composed of bedaquiline, pretomanid, linezolid (600 mg) and moxifloxacin (BPaLM) rather than the 9-month or longer (18-month) regimens in MDR/RR-TB patients. *(Conditional recommendation, very low certainty of evidence)*

Rapid approved molecular assays for MDR-TB cases

Table 3. Drug resistance determining regions targeted interrogated

Drug	Gene Target	Codon Regions	Nucleotid
Isoniazid	*inhA* promoter	NA	-1 to -32 intergenic
	katG	311-319	939-957
	fabG1	199-210	597-630
	oxyR- ahpC intergenic region	NA	-5 to -50 intergenic (or -
Ethionamide	*inhA* promoter[a]	NA	-1 to -32 intergenic
Fluoroquinolones	*gyrA*	87-95	261-285
	gyrB	531-544 (or 493-505)[12,14]	1596-1632
Amikacin, Kanamycin, Capreomycin	*rrs*	NA	1396-1417
	eis promoter	NA	-6 to -42 intergenic

a. The absence of mutations in the inhA promoter region does not exclude ETH resistance. Mutations conferring ETH re
reported to be present in genomic regions not targeted by the Xpert MTB/XDR assay.[15]

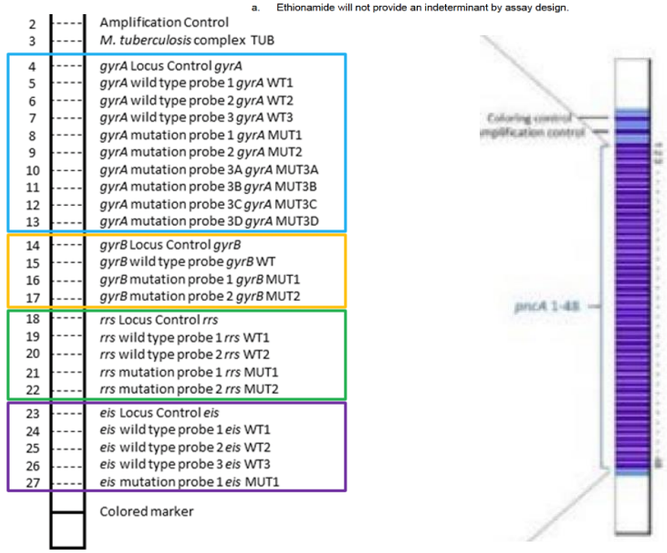
Table 2. Possible Test Results for Each Target in the Xpert MTB/XDR Assay

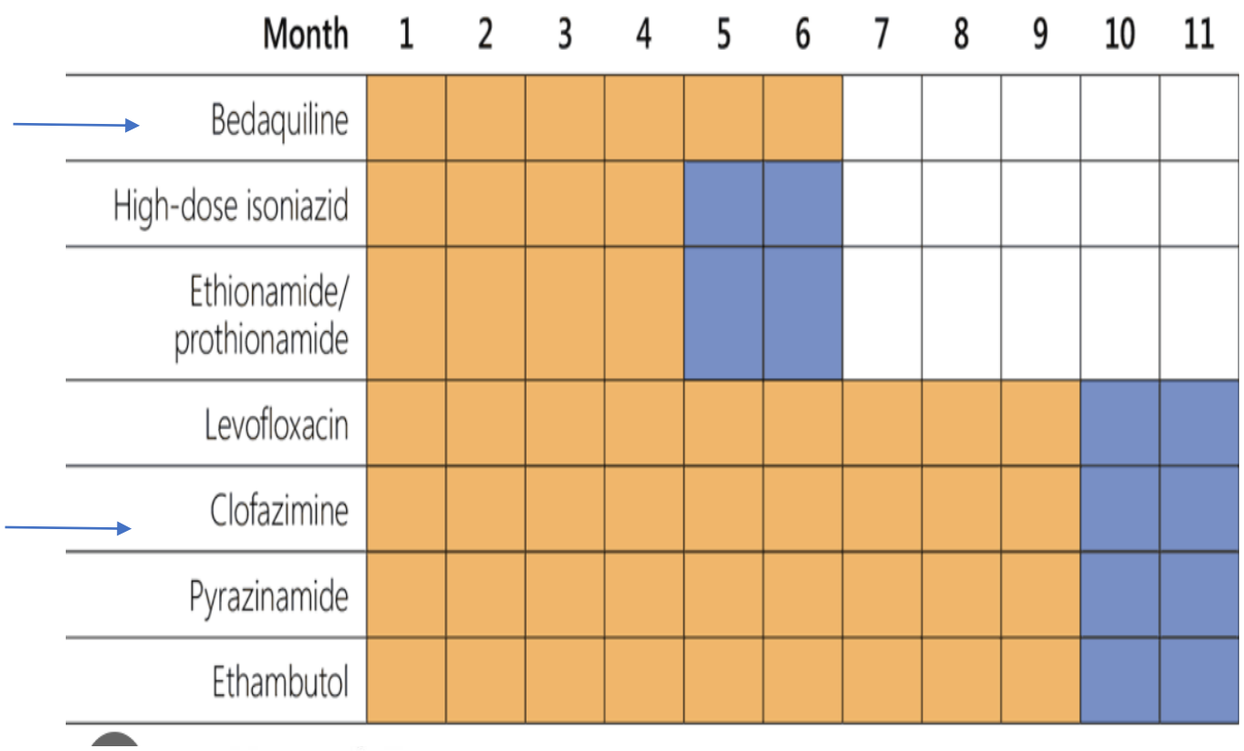
Drug Class	Result Call
N/A	INVALID/ERROR/NO RESULT
	MTB DETECTED
	MTB NOT DETECTED
Isoniazid	Low INH Resistance DETECTED
	INH Resistance DETECTED
	INH Resistance NOT DETECTED
	INH Resistance INDETERMINATE
Fluoroquinolone	Low FLQ Resistance DETECTED
	FLQ Resistance DETECTED
	FLQ Resistance NOT DETECTED
	FLQ Resistance INDETERMINATE
Amikacin	AMK Resistance DETECTED
	AMK Resistance NOT DETECTED
	AMK Resistance INDETERMINATE
Kanamycin	KAN Resistance DETECTED
	KAN Resistance NOT DETECTED
	KAN Resistance INDETERMINATE

Drug Class	Result Call
Capreomycin	CAP Resistance DETECTED
	CAP Resistance NOT DETECTED
	CAP Resistance INDETERMINATE
Ethionamide[a]	ETH Resistance DETECTED
	ETH Resistance NOT DETECTED

a. Ethionamide will not provide an indeterminant by assay design.

Targets specific mutations in the QRDR of ***gyrA*** (from codon 85 to 96) and of ***gyrB*** (from codon 536 to 541) genes for detection of <u>resistance to fluoroquinolones</u> and the ***rrs*** (nucleic acid position 1401, 1402 and 1484) and the ***eis*** promoter region (from -37 to -2 nucleotides upstream) for detection of <u>resistance to SLI drugs</u>.





http://www.who.int/iris/handle/10665/246131.

WGS DIRECTLY FROM CLINICAL SAMPLES (SPUTUM)

STRENGTHS

- reduce delays in case detection and characterization of susceptibility profiles
- generate a complete genetic drug resistance profile
- retains minority variants present in the clinical sample
- improves turnaround time for prompt appropriate treatment

CHALLENGES

- presence of large amounts of accompanying DNA
- requires enrichment procedures for high coverage
- often hampered by poor quality
- limitation on degree of confidence for resistance detection

EMPLOYED SOLUTIONS

- selectively eliminating the human DNA present in the clinical sample
- using MTB DNA enrichment systems, e.g., specific biotinylated RNA baits, in-house DNA capture platform (Myco Cap)

Lozano et al., 2021; Soundararajan et al.,2020 ; Cabibbe & Cirillo, 2016

World Health Organization

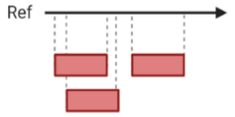
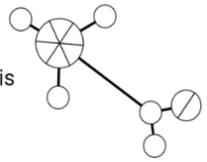
EBPU

