A cluster of MDR tuberculosis among asylum seekers in Switzerland and other European Countries

Laboratory and Epidemiological Investigation

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– No conflict of interest related to the presented data

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Table of Contents

- Observation of a cluster
- Genetic resistance detection
- DNA Fingerprinting technologies for *Mycobacterium tuberculosis*
- Whole genome sequencing
- European inter-laboratory investigation / tools
MDR tuberculosis detection in Switzerland

- *M. tuberculosis* strains resistant to at least rifampicin are sent to the National Reference Laboratory (Swiss National Center for Mycobacteria, University of Zurich)
- This is regulated by ordinance; i.e. the labs have to forward MDR strains to the Reference Laboratory
- Under mandate of the Federal Office of Public Health, the National Reference Laboratory performs second-line drug testing and DNA fingerprinting
It started in December 2015:

- MDR tuberculosis in Switzerland is rare.
- Strains resistant to rifampicin and isoniazid are considered to be multi-drug resistant.

Isolate of patient #1 from sputum of Dec/31/2015, sent to reference laboratory on Feb/02/2016:
17 yr. old male Somali

<table>
<thead>
<tr>
<th>Drug</th>
<th>Resistance mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>rpoB S531L</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>katG S315T</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>embB M306I</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>pncA W68C</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>tlyA N236K</td>
</tr>
</tbody>
</table>

→ Rare pattern, has never been reported in the international databases.
# Second isolate

Isolate of patient #2 from sputum of April/05/2016:

18 yr. old male Somali

<table>
<thead>
<tr>
<th>Drug</th>
<th>Resistance mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>\textit{rpoB} S531L</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>\textit{katG} S315T</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>\textit{embB} M306I</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td>\textit{pncA} W68C</td>
</tr>
<tr>
<td>Capreomycin</td>
<td>\textit{tlyA} N236K</td>
</tr>
</tbody>
</table>

\(\Rightarrow\) Rare, identical pattern, again.
Third isolate

Isolate of patient #3 from sputum of May/23/2016:
18 yr. old male Somali

<table>
<thead>
<tr>
<th>Drug</th>
<th>Resistance mutation</th>
</tr>
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<tbody>
<tr>
<td>Resistance to:</td>
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<tr>
<td>Rifampicin</td>
<td><em>rpoB</em> S531L</td>
</tr>
<tr>
<td>Isoniazid</td>
<td><em>katG</em> S315T</td>
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<tr>
<td>Ethambutol</td>
<td><em>embB</em> M306l</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td><em>pncA</em> W68C</td>
</tr>
<tr>
<td>Capreomycin</td>
<td><em>tlyA</em> N236K</td>
</tr>
</tbody>
</table>

→ Rare, identical pattern, again, Murphy?

→ Phonecall to Danuta Reinholz, cantonal physician Ticino

→ Start of comparative genetic analysis (MIRU-VNTR, whole genome sequencing)
Cluster

2016 186034 M. tuberculosis
2016 186046 M. tuberculosis
1417/02 Cameroon M. tuberculosis
2016 186010 M. tuberculosis
3248/02 Beijing M. tuberculosis
4486/02 Beijing M. tuberculosis
3229/02 Beijing M. tuberculosis
3550/02 Beijing M. tuberculosis
3577/02 Beijing M. tuberculosis
3556/02 Beijing M. tuberculosis
3502/02 Beijing M. tuberculosis
4408/02 Beijing M. tuberculosis
4409/02 Beijing M. tuberculosis
4446/02 Beijing M. tuberculosis
1207/02 Haarlem M. tuberculosis
3105/05 Haarlem M. tuberculosis
4130/02 Haarlem M. tuberculosis
3842/02 Haarlem M. tuberculosis
2224/09 Uganda M. tuberculosis
2329/09 Uganda M. tuberculosis

MIRU: Multilocus Variable Number Tandem Repeat Genotyping
# 24-locus MIRU-VNTR

<table>
<thead>
<tr>
<th>Locus</th>
<th>154</th>
<th>424</th>
<th>577</th>
<th>580</th>
<th>802</th>
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<th>1644</th>
<th>1955</th>
<th>2059</th>
<th>2163b</th>
<th>2165</th>
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<th>2461</th>
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</thead>
<tbody>
<tr>
<td>nzrm-Nummer</td>
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<td>2016 186004</td>
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<td>4</td>
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<td>2016 186010</td>
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<td>2</td>
<td>3</td>
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</tr>
</tbody>
</table>

