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Prevalence of drug-resistant tuberculosis assessed by next-generation sequencing: an 18-month nationwide study in Lebanon

Salam El Achkar

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**Prevalence of drug-resistant tuberculosis assessed by next-generation sequencing:
an 18-month nationwide study in Lebanon**

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Abstract

Tuberculosis (TB) is the first killer infectious disease, with 10 million new cases estimated worldwide in 2017. TB drug resistance and its diagnosis are particularly problematic. Only 25% of the 450,000 incident multidrug resistant (MDR) TB patients estimated over the same year were diagnosed and treated as such.

Although Lebanon is a low-TB burden country, significant challenges exist in terms of disease control. Lebanon is the country hosting the largest refugee population proportionally to its national population worldwide, with 1.5 million Syrian refugees as a consequence of the war in Syria, in addition to large populations of Palestinian refugees and migrant workers. Such populations are particularly vulnerable to risks of TB and emergence of drug resistance. The last national survey on the prevalence of drug resistant TB was done 15 years ago, well before the start of the Syrian crisis in 2011. Even most recent reported rates of MDR TB largely relied on estimates. Second-line drug susceptibility testing (DST) and individualized extensively drug-resistant (XDR) TB treatments were not available in the country.

In order to gain a more comprehensive view of the TB situation, we set up the first nationwide study combining phenotypic and extensive molecular testing to determine the prevalence and extent of TB drug resistance in the country. A total of 417 patients were included, corresponding to all confirmed TB cases reported to the national tuberculosis program between June, 2016 and November, 2017. Lowenstein-Jensen and/or MGIT culturing, and molecular testing using GeneXpert MTB/RIF and/or Anyplex MTB/NTM Real-time were used in Lebanon for diagnostic confirmation and DST. In Lille, we evaluated, for the first time on a nationally representative sample, a new deep sequencing assay called Deeplex-MycTB, for extensive drug resistance prediction and genotyping of patient isolates. MIRU-VNTR typing was used in combination for defining molecular clusters, potentially suggestive of endemically circulating or epidemically transmitted TB strains.

For the first time in the country, out of the 354 culture positive TB cases with available DST, 3 XDR cases, resistant to at least rifampicin (RIF), isoniazid (INH), kanamycin (KAN)/amikacin (AMI) and levofloxacin (LFX) were detected, in addition to 5 MDR (resistant to at least RIF, INH) cases and one RIF mono-resistant case. Among the remaining cases, 3.4% (12/354) had resistance to INH and streptomycin (SM), 3.4% (12/354) mono-resistance to INH,

0.3% (1/354) mono-resistance to ethambutol (EMB), 8.5% (30/354) mono-resistance to streptomycin (SM), while 81.9% (290/354) were susceptible to all 4 first line drugs. While none of MDR and XDR TB cases were found in molecular clusters, a large cluster comprising 36 other patients was identified, suggestive of a highly endemic or actively transmitted drug susceptible strain.

A total of 4184 out of 4407 (94.9%) possible phenotypes could be predicted by Deeplex-MycTB for 339/348 (97.4%) analyzable samples, of which 1282/1380 (92.9%) matched the available phenotypic results. Based on detectable resistance determinants, INH, RIF, EMB and SM resistance was concordantly predicted with 90.3%, 100%, 100%, 52.8% sensitivity, respectively, and susceptibility with 99.6%, 100%, 99.4%, 99.6% specificity, respectively. While predicted first and second-line drug resistance matched almost completely the available phenotypic profiles of the 8 MDR and XDR cases, mutations were additionally detected in all of these 8 cases in targets predicting supplementary pyrazinamide and/or ethionamide resistance, not phenotypically tested. Moreover, resistance to fluoroquinolones was also predicted in 34/339 (10%) non-MDR cases, not subjected to LFX DST. Finally, the use of advanced molecular testing allowed us to identify the first 12 (3.4%) zoonotic TB cases identified in the country.

The identification of XDR and MDR TB cases, including among refugees and migrant workers and with complex resistance patterns, calls for reinforced diagnostic means for MDR TB patients, to critically inform treatment decisions and prevent the spread of drug resistance. These findings have also ramifications for other major places of destination for refugees and migrant workers. The detection of a substantial number of cases with basal resistance to fluoroquinolones also calls for systematic testing of susceptibility to these drugs before their prioritized inclusion in treatments of RIF resistant or INH mono-resistant patients, as recently recommended by the World Health Organization. Finally, the disclosure of zoonotic TB cases indicates the existence of overlooked animal reservoirs, which are important to investigate and control.

Résumé

La tuberculose (TB) est la première maladie infectieuse mortelle, avec 10 millions de nouveaux cas estimés dans le monde en 2017. La résistance aux médicaments antituberculeux ainsi que le diagnostic de cette résistance sont particulièrement problématiques. Seulement 25% des 450 000 cas de tuberculose multi-résistante estimés au cours de la même année ont été diagnostiqués et traités comme tels.

Bien que le Liban ait un faible taux de tuberculose, le contrôle de la maladie pose d'importants problèmes. Le Liban est le pays au monde accueillant la plus grande population de réfugiés par rapport à sa population nationale. Suite à la guerre en Syrie, le pays compte 1,5 million de réfugiés syriens, ainsi qu'un nombre important de réfugiés palestiniens et de travailleurs migrants. Ces populations sont particulièrement vulnérables face aux risques de tuberculose et à l'émergence de résistance aux antituberculeux. La dernière enquête nationale de prévalence de résistance aux antituberculeux avait été réalisée il y a 15 ans, bien avant le début de la crise syrienne en 2011. Même les taux les plus récents de tuberculose multi-résistante signalés reposaient en grande partie sur des estimations. Les tests de sensibilité aux antibiotiques (DST) de seconde intention et les traitements individualisés de tuberculose ultra-résistante (XDR) n'étaient jusqu'alors pas disponibles dans le pays.

Afin d'obtenir une vue globale et actualisée de la situation de la tuberculose dans le pays, nous avons mis en place la première étude nationale combinant des tests phénotypiques et moléculaires avancés pour déterminer la prévalence et l'étendue de la résistance aux antituberculeux. Au total, 417 patients ont été inclus dans l'étude. Ils correspondent aux cas de tuberculose confirmés et signalés au programme national de lutte contre la tuberculose entre juin 2016 et novembre 2017. Des cultures en milieu Lowenstein-Jensen et/ou MGIT ainsi que des tests moléculaires GeneXpert MTB/RIF et/ou Anyplex MTB/NTM Real-Time ont été effectués au Liban pour confirmer le diagnostic et tester la sensibilité aux antituberculeux. À Lille, nous avons évalué, pour la première fois sur un échantillon national représentatif, un nouveau test de détection de résistances aux antibiotiques, Deeplex-MycTB. Ce test, basé sur le séquençage de nouvelle génération (NGS) et en profondeur de l'ADN, permet une prédiction étendue des résistances aux antituberculeux ainsi qu'un génotypage des isolats de patients. Le typage MIRU-VNTR standardisé

a été utilisé en combinaison pour définir les clusters moléculaires potentiellement évocateurs de souches de mycobactéries à circulation endémique ou à transmission épidémique.

Pour la première fois dans le pays, sur les 354 cas de tuberculose à culture positive avec DST disponible, 3 cas de XDR, résistants au moins à la rifampicine (RIF), à l'isoniazide (INH), à la kanamycine (KAN)/amikacine (AMI) et à la lévofloxacine (LFX) ont été détectés, en plus de 5 cas multi-résistants (résistant au moins à RIF, INH) et un cas mono-résistant à la RIF. Parmi les cas restants, 3,4% (12/354) présentaient une résistance à l'INH et à la streptomycine (SM), 3,4% (12/354) une mono-résistance à l'INH, 0,3% (1/354) une mono-résistance à l'éthambutol (EMB), 8,5% (30/354) une mono-résistance à la SM, tandis que 81,9% (290/354) étaient sensibles aux 4 médicaments de première intention testés. Bien qu'aucun cas de tuberculose multi- ou ultra-résistante n'ait été trouvé dans des clusters moléculaires, un cluster important comprenant 36 autres patients a été identifié, suggérant une souche non résistante hautement endémique ou transmise activement.