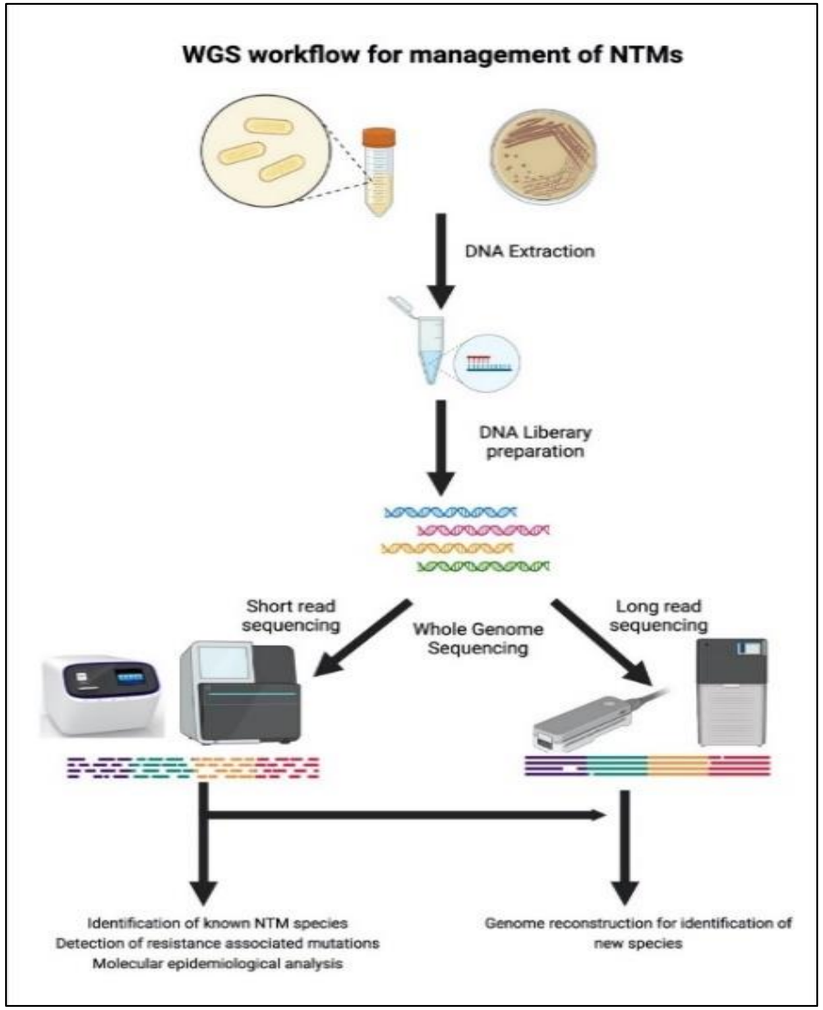
Use of targeted next-generation sequencing to detect drug-resistant tuberculosis

Rapid communication, July 2023

- Available evidence supports the use of targeted NGS to detect drug resistance after TB diagnosis, to guide clinical decision-making for drug-resistant TB treatment
  - Not a replacement of WRD, can be alternative especially where comprehensive DST is required
- Targeted next-generation sequencing was found to be accurate
- Targeted next-generation sequencing was found to be cost-effective depending on context
- Targeted next-generation sequencing was found to be acceptable and implementable under routine conditions, despite inherent complexity

NGS technologies

	SRS	LRS
Genome Coverage 	! Low coverage in repetitive regions (41/169)	✓
Variant Calling Comparative Analysis 	✓	! High-coverage to overcome error rate
Drug Resistance 	✓	✓
De Novo Assembly 	✗ High number of contigs Low NG50	✓



Di Marco et al, Front Microbiol 2023; Ghodousi A, 2023

First WHO catalogue of DR mutations in MTBC

Building components:

1. High quality phenotypic DST data

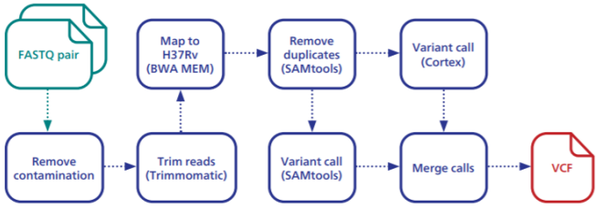
2. High quality, standardized WGS for generating unbiased raw sequence data

- CRyPTIC Consortium
- ReSeqTB
- WHO Surveillance Program Contributors
- Multinational TB researchers
- Public Health Bodies

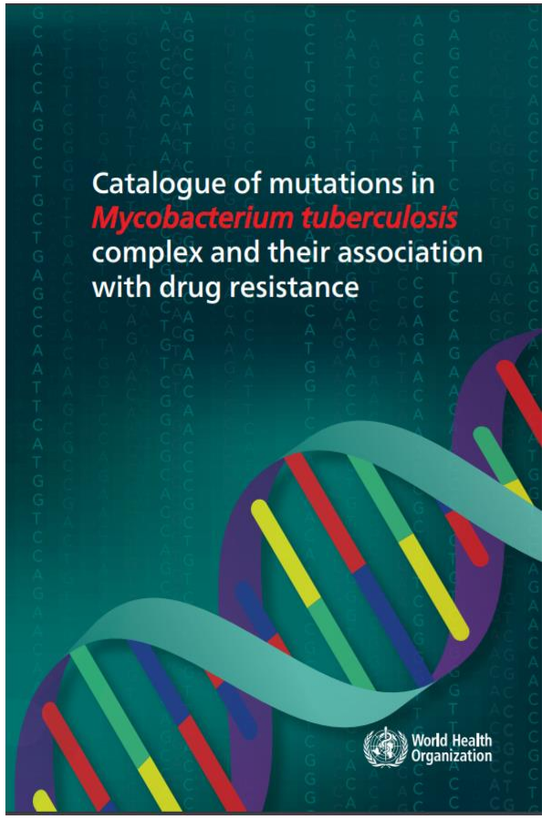
38k pDST/WGS matching data (QA/QC passed) from >40 countries

AMI, BDQ, CFZ, DLM, EMB, ETH, INH, LEV, LZD, MXF, PZA, RIF, STM (CAP, KAN)

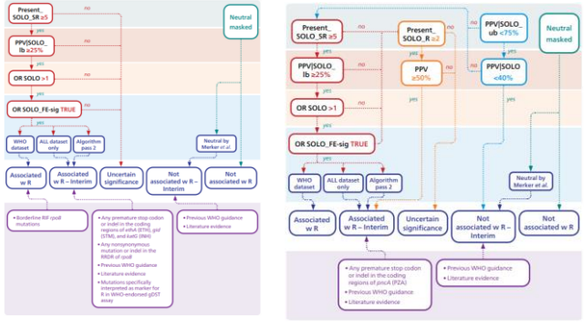
3. A standardized bioinformatics pipeline for variant detection and annotation



Clockwork pipeline
https://github.com/iqbal-lab-org/clockwork



4. A standardized and validated methodological approach for associating genotype-phenotype



- **Identification of "solo" mutations** (i.e. single mutations within a set of genes of interest that best explain the observed DR phenotype, once neutral mutations have been excluded)
- Data-driven grading based on **odd ratio** and associated p-value, and **PPV 95% CIs**
- **Additional criteria based on literature evidence and expert rules**

1200 variants associated with resistance
246 variants associated with no resistance

WHO catalogue V2

Drug	Dataset	Previous catalogue			Current catalogue			Variation
		Total	% R	(95% CI)	Total	% R	(95% CI)	Change % R
RIF	WHO	27063	24.9	(24.4–25.4)	35401	32.6	(32.1–33.1)	31
	ALL	34375	28.7	(28.2–29.2)	47730	35.3	(34.9–35.8)	23
INH	WHO	26727	31.6	(31.0–32.1)	34881	38.5	(38.0–39.0)	22
	ALL	34437	35.4	(34.9–35.9)	48706	43.0	(42.6–43.5)	21
EMB	WHO	23706	15.2	(14.8–15.7)	33240	19.8	(19.4–20.3)	30
	ALL	30708	16.0	(15.5–16.4)	45515	21.0	(20.6–21.3)	31
PZA	WHO	15903	14.6	(14.1–15.2)	19889	19.1	(18.6–19.7)	30
	ALL	15902	14.6	(14.1–15.2)	21319	20.8	(20.2–21.3)	42
LFX	WHO	10305	19.6	(18.8–20.4)	12441	22.0	(21.3–22.7)	12
	ALL	18277	17.0	(16.5–17.6)	27576	21.3	(20.8–21.8)	25
MFX	WHO	6904	15.8	(15.0–16.7)	8439	20.8	(19.9–21.7)	31
	ALL	13351	14.0	(13.4–14.6)	22783	17.7	(17.2–18.2)	26
BDQ	WHO	88	3.4	(0.7–9.6)	2165	41.7	(39.6–43.7)	1122
	ALL	8321	0.9	(0.7–1.1)	14135	7.3	(6.9–7.8)	736
LZD	WHO	1131	0.8	(0.4–1.5)	6825	2.0	(1.7–2.3)	152
	ALL	11018	1.1	(0.9–1.3)	18010	2.1	(1.9–2.3)	86
CFZ	WHO	3635	0.6	(0.4–0.9)	5027	4.3	(3.7–4.8)	576
	ALL	10179	1.2	(1.0–1.5)	14904	4.5	(4.2–4.9)	270
DLM	WHO	89	2.2	(0.3–7.9)	575	9.4	(7–11.8)	318
	ALL	7778	1.1	(0.8–1.3)	11803	2.1	(1.9–2.4)	103
AMK	WHO	8040	8.3	(7.7–8.9)	8958	12.5	(11.9–13.2)	52
	ALL	16978	7.6	(7.2–8.0)	24710	10.0	(9.7–10.4)	32
STM	WHO	9043	28.3	(27.4–29.3)	19747	39.3	(38.7–40.0)	39
	ALL	13984	33.1	(32.4–33.9)	26166	39.8	(39.2–40.4)	20
ETO	WHO	2184	40.5	(38.4–42.6)	5999	36.4	(35.2–37.6)	-10
	ALL	13918	21.3	(20.6–22.0)	20936	25.0	(24.4–25.6)	18
KAN[a]	WHO	7381	9.3	(8.7–10.0)	8014	20.1	(19.3–21.0)	116
	ALL	16154	9.2	(8.7–9.6)	24582	14.5	(14.1–15.0)	58
CAP[a]	WHO	9103	7.7	(7.2–8.3)	10025	13.1	(12.5–13.8)	70
	ALL	11526	8.4	(7.9–8.9)	17716	11.7	(11.2–12.1)	39

