The laboratory diagnosis of tuberculosis: update and news

Daniela Cirillo
Emerging Bacterial Pathogens Unit (EBPU), San Raffaele Scientific Institute, via Olgettina 58, 20132 Milan, Italy
WHOcc

22th Tunisian Congress of Infectious Diseases
2nd Arab Congress of Clinical Microbiology and Infectious Diseases

Main actions for TB Control

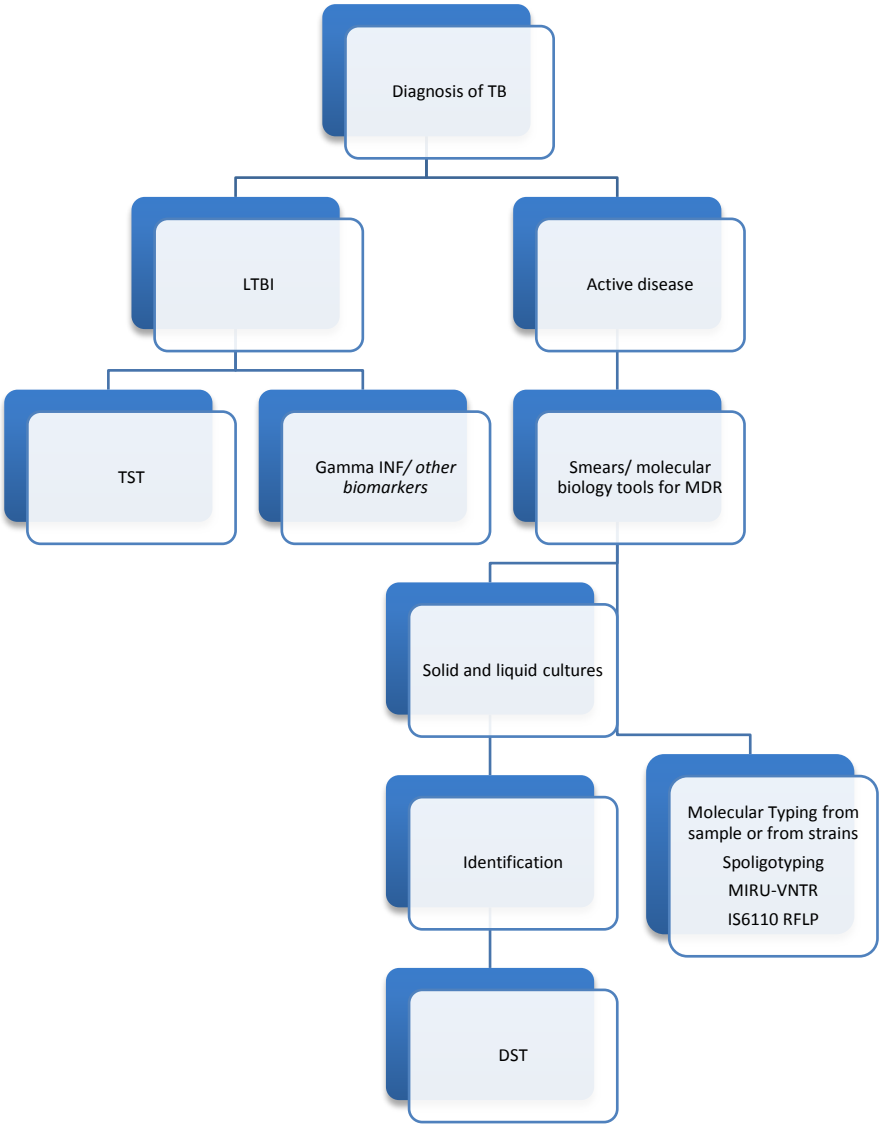
- Prevention of new infections
- Fast Detection of active infectious cases (potentially all)
- Providing effective treatment

Laboratory Diagnosis is part of the “core business” in TB control

Outline

- Diagnostic tests for TB:
  - What is new on microscopy, culture, DST
  - Molecular detection of MDRTB:
    - LPAs
    - Xpert
  - Diagnostic Algorithms
  - New Prospectives

Laboratory involvement



Role of commercial immunology based diagnostics for active TB

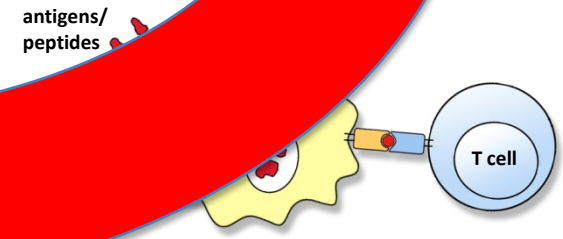
Serological tests

feron gamma release
s (IGRAs)

Antibo nst
Mtb-spe tigens

Local immune
(PPD-spe

Mtb-spec 's
(PPD, ESAT- 0)



General overview

- Diagnostic algorithms depend on TB epidemiology, human and infrastructure capacity, financial resources and sustainability
- Independently from the selected algorithm a robust and integrated network of Tb laboratories under the direction of a NRL is required
- All NRLs should aim to reach accreditation

GLI road map at :http://www.who.int/tb/dots/laboratory/policy/en

Biosafety

- Mtb is a class 3 risk pathogen
- All biosafety strategies (minimum requirements) should be based **on risk assessment**
- Based on:
  - *Bacillary load of samples and workload*
  - *Viability of bacilli*
  - *Aerosol generation*
  - *TB local epidemiology*
  - *Fitness of the staff*

Microscopy

Rapid test

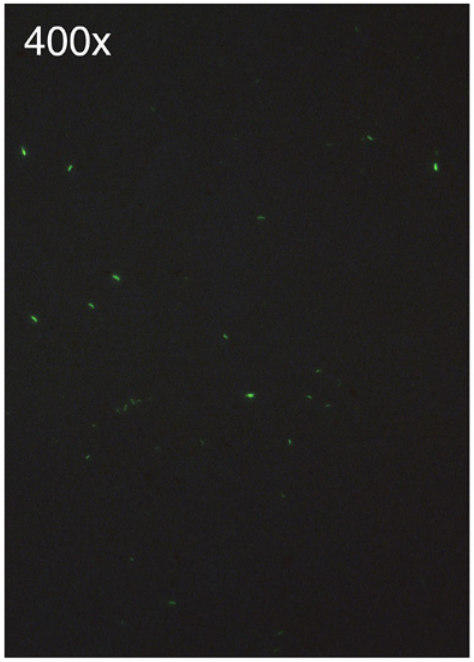
Inexpensive

Does not allow species identification

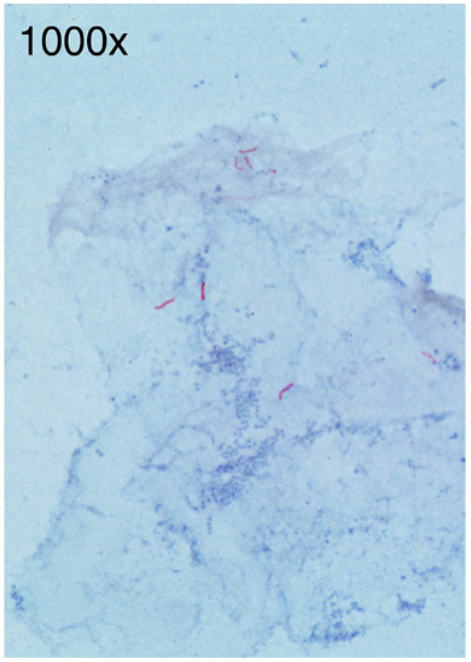
Not applicable to all samples

- Specificity for Mycobacterium spp: >95%
- Sensitivity: 25-65% (90 % of higly infectious cases)
- Positive Predictive Value for TB depends on epidemiological situation

LED microscopy recommended over light and fluorescent microscopy



Fluorescence



Ziehl-Neelsen staining

1st AFB smear	80-82 %
2nd AFB smear	10-14 %
3rd AFB smear	*5-8 %*

Microscopy: WHO 2010

- ZN light microscopy performed on UNCONCENTRATED sputum is suitable for all laboratory service levels
- Concentration of sputum is NOT recommended in programmatic settings
- **Fluorescence microscopy is recommended for increased sensitivity (add 10%)**
- **LED microscopy is recommended over conventional fluorescence**

TB Culture

Advantages

- Definitive diagnosis of TB
- Increases case finding of 30-50%
- Early detection of cases
- Provide strains for DST and epidemiological studies

Disadvantages

- Complex and expensive compared to microscopy
- Requires complex handling of specimens
- Skilled technicians
- Appropriate infrastructure and biosafety levels