Deeplex-MycTB a prédit un total de 4184 sur les 4407 (94,9%) phénotypes possibles pour 339/348 (97,4%) échantillons analysables, dont 1282/1380 (92,9%) correspondaient aux résultats phénotypiques disponibles. Sur base des déterminants de résistance détectables, la résistance à l'INH, RIF, EMB et SM était concordante avec une sensibilité de 90,3%, 100%, 100% et 52,8% respectivement, et une spécificité de 99,6%, 100%, 99,4% et 99,6% respectivement. Les résistances prédites aux médicaments de première et de deuxième intention correspondaient presque parfaitement aux profils phénotypiques disponibles chez les 8 cas de MDR et de XDR. Cependant, des mutations prédictives de résistance supplémentaire au pyrazinamide et/ou à l'éthionamide (non testées phénotypiquement) ont également été détectées chez ces 8 cas. Il est aussi important à noter qu'une résistance aux fluoroquinolones a été prédite dans 34/339 (10%) des cas non-MDR, classiquement non testés phénotypiquement pour ces antibiotiques. Enfin, l'utilisation de tests moléculaires avancés nous a permis d'identifier les 12 premiers cas de tuberculose zoonotique (3,4%) dans le pays.

L'identification de cas de TB-XDR et -MDR, incluant des réfugiés et/ou des travailleurs migrants et présentant des profils de résistance complexes, nécessite des moyens diagnostiques renforcés pour les patients atteints de TB-MDR, afin d'éclairer de manière critique les décisions thérapeutiques et de prévenir la propagation de la résistance aux médicaments. Ces résultats ont

également des répercussions pour les autres pays de destination des réfugiés et des travailleurs migrants. De plus, la détection d'un nombre important de cas avec une résistance basale aux fluoroquinolones souligne la nécessité de tests systématiques de sensibilité à ces médicaments avant leur inclusion prioritaire récemment recommandée par l'Organisation mondiale de la santé dans les traitements des patients résistants à la RIF ou mono-résistants à l'INH. Enfin, la révélation des cas de tuberculose zoonotique indique l'existence négligée de réservoirs animaux, qu'il est important d'investiguer et de contrôler.

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List of abbreviations

AFB	Acid-Fast bacilli
AMK	<i>Amikacin</i>
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin
BDQ	Bedaquiline
Bp	Base pairs
CAP	Capreomycin
CFP-10	Culture Filtrate Protein 10
CFZ	Clofazimine
CFU	Colony-Forming Unit
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Cs	Cycloserine
DLM	Delamanid
DR	Direct Repeat
DST	Drug Susceptibility Testing
EMB	Ethambutol
ESAT-6	Early Secretory Antigenic Target Protein 6
ETH	Ethionamide
FQ	Fluoroquinolones
HIV	Human Immunodeficiency Virus
IFN- γ	Interferon gamma
IGRA	Interferon-Gamma Release Assays
INH	Isoniazid
IOM	International Organization for Migration
IS	Insertion Sequence
KM	Kanamycin

LED	Light-Emitting Diode
LFX	Levofloxacin
LIN	Linezolid
LJ	Lowenstein-Jensen
LMSE	Laboratoire Microbiologie Santé et Environnement
LPA	Line Probe Assay
MDR TB	Multi Drug Resistant TB
MXF	Moxifloxacin
MGIT	Mycobacterial Growth Indicator Tube
MIRU-VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number of Tandem Repeat
MoPH	Ministry of Public Health
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> complex
NAAT	Nucleic acid amplification test
NALC	N-Acetyl-L-Cysteine
NGS	Next Generation Sequencing
NaOH	Sodium hydroxide
NTM	Non-Tuberculous Mycobacteria
NTP	National TB program
OADC	Oleic acid, Albumin, Dextrose and Catalase
OFX	Ofloxacin
PAS	Para-aminosalicylic acid
PE	Proline-Glutamate
PPD	Purified Protein Derivative
PPE	Proline- Proline- Glutamate
PRL	Palestinian Refugees in Lebanon
PRS	Palestinian Refugees from Syria

PZA	Pyrazinamide
QRDR	Quinolone Resistance Determining Region
RD	Regions of Difference
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RRDR	Rifampicin Resistance Determining Region
RR TB	Rifampicin- Resistant Tuberculosis
RT-PCR	Real- time PCR
SM	Streptomycin
SNP	Single Nucleotide Polymorphism
SR	Syrian Refugees
STB	Smooth Tubercle Bacilli
TB	Tuberculosis
TRD	Terizidone
TST	Tuberculin Skin test
UNHCR	United Nations High Commissioner for Refugees
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR TB	Extensively Drug Resistant TB

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Introduction

A. Tuberculosis: A brief historical perspective

Tuberculosis (TB) is one of the oldest known diseases in humans. Analysis of human Egyptian mummy samples dating from 2050-1650 Before Christ indicated the presence of *Mycobacterium tuberculosis* (*M. tuberculosis*) or *M. africanum* DNA (Zink *et al.*, 2003). Moreover, whole genome sequencing (WGS) of mycobacterial genomes from human skeleton from pre-Colombian Peruvian mummies indicated that these strains were related to *M. pinnipedii* known to be causing TB in seals (Bos *et al.*, 2014). The ancient Greek physician Hippocrates noted that "phthisis" was a fatal disease particularly in young adults. Between the 17th and 19th centuries, the disease was called "white plague" due to the anemic pallor of the patient, and the divesting impact that the disease had on human populations of the Western world at the time. During the 19th century, the disease has been estimated to be responsible for the death of 1 out of 7 people living in the United States and Europe (Barberis *et al.*, 2017).

On 24 March 1882, the German doctor Robert Koch identified *M. tuberculosis*, as the bacterium responsible for TB (Daniel, 2006; Barberis *et al.*, 2017). This key discovery paved the way for subsequent medico-scientific investigations and for better prevention, control and treatment of this fatal disease. Another major discovery to fight TB was the development and first use, in 1921, by Albert Calmette and Camille Guérin, of the BCG (Bacille Calmette-Guerin) vaccine. During the next decades, the vaccine was widely used, first in Britain and Europe, and later on, in many other countries worldwide (Daniel, 2006). Evidence presented BCG vaccine to be mostly protective in children against extrapulmonary forms of the disease, such as meningeal TB. In addition, it has been shown that BCG vaccine might protect against pulmonary TB when given at early age with a single criteria that children should not be already infected with *M. tuberculosis* or other mycobacterial infection (Mangtani *et al.*, 2014). However, levels of protection against pulmonary TB in adults appear overall lower and largely variable according to the cohorts studied (Abubakar *et al.*, 2013). Thus, a more effective vaccine is needed to prevent TB infection and/or disease in all age groups. Currently, 12 vaccines candidates are on phase I, II and III trials (WHO, 2018a).

Until the 1940s, rest in specialized institutions (sanatoria), with healthier conditions including improved nutrition, sun exposure and cleaner air, was the most commonly used way to favor cure of, and prevent further contagion from, TB patients. The discovery of streptomycin (SM) by

Selman Waksman in USA in 1944 as the first active antibiotics against tubercle bacilli, followed by the finding of other active anti-TB drugs in the following period (see below) progressively marked the end of the age of sanatoria (Daniel, 2006).