Cluster differences

Swiss situation November 2016

By September 2016 the NZM found a total of 5 strains with identical resistance pattern, identical MIRU-VNTR

Figure. cgMLST tree of selected 2016 Swiss tuberculosis strains

core genome multi locus sequence typing (cgMLST)

RIDOM software was used to calculate the tree

cluster 1
-> asylum seekers

Figure: courtesy of PD Dr. A. Egli, Basel
European Situation Jan 2017

21 cases confirmed by WGS

9 additional cases under investigation

★ cluster case
★ Djibouti case

Figure: courtesy of Dr. M. Merker, Borstel
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Kantonsspital St. Gallen: Prof Dr. O. Schoch

EOC Lugano: Prof. Dr. E. Bernasconi

FZ Borstel, Hamburg, Germany: Dr. K. Kranzer, Dr. M. Merker, Prof. Dr. S. Niemann

Centro Scientifico San Raffaele, Milan: Prof. Dr. D. Cirillo

ECDC: Dr. M. van der Werf

FOPH: Dr. P. Heilbling, Dr. E. Altpeter, Dr. D. Koch
Genotypic and phenotypic antibiograms and their clinical reliability

Dr. med. Peter Keller, FAMH Medical Microbiology
Deputy Head Swiss National Center for Mycobacteria

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Drug resistance in *Mycobacterium tuberculosis*

Drug resistance and clinical implications

- Genetic basis of resistance
- Principles of mycobacterial drug susceptibility testing
- Laboratory versus clinical resistance
Today’s mycobacterial laboratory report (exemple)
Reliability of laboratory versus clinical resistance (outcome)

1. Why is standard short-course therapy reportedly an effective treatment for a significant part of multidrug resistant (MDR) tuberculosis?


2. Does DOTS (directly observed therapy short-course) work as a means to control TB in populations with drug-resistant tuberculosis?

   "Even in settings with moderate rates of MDR tuberculosis, directly observed therapy (DOTS) can rapidly reduce the transmission and incidence of both drug-susceptible and drug-resistant tuberculosis."


   “We found that many control programs that have been the most successful in reducing wild-type sensitive TB (as a result of high case detection and treatment rates) have paradoxically been the most likely to evolve into hot zones of MDR.”

Principles of mycobacterial drug susceptibility testing (DST)

1. Critical proportion
   (proportion method)

2. Critical concentration
   (drug concentration)
Phenotypic DST: principle of the proportion method

- The proportion method is based on the premise that if <1% of the organisms in a given population are resistant to a drug at a given concentration (the so-called critical concentration), the population as a whole is susceptible, and conversely, if >1% of the organisms are resistant, the population as a whole is resistant.

- The critical concentration (CC) represent the lowest concentration of the agent that inhibits > 95% of the wild-type (wt; susceptible) strains.

CLSI. Susceptibility testing of Mycobacteria, Nocardiae and other aerobic actinomycetes; approved standard. 2nd ed. CLSI document M24-A2. Wayne PA: CLSI 2011.

Figure. Example: rifampicin; critical concentration 2 mg/L; concentration in serum 10 mg/L.

Drug resistance in *M. tuberculosis*

Resistance is defined as a decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug.

…, *strains that are resistant in this sense do not necessarily fail to respond.*

Mitchison, 1962
Comparison of the phenotypic DST methods

Common in most methods:

– In many commercial systems, the inoculum in the antibiotic-free medium is a 1:100 dilution of the inoculum in the antibiotic-containing medium, thus reflecting the proportion method.

– **Resistance as defined here is a technical term: it does not necessarily correspond to clinical resistance.**
Currently established critical concentrations

The critical concentrations of many antibiotics were published by WHO in 2008, with updates suggested in a meeting 2012.

Results using current critical concentrations are generally accurate and reproducible for rifampicin, and isoniazid, but less so for ethambutol and streptomycin.

Pyrazinamide testing is technically difficult, given the drug’s activity at low pH, which inhibits mycobacterial growth.
The problem of phenotypic ethambutol (EMB) testing

A drawback to the use of critical concentrations is that it assumes that there are two clearly defined populations of organisms (resistant and susceptible), and that the minimum inhibitory concentration (MIC) distributions for these two populations can be easily separated.