LIMITATIONS: need for decontamination and identification

*coverage 500.000/1000000

Culture: solid/ liquid

solid

- Low cost for reagents, not automated
- Culture level infrastructure
- Low contamination rate
- Long time to positivity
- Colony morphology
- ID required
- DST only for selected drugs

liquid

- Complex and expensive can be automated (MGIT)
- Highest infrastructure and biosafety levels
- Case finding increased 10% over solid
- Diagnostic delay reduced to days
- ID required
- DST only for selected drugs

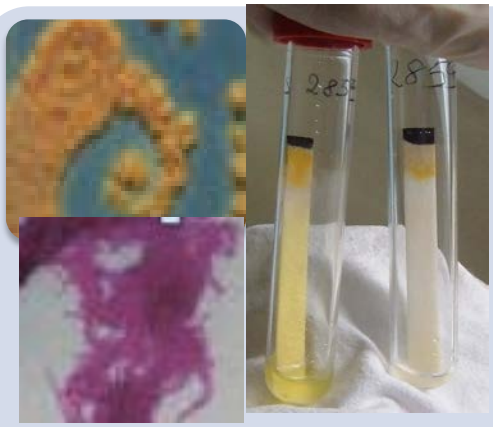
Strip speciation tests for fast ID of Tbcomplex
Molecular test for speciation of most common mycobacteria

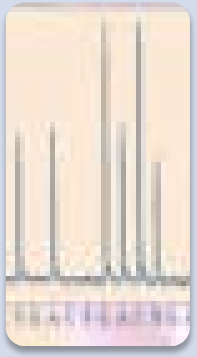
MGIT 960

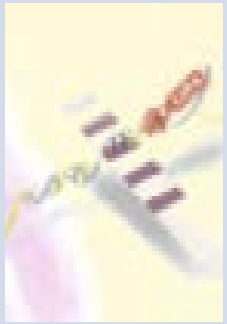
- Automatic system for mycobacteria detection
- DST automatic (1999)
- Non radiometric
- Fluorescence BBL® MGIT™
- Non invasive (no needles), totally automatic and computerized
- High workload
- No blood culture

- ❑ Mycobacteria liquid medium
- ❑ Rutenium salt registers oxygen variation
- ❑ Oxygen consumption by bacterial metabolism releases fluorescence
- ❑ Fluorescence is detected manually by UV lamp or automated

M. tuberculosis identification



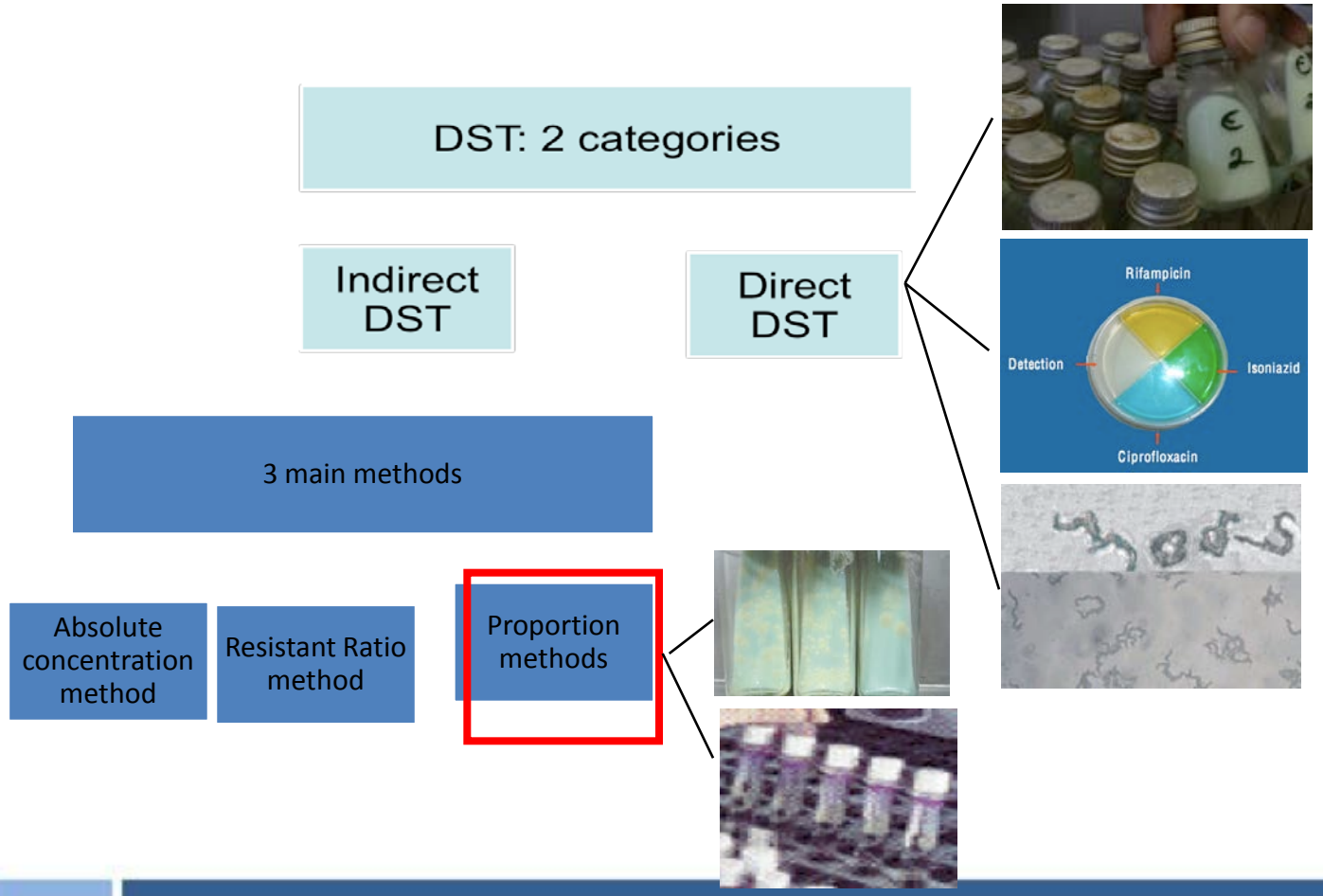




Morphology/Biochemical tests	Molecular tests	Immuno-cromatographic test
	LPAs	
	Probes on liquid phase	
	Sequencing	
	Spoligotyping	
	Enzyme restriction	

DST

- Definitive diagnosis of DRTB



Liquid/solid media comparison

Advantages compared to solid media:

- more rapid
- high quality of media
- fully automated system
- testing of 1st, 2nd , and new drugs (Linezolid)
- safety: plastic tubes
- pyrazinamide sensitivity test

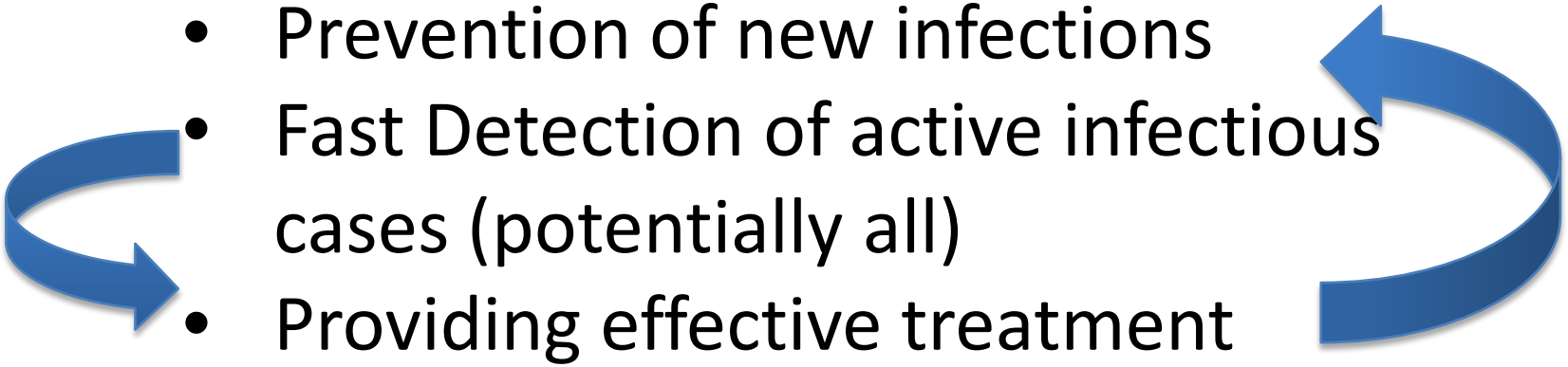
Disadvantages:

- expensive
- higher contamination rate
- dependency on a company
- no DST for Cycloserine

Break points for 2nd line drugs recently revised
Still poor correlation with clinical outcome, some testing not fully reliable

Main action for TB Control

- Prevention of new infections
- Fast Detection of active infectious cases (potentially all)
- Providing effective treatment



Conventional tools are often too slow to fulfil the task

Commercial Molecular tests for DR detection

GenoType MTBDR*plus*, InnoLiPA Rif.TB

- Reverse hybridization, colorimetric reaction
- Results in 6-7 h
- some flexibility (n probes/strip: 30-40)
- Technical expertise: some
- Biosafety lev 2

Xpert MTB/RIF

- Integrated/automated qPCR
- Results in 2h
- Closed system (limited number of probes: <10)
- Technical expertise: none

Main mutations responsible for DR in MTB

	Drug (year of discovery)	MIC µg/ml	Gene(s) involved in resistance	Gene function	Role	Mechanism of action	Mutation frequency %
First-line drugs	Isoniazid (1952)	0.02–0.2	*katG* *inh*A	Catalase-peroxidase Enoyl ACP reductase	Pro-drug conversion Drug target	Inhibition of mycolic acid biosynthesis and other multiple effects	50–95 8–43
	Rifampicin (1966)	0.05–1	*rpo*B	β subunit of RNA polymerase	Drug target	Inhibition of RNA synthesis	95
	Pyrazinamide (1952)	16–50 (pH 5.5)	*pnc*A	Nicotinamidase/pyrazinamidase	Pro-drug conversion	Depletion of membrane energy	72–97
	Ethambutol (1961)	1–5	*emb*B	Arabinosyl transferase	Drug target	Inhibition of arabinogalactan synthesis	47–65
	Streptomycin (1944)	2–8	*rps*L *rrs* *gid*B	S12 ribosomal protein 16S rRNA rRNA methyltransferase (G527 in 530 loop)	Drug target Drug target Drug target	Inhibition of protein synthesis	52–59 8–21 ?
Second-line drugs	Amikacin/kanamycin (1957)	2–4	*rrs*	16S rRNA 16S rRNA	Drug target	Inhibition of protein synthesis	76
	Capreomycin (1960)		*tly*A	2′-O-methyltransferase			
	Quinolones (1963)	0.5–2.5	*gyr*A *gyr*B	DNA gyrase subunit A DNA gyrase subunit B	Drug target	Inhibition of DNA gyrase	75–94
	Ethionamide (1956)	2.5–10	*eta*A/*eth*A *inh*A	Flavin monooxygenase	Prodrug conversion Drug target	Inhibition of mycolic acid synthesis	37 56
	PAS (1946)	1–8	*thy*A	Thymidylate synthase	Drug activation?	Inhibition of folic acid and iron metabolism?	36

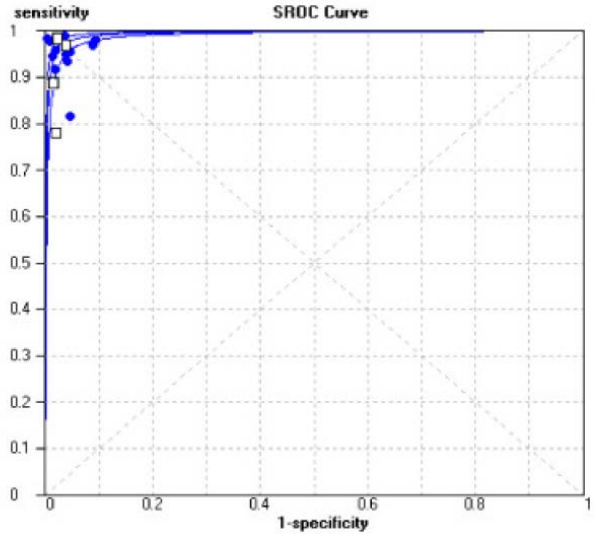
MIC = minimum inhibitory concentration; ACP = acyl carrier protein; PAS = para-aminosalicylic acid.