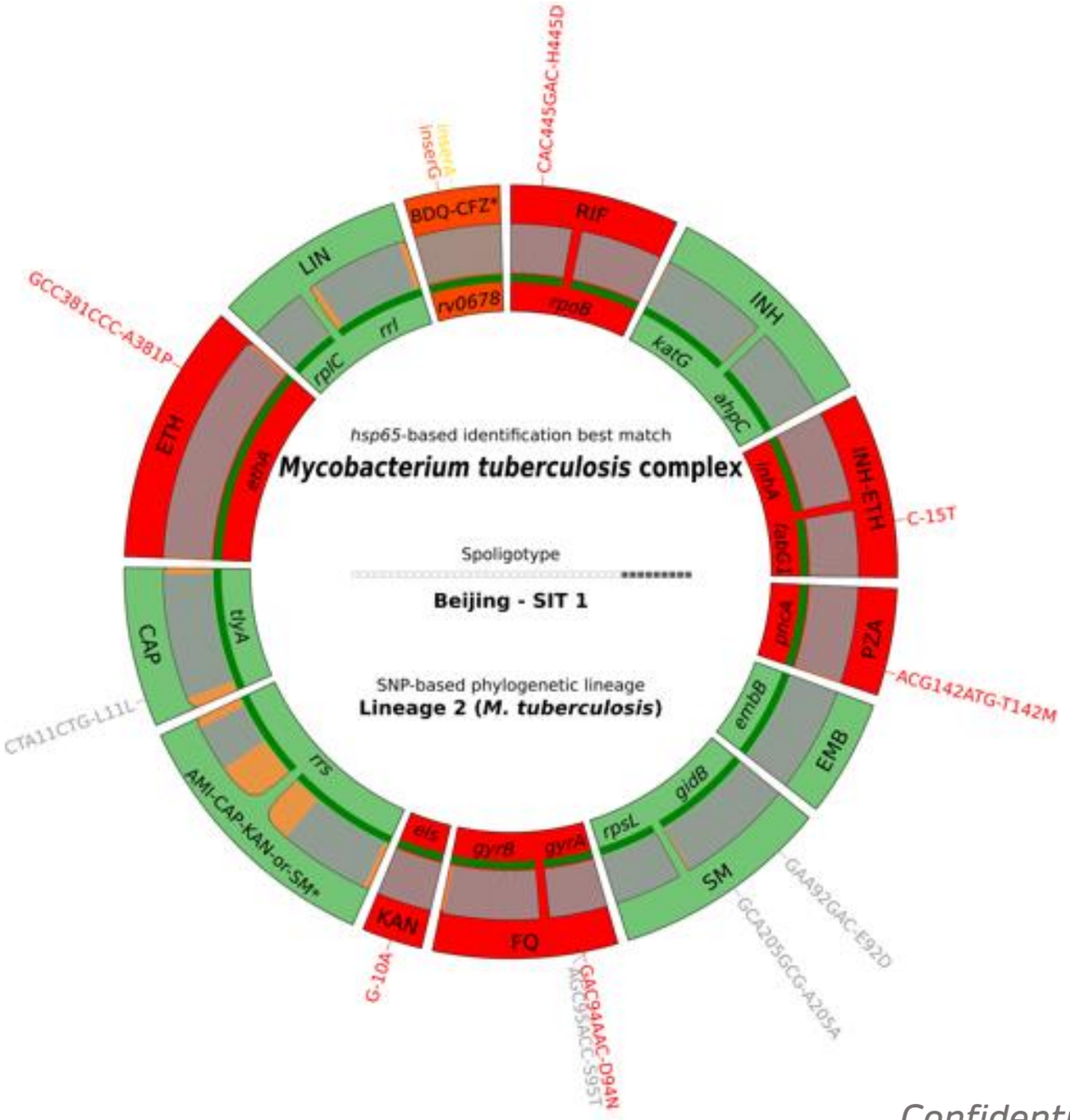
Provisional numbers

Incresead geographical representation

Increased number of genomes: from 38 to 53.000!

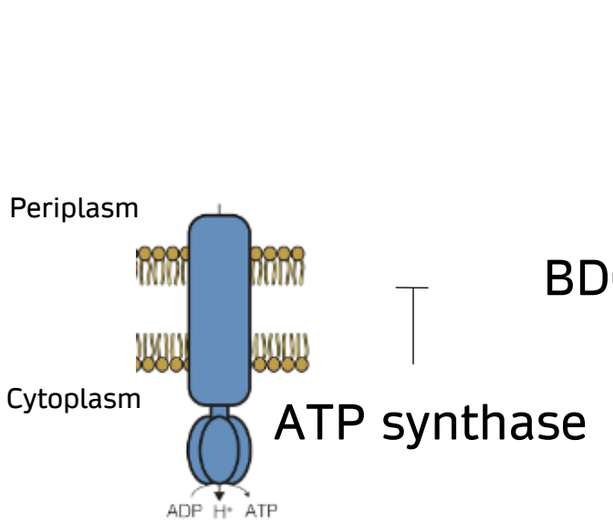
- Increased percentages of resistant strains for new/repurposed drugs
- Increased sensitivity for Bd, Cz, L, DLM
- No data on pretomanid
- Evaluation of more relaxed rules for interpretation associated to mutations targeting non-essential genes
- Epistasis and effect on genetic interpretation of DR

Deeplex Myc-TB



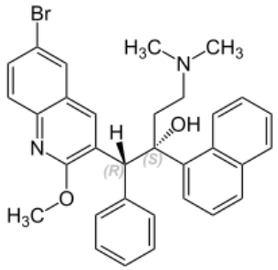
- Single 24-plex PCR
- 18 drug resistance-associated gene targets comprising *Rv0678* for BDQ

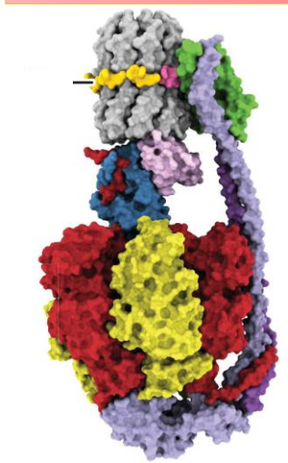
Bedaquiline resistance mechanisms

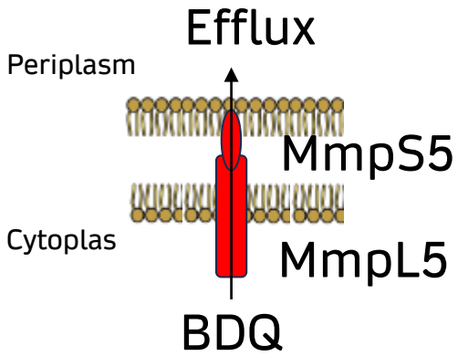


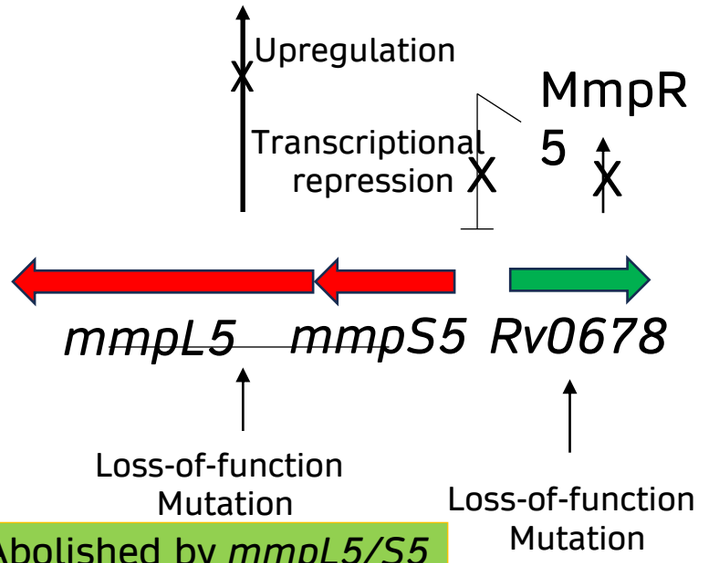
High level resistance: ATP synthase C chain

BDQ









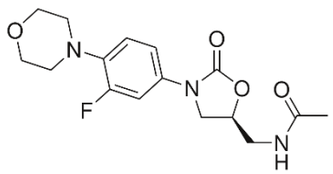
Main resistance target in clinical isolates: Rv0678 encoding repressor of MmpL5-mmpS5 efflux pump

- Abolished by *mmpL5/S5* LoF mutations
- Resistance mutations also in pepQ
- Cross-resistance to clofazimine

