ReSeqTB
Relational Sequencing TB Data Platform

Update on Phenotypic and NGS-based Advances for Drug Susceptibility Testing and Their Use in Context of Clinical Trials

Marco Schito
Critical Path Institute

Outline

ReSeqTB
Relational Sequencing TB Data Platform

1. **CRyPTIC and the need to capture MIC data for NGS**

2. **Microdilution-based MIC detection for old and new anti-TB drugs**
   - Plate and study design
   - Validation study
   - Preliminary data analysis

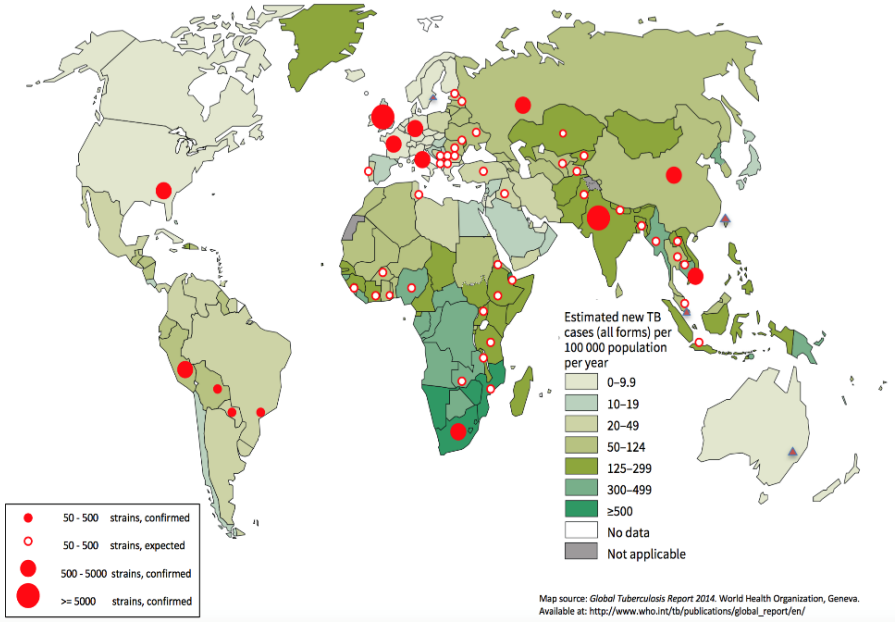
3. **Next generation sequencing**
   - ReSeqTB data sharing platform
     - Standardized and curated drug resistance database
   - Sequencing in clinical trials
     - Heteroresistance
     - Pharmacogenomics

CRyPTIC

Comprehensive Resistance Prediction for Tuberculosis: an International Consortium

Aim:

- Achieve sufficiently accurate genetic prediction of resistance to most of the anti-TB drugs, so that whole genome sequencing replaces culture-based DST



Tools:

- Extremely large number of strains run through the pipeline to capture all possible spectra
- **Handy tool to capture MIC data on subset of strains**
- **Collaboration with CPTR to develop a new lyophilized microdilution Trek plate containing new and repurposed TB drugs**

Use of MICs for optimizing TB treatment

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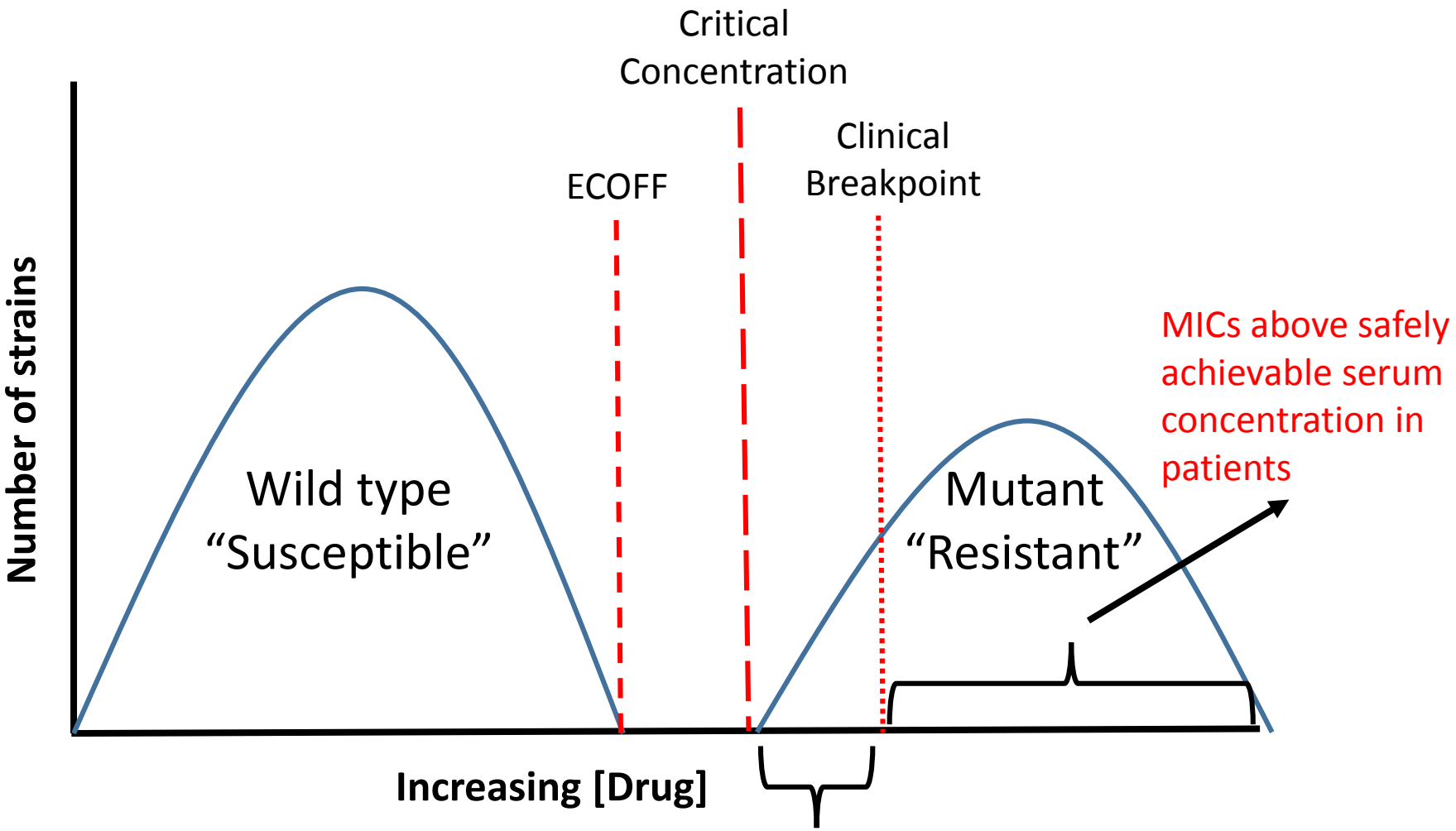

Lost opportunity for optimized dosing if CC is only used

MICs of moxifloxacin by mutation

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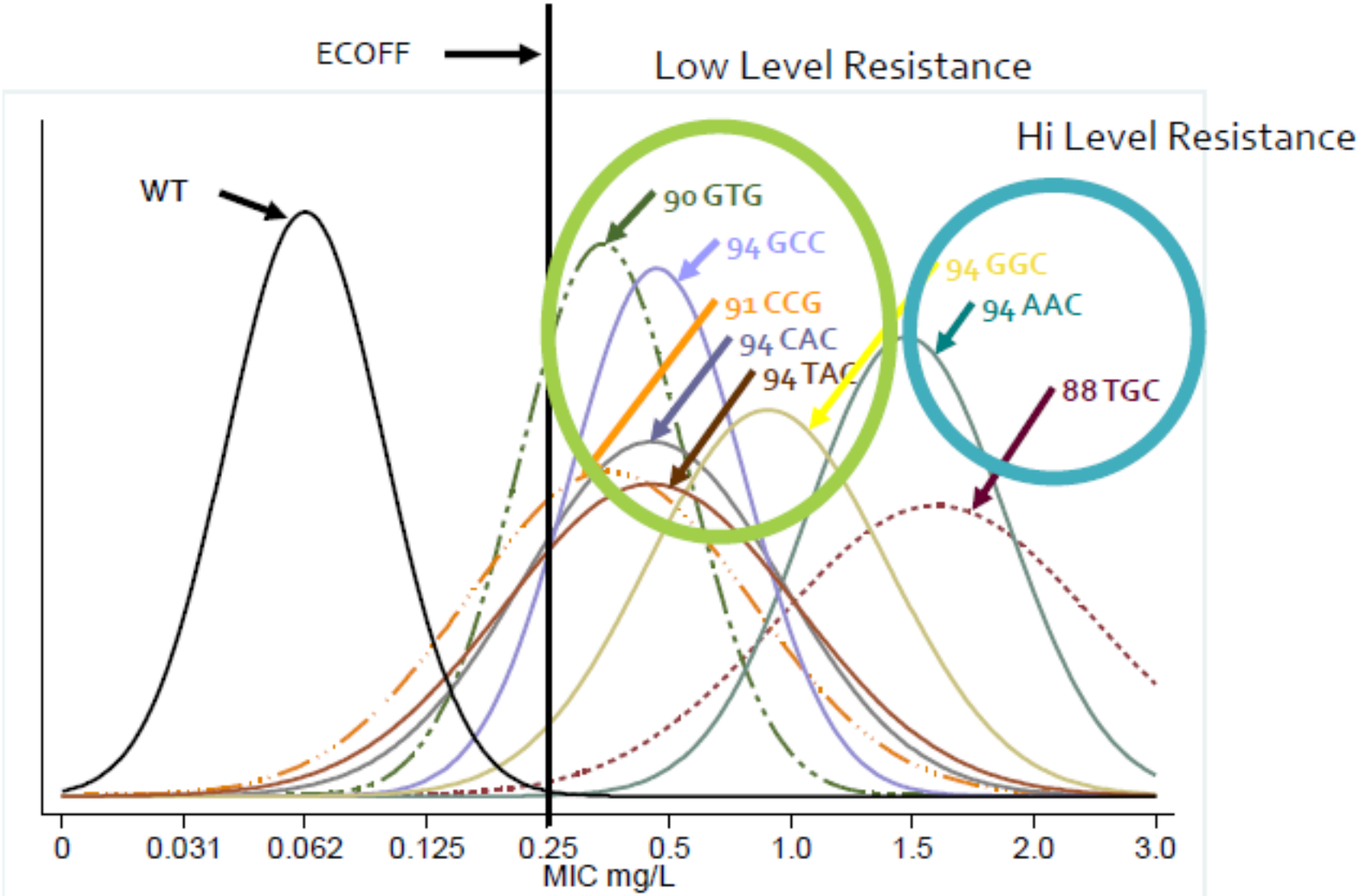