Zhang Y et al 2009. IJTLD 13(11):1320–1330

LPA performance in isolates and clinical samples

Inno-LiPA Rif.TB

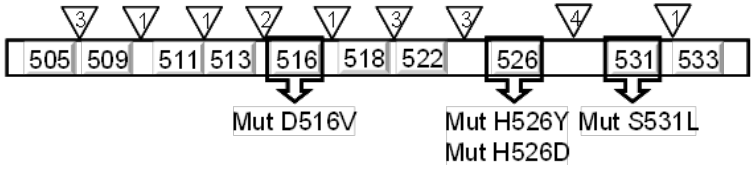
Hot-spot *rpoB gene*

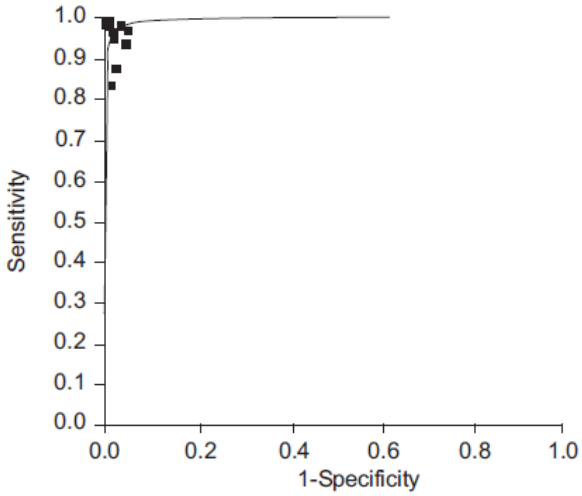


Morgan M et al 2005. BMC Infect Dis 5:62

Sensitivity 95-98%
Specificity 98-100%

GenoType MTBDR*plus**



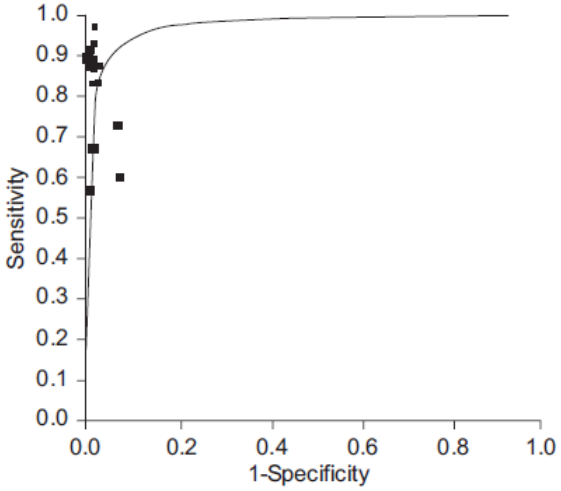


Ling DI et al 2008. Eur Respir J 32:1165-1174

Sensitivity 95-99%
Specificity 97-100%

GenoType MTBDR*plus*

cod. 315 *katG gene*
nt -8,-15,-16 *inhA gene*



Ling DI et al 2008. Eur Respir J 32:1165-1174

Sensitivity 82-93%
Specificity 95-100%

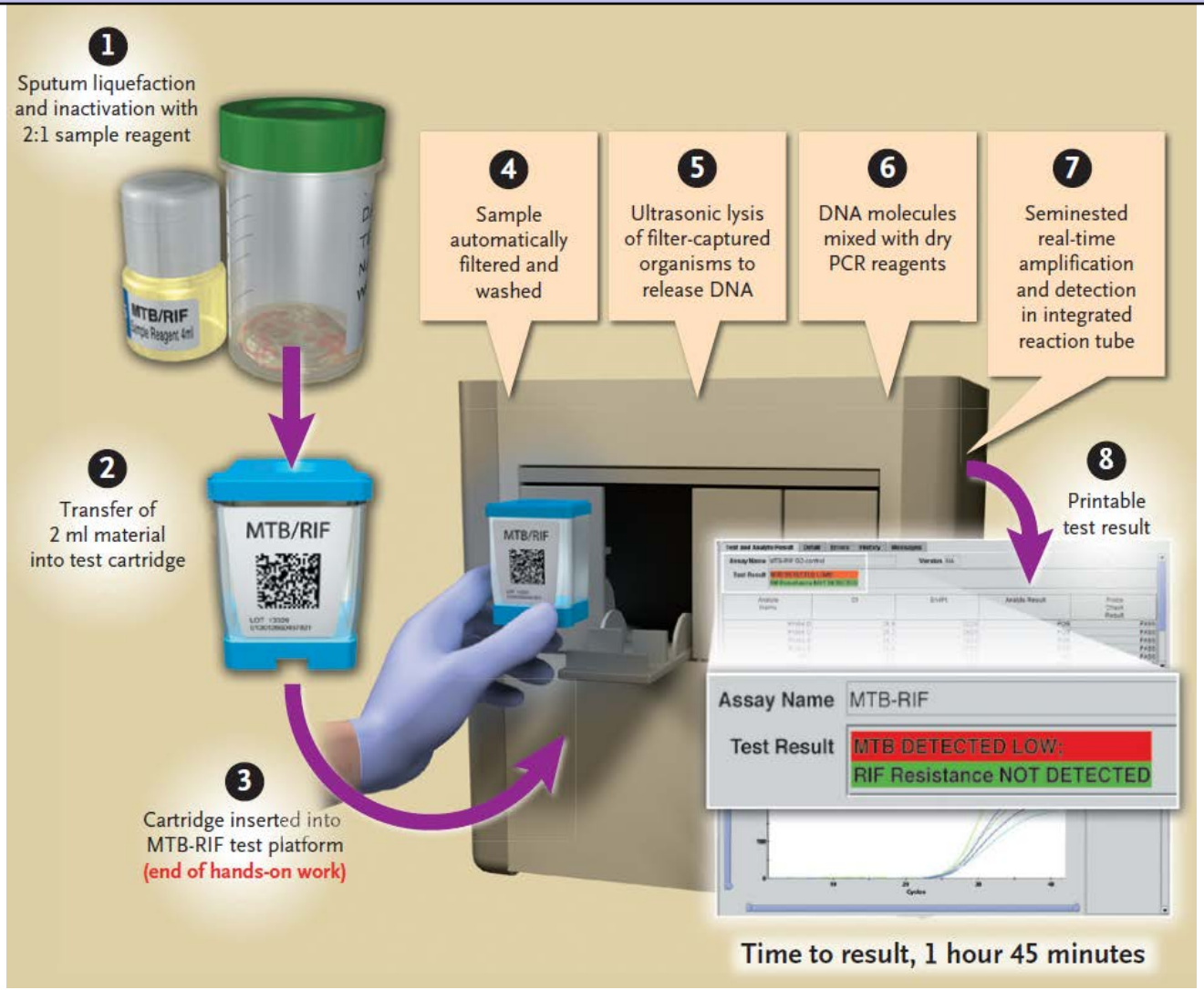
Decontaminated clinical specimens (AFB-positive)

Sensitivity 95-99%
Specificity 97-99%

Dec. clin. spec. (AFB-pos)

Sensitivity 72-92%
Specificity 96-99%

Xpert MTB/Rif: workflow

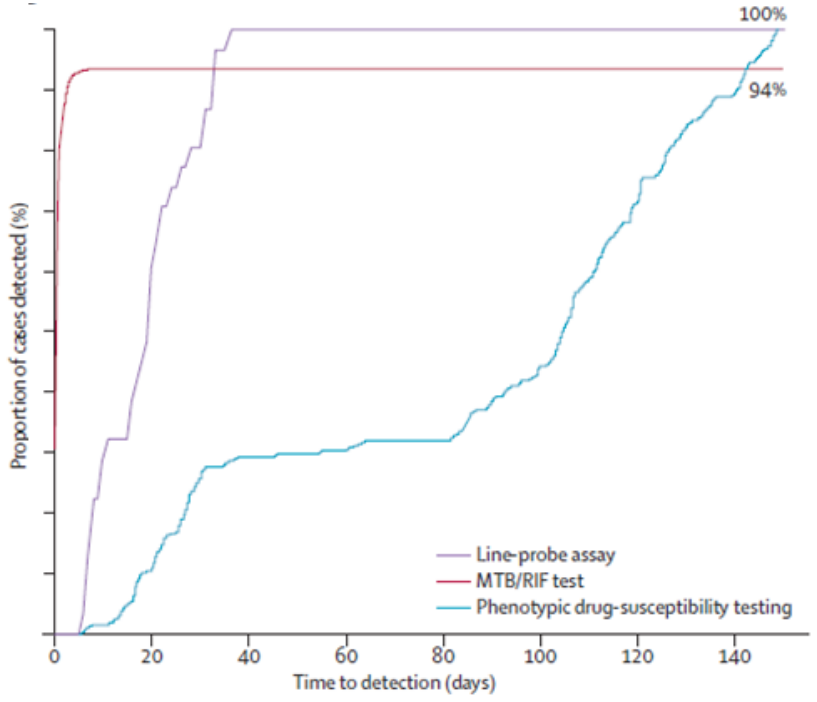


Boehme CC et al 2010. N Engl J Med 363(11):1005-15

TAT to Rif –R detection and reporting

RIF-R detection

Boehme CC et al 2011. Lancet 377(9776):1495-505

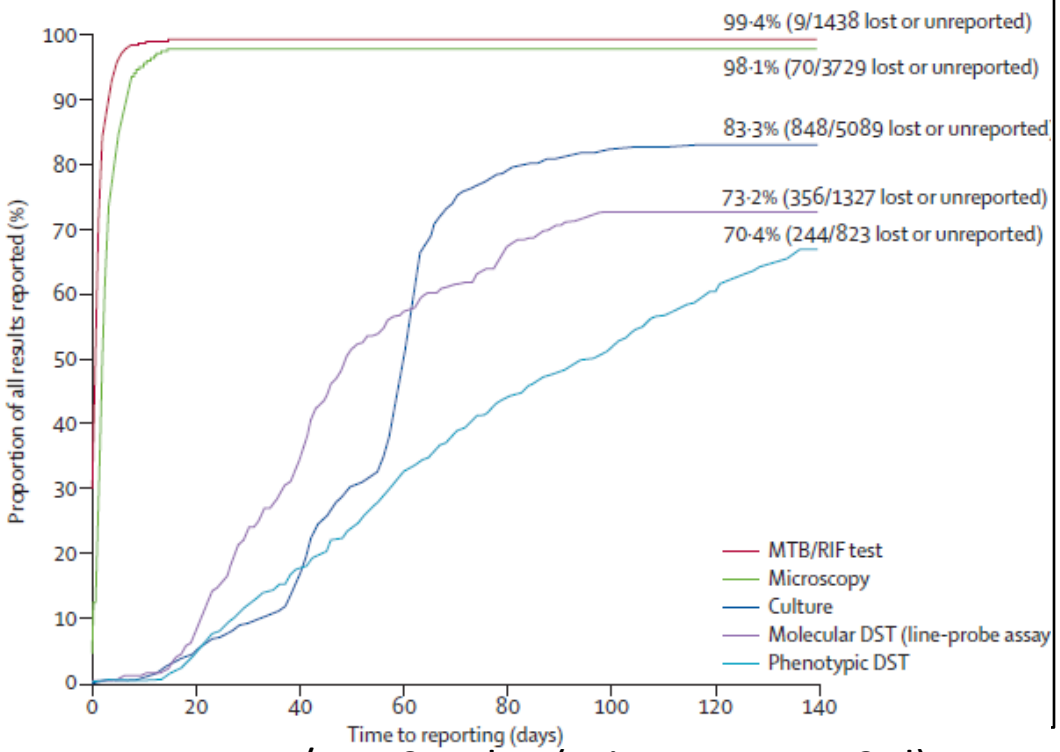


Xpert MTB/RIF: 0-1 d

LPA: 10-26 d*

Culture DST: 30-124 d**

Time to report to treatment center



Xpert MTB/RIF: 0-1 d (Microscopy: 1-2 d)

LPA: 27-53 d*

Culture DST: 38-102 d** (culture: 42-62 d)