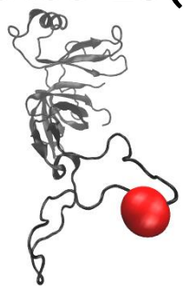
Haseboehrl et al., Front Cell Inf Microbiol, 2021; Guo et al., Nature, 2021; Kadura et al., JAC, 2020

M. tuberculosis Linezolid-resistance associated mutation mechanism: C154R (rplC) or G2814T/G2270T (rrl)

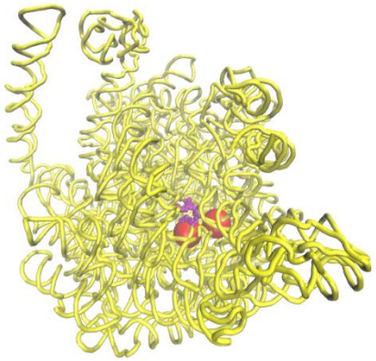
Linezolid

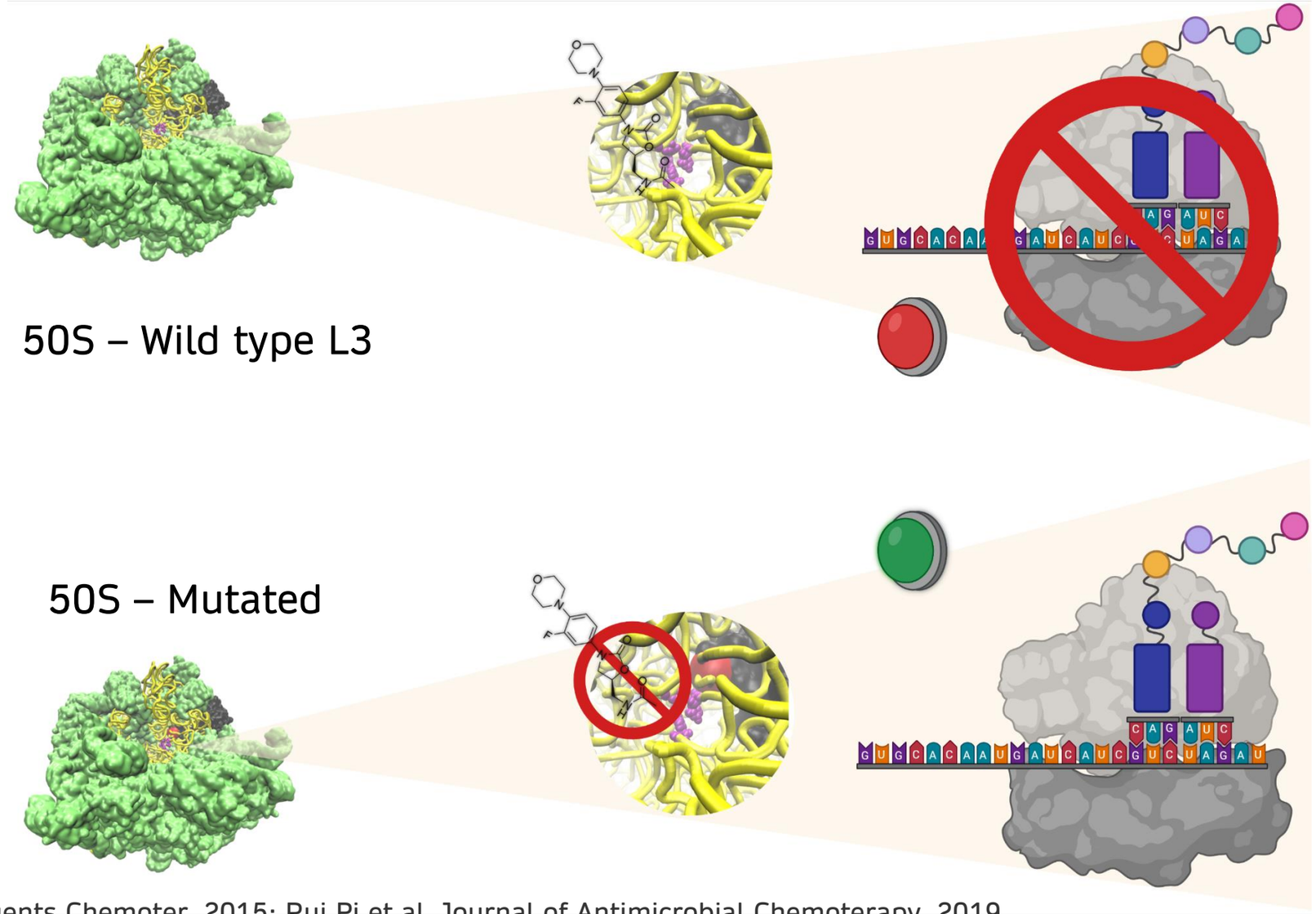


Mutated L3(C154R)



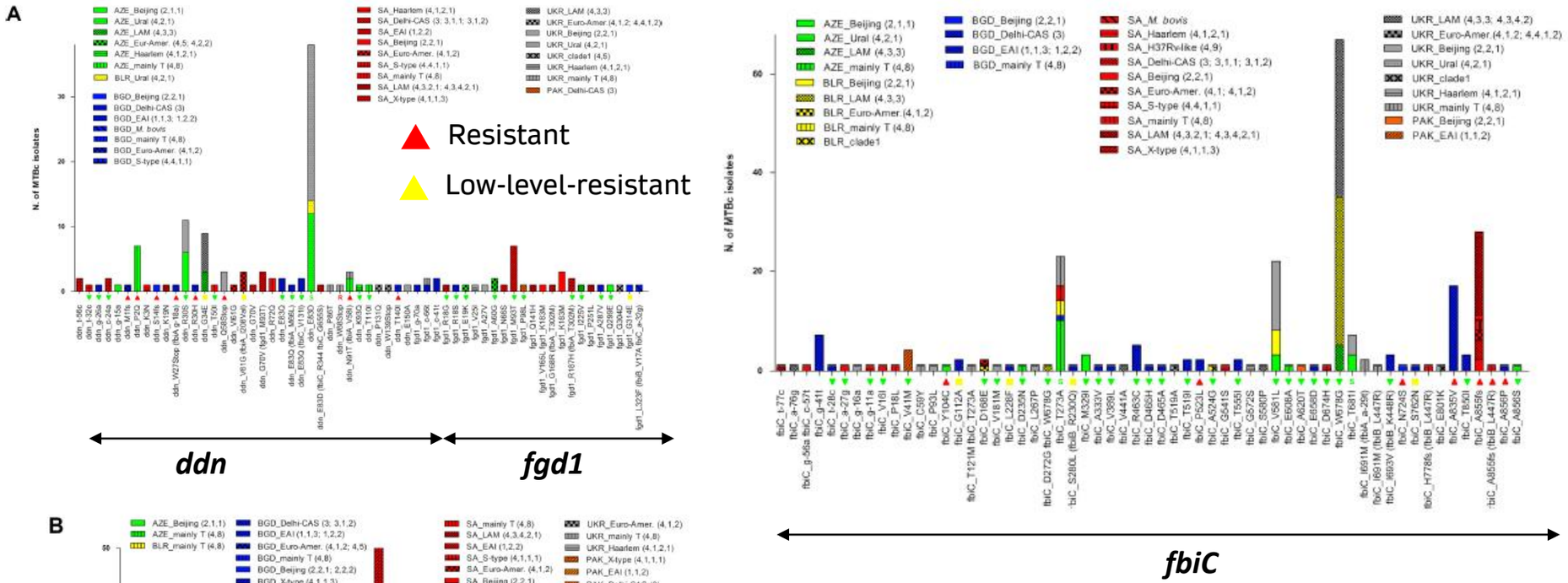
Mutated 23S (G2814T/G2270T)





Klitgaard et al, Antimicrob Agents Chemoter, 2015; Rui Pi et al, Journal of Antimicrobial Chemoterapy, 2019