However, despite the important progresses made on the fight against TB over the last century, this curable disease is still far from being eradicated, as illustrated by the epidemiological situation described below (page 13). This fact can be largely explained by remaining diagnostic, therapeutic and prophylactic challenges, as well as important associated risk factors such as poverty and human immunodeficiency virus (HIV) infection that continue to prevail (CDC, 2018).

B. The mycobacteria

The *Mycobacterium* genus belongs to the *Mycobacteriaceae* family, the Actinomycetales order and the Actinobacteria phylum (Stackebrandt *et al.*, 1997).

Mycobacterium species are non-motile, non-flagellated and non-spore forming Gram-positive aerobe. The bacilli are straight or slightly curved measuring 0.2 to 0.6 μ m in diameter and 1 to 10 μ m in length (Murray *et al.*, 2003). One of the major characteristics of mycobacteria is their exceptionally rich lipid walls. These species harbor a unique, waxy layer composed of long chained mycolic acid, which gives high impermeability to the cell wall, making them naturally resistant to many antibiotics and helping them to escape immune clearance (McMurray, 1996). In addition, this gives them the ability of resisting to disinfectants, acids, and strong bases. From a diagnostic perspective, this lipid-rich cell wall enables the differentiation from most other bacteria by staining techniques, as mycobacteria resist decolorization with an acid-alcohol wash, hence their name of acid-fast bacilli (AFB) (Farver and Jagirdar, 2018). Another major characteristic of mycobacteria is their (relatively) slow growth when compared to other bacteria, as at least 5-7 days of culture are required to detect growth (Forbes *et al.*, 2018).

Results from phylogenetic analysis support the taxonomic division of *Mycobacterium* species into slow-growers and fast-growers, requiring respectively, more or less than 7 days to grow on Lowenstein-Jensen (LJ) solid media (Gutierrez *et al.*, 2009). Furthermore, mycobacterial sequence

comparisons of 16S rRNA sequences suggest the evolution of the slow-growing sub-group from a common fast-growing ancestor (Rogall *et al.*, 1990).

Among the 170 or so slow- and rapid-growing *Mycobacterium* species described so far, the great majority were isolated from the environment. All but those included in the so-called *Mycobacterium tuberculosis* complex (MTBC) and *M. canettii* (also collectively called the TB bacilli) are designated as non-tuberculous mycobacteria (NTM) (Fedrizzi *et al.*, 2017). While most fast-growing NTM are considered as harmless saprophytic organisms, a number of fast-growing NTM as *M. abscessus*, as well as slow-growing species as *M. avium*, *M. marinum*, *M. xenopi*, *M. gordonae* and *M. kansasii* can cause disease, mostly in immuno-compromised individuals (Figure 1). In contrast, the TB bacilli and the other human pathogens *M. ulcerans* and *M. leprae* are slow growers (Gutierrez *et al.*, 2009).

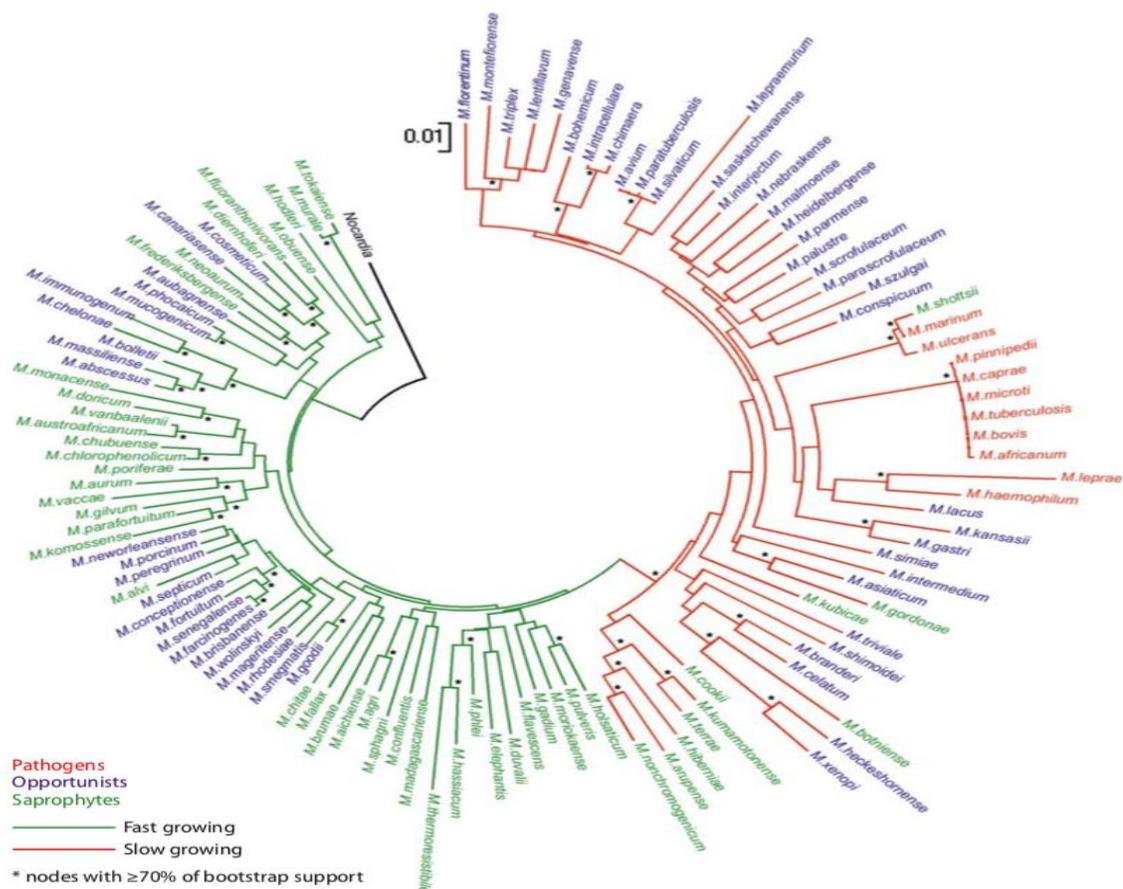


Figure 1: Global phylogenetic structure of 119 mycobacterial species based on 16S rRNA, *hsp65* and *rpoB* genes (Gutierrez *et al.*, 2009).

C. *M. tuberculosis* genome, evolution and phylogeny

In addition to the principal agent of human TB *M. tuberculosis*, the MTBC comprise several additional members. *M. bovis* was the first animal-associated MTBC member discovered in 1896 and is primarily characterized as a pathogen of cattle. However, *M. bovis* has been isolated from a range of other mammals and can also cause TB in humans (Smith *et al.*, 2009). Other animal-associated MTBC members often appear to have a stricter host range and include (preferred host in brackets): *M. microti* (voles), *M. pinnipedii* (seals and sea lions), *M. orygis* (antelopes), *M. mungi* (banded mongooses), *M. suricattae* (meerkats), the dassie bacillus (hyrax), and the chimpanzee bacillus (Niemann *et al.*, 2016; Gagneux, 2018).

The MTBC members all share identical 16S rRNA gene sequences, have more than 99% identity at the nucleotide level, and possess highly syntenic genomes. For these reasons, they are regarded as host- (human- or animal-) adapted variants (also termed ecotypes) rather than different species (Smith *et al.*, 2009).

The first genome representative of the MTBC that was completely sequenced was from the type strain of *M. tuberculosis* H37Rv, in 1998 in collaboration between the Institut Pasteur in Paris, France, and the Sanger Institute in Hinxton, United Kingdom. This genome comprises 4411532 base pairs (bp), about 4000 genes and a G+C rich content (65.6%) (Cole *et al.*, 1998). Among its other characteristics, it is rich in repetitive sequences, comprising in particular numerous genes encoding the so-called PE (Proline-Glutamate)/ PPE (Proline- Proline- Glutamate) protein families (see below) (Mathema *et al.*, 2006).