Some results not reported/lost

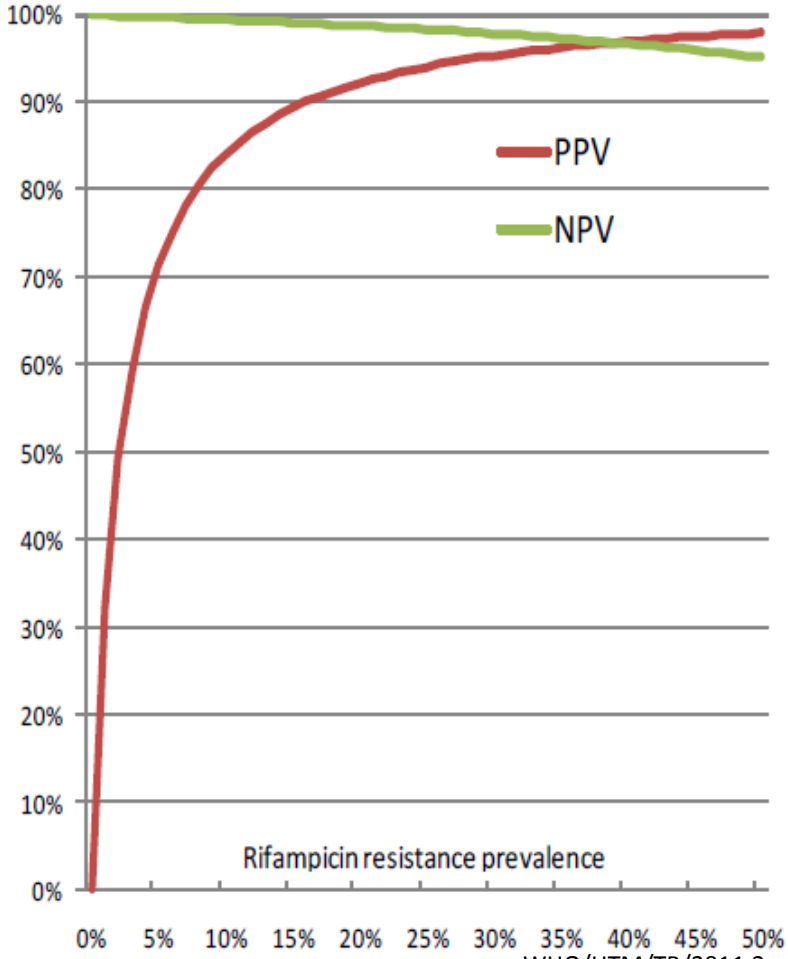
* test on AFB-pos clinical specimen + test on clinical isolate for AFB-neg cases

** DST performed by MGIT + DST performed on LJ

PPV and NPV for Rif resistance at different prevalence of Rif resistance

Rifampicin resistance prevalence	PPV	NPV	True positive*	False negative*	False positive*	True negative*
1%	32.4%	99.9%	9.5	0.5	19.8	970.2
2%	49.2%	99.9%	19	1	19.6	960.4
3%	59.5%	99.8%	28.5	1.5	19.4	950.6
4%	66.4%	99.8%	38	2	19.2	940.8
5%	71.4%	99.7%	47.5	2.5	19	931
6%	75.2%	99.7%	57	3	18.8	921.2
7%	78.1%	99.6%	66.5	3.5	18.6	911.4
8%	80.5%	99.6%	76	4	18.4	901.6
9%	82.4%	99.5%	85.5	4.5	18.2	891.8
10%	84.1%	99.4%	95	5	18	882
11%	85.4%	99.4%	104.5	5.5	17.8	872.2
12%	86.6%	99.3%	114	6	17.6	862.4
13%	87.7%	99.2%	123.5	6.5	17.4	852.6
14%	88.5%	99.2%	133	7	17.2	842.8
15%	89.3%	99.1%	142.5	7.5	17	833
20%	92.2%	98.7%	190	10	16	784
25%	94.1%	98.3%	237.5	12.5	15	735

* Sensitivity (95%) and specificity (98%) for Xpert MTB/RIF rifampicin resistance, compared with reference method (culture)



WHO/HTM/TB/2011.2

WHO Policies

MOLECULAR LINE PROBE ASSAYS FOR RAPID SCREENING OF PATIENTS AT RISK OF MULTIDRUG-RESISTANT TUBERCULOSIS (MDR-TB)

- Strains or AFB positive respiratory samples
- Adequate infrastructures (biosafety, molecolar biology)
- Technical capacities (supervision, QC)
- Appropriate transport and storage of reagents
- Central or Regional level
- INH drug-sensitive cases need to be confirmed by culture

AUTOMATED REAL-TIME NUCLEIC ACID AMPLIFICATION TECHNOLOGY FOR RAPID AND SIMULTANEOUS DETECTION OF TUBERCULOSIS AND RIFAMPICIN RESISTANCE: Xpert MTB/RIF SYSTEM

- Approved for smear-negative cases
- Biosafety at microscopy level
- No technical skill required
- Annual module's calibration
- District peripheral labs
- Appropriate transport and storage of reagents
- High NPV (99%)
- RIF-R cases to be re-confermed by LPA /colture if prevalence of RIF-R <10%

❑ **Test to be adopted in settings with adequate capacity and resources in agreement with local NTP and WHO reccomandations**

❑ **Reference test for MDR suspects and for TB/HIV**

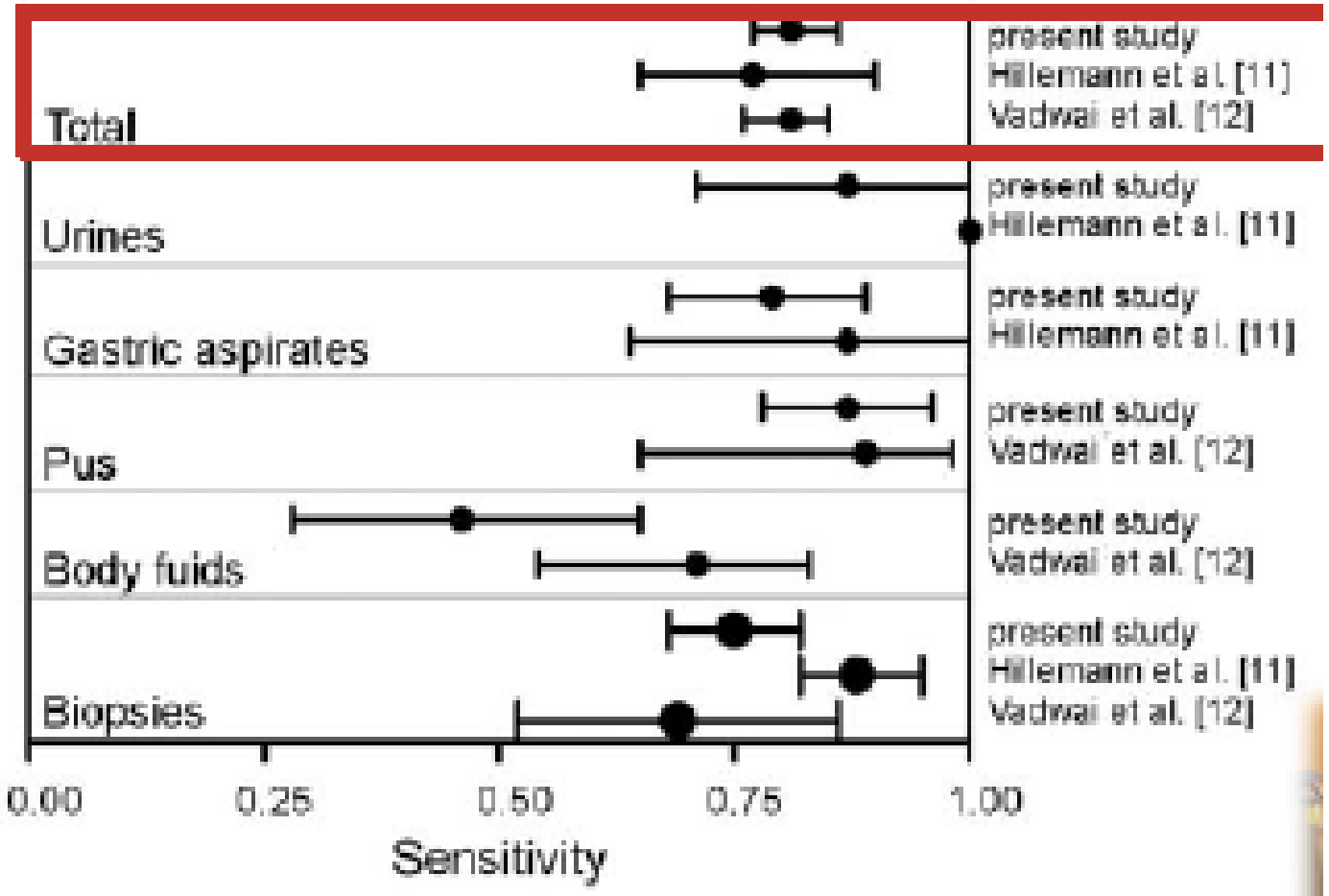
World Health Organization

Potential limits of Xpert technology

- Unknown performance at a district level
- Unknown performance in children
- If RIF resistance is diagnosed in a low level MDR-TB prevalence setting, the assay needs to be confirmed
- Need to perform a culture for DST to evaluate other drug resistance
- Need to perform smear/ culture for monitoring issue (conversion)
- It requires uninterrupted and stable electronic power supplies and yearly calibration
- Storage of reagents