Delamanid resistance targets and mutations



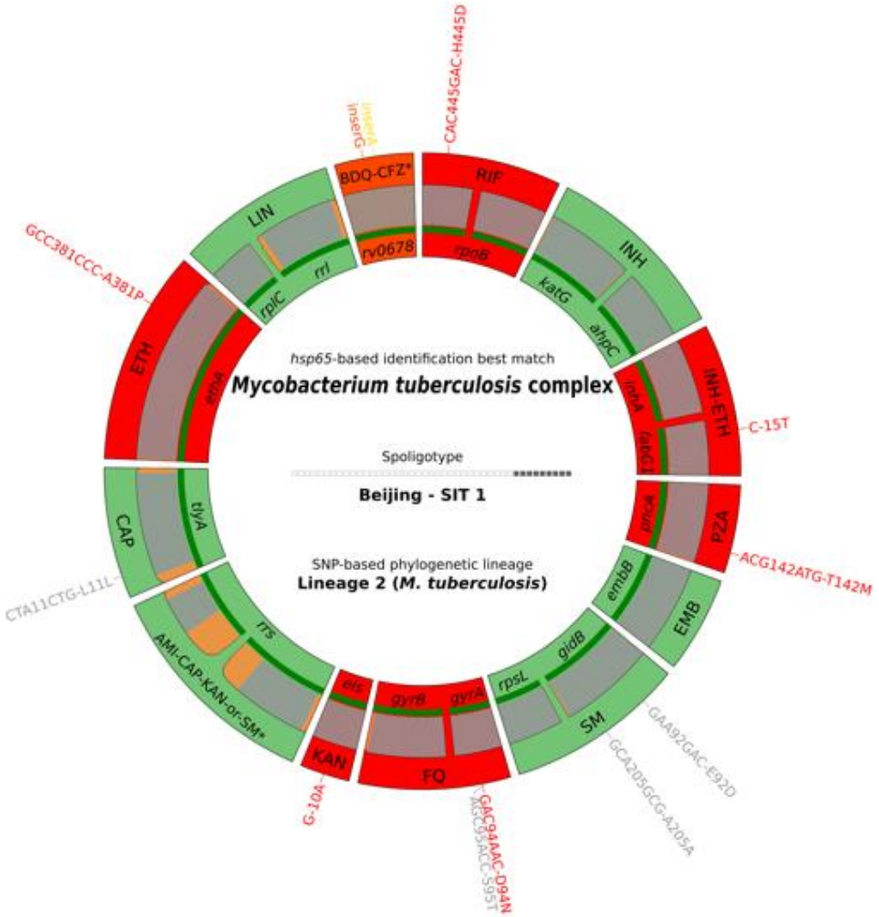
WGS analysis of 4,795 isolates from patients never exposed to DLM/PTM:

DLM resistance and low-level-resistance mutations identified in different genetic backgrounds across the entire coding sequence of each target

Battaglia et al., J. Clin. Microbiol., 2020

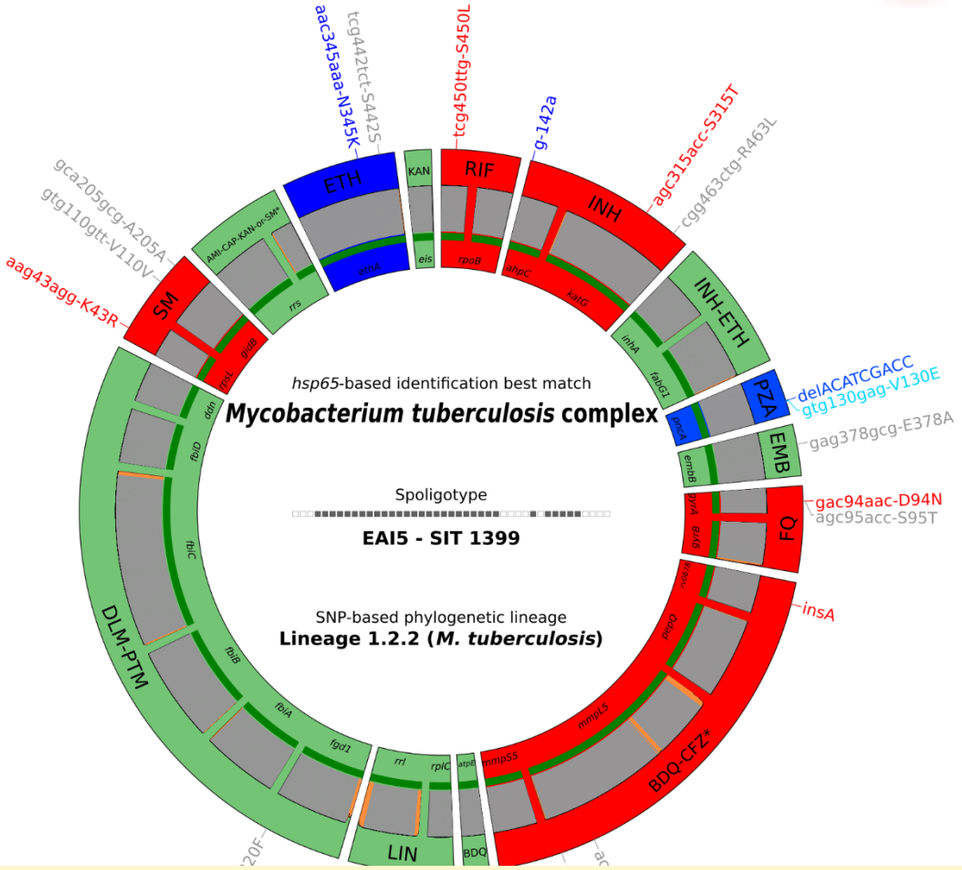
Courtesy of P. Supply

Deeplex Myc-TB



- Single 24-plex PCR
- 18 drug resistance associated gene targets comprising *Rv0678* for BDQ

Deeplex Myc-TB XL



- Single 42-plex PCR
- 27 drug resistance associated gene targets incl.:
  - Full *ddn*, *fgd1*, *fbiA*, *fbiB*, *fbiC*, *fbiD* genes for PTM/DLM
  - Full *Rv0678*, *atpE*, *pepQ*, *mmpL5*, *mmpS5* for BDQ
  - Optimized target design for other drugs
- Enhanced limit of detection on paucibacillary samples

OSR report template WGS

In case of no mutation, resistance cannot be excluded

OSPEDALE SAN RAFFAELE

Servizio di Medicina di Laboratorio
Ospedale San Raffaele
Direttore: Dott. Massimo Locatelli
Unità Patogeni Batterici Emergenti
Responsabile: Dott.ssa Daniela Cirillo

Protocollo: IT-1187 — Allegato al Referto del: NA

Lineage: LAM (4.3.3) — Campione arrivato il: 09-05-2019

Coverage: 113.4 — Cluster: Isolato NON in cluster*

*Database di riferimento includono tutti gli isolati sequenziati presso Emerging Bacterial Pathogens Unit- IRCCS San Raffaele – Milano

Farmaco	Regione	Gene	Mutazione [aminoac.]	Interpretazione	Altre Informazioni**
Rifampicina	Rv0667	*rpoB*	Ser450Leu	Resistente	
Isoniazide	Rv1483	*inhA*	c-15t	Resistente	Mutazione associata a bassi livelli di resistenza al farmaco isoniazide;
	Rv1908c	*katG*	/		
Etambutolo	Rv3795	*embB*	Met306Ile	Resistente	
Pirazinamide	Rv2043c	*pncA*	His45Arg	Resistente	
Fluorochinoloni (MXF;LEV;OFL)	Rv0006	*gyrA*	Glu21Gln Ser95Thr Gly247Ser Gly668Asp	Indeterminato	- Mutazioni legate al genotipo; - Arg446His (gyrB): Mutazione osservata sia in fenotipi sensibili che resistenti: non ci sono quindi evidenze che tale mutazione sia associata a farmacoresistenza; si raccomanda di eseguire il test fenotipico di conferma;
	Rv0005	*gyrB*	Arg446His		
Amikacina	Rvnr01	*rrs*	/	Sensibile	
	Rv2416c	*eis*	/		
Kanamicina	Rvnr01	*rrs*	/		
	Rv2416c	*eis*	/		
Capreomicina	Rvnr01	*rrs*	/		
	Rv1694	*tlyA*	(sil)Leu11		
Etionamide	Rv3854	*ethA*	/	Resistente	
	Rv3855	*ethR*	/		
	Rv1483	*inhA*	c-15t		
Bedaquilina	Rv1305	*atpE*	/	Sensibile	
	Rv0678		/		
Delamanid	Rv3261	*fbiA*	/	Sensibile	
	Rv3262	*fbiB*	/		
	Rv1173	*fbiC*	/		

Mod. EBP DMA 10

	Rv0407	*fgd1*	/
	Rv3547	*ddn*	/
Linezolid	Rv0701	*rplC*	/
Linezolid	Rvnr02	*rrl*	/
Clofazimine	Rv0678		/
	Rv1979c		/

Sensibile

Sensibile

"/" Mutazioni non rilevate; wild-type

§ Pipeline di analisi del genoma: MTBseq. Kohl TA, Utpatel C, Schleusener V, De Filippo MR, Beckert P, Cirillo DM, Niemann S. 2018. MTBseq: a comprehensive pipeline for whole genome sequence analysis of *Mycobacterium tuberculosis complex* isolates. PeerJ 6:e5895 DOI 10.7717/peerj.5895

** Eccezioni o variazioni rispetto alle condizioni standard di analisi MTBseq sono riportate in questa sezione.

° Il non rilevamento di mutazioni in una particolare regione del genoma (Rv), non esclude la possibilità di farmacoresistenza per il relativo farmaco.