Fig. 1. Wild-type minimum inhibitory concentration (MIC) distribution for ethambutol (Middlebrook 7H10 agar proportion method)

ECOFF, epidemiological cut-off. Note: Sequencing data suggest that approximately 50% of isolates at a MIC of 4 mg/L have genetic resistance mechanisms, whereas none of the isolates at a MIC of 2 mg/L and all of the isolates at a MIC of 8 mg/L have genetically detectable resistance mechanisms. Thus, the current critical concentration of 5 mg/L for Middlebrook 7H10 medium (indicated by an arrow) splits the upper end of the wild-type MIC distribution, which leads to poor reproducibility.

Conclusions: phenotypic DST

1. Phenotypic DST is still commonly regarded as the gold standard for determining the susceptibility of *M. tuberculosis* to various drugs.

2. The DST results for some drugs (such as INH and RMP) are more reliable than for other drugs (such as EMB).

3. There is a lack of good clinical outcome data to correlate with the phenotypic DST results for some agents.

4. A good understanding of local epidemiology and molecular resistance mechanisms is important to appropriately interpret phenotypic DST results.
Genetic basis of resistance in *M. tuberculosis*

- Resistance in *M. tuberculosis* is mainly due to **single nucleotide mutations** (SNMs) that accumulate over time on specific genes.
- For some antibiotics the association between the mechanisms of resistance and the responsible genes are very well known, whereas for others we still have incomplete knowledge.

**Precaution:**
- Not all of the SNMs detectable in strains showing a resistant phenotype are responsible for drug resistance: some are phylogenetic markers.
Genetic basis of resistance - continued

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanism of drug action</th>
<th>Resistance mechanism</th>
<th>Function of gene product</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 drugs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EMB</td>
<td>Interferes with cell wall synthesis</td>
<td><em>embA, embB</em></td>
<td>Arabinosyl transferase</td>
<td></td>
</tr>
<tr>
<td>INH</td>
<td>Interferes with mycolic acid synthesis</td>
<td><em>katG, inhA</em></td>
<td>Catalase/peroxidase (katG), enoyl reductase (inhA)</td>
<td><em>inhA</em> mutations confer low grade phenotypic resistance</td>
</tr>
<tr>
<td>PZA</td>
<td>Unclear</td>
<td><em>pcnA</em></td>
<td>Pyrazinamidase</td>
<td>Cross-resistance between members of this family</td>
</tr>
<tr>
<td>RMP/ RBT/RPT</td>
<td>Inhibits RNA polymerase</td>
<td><em>rpoB</em></td>
<td>RNA polymerase (beta subunit)</td>
<td></td>
</tr>
</tbody>
</table>

Genetic basis of mycobacterial resistance

For important first and second line drugs there are mutational hot-spots.

What is the added value of molecular detection of mutations?

− Molecular detection of the SNMs associated with drug resistance is the fastest way to design a personalised treatment regimen, and it also has the potential to become a bedside technology.

− WHO endorsed commercial methods for drug resistance detection include line probe assays and the Xpert® MTB/RIF assay.

Figure. Mutations covered by line blot assays.

Reliability of genotypic testing: first line drugs; rifampicin (RMP)

Different commercial assays detect RMP resistance by targeting the RMP resistance-determining region (81 bp), which harbours more than 95% of mutations responsible for RMP resistance.

**Figure.** Mutations in *rpoB* (RIF target) and minimum inhibitory concentration in 7H10 agar proportion testing: Strains with wild-type *rpoB* sequence show MIC values <0.5 mg/L whereas strains with mutations show elevated MICs >0.5 mg/L.

Blöchliger N., SNF consortium (Gagneux S., Egger M., Böttger E.C.), unpublished
Xpert MTB/RIF (genetic RIF resistance testing)

- The PCR targets the 81 bp region of the rpoB gene.
- The assay flags the presence of resistance in the absence of binding of wild-type probes to the target sequence.
- The pooled sensitivity and specificity of Xpert® MTB/RIF for RMP resistance detection are 95% (95% confidence interval 90-97) and 98% (95% CI 97-99).

Reliability of genotypic testing: pyrazinamide (PZA)

PZA is a prodrug that is converted to the active form pyrazinoic acid (POA) by PZase/ nicotinamidase encoded by the pncA gene in *M. tuberculosis*.