Testing only for Rifampicin resistance

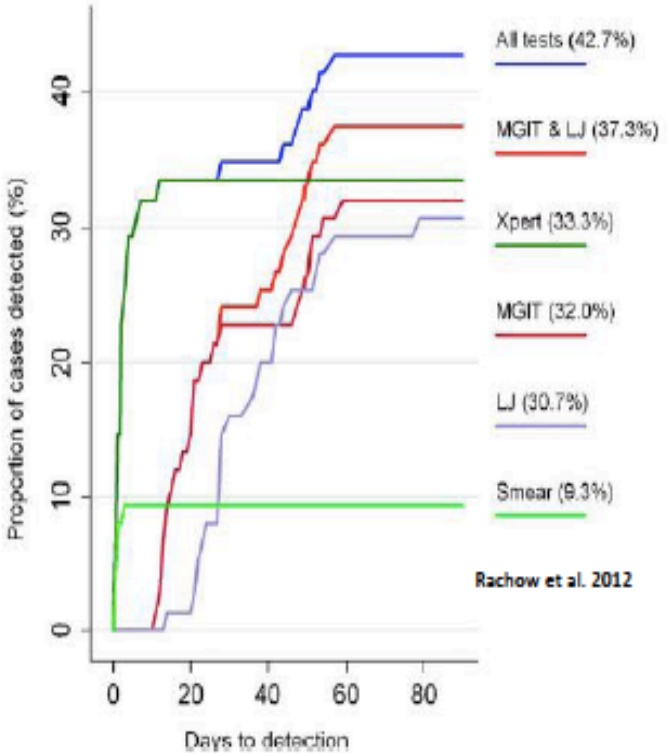
Sensitivity in Extrapulmonary TB



Tortoli et al., ERJ 2012

Performance in children

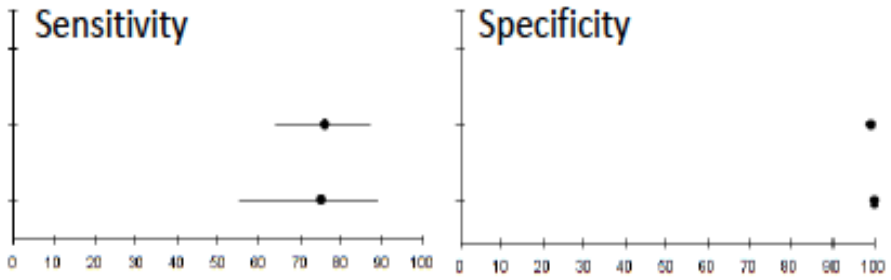
Baseline characteristics				
	Nr of participants	Median age	HIV infection	Specimens
Nicol et al, Lancet ID 2011	452	1.6 years	24%	Induced
Rachow et al, CID 2012	164	5.8 years	51.2%	Induced and spontaneous



Rachow et al. 2012

Sensitivity and specificity for pediatric TB detection		
	Sensitivity in C+ (95 CI)	Specificity in C- (95 CI)
Nicol et al, Lancet ID 2011	76% [64-87]	99% [98 - 100]
Rachow et al, CID 2012*	75% [55-89]	100% [99 - 100]

*4/47 (8.5%) Xpert positive among highly probable TB



Open Issues

- How to monitor the response to therapy?
  - Sputum smear is still guiding decisions on admission and discharge
  - Sputum culture is still the only reliable monitoring tool for MDR patients
- Patients with H monoresistance may go undetected, in R res H should be left until proven R?
- Are all the mutations in rpoB equally contributing to resistance?
- Long term sustainability outside research and cooperation projects
- First diagnostic step? Or should smear microscopy be kept as the first step to reduce the use of cartridges?

Interpretative problems

- *Uncommon rifampicin mutations*
- *inhA - 15 alone: increased mic, needs to follow closely over time*
- *Resistance to Ethionamide*
- *Eth 306: main mechanism fot ETH resistance*

ERDR seq.[c]	Conventional	MIC (MABA)		n
	DST	µg/mL	Result	
M306V	S	32	R	1
		16	R	5
		8	R	4
		4	R	1
M306I	S	8	R	5
		4	R	4
M306I[a]	S	16	R	2
		8	R	2
		4	R	2
		2	S	1
M306I[b]	S	8	R	1
		2	S	1
S297A	S	16	R	1
		4	R	1
S298N	S	8	R	1
S347I	S	8	R	1

MABA: microplate Alamar blue assay; R: resistant; S: susceptible; [a]: atg→atc; [b]: atg→att.

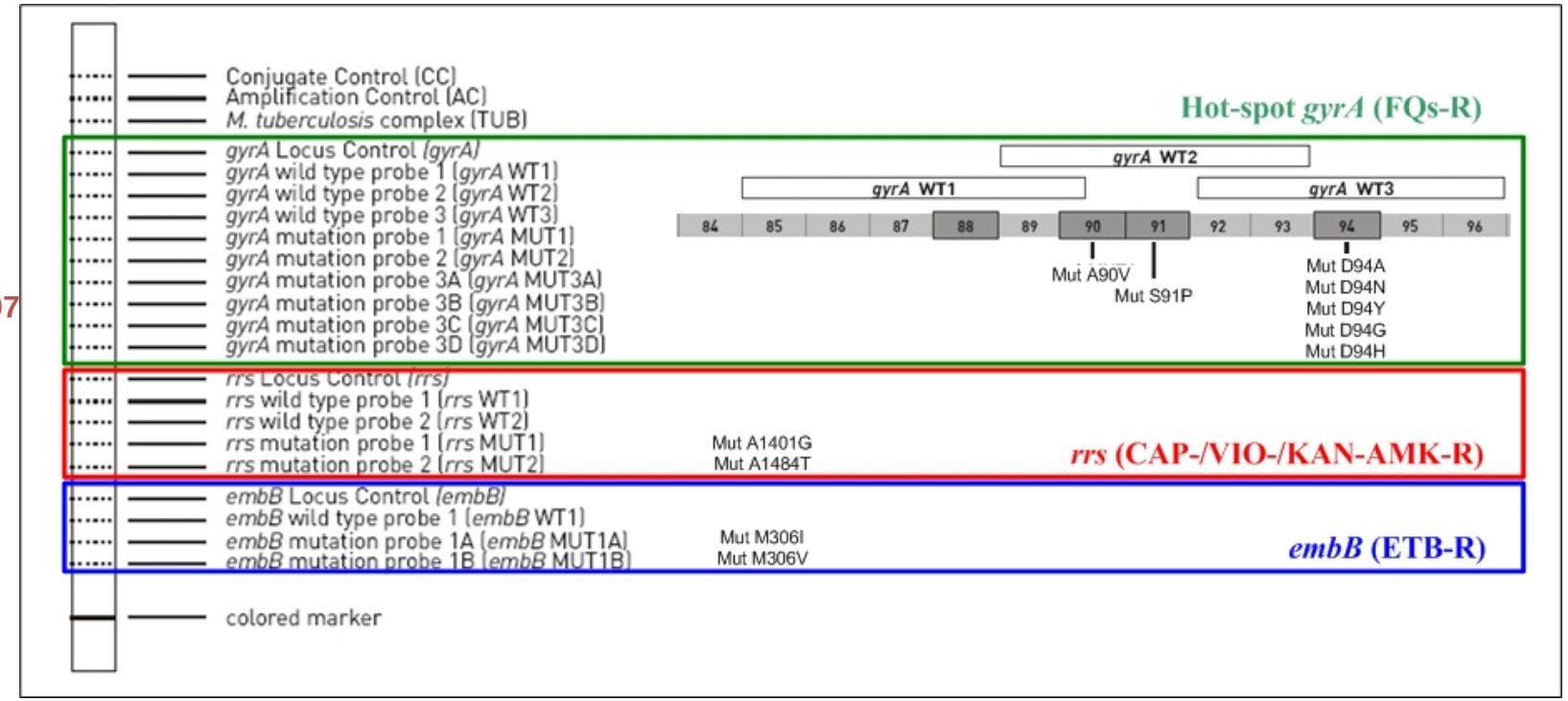
Molecular DST for drugs other than R and H