With 4.34 Mbp, the genome of *M. bovis* is slightly smaller, reflecting deletions of some genome regions relatively to *M. tuberculosis*. The presence of such deletions (also termed regions of difference (RDs)) in the genomes of strains of *M. bovis* and other animal-adapted MTBC members argues against the zoonotic origin of TB. The characterization of the sequences and the distribution of these RDs among MTBC and *M. canettii* strains suggests that all MTBC strains derive by reductive evolution from a single clonal progenitor resembling more to *M. tuberculosis* or *M. canettii* (Brosch *et al.*, 2002).

This scenario is further supported by results from comparative genomics of *M. canettii* strains, which correspond to exceptional TB clinical isolates from East Africa. These strains have slightly

larger genomes than *M. tuberculosis*, specific CRISPR-Cas systems, much greater genetic diversity, and multiple traces of horizontal DNA exchange contrasting with the strong clonality of *M. tuberculosis* (Supply *et al.*, 2013; Boritsch *et al.*, 2016). These data suggest that *M. canettii* represents evolutionarily early branching lineages of TB bacilli. In addition, mouse infection experiments showed that smooth tubercle bacilli (STB) strains are less persistent and virulent than *M. tuberculosis*. These results suggest that the MTBC ancestor emerged as a professional pathogen from an ancestral *M. canettii*-like pool of mycobacteria, putatively associated with an environmental reservoir in East Africa, by gain of persistence and virulence mechanisms (Supply *et al.*, 2013). One such mechanism consists of a change in cellular morphotype due to recombination in the *pks5* locus in *M. tuberculosis*, revealing a key step in pathoadaptation (Boritsch and Brosch, 2016).

In contrast, the MTBC and *M. canettii* genomes are substantially smaller than those of the most closely related NTM, i.e. 6.4 Mbp for *M. kansasii* and 6.6 Mbp for *M. marinum* (Wang *et al.*, 2015; Gagneux, 2018). This is attributable to the deletion of genes putatively superfluous for MTBC pathogenic lifestyle, only partly compensated by the acquisition of new genes through horizontal gene transfer (Veyrier *et al.*, 2011). However, the MTBC pathogenicity does not appear to be simply determined by the absence or presence of known virulence factors. For example, the PhoPR two-component system, the DosR/S/T regulon, the mce-associated genes, and the early secretory antigenic target protein 6 (ESAT-6) secretion are also present in non-pathogenic NTMs (Wang *et al.*, 2015). In the same way, the PE and PPE protein families, which are associated with virulence modulation and host immune responses in MTBC are also found in non-pathogenic NTMs (Cole *et al.*, 1998; Gey van Pittius *et al.*, 2006; Ates *et al.*, 2018).

Detailed genetic and genomic studies have shown that the major causative agents of human TB, *M. africanum* and *M. tuberculosis sensu stricto* can further be divided into seven phylogenetic lineages: L1 (East African Indian), L2 (Beijing), L3 (Delhi/CAS), L4 (Euro-American), L7 (Ethiopia) fit in *M. tuberculosis sensu stricto* while L5 (West African 1) and L6 (West African 2) are part of *M. africanum* (Niemann *et al.*, 2016; Cadena *et al.*, 2017). The human-adapted MTBC lineages have different phylogeographical distributions, with strains found either worldwide or more restricted to a unique geographical region (Coscolla and Gagneux, 2014). L5 and L6 are lineages essentially restricted to patients living in or originating from West Africa. Likewise, L7 is

solely found in Ethiopia. L1 and L3 are found mainly in regions around the Indian Ocean. In contrast, L2 and L4 are considered as more universal lineages (Figure 2) (Gagneux, 2018).

While L2 is strongly dominating in East Asia but is also present on other continents (Merker *et al.*, 2015), L4 globally has the widest geographic distribution (Stucki *et al.*, 2016; Gagneux, 2018). However, whole genome sequencing analyses have shown the ability to further subdivide L4 into multiple sublineages, also characterized by unequal geographic distributions (Coll *et al.*, 2014; Stucki *et al.*, 2016) (Figure 3a). The so-called L4.3/LAM, L4.1.2/Haarlem, L4.10/PGG3 show the largest geographical spread, while L4.1.3/Ghana, L4.5/New1, L4.6.1/Uganda, and L4.6.2/Cameroon are specifically found in Africa or Asia, and are almost completely absent in Europe and America (Figure 3b) (Stucki *et al.*, 2016; Gagneux, 2018). Such contrast in epidemiological success between (sub) lineages that can be viewed as generalist or specialist has been proposed to potentially reflect differential biological adaptation of the corresponding strains to their sympatric host population, as well as the influence of human migration and colonization (Stucki *et al.*, 2016).

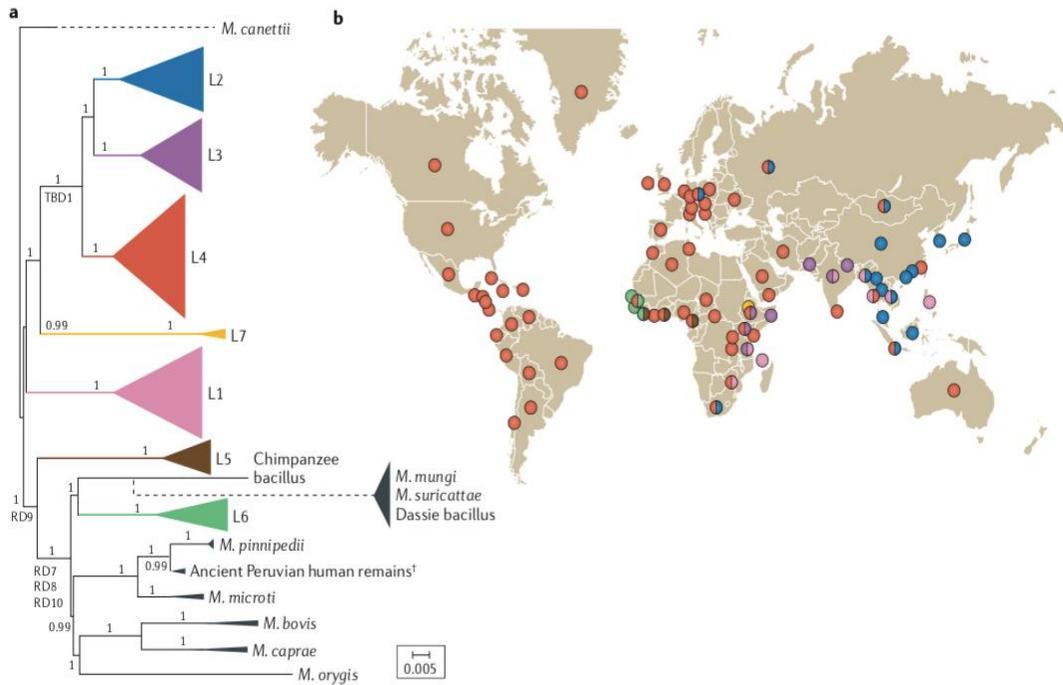


Figure 2: Worldwide phylogeography of human-adapted MTBC (Gagneux, 2018).

a. *Mycobacterium tuberculosis* complex (MTBC) genome-based phylogeny rooted according to *Mycobacterium canettii*. The MTBC consists of seven human-adapted lineages (in colour) and some animal-adapted lineages (in grey). Branches of the main lineages are collapsed to improve clarity (indicated by triangles). The absence of *M. tuberculosis*-specific deletion 1 (TBD1) is common between lineage 2 (L2), L3 and L4 strains (Brosch *et al.*, 2002). Likewise, deletion of the region of difference 7 (RD7), RD8, RD9 and RD10 is designated for strains under the corresponding branches. Phylogenetic relationship between *Mycobacterium mungi*, *Mycobacterium suricattae* and the dassie bacillus and members of MTBC members is marked by grey dotted line. Sharp edge is representative of an old genome from human DNA in Peru dating from ~1,000 year (Bos *et al.*, 2014). **b.** The worldwide distribution of human-adapted MTBC.