Resistance to PZA

Although PZA resistance in *M. tuberculosis* was shown by McDermott’s group to be related to loss of nicotinamidase and pyrazinamidase in 1967, the mechanism of PZA resistance was not known until 1996 when mutation in the *pncA* gene encoding nicotinamidase and pyrazinamidase was demonstrated to cause PZA resistance (*Scorpio A, Zhang Y. Nat Med. 1996; 2:662-7.*).

**Problems in phenotypic testing:**
- PZA is only active in vitro at low pH (< 6.0).
- Some *M. tuberculosis* strains do not grow well at low pH
- This might suggest false susceptibility *in vitro.*

**Solution:**
Molecular tests targeting mutations associated with drug resistance have high specificity and sensitivity when compared to DNA sequencing as gold standard.

In the Swiss epidemiology line probe assays may be used as rule out tests for resistance against INH, RIF, EMB, fluoroquinolones, and second line injectables (AMK, KAN, CAP).

Line blot assay layout/ targets

Module 1: Isoniazid (INH) / Rifampin (RIF)
- Conjugate control
- Amplification control
- M. tuberculosis complex
- INH wt (inhA -16, -15, -8)
- INH mut (inhA -16G, -15T, -8A, -8C)
- INH wt (KatG 315)
- INH mut (KatG S315T)
- RIF wt (RpoB 513-516)
- RIF mut (RpoB D516V, D516Y)
- RIF wt (RpoB S522-526)
- RIF mut (RpoB H526Y, H526D, H526R)
- RIF wt (RpoB S531L, S531W)

Module 2: Streptomycin (STR)/ Amikacin (AMK)
- Conjugate control
- Amplification control
- M. tuberculosis complex
- STR wt (RpsL 43)
- STR mut (RpsL K43R)
- STR wt (RpsL 88)
- STR mut (RpsL K88R)
- STR wt (rs513-517)
- STR mut (rsC513T)
- STR mut (rsA514C)
- STR mut (rsG515C)
- AMK/CAP wt (rsA1401G)
- AMK/CAP wt (rsA1401T)
- AMK/CAP wt (rsG1484C/T)

Module 3: Fluoroquinolones (FQ)/ Ethambutol (EMB)
- Conjugate control
- Amplification control
- M. tuberculosis complex
- FQ wt (GyrA 90, 91, 94)
- FQ mut (GyrA A90V)
- FQ wt (GyrA S91P)
- FQ mut (GyrA D94A)
- FQ wt (GyrA D94N)
- FQ mut (GyrA D94Y)
- FQ mut (GyrA D94G)
- EMB wt (EmbB 306)
- EMB wt (EmbB M306V)
- EMB wt (EmbB M306I; G918A)
- EMB wt (EmbB M306I; G918C)
- EMB wt (EmbB M306I; G918T)

Note:
1. RpoB numbering according E. coli; all other protein / gene numbering according M. tuberculosis strain H37Rv.
2. rsrs M. tuberculosis H37Rv positions 513, 514, 515, 517, 1401, 1402 and 1484 correspond to rsrs E. coli positions 522, 523, 524, 526, 1408, 1409 and 1491, respectively.

modular design
fast (6-8 hrs)

Platforms for simultaneous detection of multiple mutations

- Knowledge about mutations associated with resistant phenotype is increasing with widely available next generation sequencing (NGS).
- This technology allows the collection of a large amount of *M. tuberculosis* sequencing data at relatively low cost.
- Most studies published so far, perform whole genome sequencing from cultured isolates


- Brown et al. reported the successful and accurate sequencing of *M. tuberculosis* genomes directly from uncultured sputum.

Challenges with regards to genome based resistance detection

1. Lack of studies assessing resistance mutation candidates with quantitative MIC data.
2. Lack of international *bona fide* resistance mutation reference databases.
3. Lack of clinical correlation studies for suggested resistance mutations.