Rodwell et al., unpublished

Rationale: Proportion of patient population reaching therapeutic target for MXF

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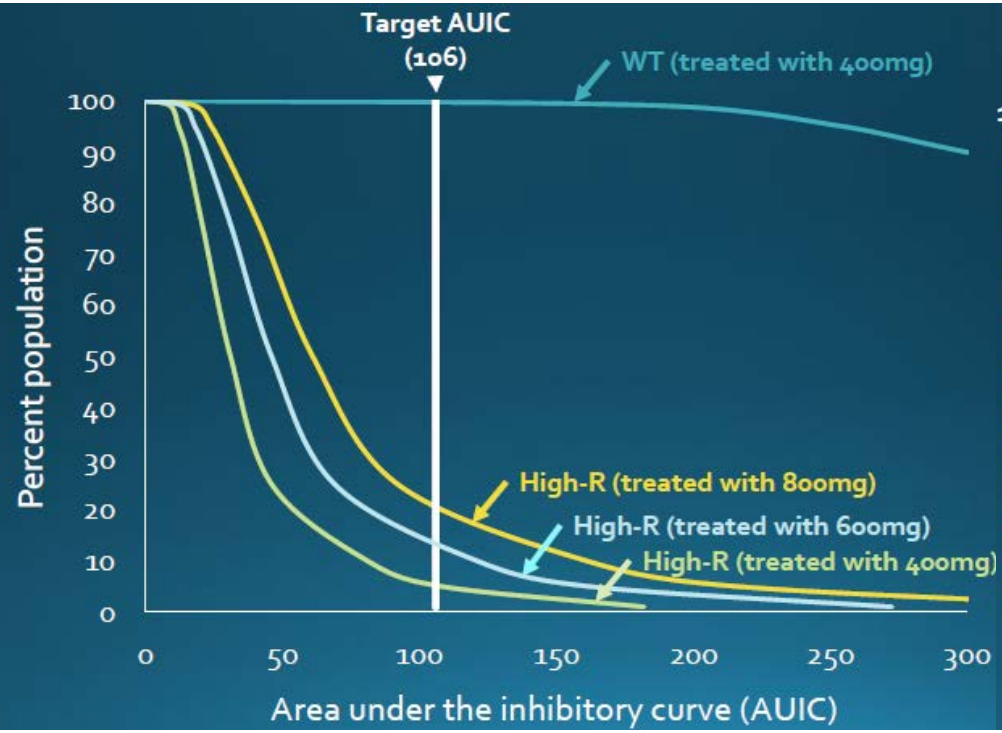
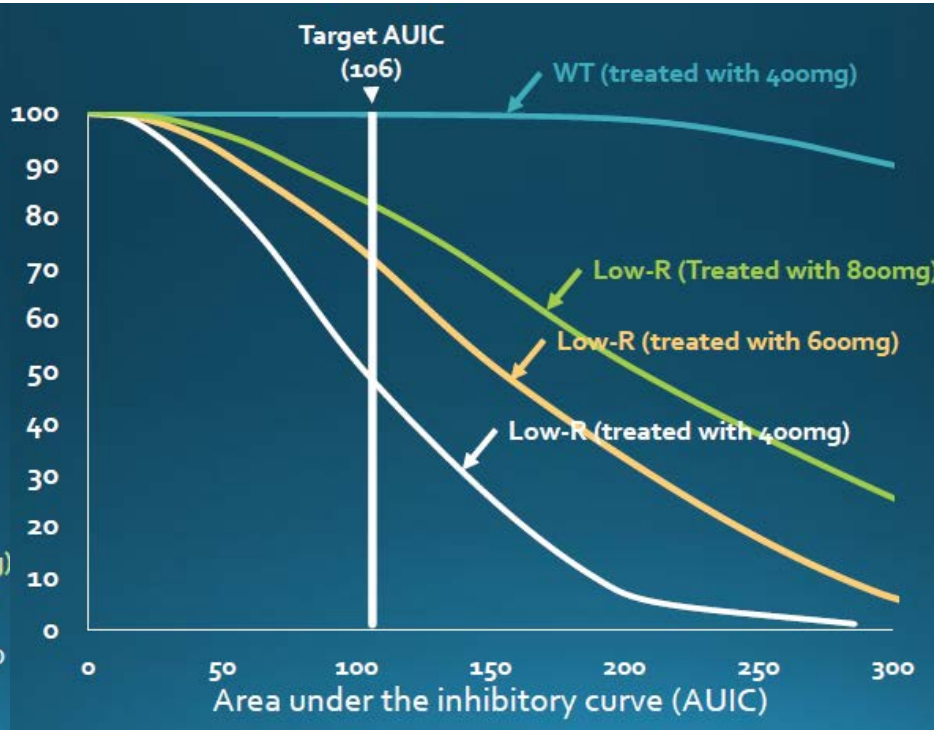
High level *gyrA* resistance mutations



Low level *gyrA* resistance mutations



- Knowing the SNP mutation can dramatically alter patient management by optimizing treatment

Rodwell et al., unpublished

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Trek microtiter validation study design

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- Phase 1: assessment of reference strain H37Rv ATCC 27294, to be tested in 10 replicates

- Phase 2: assessment of external quality control (EQC) panel consisting of 30 strains, CRY1-CRY30 (10 duplicated strains and 10 unique ones, all genotypically and phenotypically characterized), to be tested in duplicates;

- Phase 3: testing of 4,500 clinical isolates

Workflow

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PZA MGIT
DNA extraction
Preparation of mycobacterial inoculum of turbidity ~0.5McF

ThermoFisher SCIENTIFIC

70 assays per center
H37Rv +30 (x2) Clinical strains

3 methods

2 independent readers

4 time points

DAY 7	DAY 10	DAY 14	DAY 21

TOTAL NUMBER OF OBSERVATIONS: 70 * 2 * 4 * 3 = 1,680 X 7 = 11,760 (3,902 images)

Phase 1: Microtitre plate design and distribution ranges of H37Rv over time

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H37Rv ATCC 27294

	1	2	3	4	5	6	7	8	9	10	11	12
A	BDQ 2	KAN 16	KAN 8	KAN 4	KAN 2	KAN 1	ETH 8	ETH 4	ETH 2	ETH 1	ETH 0.5	ETH 0.25
B	BDQ 1	AMI 8	EMB 8	INH 1.6	LEV 8	MXF 4	DLM 1	LZD 2	CFZ 4	RIF 4	RFB 2	PAS 4
C	BDQ 0.5	AMI 4	EMB 4	INH 0.8	LEV 4	MXF 2	DLM 0.5	LZD 1	CFZ 2	RIF 2	RFB 1	PAS 2
D	BDQ 0.25	AMI 2	EMB 2	INH 0.4	LEV 2	MXF 1	DLM 0.25	LZD 0.5	CFZ 1	RIF 1	RFB 0.5	PAS 1
E	BDQ 0.125	AMI 1	EMB 1	INH 0.2	LEV 1	MXF 0.5	DLM 0.125	LZD 0.25	CFZ 0.5	RIF 0.5	RFB 0.25	PAS 0.5
F	BDQ 0.06	AMI 0.5	EMB 0.50	INH 0.1	LEV 0.5	MXF 0.25	DLM 0.06	LZD 0.125	CFZ 0.25	RIF 0.25	RFB 0.125	PAS 0.25
G	BDQ 0.03	AMI 0.25	EMB 0.25	INH 0.05	LEV 0.25	MXF 0.125	DLM 0.03	LZD 0.06	CFZ 0.125	RIF 0.125	RFB 0.0625	PAS 0.125
H	BDQ 0.015	EMB 0.0625	EMB 0.125	INH 0.025	LEV 0.125	MXF 0.0625	DLM 0.015	LZD 0.03	CFZ 0.0625	RIF 0.0625	POS control	POS control

Concentrate on clinically relevant and available drugs
Missing is STM, CM, ETO, CS, AMX, IPM, CLR

Reproducibility testing highlighted training needs and advantages of image capture