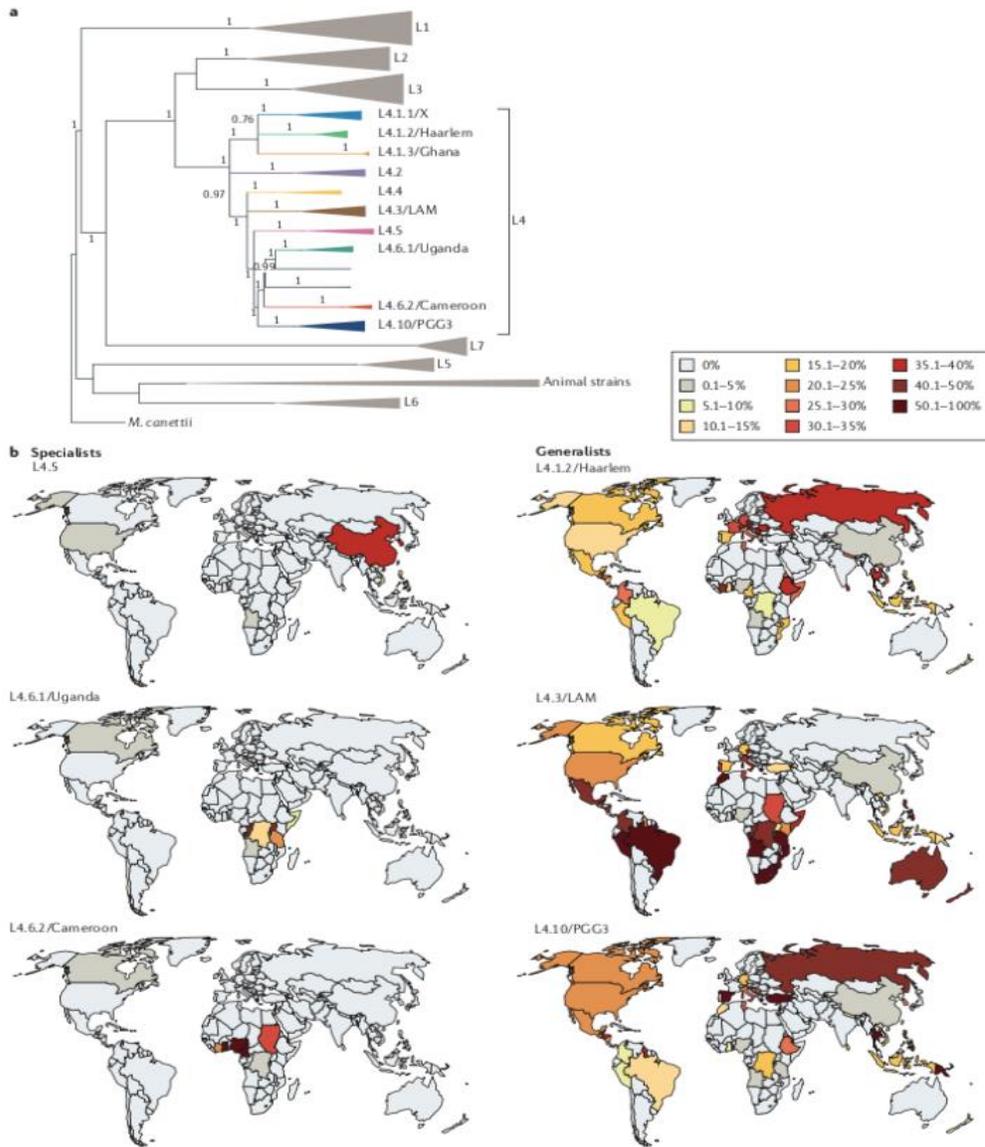


Figure 3: MTBC L4 sublineages and worldwide distribution (Gagneux, 2018).

a. MTBC L4 genome-based phylogeny reveals at least ten sublineages. To improve clarity, branches of MTBS lineages and sublineages are collapsed (indicated by triangles). **b.** Genotypic screening of L4 isolates from 100 countries showed the geographical distribution of L4 sublineages being either geographically restricted or distributed globally. Percentages indicates the proportion of the respective L4 sublineage in a given country. LAM, Latin American- Mediterranean. PGG3, principle genotypic group.

D. Tuberculosis pathology and infection cycle

TB is predominantly a pulmonary disease but can also exhibit extra-pulmonary forms resulting from hematogenous dissemination and affecting e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints, and bones, or meninges (Lee, 2015). Symptoms vary by the site of infection, but the most common ones, associated with the dominant pulmonary forms, are a cough with bloody sputum, chest pains, weakness, weight loss, fever and night sweats (WHO, 2018e).

Infection usually starts with the inhalation of contaminated droplets, emitted into the air by a patient with pulmonary TB especially while coughing. After coughing or sneezing, the infectious particles can remain in the air for several hours and the minimal infectious dose can be very low, ranging from 1 to 10 bacilli (Russell *et al.*, 2009). Tubercle bacilli can then be inhaled by contacts of TB patients and dragged by the mucosal surface to the lungs. In the case of TB caused by *M. bovis*, infection can occur via the digestive tract after the consumption of e.g. contaminated milk products (Dietrich and Doherty, 2009).

In the lungs, TB bacilli can reach the pulmonary alveoli where they are phagocytized by resident alveolar macrophages (Kaufmann, 2001). The infected macrophages will invade the adjacent lung epithelium, causing a localized pro-inflammatory response leading to the recruitment of additional monocytes and macrophages from neighboring blood vessels. These recruited cells will in turn be infected with mycobacteria, and will migrate to the lymphoid organs for the establishment of an adaptive immune response, but also can contribute to the dissemination of TB bacilli in the whole body (Russell, 2007).

At the pulmonary epithelium level, the aggregation of macrophages and lymphocytes leads to the formation of a pluricellular structure characteristic of TB known as the granuloma. This structure adopts an organized and stratified architecture: the infected macrophages are located in the center of the granuloma and surrounded by giant multinucleated cells resulting from the fusion of several macrophages. A layer of lymphocytes, predominantly T cells, associated with a fibrous sheath delineates the periphery of the granuloma (Ulrichs and Kaufmann, 2006; Russell *et al.*, 2009).

This step marks the end of the rapid replication phase of bacteria in the host and the evolution to a so-called "containment" phase during which the disease does not clinically manifest and the

host is not contagious. At this stage, the granuloma is highly vascularized and the cells of the immune system are actively recruited to the granuloma. In the center of the granuloma, the intervention of the immune system cells will cause necrosis of infected macrophages, leading to the formation of a characteristic necrotic center, the caseum. At a later stage, the granuloma develops a more marked fibrous sheath and the number of blood vessels that penetrate the structure decreases considerably; the center of the granuloma then becomes hypoxic. In this caseous granuloma, bacteria can persist for many years. This stage is known as latent tuberculosis infection (LTBI) (Figure 4) (Ulrichs and Kaufmann, 2006; Russell *et al.*, 2009).