Conclusion NGS-based resistance detection:

Only full genome sequencing on an extremely large number of strains collected worldwide, coupled with phenotypic DST results, drug treatment and clinical outcome data, can provide the appropriate statistical power to identify the subset of mutations predictive of treatment failure to any given drug.
Consensus recommendations of TBNET/RESIST-TB on molecular DST

- **RMP**: Molecular methods for the detection of **RMP resistance should be considered a standard** for the diagnostic evaluation of patients with presumptive MDR-TB.
- **INH**: Although >90% of RMP-resistant strains are also resistant to INH, **molecular testing for INH resistance is important**.
- **INH**: In case of *inhA* promoter mutations, level of resistance should be confirmed by phenotypic methods.
- **PZA**: mutations associated with resistance can be detected by sequencing of the *pncA* gene.
- **EMB**: the clinical implications of EMB resistance, which is mostly low or moderate are not clear at present, nor are those of *embB* mutations.
- **Second line drugs**: All patients with *rpoB* mutations in a direct specimen should receive molecular DST for second-line drugs.

If the results of molecular and culture-based drug susceptibility testing differ, what is the gold standard?

- The level of discordance between molecular and culture-based detection depends on the drug and the genomic region evaluated.
- Despite the fact that the results of phenotypic methods do not always correspond to response to clinical treatment, culture-based methods are still regarded as gold standard for DST by most experts.
Clinical implications of mutations detected by molecular methods (INH)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Drug</th>
<th>Association with in vitro phenotypic resistance</th>
<th>Association with clinical resistance</th>
<th>Frequency among strains categorised as resistant on the basis of critical concentration testing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>katG S315T</strong></td>
<td>INH</td>
<td>-</td>
<td>S315T confers high-level INH resistance (MIC &gt;1 mg/l), but does not affect susceptibility to ETH</td>
<td>Indirect evidence strongly suggests that high-level resistance affects clinical outcomes. katG S315T mutations are associated with multidrug resistance (see e.g.). Limited data on direct association between katG S315T mutation and clinical outcome suggest increased risk of first-line treatment failure, death and relapse</td>
</tr>
<tr>
<td></td>
<td>ETH</td>
<td>+</td>
<td>Note: there are additional mutations in <em>inhA</em> or <em>ethA</em>, which confer ETH resistance</td>
<td>In a systematic review of 52 studies, 5–98% of INH-resistant isolates showed katG S315T mutations (median 64%, interquartile range 54–79) (Hooijer et al. unpublished)</td>
</tr>
<tr>
<td><strong>inhA</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>-16G</td>
<td>INH</td>
<td>+</td>
<td><em>inhA</em> promoter mutations confer low-level INH resistance (MIC &lt;1 mg/l), but significantly affect ETH susceptibility</td>
<td>Limited direct and indirect data, suggesting no effect on cure rates for standard first-line treatment. One study showed increased relapse rates with INH-EMB (6 months) in the continuation phase. <em>inhA</em> promoter mutations are not associated with multidrug resistance when compared to the katG S315T mutation, but have been associated with XDR-TB in South Africa</td>
</tr>
<tr>
<td>-15T</td>
<td>ETH</td>
<td>-</td>
<td>Note: there are additional mutations in the structural <em>inhA</em> gene, which together with <em>inhA</em> promoter mutations result in INH MIC levels &gt;1 mg/l</td>
<td>In various studies, 12–42% of INH-resistant isolates had <em>inhA</em> promoter region mutations</td>
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<tr>
<td>-8A/C</td>
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### Example of clinical implications for mutations affecting fluoroquinolones

<table>
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<tr>
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<th>MFX</th>
<th>OFX</th>
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</thead>
<tbody>
<tr>
<td>gyrA</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>D94mut</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mutations in gyrA affect MFX and OFX susceptibility. Mutations of residue D94 confer clinical resistance; mutations affecting codon A90 are discussed controversially. For mutations of codon S91, few data are available, most likely similar to mutations of residue A90.</td>
<td></td>
</tr>
<tr>
<td>A90mut</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Strong indirect and some direct evidence for association of gyrA codon 94 mutations with clinical resistance to OFX. D94mut and A90mut in 40–58% and 20–30%, respectively, of OFX- or MFX-resistant isolates.</td>
<td></td>
</tr>
</tbody>
</table>
Conclusions

- Molecular DST allows rapid (2-3 days) identification of >90% of 1st line therapeutic agents resistance.
- Low-level resistance and high-level resistance can be separated; the drugs might still be used in case of low-level resistance.
- WGS allows for identification of heteroresistance, drug resistance targets (if subsequent isolates are available).
- Phenotype/ genotype agreement studies are needed.

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