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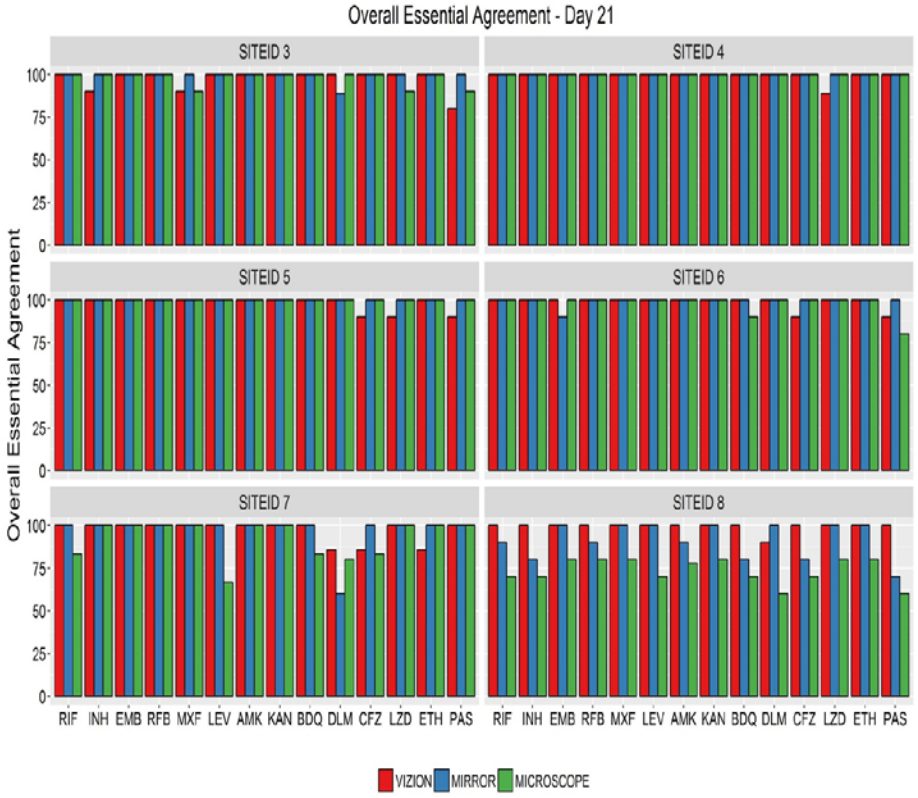
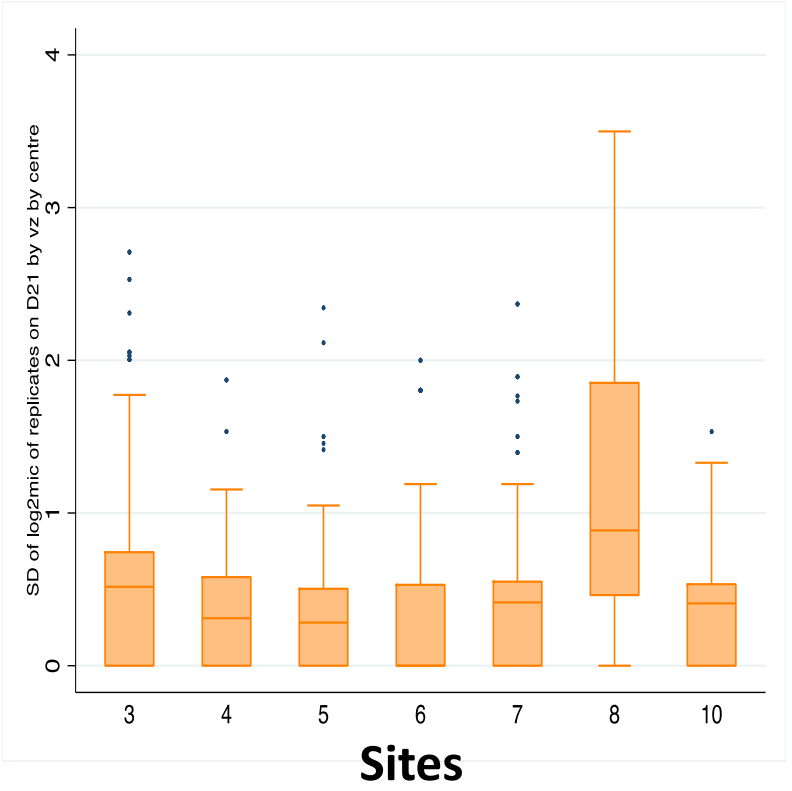
All sites at day 21 – by drug (H37Rv)



Vizion is the most reproducible method in all sites

Standard deviation of Log2 MIC for pooled strains and drugs on Day 21 using Vizion



Consistency in reading across centers is good with one exception

Phase 2: Log2 MIC for each drug on day 21 using Vizion and stratified by MGIT results

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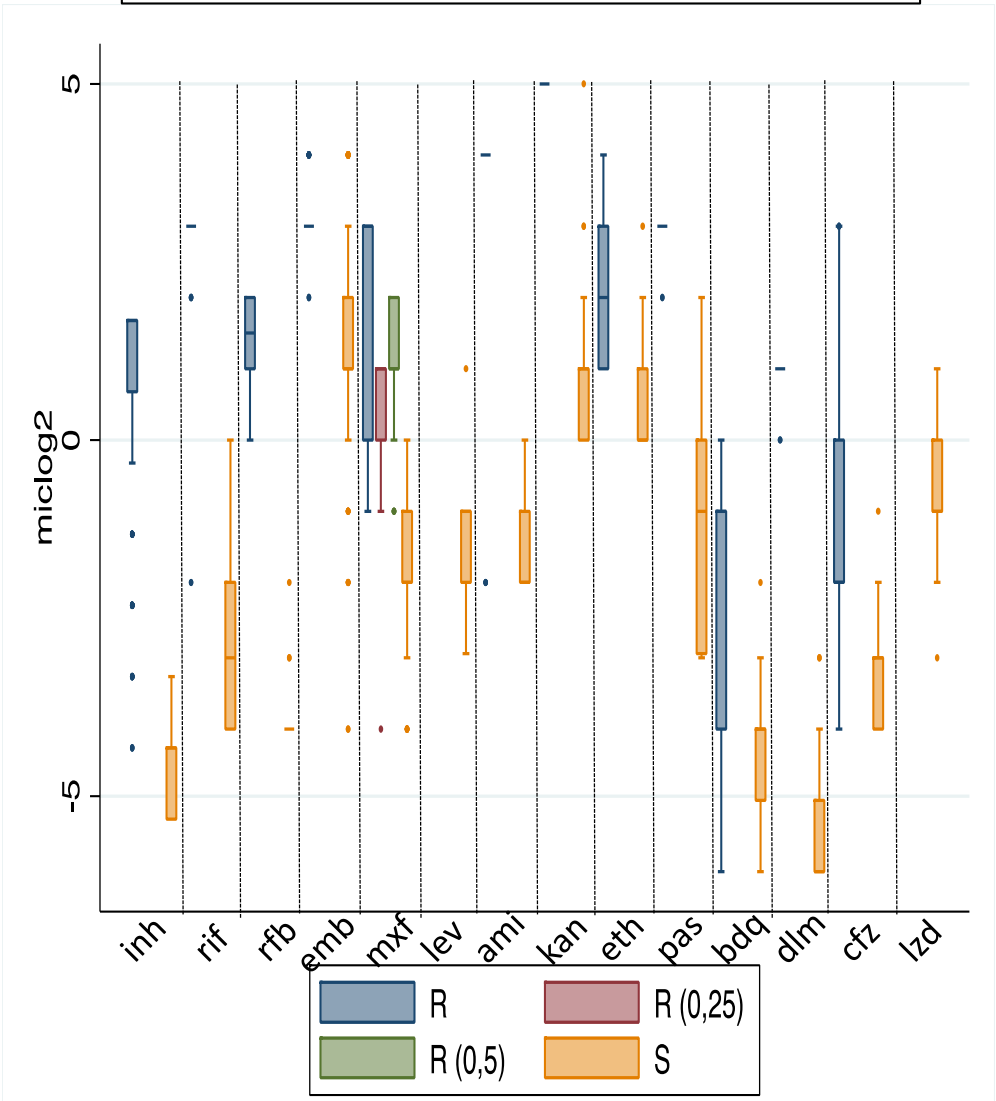
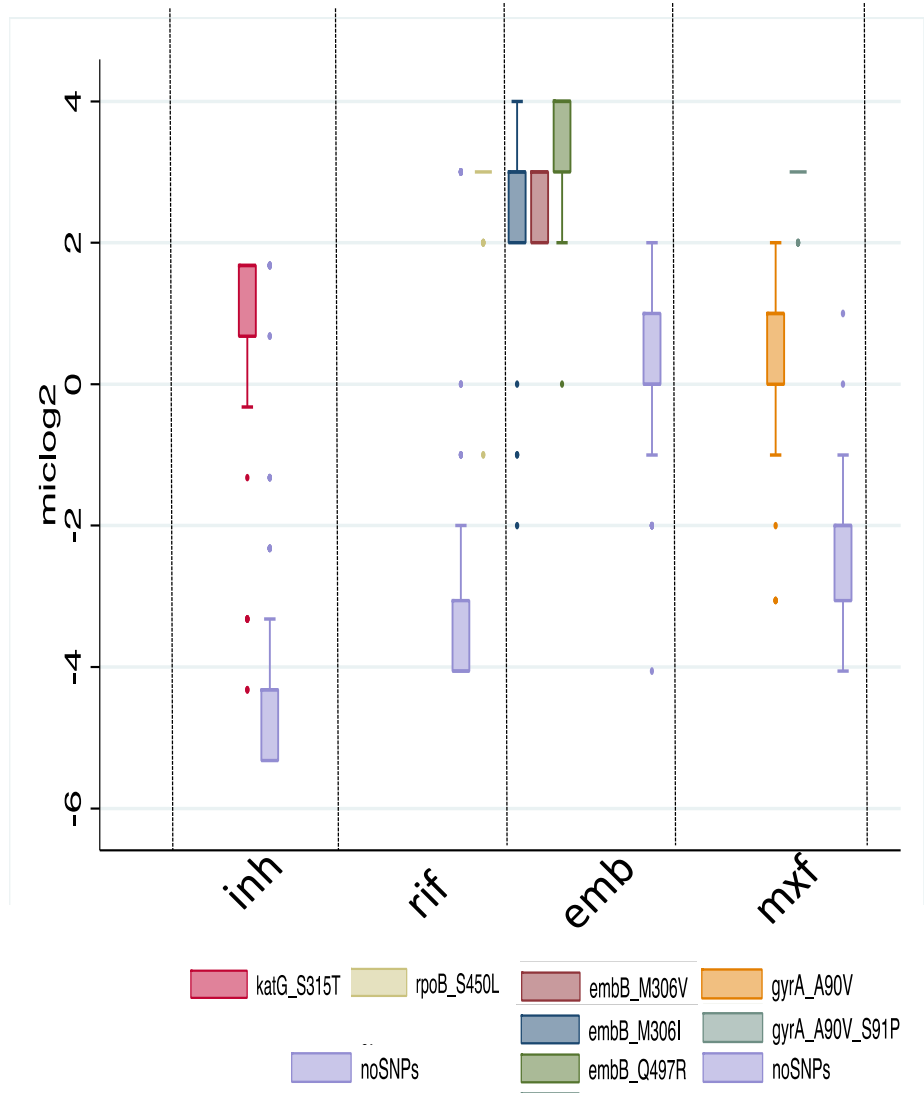
Good separation between S and R