In 90% of the infected individuals, the immune system will successfully keep the bacilli dormant at this stage and prevent the development of the disease. In the remaining 10% of individuals, failure of the “containment” phase will occur, also possibly favored by various factors affecting the patient’s immune system, such co-infection with HIV, malnutrition or diabetes, or tobacco use. As a consequence, the granuloma caseates, breaks, and spills the infectious bacilli into the respiratory system of the patients. The patients will develop active TB and become contagious (Russell, 2007). For patients who do not suffer from immuno-deficiency, half of the total risk of progressing to active TB over the lifetime is concentrated over the first 2-3 years after primo-infection (Blower *et al.*, 1995). Such time window is therefore often used as a study period in molecular epidemiological studies to detect potential recent transmission of TB in a patient population.

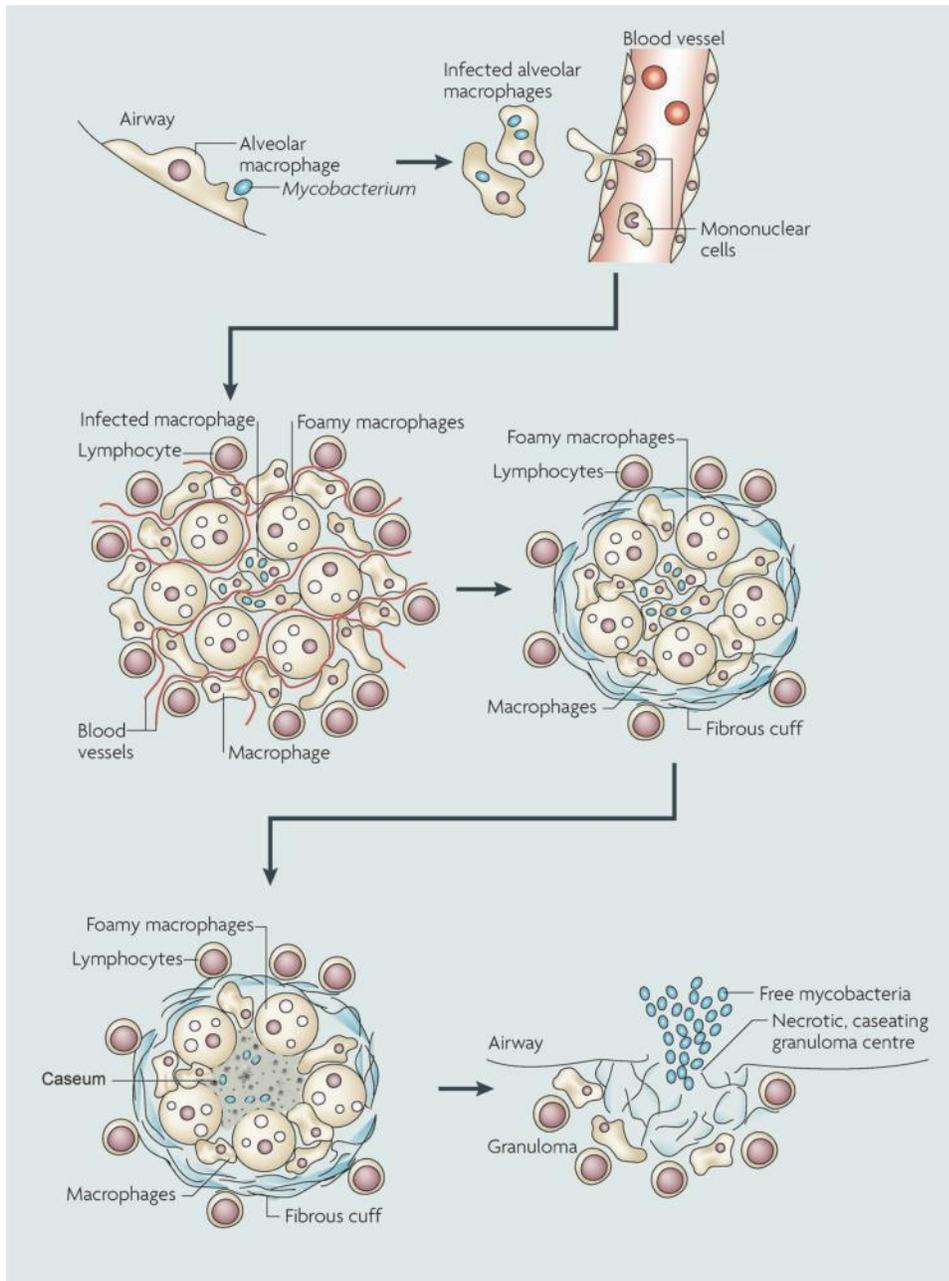


Figure 4: The pathology of the granuloma (Russell *et al.*, 2009).

E. Global tuberculosis epidemiology

TB represents a major global public health problem. It is one of the top 10 leading causes of death worldwide, and the first infectious killer ranking above the human HIV infection. According to the latest estimate, one-fourth of the world population is infected by the TB pathogen (Houben and Dodd, 2016). According to the World Health Organization (WHO), in 2017, there were about 10 million incident TB cases including 1 million children and 1.6 million people died from the disease including 230,000 children (Figure 5) (WHO, 2018a).

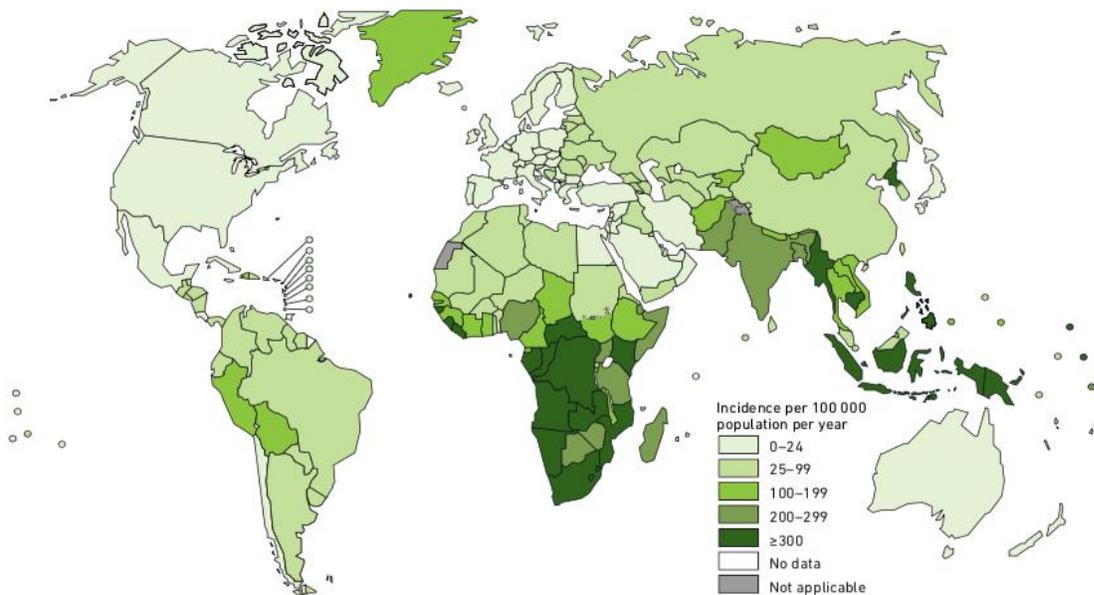


Figure 5: Worldwide estimated TB incidence, 2017 (WHO, 2018a).

According to WHO, 30 countries are classified as high TB burden countries and accounted for 87% of new TB cases in 2016. Among these, eight countries represent 66% of the new TB cases starting with India, followed by China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh and South Africa (WHO, 2018a).

F. Tuberculosis treatment and drug resistance

1. First line treatment

While most bacterial infections are cured after one or two weeks of treatment, the disease caused by *M. tuberculosis* requires six months of first-line treatment, when the infecting strain is drug sensitive. A long duration of treatment is required for favoring complete eradication of the infecting bacilli. The treatment regimen includes an intensive phase and a continuation phase.