Diagnosing XDR-TB by molecular assays: the GenoType MTBDR*sl* (Hain Lifescience)

gyrA : cod. 85-97

rrs: nt 1401, 1402,1484

embB: cod. 306



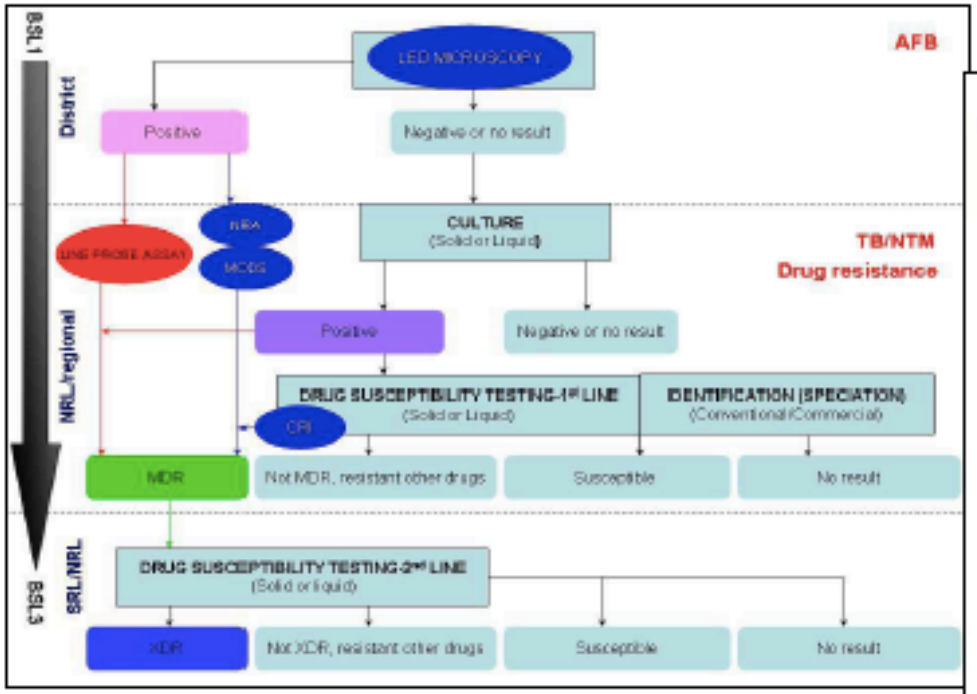
Performances of Geno Type *MTBDRsl*

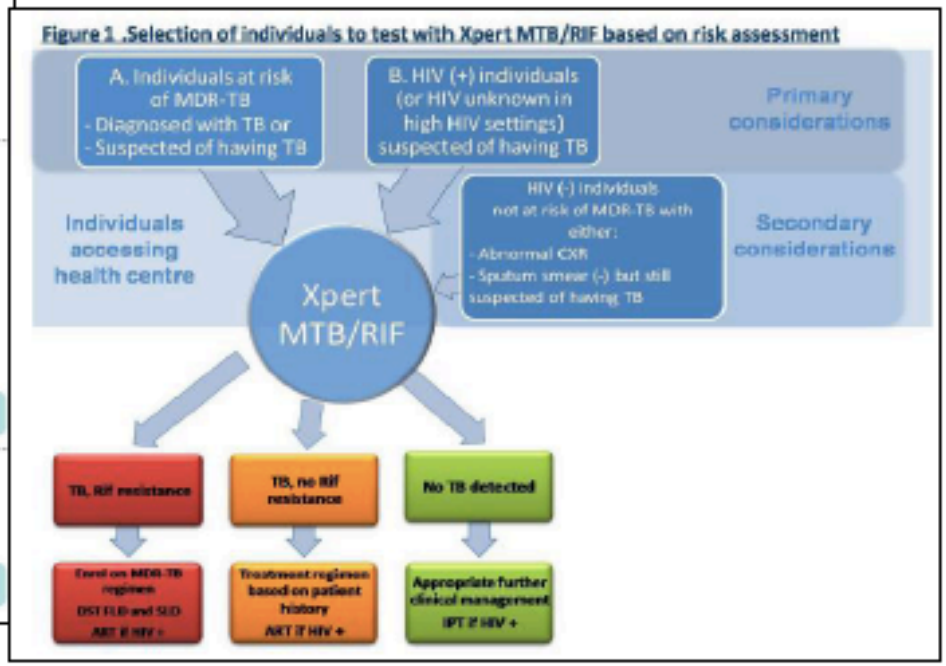
		Clinical isolates		Clinical specimens	
		MTB Detection % (95% CI)	DST % (95% CI)	MTB Detection % (95% CI)	DST % (95% CI)
Fluoroquinolones					
	Sensitivity	99,4 (96,8-99,9)	73,7 (61,0-83,4)	94,6 (85,4-98,2)	100 (61,0-100)
	Specificity	-	99,2 (95,3-99,9)	-	100 (92,4-100)
	PPV	-	97,7 (87,9-99,6)	-	100 (61,0-100)
	NPV	-	88,6 (82,0-92,9)	-	100 (92,4-100)
	Diagnostic accuracy	-	90,8 (85,6-94,3)	-	100 (93,2-100)
Second-line injectables					
	Sensitivity	99,4 (96,8-99,9)	71,4 (61,2-80,0)	94,6 (85,4-98,2)	80,0 (37,6-96,4)
	Specificity	-	100 (95,9-100)	-	89,1 (77,0-95,3)
	PPV	-	100 (94,0-100)	-	44,4 (18,9-73,3)
	NPV	-	79,0 (70,6-85,4)	-	97,6 (76,6-94,5)
	Diagnostic accuracy	-	86,2 (80,3-90,6)	-	88,2 76,6-94,5)
Ethambutol*					
	Sensitivity	99,4 (96,8-99,9)	69,7 (61,0-77,1)	94,6 (85,4-98,2)	84,9 (69,1-93,4)
	Specificity	-	96,2 (87,0-98,9)	-	100 (83,9-100)
	PPV	-	97,7 (92,0-99,4)	-	100 (87,9-100)
	NPV	-	57,5 (47,0-67,3	-	80,0 (60,9-91,1)
	Diagnostic accuracy	-	77,6 (70,8-83,2)	-	90,6 (79,8-95,9)

- High PPV and specificity → rapid identification of resistant cases
- Low sensitivity and NPV → need to confirm SENSITIVE cases by conventional DST
- Can be used for screening MDR-TB cases at high risk to develop XDR-TB
- For ETB sensitivity is increased (15-20%) when using the presence of mutations as marker for resistance
- *Overall diagnosis of XDR-TB: 44.4% → additional studies and markers are needed*

Miotto P et al. ERJ 2012

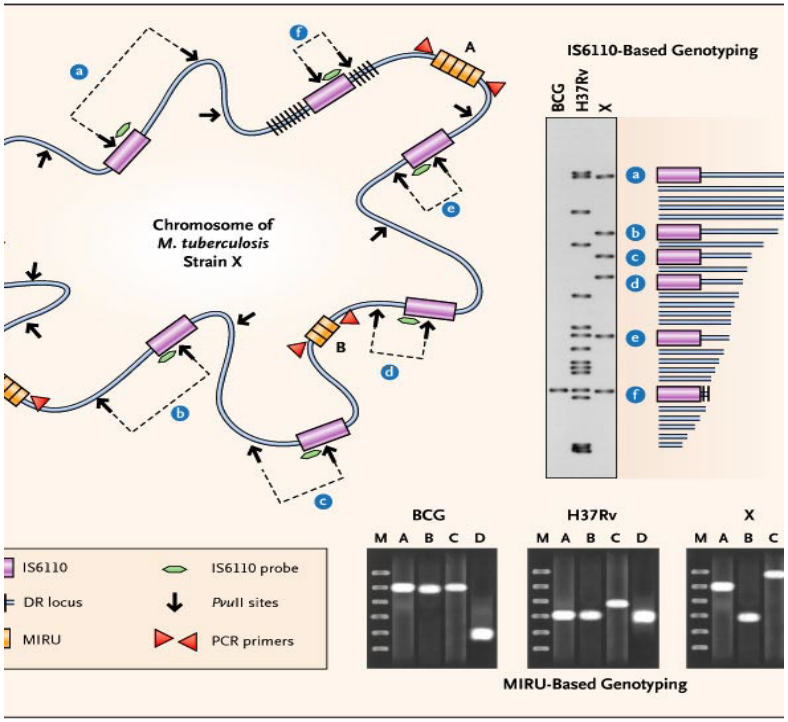
Tools in different algorithms

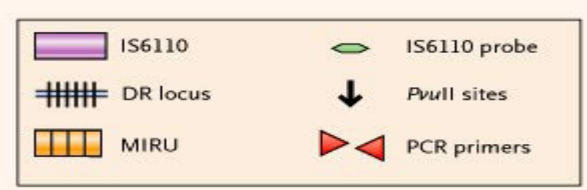




One size no longer fits all

Role of Molecular typing





- To identify epidemiological links between TB patients to detect and control outbreaks early and rapidly
- Rule out suspected outbreaks and confirm transmission has NOT occurred
- To identify incorrect TB diagnosis based on false-positive cultures and thus avoid unnecessary investigation and treatment
- To distinguish exogenous re-infection from endogenous reactivation in patients with a past history of TB
- Discover unusual transmission settings and transmission between different regions
- Monitor the size of clusters and thus monitor progress towards TB elimination
- Vaccine and DR detection implications