Correlation with WGS data



Phase 3: MIC from drug resistant Pakistan clinical isolates read on Day 14 using Vizion

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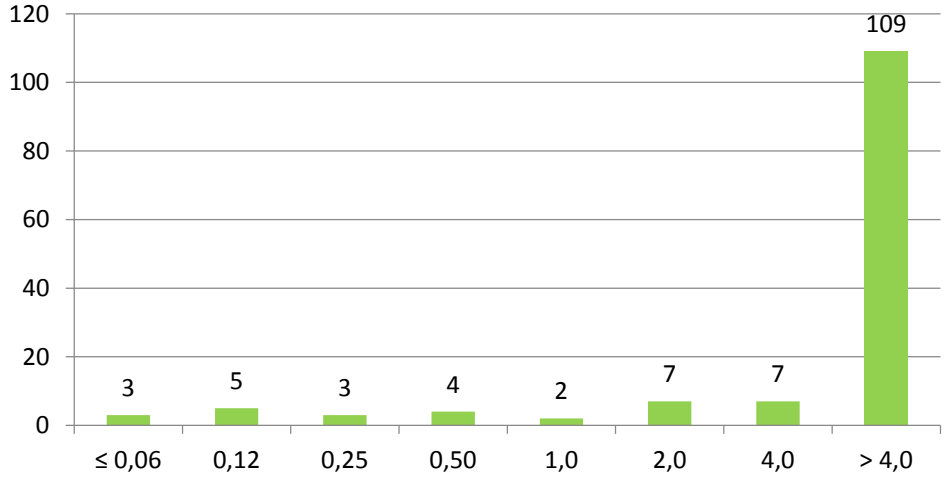
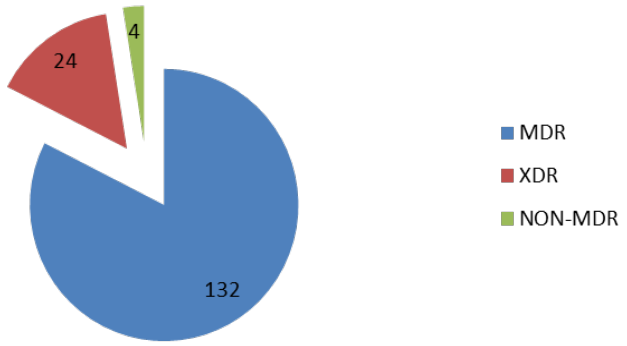
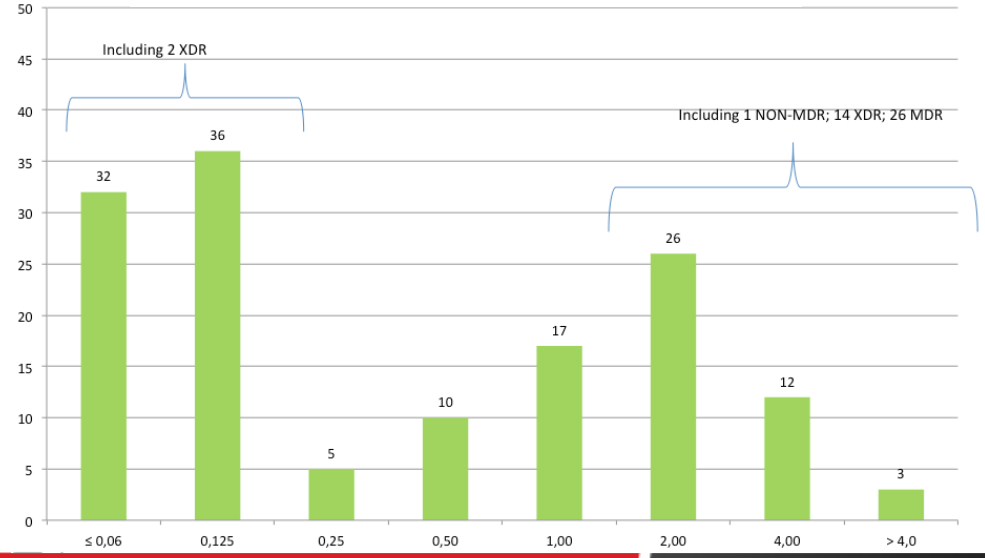
RIF MICs - Vizion ™ - Day 14



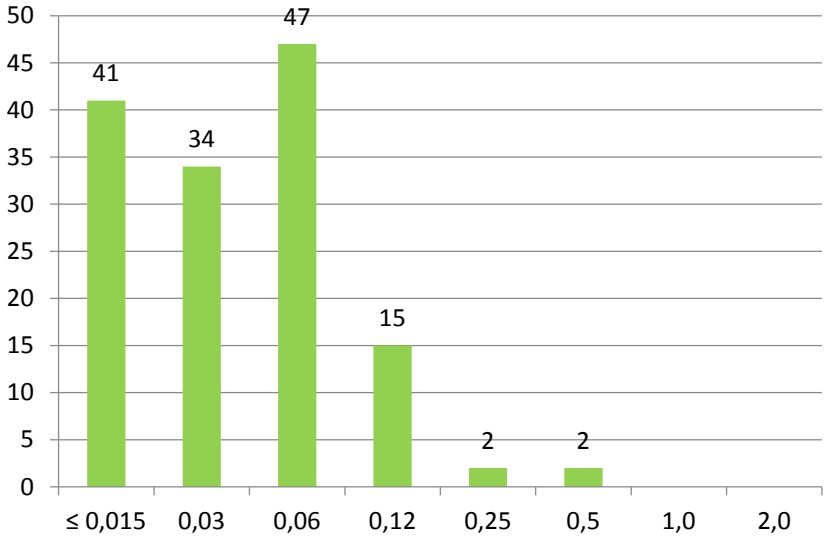
Resistance proile of tested isolates (Pakistan)



MXF MICs – Vizion ™ – Day 14



BDQ MICs - Vizion ™ - Day 14



Microtiter plate conclusions

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- The Thermofisher Vizion™ image capture
  - More concordant readings amongst the three interpretation methods
  - Days 14 and 21 give more reproducible results
  - Automatic reading promising and planned for future

- Despite good reproducibility in phase I, the EQA panel identified training issues in phase II even when using the Thermofisher Vizion™ reader

- More “in site” training is needed to address variation across sites
  - For highly trained sites:
    - agreement was excellent for all drugs and by any interpretation method
    - inter-operator variability is very low both for H37Rv and all the validation strains

- The overall performance of the plates is good at first analysis using both phenotypic (MGIT CC, agar and REMA MICs) and genotypic comparators

- Additional analysis on going

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What are the pros and cons between whole genome and targeted NGS?

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Whole Genome Sequencing

- Strengths
  - Full genome
  - Comprehensive
- Weakness
  - Slow
  - Culture dependent
  - Expensive
  - Bioinformatics

Targeted Next Gen Sequencing

- Strengths
  - Sequence direct from sputum
  - Simpler and faster
  - Deeper sequencing
  - Up to several hundred loci
- Weakness
  - Less information
  - Prior knowledge of targets
  - Optimization

Need for a comprehensive database to provide a priori information regarding *Mtb* drug loci and mutations associated with drug resistance

Who Built ReSeqTB?