The intensive phase lasts for two months. This phase consists of the simultaneous intake of rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA), and ethambutol (EMB). The purpose of the intensive phase is to quickly kill most of the bacilli in order to rapidly improve the patient's pathological situation and prevent prolonged contagiousness. It is followed by a continuation phase, which comprises the intake of RIF and INH during 4 months to sterilize the lesions and prevent case relapses (WHO, 2017a).

According to the latest WHO report, a daily therapy is preferred over a three-time weekly dosing. In addition to that, a fixed dose with combined drug tablets is favored over a single drug formulation. To note, children, pregnant women, and TB/HIV patients have different treatment regimens (WHO, 2017a). The recommended treatment of patients diagnosed with an *M. bovis* infection also differs, comprising RIF, INH and EMB over a total of 9 months. This modification is to take into account the natural resistance of *M. bovis* to PZA (Lan *et al.*, 2016).

In addition to the susceptibility of the infecting strain to the first-line drugs, patient's education and counseling on the severity of the disease and the importance of treatment compliance are main factors favoring an effective treatment. In 2016, 82% of newly diagnosed cases worldwide successfully completed their treatment (WHO, 2018a).

Crucially and ideally, a rapid and effective diagnosis including drug susceptibility testing (DST) (see further below) should be performed in order to ensure that the patient is placed on effective treatment, or to possibly modify the treatment if drug resistance is detected.

2. Drug resistance and second line treatment

The first drug that was shown to be effective for treating TB was SM. However, drug resistance already emerged, just a few years after the molecule started to be used as a mono-therapy (Crofton and Mitchison, 1948).

As a reflection of the successive addition of new drugs in anti-TB treatments over the following decades, multidrug resistant (MDR; defined as resistance to at least RIF and INH) and extensively drug resistant (XDR; defined as MDR plus resistance to at least one aminoglycoside drug and to any fluoroquinolone (FQ)) TB forms have evolved and spread worldwide, greatly complicating diagnostics, treatment and failure rates. The selection of such resistant forms was globally favored by the frequent lack of adequate diagnostics and/or use of inappropriate treatments and/or poor patient's compliance, leading to resistance acquired and/or amplified during treatment. However, several lines of evidence indicate that the MDR TB epidemics is now mainly driven by transmission of drug resistance, leading to TB cases defined as primary resistant (Marais, 2016).

The classical longer MDR TB treatment lasts for at least 18 months, modified according to patients' response. As recommended by WHO, the intensive phase (6 months) comprises the use of all 3 drugs from Group A (FQ, bedaquiline (BDQ) and linezolid (LIN)) and one drug from Group B (clofazimine (CFZ) or cycloserine (Cs)/ Terizidone (TRD)), all continued for the rest of the treatment (12 months) except for BDQ that is stopped at 6 months (Table 1). If the minimum number of effective TB drugs cannot be composed as described above (because of detected resistance or intolerance) with only one or two drugs from Group A can be used, both group B agents can be added to the regimen. To monitor treatment response, it is strongly recommended to perform culture and smear microscopy at a monthly interval (WHO, 2018b).

Table 1: Recommended anti-TB drugs used for longer MDR TB regimen (WHO, 2018b).

GROUPS & STEPS	MEDICINE	
Group A: Include all three medicines	Levofloxacin <u>OR</u> Moxifloxacin	Lfx Mfx
	Bedaquiline ^{2,3}	Bdq
	Linezolid ⁴	Lzd
	Clofazimine	Cfz
Group B: Add one or both medicines	Cycloserine <u>OR</u> Terizidone	Cs Trd
	Ethambutol	E
Group C: Add to complete the regimen and when medicines from Groups A and B cannot be used	Delamanid ^{3,5}	Dlm
	Pyrazinamide ⁶	Z
	Imipenem-cilastatin <u>OR</u> Meropenem ⁷	Ipm-Cln Mpm
	Amikacin (<u>OR</u> Streptomycin) ⁸	Am (S)
	Ethionamide <u>OR</u> Prothionamide ⁹	Eto Pto
	<i>p</i> -aminosalicylic acid ⁹	PAS

In 2016, WHO supported the alternative use of a standardized shorter MDR TB regimen under eligibility criteria, including absence of resistance to a second-line drug included in the regimen, pregnancy and extrapulmonary TB. The duration is reduced to 9-12 months by the use of seven anti-TB drugs (WHO, 2017b), comprising kanamycin (KM), moxifloxacin (MFX), ethionamide (ETH), CFZ, INH at high dose, PZA and EMB during 4 to 6 months, followed by MFX, CFZ, PZA and EMB for 5 months. In case of failure of this shorter MDR TB regimen, development of drug intolerance, if the patient interrupts the treatment for more than 2 months, or some exclusion criteria emerge, WHO recommends shifting to the long MDR TB regimen (Figure 6) (WHO, 2016c).

More limited treatment options are offered for the treatment of XDR TB patient, which most often needs customization depending on extensive drug resistance profiling and previous treatment history. In addition, the high cost of the medicines makes them inaccessible to many low- and middle-income countries without subsidizing. According to WHO, the worldwide success rates of treatments in patients with MDR/Rifampicin Resistant (RR) TB and XDR TB who started

treatment in 2014 were only 54% and 30 % respectively whereas success rates reached 83% in patients with drug-susceptible TB. Furthermore, 16% of MDR/RR TB patients died, 8% had treatment failure status and the remaining 22% were lost to follow up (WHO, 2017b).

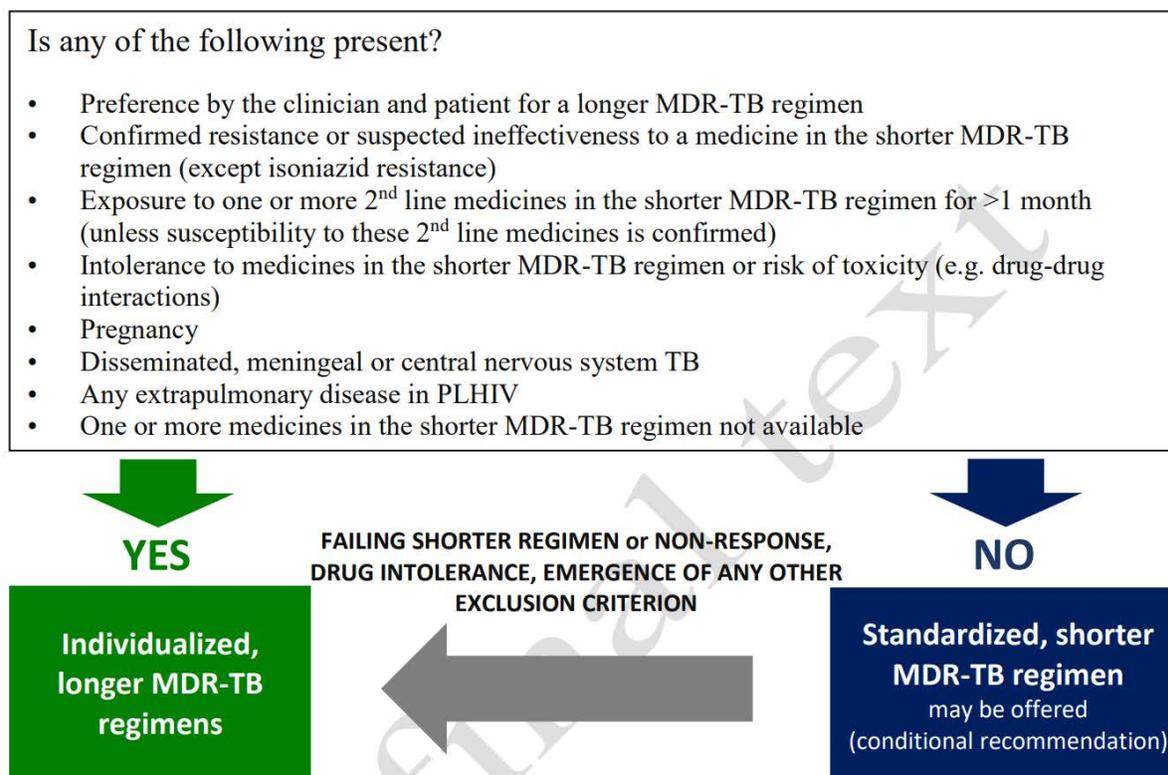


Figure 6: WHO recommendations for a shorter MDR TB regimen (WHO, 2018b).