Milano, 20-05-2019

Mutation linked to low level resistance

Mutation observed among R and S.
No evidence of its role in resistance.
Phenotypic testing recommended

Phenotypic test for Bd, L, Cl to be referred id resistance is suspected

Person diagnosed with TB

RIF or RIF/INH molecular test on smear positive sample

RIF/INH S TB

Liquid Culture positive

RIF R/TB

Treatment:
2HRZE/4HR (6 month regimen)
or 2HPMZ/2HPM (4 month regimen

WGS

Treatment:
BPaLM
6 months

TNGS testing for drug resistances:

- Fluoroquinolone (FQ)* (Levofloxacin (LFX), Moxifloxacin (MXF))
- Bedaquiline (BDQ)*
- Linezolid (LZD)*
- Pretomanid (Pa) – NO DATA EXPECTED
- Delamanid (DLM) – NO DATA EXPECTED
- Clofazimine (CFZ)*
- Amikacin (AMK)*
- INH, Ethambutol, PZA (first-line drugs)

NO SNPs IN/DEL
Detected in target genes

any SNPs IN/DEL
Detected in target genes

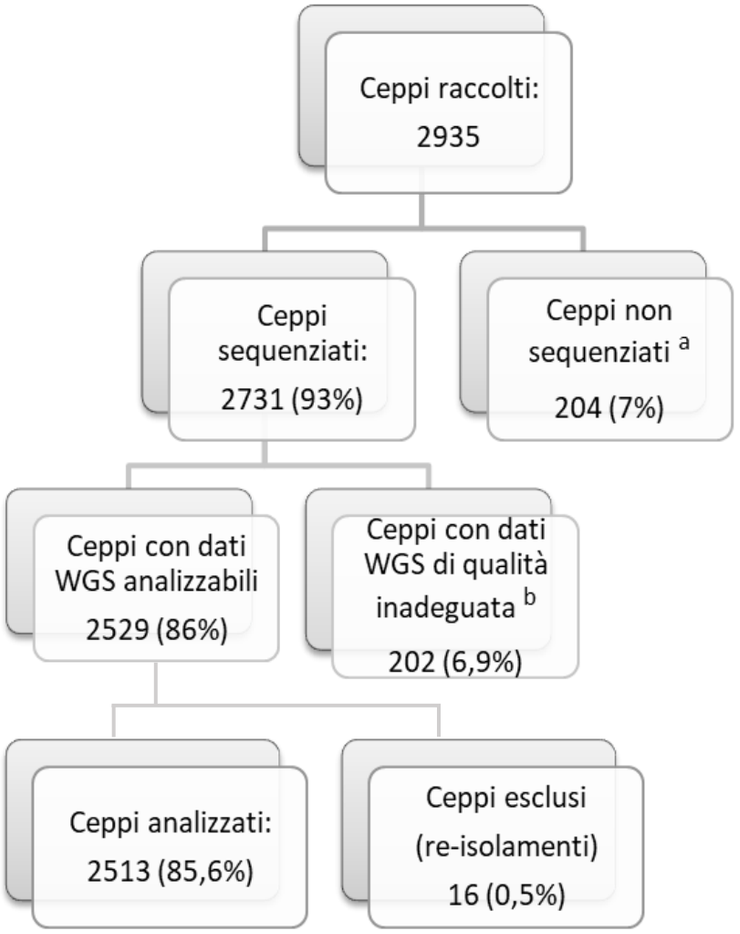
Treatment:
BPaL
6-9 months

Stop

Additional resistance, personalized regimen

report based on grading and pDST

Progetto CCM 2018: Definizione di strategie di controllo della tubercolosi associata ad HIV in Italia nel contesto di una strategia di eliminazione della malattia tubercolare



a: esclusi a causa di contaminazione, assenza di crescita in coltura, insufficiente quantità di DNA estratto; (ceppi provenienti esclusivamente da Emilia-Romagna)
b: esclusi perché non MTBc, mixed con altre specie, o basso sequencing coverage (<25x) (ceppi provenienti da Lombardia (51%), Lazio (285), Piemonte (19%)).

- **AIM**: Utilizzare i **dati genomici (WGS) dei ceppi di *M. tuberculosis*** complex raccolti in 4 regioni italiane (Lombardia, Emilia-Romagna, Piemonte, Lazio) nel quadriennio 2017-2020 a fini epidemiologici per **determinare i maggiori cluster di trasmissione circolanti in Italia e caratterizzarne i profili di resistenza** ai farmaci antitubercolari.

Numero e percentuale dei ceppi raccolti ed analizzati nello studio rispetto ai casi notificati per regione (01/2017- 06/2020)

Regione	Numero casi TB notificati	Numero (%) colture positive	Numero (%) di ceppi raccolti	Numero (%) di ceppi analizzati	% di casi analizzati rispetto alle colture positive
Lombardia	3642#	2213 (60,8)	1219 (33,5)	1116 (30,6)	50,4%
Emilia-Romagna*	1394	1020 (73,2)	600 (43,0)	391 (28,0)	38,3%
Lazio	1964	958 (48,8)	863 (43,9)	807 (41,1)	84,2%
Piemonte	1140	619 (54,3)	253 (22,2)	215 (18,9)	34,7%

Basato su numero di casi notificati per gli anni 2017, 2018 e 2019, e numero di notifiche stimate per il 2020.

* Buona parte delle colture sequenziate provenienti dal laboratorio di Bologna non hanno permesso un sequenziamento soddisfacente in quanto conservate in modo improprio.

Sensibilità e specificità del WGS nel predire il profilo di resistenza ai farmaci di 1a linea

	Rifampicina	Isoniazide	Etambutolo	Pirazinamide
Sensibilità (95%CI)	88,6 (79,7 - 94,1)	79,6 (73,3 - 84,8)	54,8 (41,8 - 67,3)	53,2 (44,1 - 62,0)
Specificità (95%CI)	99,4 (99,0 - 99,7)	99,3 (98,9 - 99,6)	99,0 (98,5 - 99,3)	98,5 (97,8 - 98,9)

- **Limiti del test fenotipico** (*i.e.,* mutazioni borderline, MIC elevate in alcuni lineages…)

Fluorochinoloni

- 100% (N= 36) profilo di resistenza fenotipico-genotipico concordante.
- 100% Sensibilità e specificità, ma numero di ceppi con profilo fenotipico ridotto (test non eseguito/non disponibile per tutti i ceppi con resistenza a rifampicina e isoniazide).

Profilo genotipico di resistenza ai farmaci antitubercolari dei ceppi analizzati (n=2513)

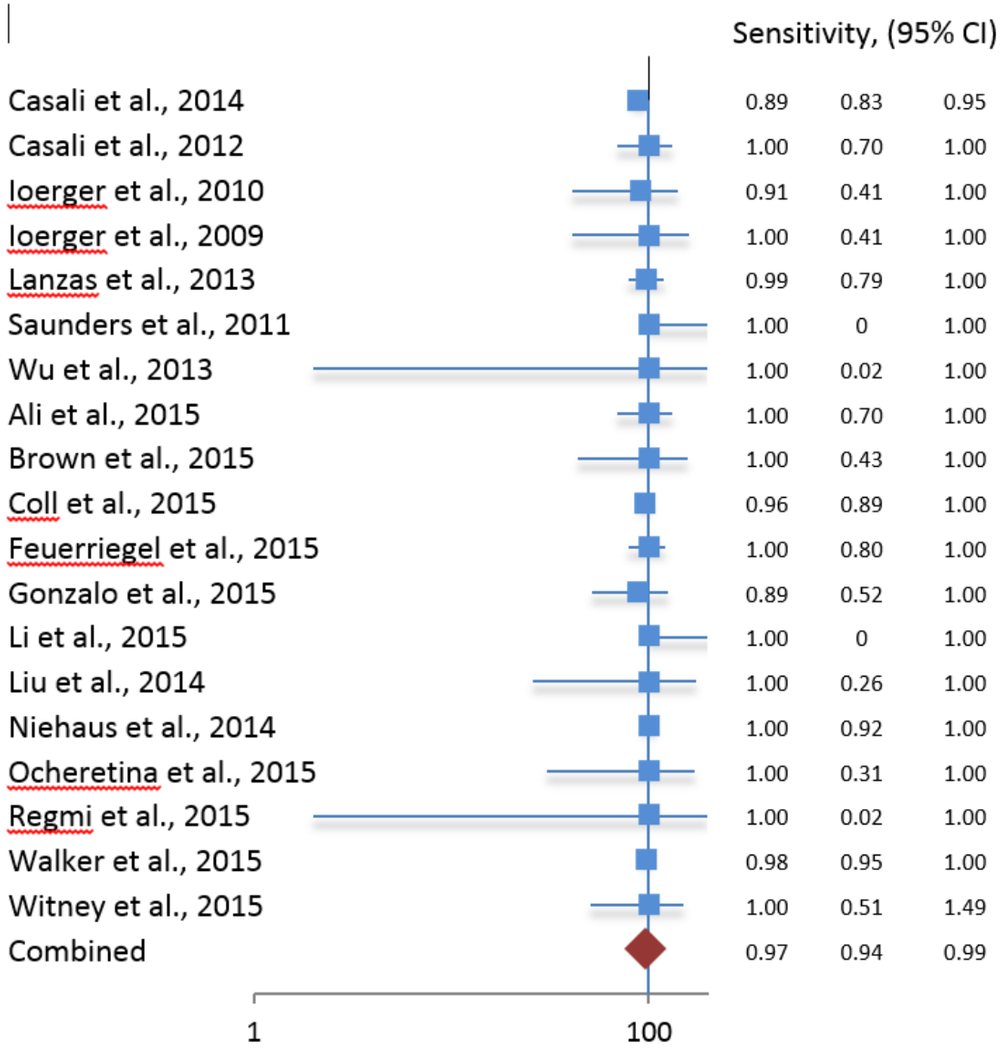
	RS-TB (n=2419)	RR-TB (n=94)
INH	104 (4,3%)	83 (88.2%)
FQ	39 (1,6%)	22 (23.4%)
PZA	61 (2,5%)	46 (48,9%)
INH+FQ	2 (0.1%)	22 (23,4%)
INH+FQ+PZA	0 (0%)	14 (14,9%)