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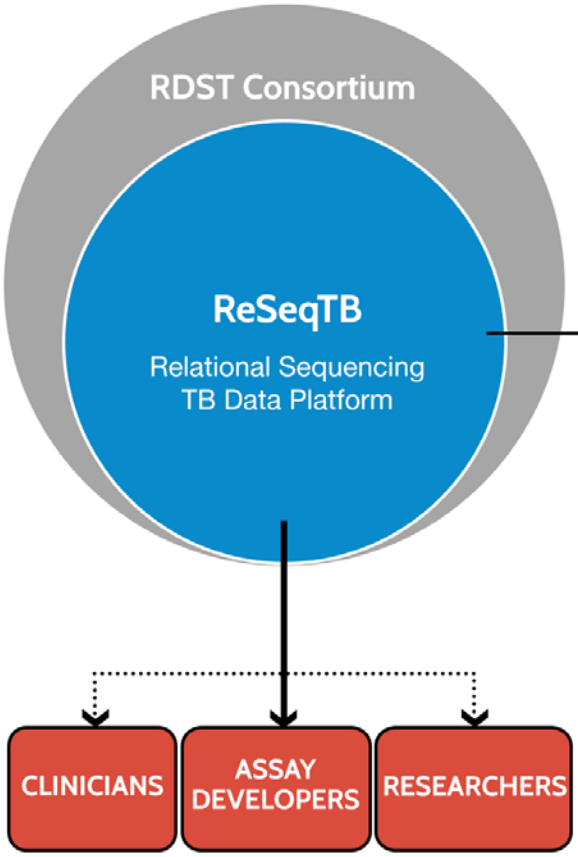


CRITICAL PATH INSTITUTE	C-Path has 10 years of experience with the design and implementation of global data platforms in nine major disease areas, representing data from more than 30,000 patients and growing.
FIND	Over the past 12 years, FIND collaborations have led to the delivery of 11 new diagnostic tools, created an enabling environment for countless more through specimen banks, reagent development and increased market visibility, and supported scale-up of diagnostics through quality assurance and lab strengthening.
World Health Organization	The WHO Global TB Programme aims to advance universal access to TB prevention, care and control, guide the global response to threats, and promote innovation.
CDC Division of Tuberculosis Elimination	The mission of the CDC Division of Tuberculosis Elimination (DTBE) is to promote health and quality of life by preventing, controlling, and eventually eliminating tuberculosis from the United States by collaborating with other countries and international partners in controlling global tuberculosis.
Stop TB Partnership New Diagnostics Working Group	As a Stop TB Partnership working group, the New Diagnostics Working Group (NDWG) is a network of global experts representing academia, governmental and technical agencies, NGOs, diagnostic manufacturers, national TB programmes and the patient community. The mission of the NDWG is to foster development and evaluation of new TB diagnostics by serving as a coordination, communication, and advocacy platform for effective collaboration of all stakeholders in TB diagnostic research and development.
The National Institute of Allergy and Infectious Diseases	The National Institute of Allergy and Infectious Diseases (NIAID) conducts and supports basic and applied research to better understand, treat, and ultimately prevent infectious, immunologic, and allergic diseases.

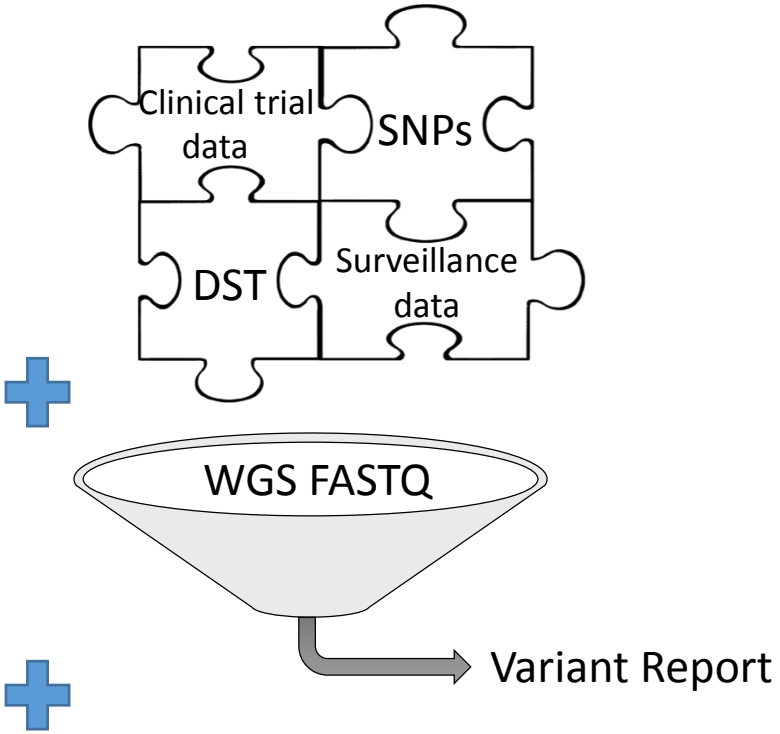
What is ReSeqTB?

ReSeqTB
Relational Sequencing TB Data Platform

1. A curated database of standardized global genotypic, phenotypic and clinical data regarding TB drug resistance

2. A "regulatory-grade" variant detection pipeline standardized and validated for the TB genome

3. A cloud-based data analysis, clinical interpretation and data sharing platform designed for controlled global access by expert and non-expert users



ReSeqTB statistical interpretation with inclusion of homoplasy and "expert" rules

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http:/platform.reseqtb.org

p-value	LR	Interpretation	Symbol
<0.05	≥ 10	**High (Hi) confidence** for association with resistance – strong association of the mutation with phenotypic drug resistance; sufficient evidence that the mutation confers or is strongly associated with drug resistance	
<0.05	5 ≤ … < 10	**Moderate (Mo) confidence** for association with resistance – moderate association of the mutation with phenotypic drug resistance; additional data desirable for improved evidence that the mutation confers or is strongly associated with drug resistance	
<0.05	1 ≤ … < 5	**Minimal (Mi) confidence** for association with resistance – weak association of the mutation with phenotypic drug resistance; inconclusive evidence that the mutation confers or is strongly associated with drug resistance. Substantial additional data required	
<0.05	< 1	**No association with resistance** – No evidence of association between the mutation and drug resistance	
≥0.05	-	**Indeterminate** – no statistically significant threshold reached; additional data required	Indeter

ReSeqTB statistical interpretation: a systematic review of literature (Sanger)

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Drug (phenotypic testing)		Gene	High confidence mutations	Moderate confidence mutations	Minimal confidence mutations	No association with resistance
First-line Rx	RIF	rpoB	D516A, D516F, D516G, D516G+L533P, D516ins, D516N, D516V, D626E, Del N518, F505V+D516Y, F514dupl, H526C, H526D, H526F, H526G, H526L, H526R, H526Y, M515I+D516Y, Q513-F514ins, Q513H+L533P, Q513K, Q513L, Q513P, S512T, S522Q, S531F, S531L, S531Q, S531W	D516Y, H526P, L533P, S522L	H526N, I572F, L511P	
	INH	inhA-mabA	g-102a$^{G\text{-}NC}$	c-15t		g-47c, t-80g, T4I
		katG	S315I, S315N, S315T, Pooled frameshifts and premature Stop codons			A110V, L499M, R463L
		furA				L68F
		mshA		A187V$^{G\text{-}NC}$		N111S
Second-line Rx (group A)	MFX	gyrA	A90V, D94A, D94G, D94N, D94Y, G88C, S91P			E21Q, G247S, G668D, S95T, V712L
	OFX/ LFX	gyrA	A90V, D94A, D94G, D94H, D94N, D94Y, G88A, G88C, S91P	D89N		E21Q, G247S, G668D, S95T, T80A, V712L
		gyrB	A504V, E459K			
Second-line Rx (group B)	AM	rrs	a1401g, g1484t			
	KM	eis	c-14t, g-10a		c-12t, g-37t	a1338c
		rrs	a1401g, a514c^{NC}, c1402t, g1484t			
		rrs+eis	rrs c517t^{NC} + eis g-37t			
	CM	rrs	a1401g, c1402t, g1484t			c517t

Miotto et al. Lancet Respir Med submitted

Sequencing clinical trials

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- Transmission
- Relapse vs re-infection
- Heteroresistance

OPEN ACCESS Freely available online

Elucidating Emergence and Transmission of Multidrug-Resistant Tuberculosis in Treatment Experienced Patients by Whole Genome Sequencing

Taane G. Clark[1,2], Kim Mallard[1], F
Ogwang[4], Francis Mumbowa[4,5], B
Eisenach[7,8], Moses Joloba[5], Stepl
López[9], Ruth McNerney[1*]

1 Faculty of Infectious and Tropical Diseases, L
Population Health, London School of Hygiene &
Kingdom, 4 Joint Clinical Research Centre, Kan
Kampala, Uganda, 6 Mulago Hospital Tuberculosi
Sciences, Little Rock, Arkansas, United States of
of Infectious Diseases, Department of Medicine,
America

Abstract

Background: Understanding the e
its control. MDR-TB in previously t
during inadequate therapy rather t
sufficient to distinguish strains in po
assess the degree of transmissio
tuberculosis strains isolated from tre

Methods and Findings: We u
polymorphisms and large deletions
patients attending a TB referral anc
of MDR-TB cases identified over th
individual patients (2-15 SNPs). Clu
comprising a total of 8 patients we
resistance to rifampicin and isoni
susceptible disease were found to h
another patient in the cohort.

Conclusions: Whole genome seq
patient. The transmission of multi
importance of early detection and r
resistance in patients undergoing tr
avoid onward transmission.