Conclusions

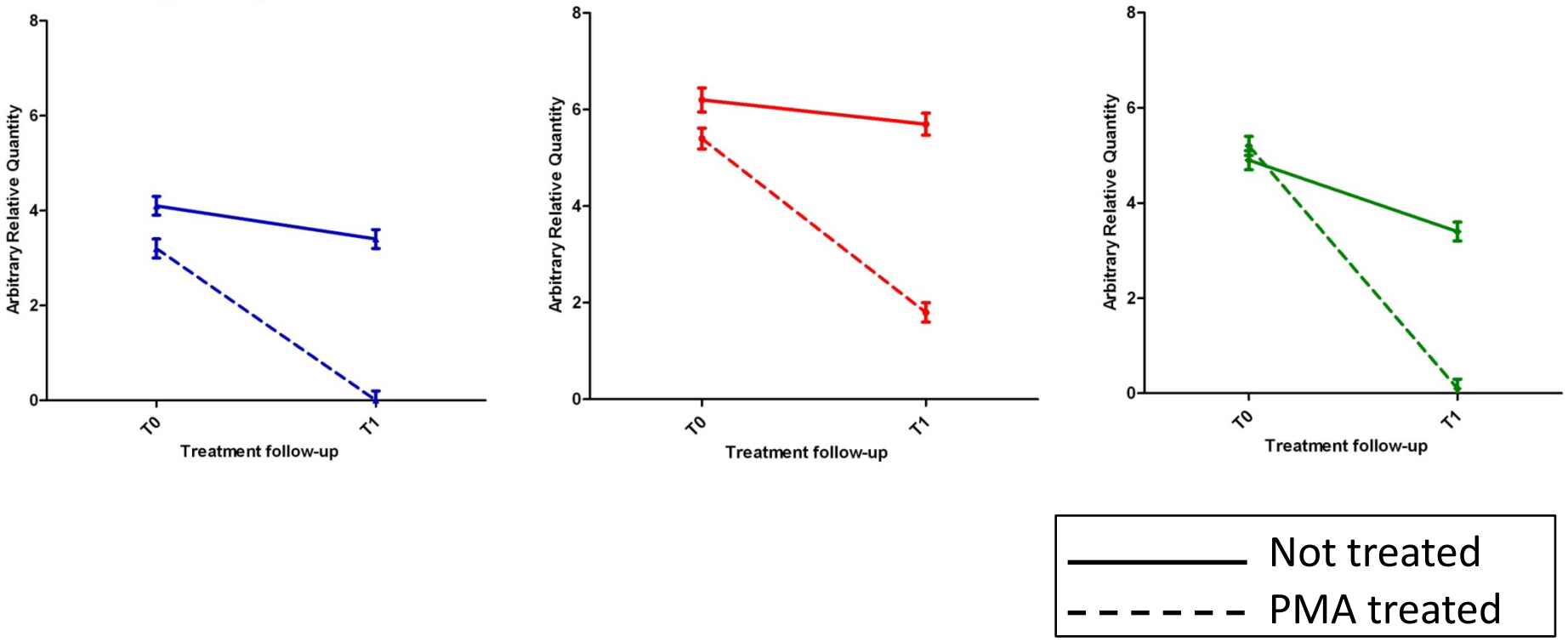
- Appropriate implementation of current diagnostic tools will highly contribute to reaching the 2015 target
- Large effort is needed in:
  - improvement of diagnosis in Children
  - Biomarkers discovery with the potential to become a point of care

Future perspectives

- Adapting molecular tests to therapy monitoring
- Including new clinically relevant mutations in medium density/user friendly molecular platforms:
- AMK/KAN/CAP: *Rv3919c* (*gidB*), *Rv2416* (*eis*)
- Characterization of mutations occurring in genes encoding putative targets for new drugs (nitroimidazopyran, linezolid)
- Compensatory mutations in MDR-TB strains (Comas et al. 2011, Nat Genetics)
- Better understanding of genetic diversity and drug resistance relationships
- Full genome sequencing?
- Biomarkers? LAM, cytokines,TNFαMTB specific CD4 miRNAs, volatile molecules
- Point of care tests?

PMA for selective amplification of vital MTB

Comparison between DNA amplified from PMA treated (- -) and untreated (—) sputum samples collected at diagnosis (t_0) and at 14 days from beginning of antitubercular therapy

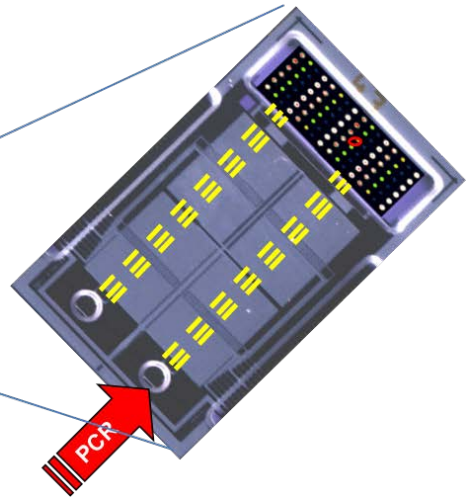


Miotto P et al. ERJ 2012

Lab-on Chip for molecular diagnostics

PCR:

- *Ultra-Fast PCR*
- *Asymmetric Cy-5 PCR strategy*



Microarray:

- *Orientation probes*
- *Hybridization Control probes*
- *Hybridization Negative Controls probes*

Current Lay out:
ID of MTBC, relevant NTMs
MDR phenotype

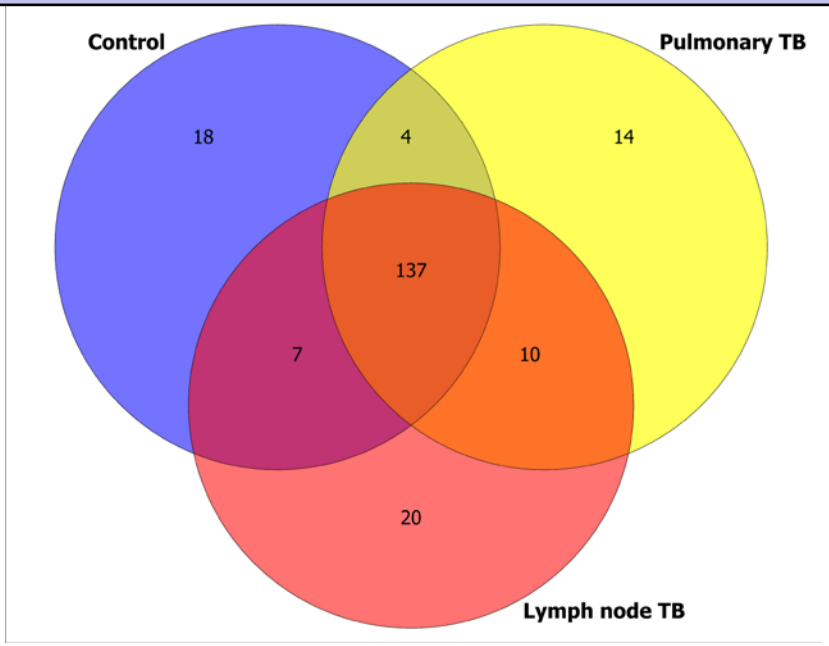
Lab-on-chip architecture

2 PCR reactors of 12.5 uL volume each (Total 25 ul)
1 Hybridization chamber of 30 uL
A 126 spots DNA microarray
2 in-let ports compatible with standard micro-pipettor tips
Integrated Heaters and Sensors

All the reaction modules are fluidically integrated

Serum miRNAS as stable biomarkers for TB

Proof-of-principle: analysis of 667 miRNAs serum expression profile in pooled samples from patients with active pulmonary, lymph node TB and pooled healthy subjects.
(low-density TaqMan® arrays)



Outcome:

1. This new approach has the potential to revolutionize present clinical management, including revisiting TB classification and predicting therapy response.
2. miRNAs expression pattern profiles may allow developing decision-tree classifier/algorithms.
3. Serum allows easy testing in paucibacillary patients, in particular HIV-positive patients and children.

Conclusions

- Appropriate implementation of current diagnostic tools will highly contribute to reaching the 2015 target
- Large effort is needed in:
  - improvement of diagnosis in Children
  - Biomarkers discovery with the potential to become a point of care

Acknowledgements

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