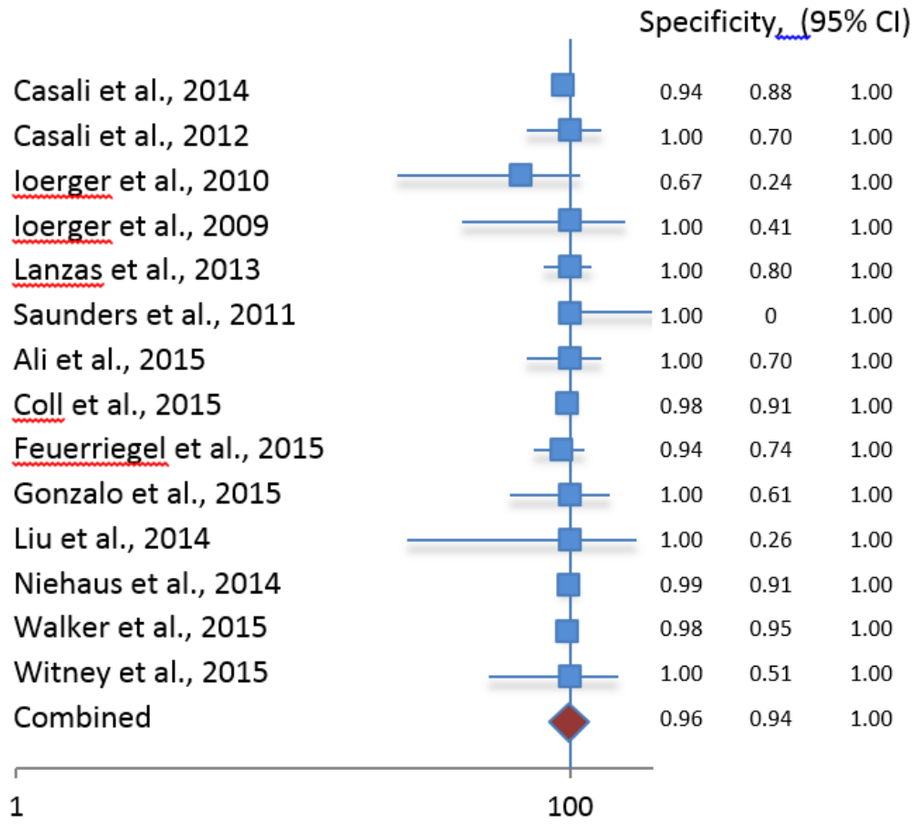
Casi di TB rifampicina resistente (n=94) identificati nello studio per anno (2017-2020)

	2017	2018	2019	2020	Totale
RR-TB	3	1	5	2	11
MDR-TB	22	15	12	12	61
preXDR-TB	4	7	5	0	16
XDR-TB	1	3	2	0	6
Totale	30	26	24	14	94
RR/MDR-TB	25	16	17	14	72
% RR/MDR-TB	83,3	61,5	70,8	100,0	76,6

5 casi resistenti a BDQ ed 1 caso resistente a LZD

TB WGS for DST (RIF)





Papaventsis et al., CMI 2017

Accuracy of DST prediction

Table 2. Prediction of Phenotypes of Resistance or Susceptibility to Individual Drugs.*

Analysis and Drug	Resistant Phenotype					Susceptible Phenotype					Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
	R	S	U	F	Total	R	S	U	F	Total				
	number of isolates													*percent*
WGS, all isolates														
Isoniazid	3067	90	93	44	3294	65	6313	215	117	6710	97.1 (96.5–97.7)	99.0 (98.7–99.2)	97.9 (97.4–98.4)	98.6 (98.3–98.9)
Rifampin	2743	69	7	84	2903	85	6763	232	147	7227	97.5 (96.9–98.1)	98.8 (98.5–99.0)	97.0 (96.3–97.6)	99.0 (98.7–99.2)
Ethambutol	1410	81	94	55	1640	468	6835	781	70	8154	94.6 (93.3–95.7)	93.6 (93.0–94.1)	75.1 (73.0–77.0)	98.8 (98.5–99.1)
Pyrazinamide	863	82	117	77	1139	204	6146	197	108	6655	91.3 (89.3–93.0)	96.8 (96.3–97.2)	80.9 (78.4–83.2)	98.7 (98.4–99.0)
WRAs, all isolates‡														
Isoniazid	2886	355	—	53	3294	27	6675	—	8	6710	89.0 (87.9–90.1)§	99.6 (99.4–99.7)§	99.1 (98.7–99.4)§	95.0 (94.4–95.5)§
Rifampin	2669	143	—	91	2903	129	6826	—	272	7227	94.9 (94.0–95.7)§	98.1 (97.8–98.4)¶	95.4 (94.5–96.1)¶	97.9 (97.6–98.3)§
Ethambutol	961	641	—	38	1640	241	7895	—	18	8154	60.0 (57.5–62.4)§	97.0 (96.6–97.4)§	80.0 (77.6–82.2)¶	92.5 (91.9–93.0)§
WGS, unenriched‖														
Isoniazid	314	8	9	4	335	15	3770	104	90	3979	97.5 (95.2–98.9)	99.6 (99.3–99.8)§	95.4 (92.6–97.4)¶	99.8 (99.6–99.9)§
Rifampin	126	0	0	9	135	31	3958	103	116	4208	100.0 (97.1–100.0)	99.2 (98.9–99.5)**	80.3 (73.2–86.2)§	100.0 (99.9–100.0)§
Ethambutol	72	1	0	0	73	47	3711	458	36	4252	98.6 (92.6–100.0)	98.7 (98.3–99.1)§	60.5 (51.1–69.3)§	100.0 (99.8–100.0)§
Pyrazinamide	109	6	4	6	125	30	4003	14	58	4105	94.8 (89.0–98.1)	99.3 (98.9–99.5)§	78.4 (70.6–84.9)	99.9 (99.7–99.9)§
WRAs, unenriched††														
Isoniazid	295	36	—	4	335	10	3965	—	4	3979	89.1 (85.3–92.3)§	99.7 (99.5–99.9)	96.7 (94.1–98.4)	99.1 (98.8–99.4)§
Rifampin	114	11	—	10	135	22	3957	—	229	4208	91.2 (84.8–95.6)§	99.4 (99.2–99.7)	83.8 (76.5–89.6)	99.7 (99.5–99.9)§
Ethambutol	57	16	—	0	73	29	4220	—	3	4252	78.1 (66.9–86.9)§	99.3 (99.0–99.5)**	66.3 (55.3–76.1)	99.6 (99.4–99.8)§

Walker et al., NEJM 2018

A

Analysis and Drug	Resistant Phenotype					Sensitive Phenotype					Prediction			
	R	S	U	F	Total	R	S	U	F	Total	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Isoniazid	86	1[§]	5	0	92	0	972	11	0	983	100% (99.6%, 100%)	98.85% (93.77%, 99.79%)	99.90% (99.42%,99.98%)	100% (95.72%, 100%)
Rifampicin	36	2*	0	0	38	0	1011	26	0	1037	100% (99.62%, 100%)	94.74% (82.71%, 98.54%)	99.80% (99.28%, 99.94%)	100% (90.35%, 100%)
Ethambutol	21	3[#]	2	0	26	2	991	56	0	1049	99.8% (96.26%, 99.94%)	86.96% (67.87%, 95.46%)	99.70% (99.11%, 99.89%)	90.91% (72.18%, 97.47%)
Pyrazinamide	28	29	1	0	58	0	1017	0	0	1017	100% (99.62%, 100%)	47.37% (34.98%, 60.08%)	97.13% (95.93%, 97.98%)	100% (87.54%, 100%)