PLOS ONE

Whole-genome sequencing t re-infection with *Mycobacter* observational study

oa

Josephine M Bryant, Simon R Harris, Julian Parkhill, Rodney Dawson, Charoen Chuchot taworn, Ian M Sanne, Cheryl Louw, Martin J Boeree, Solomon Mwaigwisya, Laura Wright, Stephen H Gillespie, Stephen D

Lancet Respir Med 2013; 1: 786–92

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See Comment page 759

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Summary

Background Recurrence of tuberculosis after treatment r
treatment efficacy. Two processes can cause recurren
exogenous strain. Although re-infection can and does
biological basis is still debated. We used whole-geno
typing used to date—to assess the frequency of recurre

Methods We assessed patients from the REMoxTB trial
enrolled previously untreated participants with *Mycoba*
Thailand. We did whole-genome sequencing and m
tandem repeat (MIRU-VNTR) typing of pairs of isolate
another from either the end of failed treatment at 17 w
number and location of SNPs between isolates collecte

Findings We assessed 47 pairs of isolates. Whole-geno
(0–6 SNPs) between strains, deemed relapses, and thr
1419 SNPs, deemed re-infections. Six cases of relapse
whole-genome sequencing and MIRU-VNTR. We detec
least two negative cultures) without clinical evidence of d

Interpretation Whole-genome sequencing enables the
resolution than do genotyping methods used at present
of recurrence. The additional clarity provided by whole
for clinical trials.

Wellcome Trust Sanger Institute, Hinxton, UK (J M Bryant BSc, S R Harris PhD, Prof J Parkhill PhD, S D Bentley PhD); Division of Pulmonology, University of Cape Town, Cape Town, South Africa (Prof R Dawson MD); DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Stellenbosch University, Tygerberg, South Africa (Prof A H Diacon MD, Prof P van Helden PhD); South African Medical Research Council and KwaZulu Research Institute for TB and HIV,

Funding Wellcome Trust, European Union, Medical Research Council, Global Alliance for TB Drug Development, European and Developing Country Clinical Trials Partnership.

PLOS ONE

RESEARCH ARTICLE

Detection of Low-Level Mixed-Population Drug Resistance in *Mycobacterium tuberculosis* Using High Fidelity Amplicon Sequencing

Rebecca E. Colman[1], James M. Schupp[1], Nathan D. Hicks[1], David E. Smith[1], Jordan L. Buchhagen[1], Faramarz Valafar[2], Valeriu Crudu[3], Elena Romancenco[4], Ecaterina Noroc[3], Lynn Jackson[4], Donald G. Catanzaro[5], Timothy C. Rodwell[4], Antonino Catanzaro[4], Paul Keim[1,6], David M. Engelthaler[1]*

1 Translational Genomics Research Institute, Flagstaff, AZ, United States of America, 2 San Diego State University, San Diego, CA, United States of America, 3 Phthisiopneumology Institute (PPI), Chisinau, Republic of Moldova, 4 University of California San Diego, San Diego, CA, United States of America, 5 University of Arkansas College of Education and Health Professions, Fayetteville, AR, United States of America, 6 Center for Microbial Genetics & Genomics, Northern Arizona University, Flagstaff, AZ, United States of America

* dengelthaler@tgen.org

OPEN ACCESS

Citation: Colman RE, Schupp JM, Hicks ND, Smith DE, Buchhagen JL, Valafar F, et al. (2015) Detection of Low-Level Mixed-Population Drug Resistance in *Mycobacterium tuberculosis* Using High Fidelity Amplicon Sequencing. PLoS ONE 10(5): e0126626. doi:10.1371/journal.pone.0126626

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Data Availability Statement: The analysis software is found at https://github.com/TGenNorthSMOR, as referenced in the manuscript, and the amplicon read data is deposited in NIH short read archive (bioproject # PRJNA271805).

Abstract

Undetected and untreated, low-levels of drug resistant (DR) subpopulations in clinical *Mycobacterium tuberculosis* (*Mtb*) infections may lead to development of DR-tuberculosis, potentially resulting in treatment failure. Current phenotypic DR susceptibility testing has a theoretical potential for 1% sensitivity, is not quantitative, and requires several weeks to complete. The use of "single molecule-overlapping reads" (SMOR) analysis with next generation DNA sequencing for determination of ultra-rare target alleles in complex mixtures provides increased sensitivity over standard DNA sequencing. Ligation free amplicon sequencing with SMOR analysis enables the detection of resistant allele subpopulations at ≥0.1% of the total *Mtb* population in near real-time analysis. We describe the method using standardized mixtures of DNA from resistant and susceptible *Mtb* isolates and the assay's performance for detecting ultra-rare DR subpopulations in DNA extracted directly from clinical sputum samples. SMOR analysis enables rapid near real-time detection and tracking of previously undetectable DR sub-populations in clinical samples allowing for the evaluation of the clinical relevance of low-level DR subpopulations. This will provide insights into interventions aimed at suppressing minor DR subpopulations before they become clinically significant.

Single Molecule Over-lapping Reads (SMOR): Getting past sequencing error

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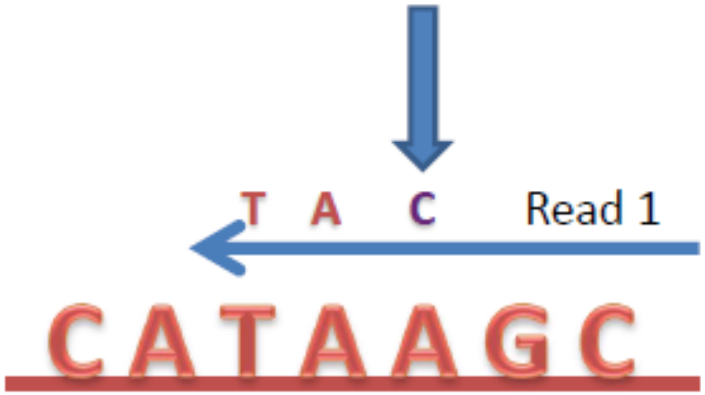
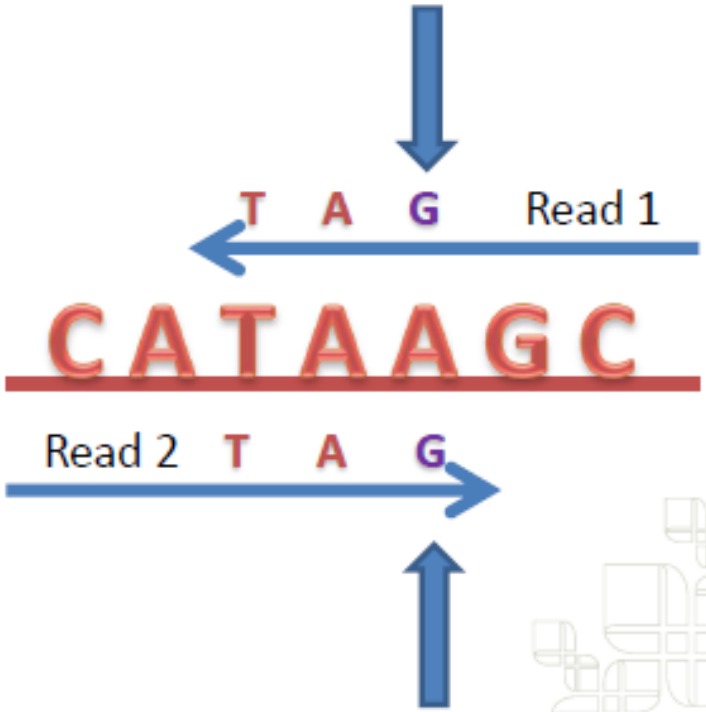
- Standard baseline error for Illumina ~$1X10^{-2}$
- SMOR Error = error on both reads: Product rule





$1X10^{-2}$

$1X10^{-2} \times 1X10^{-2} = 1X10^{-4}$

Colman et al. 2015. PLoS One 10(5):e0126626
Colman et al. 2016. J Clin Microbiol 54(8):2058-67

Potential for SMOR to be more sensitive than culture

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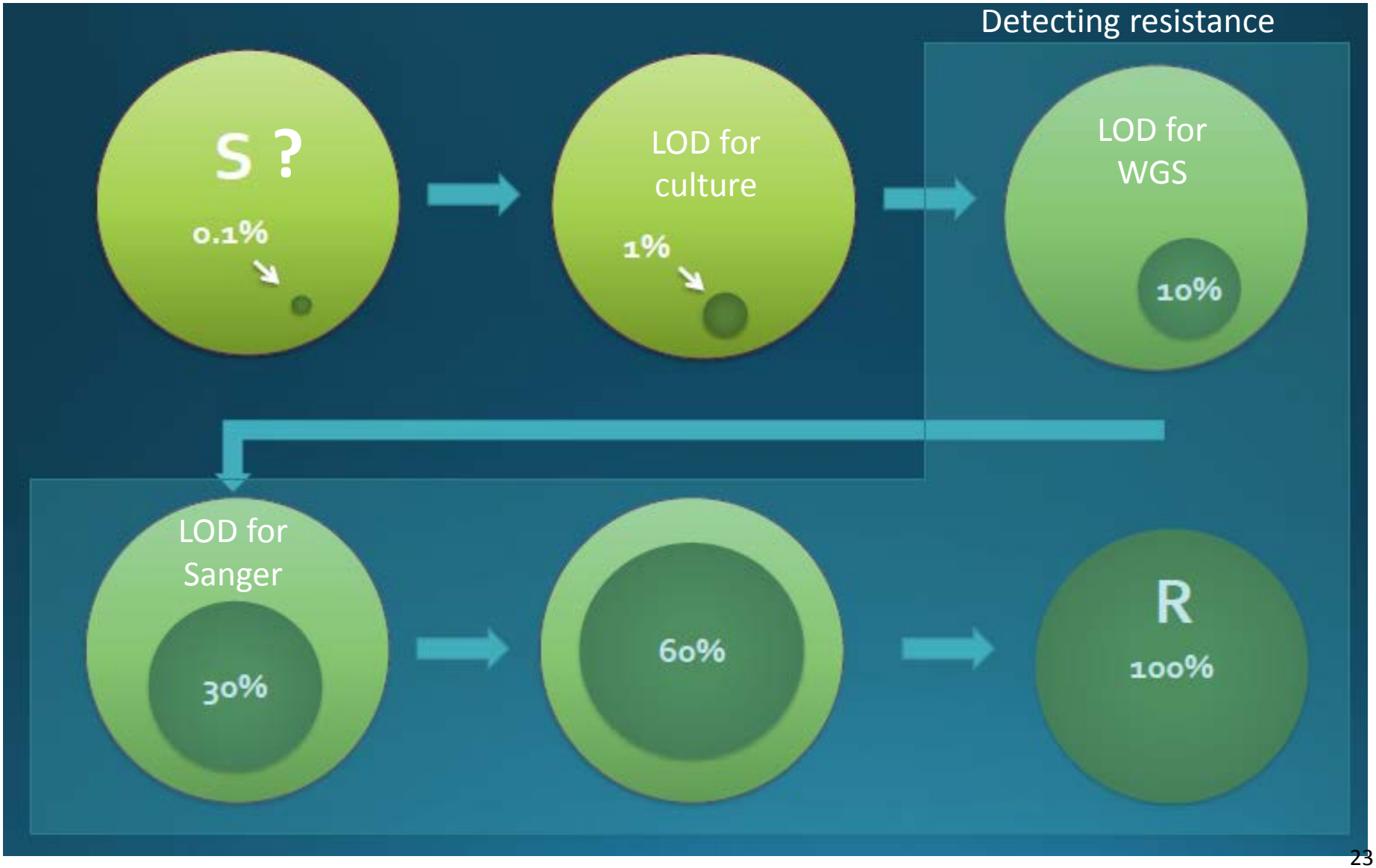

Monitoring heteroresistance (SMOR)

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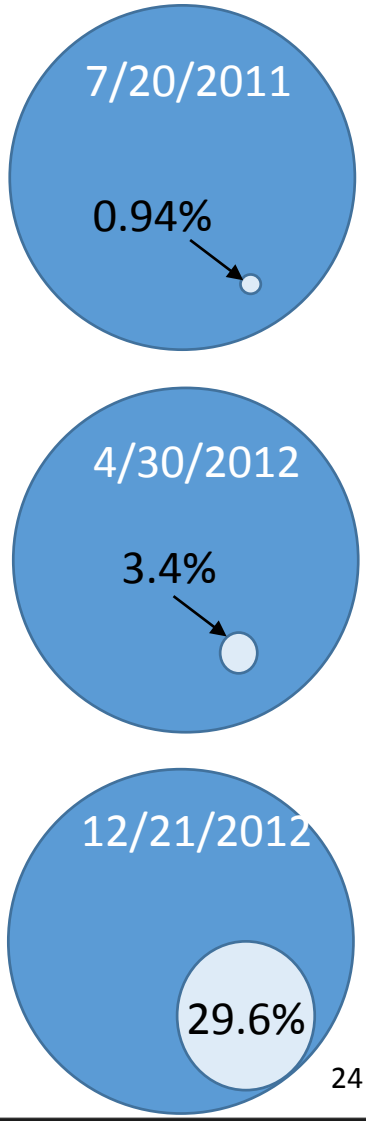
- Serial sputa from Moldova (Crudu)
- DNA extracted from decontaminated sediments

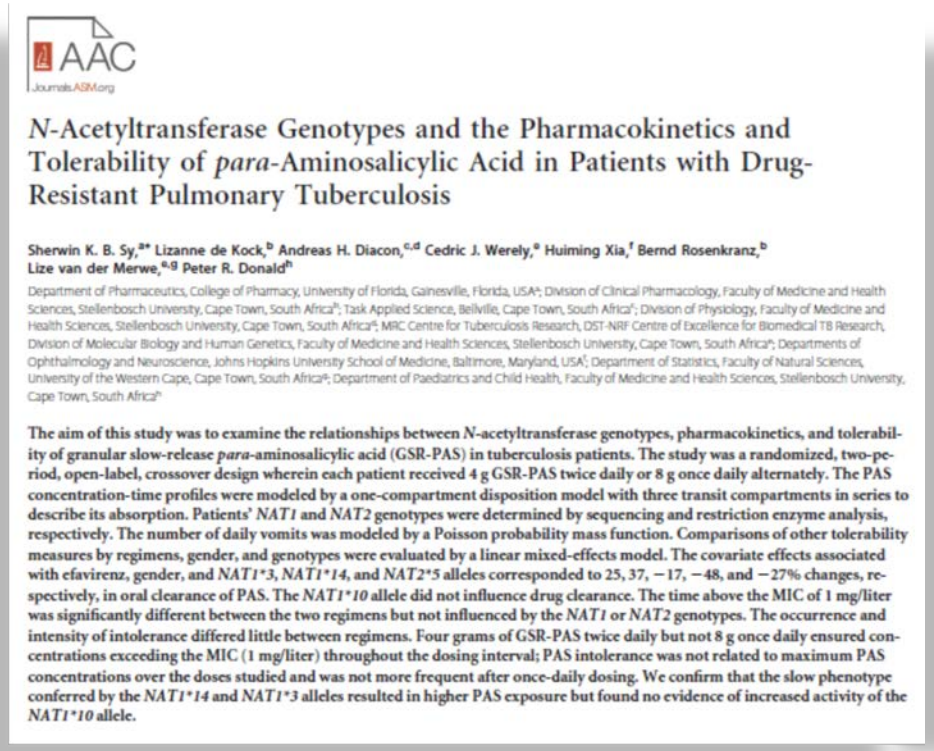
Sample Date	Resistance	AMK DST		rrs SNP (% R Allele)
		Phenotypic	Genotypic	
10/1/2008	MDR	S	S	none
11/19/2008	MDR	S	S	none
10/15/2009	unclassified	S	S	none
1/19/2010	MDR	S	S	none
7/20/2011	MDR	S	S	1401G (0.94%)
9/27/2011	pre-XDR	S	S	none
4/30/2012	pre-XDR	S	S	1401G (3.4%)
12/21/2012	XDR	R	R	1401G (29.6%)



Engelthaler, Metcalfe, Warren. Unpublished data

Can NGS be used to assess host pharmacogenetics?

ReSeqTB
Relational Sequencing TB Data Platform



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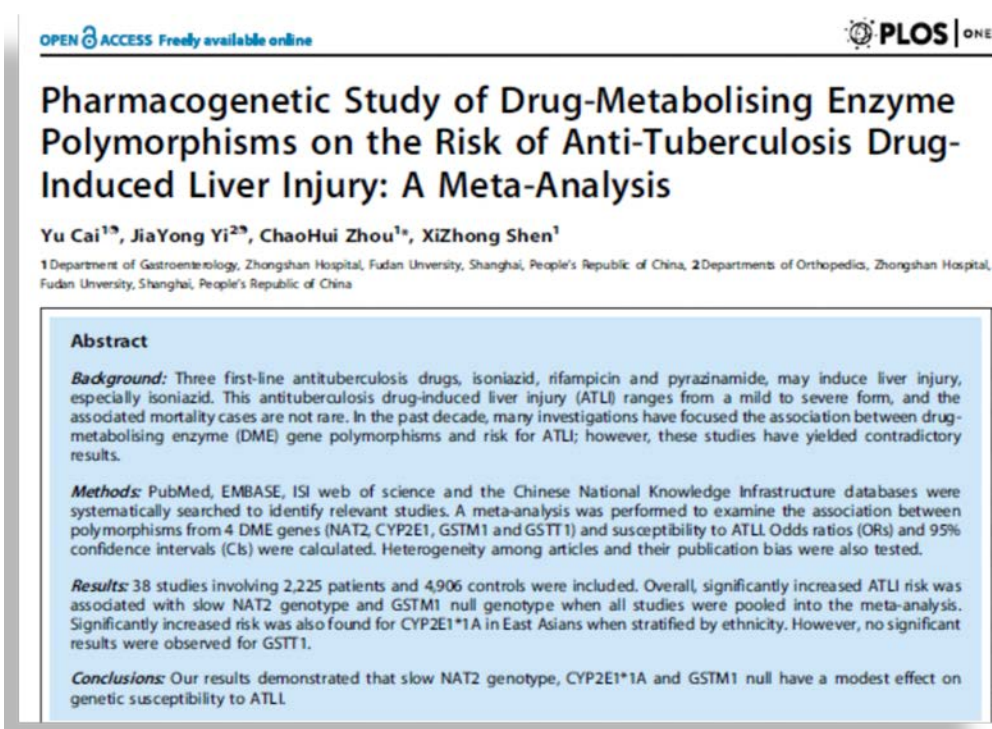
N-Acetyltransferase Genotypes and the Pharmacokinetics and Tolerability of *para*-Aminosalicylic Acid in Patients with Drug-Resistant Pulmonary Tuberculosis

Sherwin K. B. Sy,[a*] Lizanne de Kock,[b] Andreas H. Diacon,[c,d] Cedric J. Werely,[e] Huiming Xia,[f] Bernd Rosenkranz,[b] Lize van der Merwe,[g,g] Peter R. Donald[h]

Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, Florida, USA[a]; Division of Clinical Pharmacology, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa[b]; Task Applied Science, Bellville, Cape Town, South Africa[c]; Division of Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa[d]; MRC Centre for Tuberculosis Research, DST-NRF Centre of Excellence for Biomedical TB Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa[e]; Departments of Ophthalmology and Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA[f]; Department of Statistics, Faculty of Natural Sciences, University of the Western Cape, Cape Town, South Africa[g]; Department of Paediatrics and Child Health, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa[h]

The aim of this study was to examine the relationships between *N*-acetyltransferase genotypes, pharmacokinetics, and tolerability of granular slow-release *para*-aminosalicylic acid (GSR-PAS) in tuberculosis patients. The study was a randomized, two-period, open-label, crossover design wherein each patient received 4 g GSR-PAS twice daily or 8 g once daily alternately. The PAS concentration-time profiles were modeled by a one-compartment disposition model with three transit compartments in series to describe its absorption. Patients' *NAT1* and *NAT2* genotypes were determined by sequencing and restriction enzyme analysis, respectively. The number of daily vomits was modeled by a Poisson probability mass function. Comparisons of other tolerability measures by regimens, gender, and genotypes were evaluated by a linear mixed-effects model. The covariate effects associated with efavirenz, gender, and *NAT1*3*, *NAT1*14*, and *NAT2*5* alleles corresponded to 25, 37, −17, −48, and −27% changes, respectively, in oral clearance of PAS. The *NAT1*10* allele did not influence drug clearance. The time above the MIC of 1 mg/liter was significantly different between the two regimens but not influenced by the *NAT1* or *NAT2* genotypes. The occurrence and intensity of intolerance differed little between regimens. Four grams of GSR-PAS twice daily but not 8 g once daily ensured concentrations exceeding the MIC (1 mg/liter) throughout the dosing interval; PAS intolerance was not related to maximum PAS concentrations over the doses studied and was not more frequent after once-daily dosing. We confirm that the slow phenotype conferred by the *NAT1*14* and *NAT1*3* alleles resulted in higher PAS exposure but found no evidence of increased activity of the *NAT1*10* allele.



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PLOS ONE

Pharmacogenetic Study of Drug-Metabolising Enzyme Polymorphisms on the Risk of Anti-Tuberculosis Drug-Induced Liver Injury: A Meta-Analysis

Yu Cai[1☯], JiaYong Yi[2☯], ChaoHui Zhou[1*], XiZhong Shen[1]

1 Department of Gastroenterology, Zhongshan Hospital, Fudan University, Shanghai, People's Republic of China, 2 Departments of Orthopedics, Zhongshan Hospital, Fudan University, Shanghai, People's Republic of China

Abstract

Background: Three first-line antituberculosis drugs, isoniazid, rifampicin and pyrazinamide, may induce liver injury, especially isoniazid. This antituberculosis drug-induced liver injury (ATLI) ranges from a mild to severe form, and the associated mortality cases are not rare. In the past decade, many investigations have focused the association between drug-metabolising enzyme (DME) gene polymorphisms and risk for ATLI; however, these studies have yielded contradictory results.

Methods: PubMed, EMBASE, ISI web of science and the Chinese National Knowledge Infrastructure databases were systematically searched to identify relevant studies. A meta-analysis was performed to examine the association between polymorphisms from 4 DME genes (NAT2, CYP2E1, GSTM1 and GSTT1) and susceptibility to ATLI. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Heterogeneity among articles and their publication bias were also tested.

Results: 38 studies involving 2,225 patients and 4,906 controls were included. Overall, significantly increased ATLI risk was associated with slow NAT2 genotype and GSTM1 null genotype when all studies were pooled into the meta-analysis. Significantly increased risk was also found for CYP2E1*1A in East Asians when stratified by ethnicity. However, no significant results were observed for GSTT1.

Conclusions: Our results demonstrated that slow NAT2 genotype, CYP2E1*1A and GSTM1 null have a modest effect on genetic susceptibility to ATLI.

We observed the slow phenotype of NAT1*14 and NAT1*3 alleles was associated with greater PAS exposure. (Antimicrob Agents Chemother. 2015; 59(7): 4129-38)

…our meta-analysis indicates that CYP2E1, NAT2 and GSTM1 genetic variation is significantly associated with anti-tuberculosis drug-induced liver injury. (PLoS One. 2012; 7(10): e47769.)

Human genes associated with anti-TB drug-induced adverse reactions

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Drug	Adverse Drug Reaction	Gene	Gene class	# SNPs *
RIF	Thrombocytopenia	*GPIX*	Receptor	2
INH	Hepatotoxicity	*NAT2*	DME-1	11
		CYP2E1	DME-1	10
		GSTM1	DME-2	3
		GSTT1	DME-2	2
PZA	Hepatotoxicity	*XDH*	DME	8
	Nephrotoxicity	*SLC22A12*	Transporter	10
EMB	Optic neuritis	*OPA1*	GTPase	12
AG	Nephrotoxicity	*LRP2*	Receptor	12
	Ototoxicity	*MYO7a*	Transporter	5

* SNP frequencies are population dependent

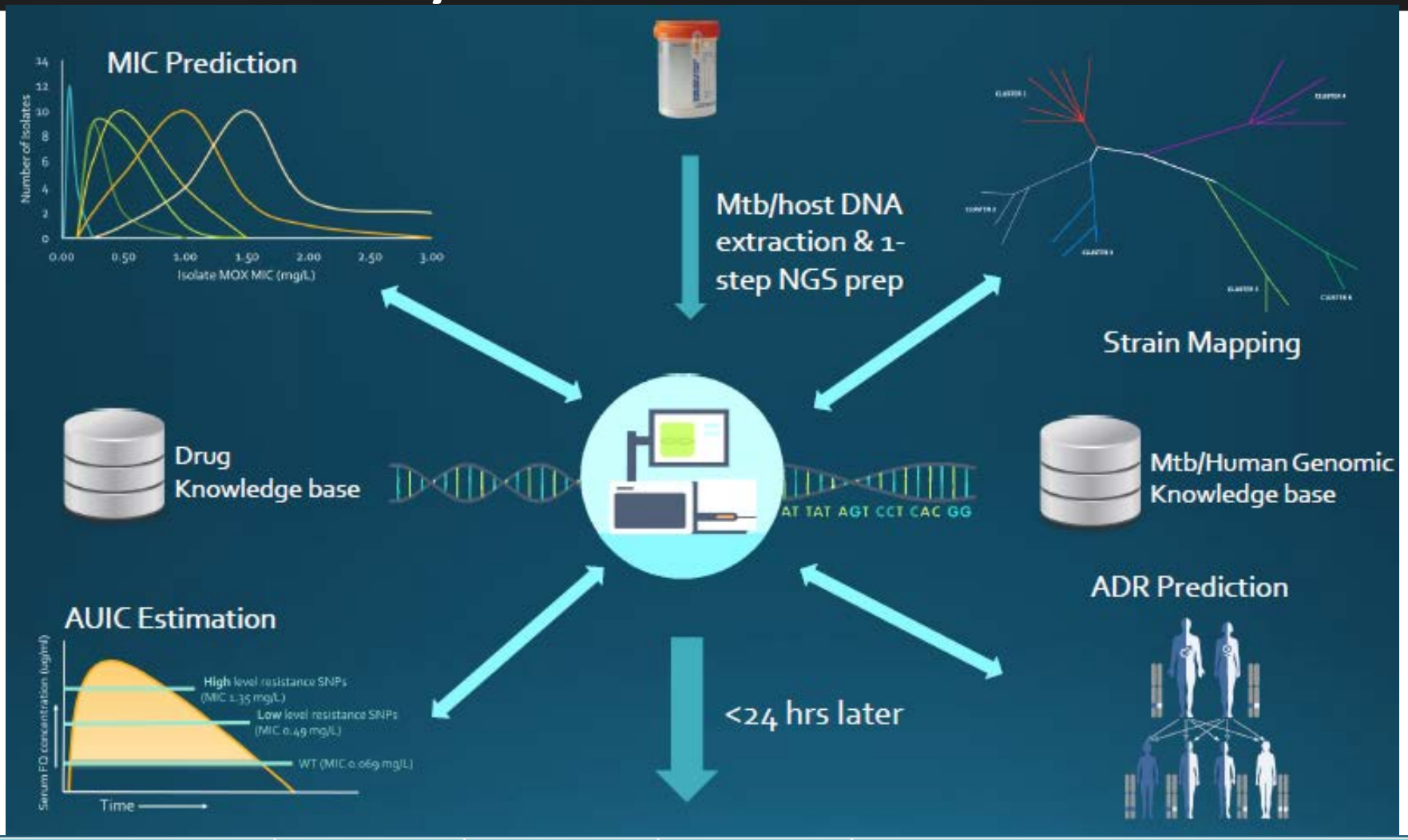
Plasma levels of drug -> influence therapeutic window

1. If levels increase -> approach MTD and accumulation of **toxic** metabolites
2. If levels decrease -> reduce treatment efficacy
   - Incomplete eradication of bacilli -> prolonged treatment and **relapse**
   - Increase chance of developing DR

Adapted from Sahu et al. 2015. Curr Drug Metabol. 16(7): 538-52

Targeted NGS technology to optimize treatment efficacy and reduce AE

ReSeqTB
Relational Sequencing TB Data Platform



Mtb Mutation	Predicted MOX MIC	Outbreak Strain	G6PD Deficiency	Recommendation
15% gyrA 91 CCG	0.3-0.5	N	N	MOX 800 mg (83% prob. of efficacy)

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