B

GENOTYPIC DRUG PROFILE PREDICTIONS OF PANSUSCEPTIBILITY

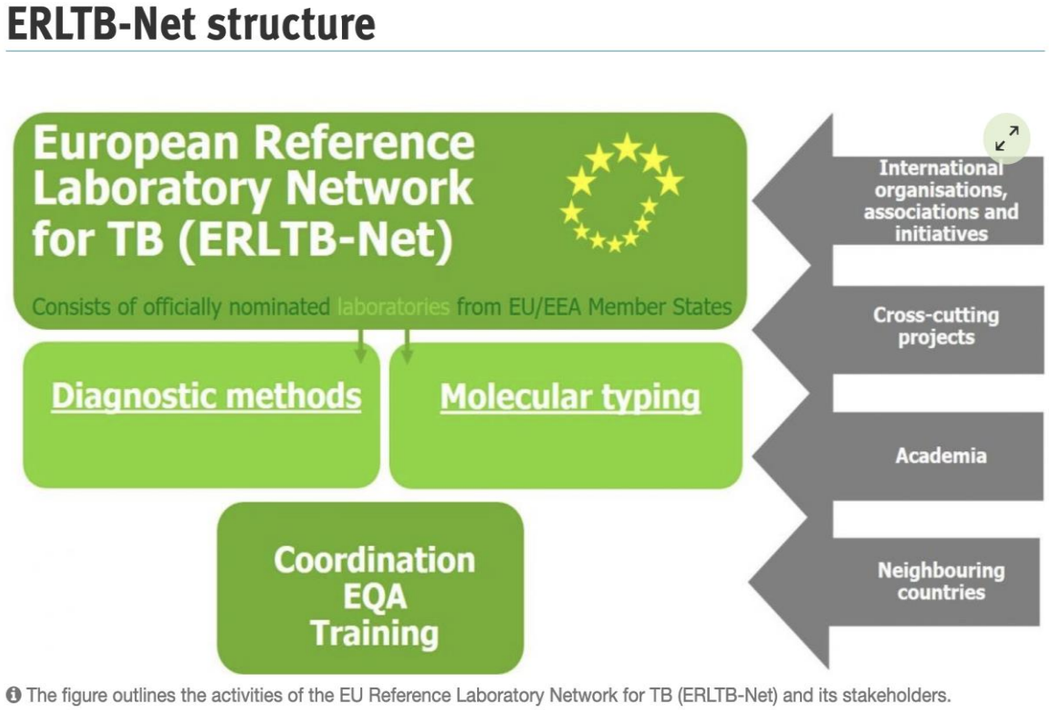
Prediction and Genotypic Drug Profile				No. of Isolates Predicted to Have Profile	No. of Phenotypically Pansusceptible Isolates Predicted to Have Profile (%Predicted Correctly)
Isoniazid	Rifampicin	Ethambutol	Pyrazinamide		
Predicted to be pansusceptible					
S	S	S	S	901	873 (96.89%)
Predicted to be pansusceptible when U mutations are inferred to be consistent with susceptibility					
S	S	S	U	0	0
S	S	U	S	39	39 (100%)
S	S	U	U	0	0
S	U	S	S	23	22 (95.65%)
S	U	U	S	2	2 (100%)
Total				64	63 (98.43%)
Predicted to have some phenotypic resistance					
R	S	R or S, no U or F	R or S, no U or F	44	0
S	At least one R, no U or F			8	0
R	R	R or S, no U or F	R or S, no U or F	27	0
Total				79	0
No prediction made; drug profile prediction incomplete					
U	S or U	S or U	S or U	15	9 (60%)
At least one F, no R				0	0
At least one R and one U, no F				18	0
At least one R and one F, no U				0	0
At least one R and one F and one U				0	0
Total				33	9 (27%)

> 90% of DST to be saved on fully sensitive strains

ERLN TB-NET3

Continue to support the EU/EEA Member States, the EU Enlargement Countries in ensuring the provision of reliable, high quality and timely TB diagnostic services with the specific focus on the challenges of TB control and elimination in the EU settings

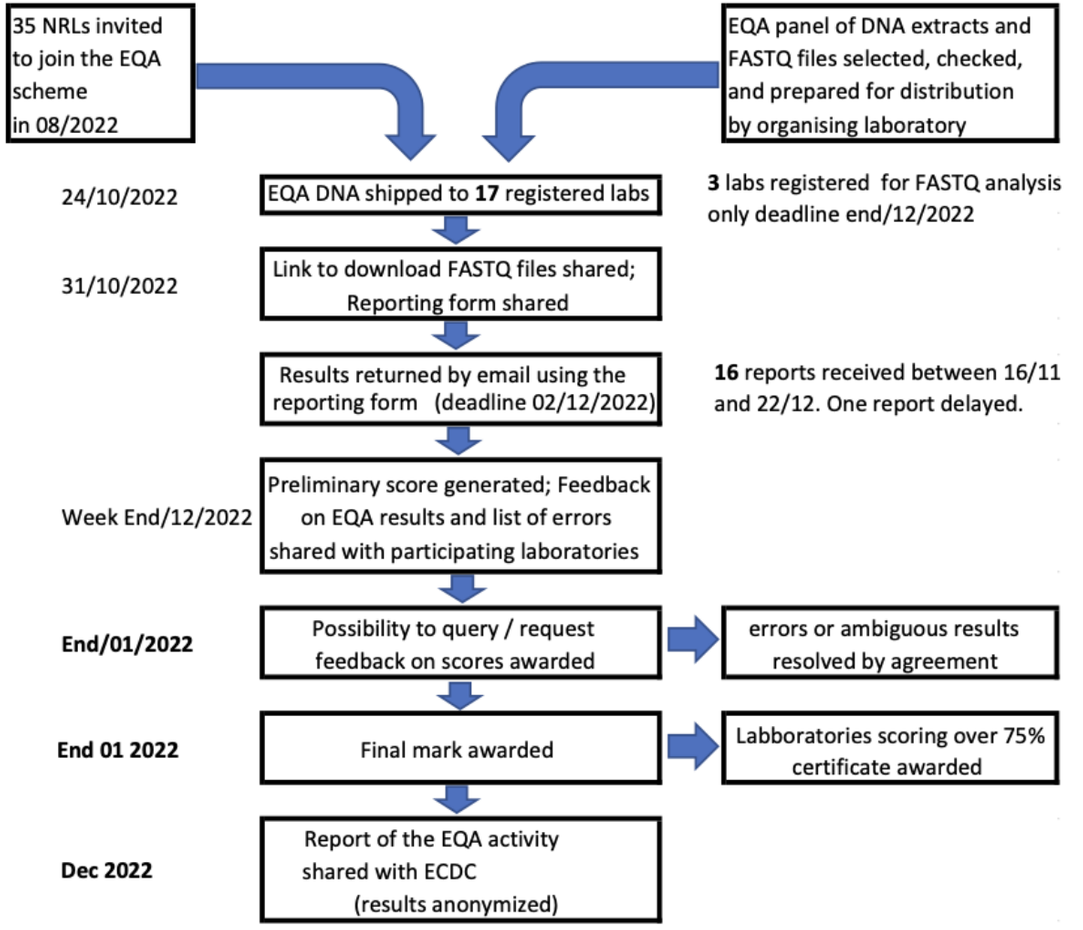
- Establish the current performance of the ERLTB-Net laboratories and identify training needs and areas for improvement
- Support molecular surveillance and related public health needs in the field of TB
- Further develop continuity/resilience arrangements and schemes
- Provide support to the Member States, EU Enlargement Countries in implementation of standardised TB laboratory techniques
- Further develop and implement training schemes
- Strengthen TB laboratory networks and improve TB laboratory diagnosis at community, national and regional levels through building synergies and coordinating actions with other EU and global TB initiatives



EQA

Lab code	Score 2022	2021	2020	2019	2018	2017
1	100	90	98	80	100	90
2	100	86	91	80*	100	85
3	75	90	81	85	88	87
4	95	81	79	80	85	87
5	98	83	100	85**	97	78
6	100	100	100	85		90
7		84	79		49	87
8	98	82	100	90	88	87
9	100	74	79	85	85	89
10		91***	79***	NA***		87
11	100	77****	81	85	100	88
12	100	82****	81	85	100	88
13	90	93	98	90	97	88
14				90	66	
15	90	82	88	95		
16	100	79	88	90		
17	Delayed	77	83			
22	90					
23	100					
24	80					

Type of failure/error	Loss of points
Failure to detect and report a significant mutation.	10
Incorrectly report resistance (resistant without sufficient or no evidence in the WHO catalogue, or no resistance for a mutation associated with resistance).	5
Failure to correctly classify a mutation correctly identified.	2
Genotyping: Failure to identify a sample as a member of a cluster or the incorrect inclusion of a sample in a cluster.	10



NRL = EU/EEU National TB reference laboratories
EQA = External quality assessment

Considerazioni per la discussione

- WGS rappresenta il metodo con **più alta risoluzione per la genotipizzazione e l'analisi filogenetica** dei ceppi di MTBC, consentendo di identificare in modo rapido le catene di trasmissione dell'infezione tubercolare.
- In un paese a bassa incidenza quale l'Italia, un progetto **di sorveglianza molecolare della TB** è **necessario sia per interventi mirati ad interrompere catene di trasmissione**, sia per la sorveglianza delle resistenze.
- Per essere efficace, **dati WGS devono essere forniti in tempi rapidi** e i dati **analizzati in centri di riferimento nazionali e non solo a livello regionale**.
- Dati WGS devono essere **trasferiti sulla piattaforma ECDC (EpiPulse) per un'analisi transnazionale**.
- Dati italiani e europei mostrano come l'implementazione in routine del WGS in centri qualificati con EQA adeguato permetterebbe di non effettuare il DST fenotipico in circa il 90% dei casi.
- tNGS nei casi R resistenti o in cui si sospetti resistenza ad isoniazide (contatti, diagnosi molecolare iniziale)