3. Drug resistance mechanisms and targets

The major mechanism behind drug resistance in *M. tuberculosis* is the selection of mutations in chromosomal genes that code for drug targets or pro-drug activating enzymes. The modes of actions and main known gene targets of first line and mainly used second line drugs are listed below (Figure 7).

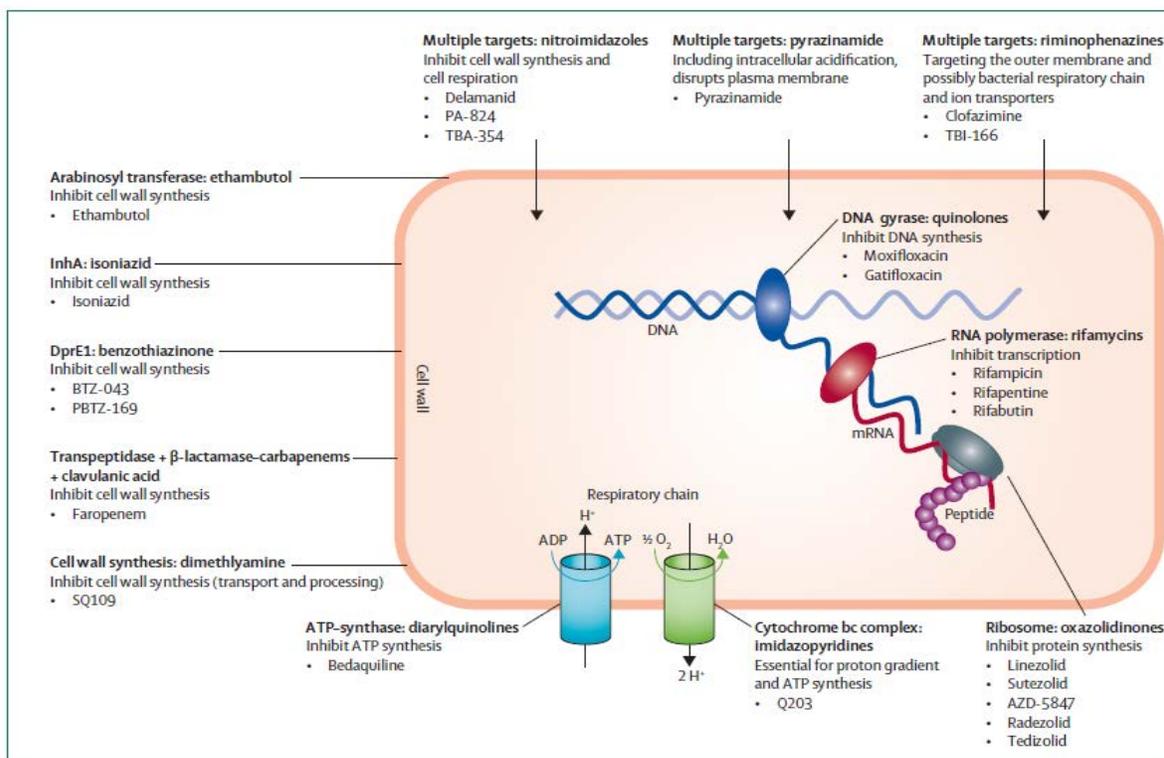


Figure 7: Mechanism of action of anti-TB drugs (Zumla *et al.*, 2014).

a. First line anti-TB drugs

INH inhibits cell wall mycolic acid synthesis, leading to intracellular accumulation of long chain fatty acids and subsequent cell death. As INH is a prodrug, it needs to be activated by a mycobacterial catalase-peroxidase enzyme, encoded by *katG*. The activated product then inhibits the enzyme InhA. Two main molecular mechanisms are thus behind INH-resistant *M. tuberculosis*: (i) inactivation of KatG, (ii) overexpression of InhA. Mutations in the *katG* gene are the most common mechanism of INH resistance. For example, a KatG S315T mutation, which is the most common mutation in INH-resistant isolates, decreases the ability to form INH-NAD adduct rendering KatG unable to activate INH. Noteworthy, mutations in *katG* confer a high level of resistance (Zhang and Yew, 2015). In contrast, mutations in *inhA* promoter at -15T and -8A positions cause an overexpression of InhA, which confers a low level of INH resistance and a cross-resistance to ETH (Cohen *et al.*, 2014).

RIF inhibits the transcription by binding to the β subunit of the RNA polymerase, which is encoded by the *rpoB* gene. While the resistance to INH is caused by several mutations affecting different genes, the mechanism of RIF resistance is way simpler, as almost all resistant strains present mutations in the *rpoB* gene. In addition, about 95% of these mutations are clustered within a small 81 bp region spanning codons 507–533 of the *rpoB* gene, known as the Rifampicin Resistance Determining Region (RRDR), resulting in a high level of RIF resistance. In the majority of the studies, mutations in codons 526 and 531 (*Escherichia coli*-based nomenclature; alias 445 and 450 codons according to the *M. tuberculosis*-based nomenclature (Andre *et al.*, 2017)) are the most commonly associated mutations with RIF resistance (Silva *et al.*, 2011; Palomino and Martin, 2014; Dookie *et al.*, 2018). However, strains bearing mutations outside the RRDR, such as I572F (*Escherichia coli*-based nomenclature; alias I491F according to the *M. tuberculosis*-based nomenclature (Andre *et al.*, 2017)), have been identified at high prevalence in some world regions, e.g. in 30% of MDR TB strains in survey done in Swaziland in 2009 (Sanchez-Padilla *et al.*, 2015). A recent study involving my hosting laboratory showed that at least two lineages of I491F-bearing strains also circulate in South African provinces, only one which represents the longitudinal extension of the outbreak involving such strains in Swaziland (Makhado *et al.*, 2018).

PZA is a nicotinamide analog and a critical first-line TB-drug. Its incorporation in the TB therapy allowed the reduction of treatment period from 9 to 6 months. One unique characteristic of this anti-TB drug is its capability to inhibit dormant bacilli living in an acidic environment (Silva *et al.*, 2011). As a prodrug, it needs to be converted into its active form, pyrazinoic acid, by the pyrazinamidase/nicotinamidase (PZase) enzyme encoded by the *pncA* gene in *M. tuberculosis*. Once in its active form, it has the ability to disrupt bacterial membrane energetics inhibiting thus membrane transport functions (Zhang and Mitchison, 2003). As a reflection of this mode of action, the main mechanism of pyrazinamide resistance in *M. tuberculosis* is the occurrence of mutations in the *pncA* gene. In contrast to RIF resistance mutations that largely concentrate in the RRDR of *rpoB*, PZA resistance mutations can occur over some 300 different nucleotide positions in *pncA* (Yadon *et al.*, 2017). To note, *M. bovis* has a natural resistance to PZA, caused by a phylogenetically conserved H57D mutation (Sreevatsan *et al.*, 1997).

EMB, a bacteriostatic drug, inhibits the arabinosyl transferases encoded by the *embCAB* operon genes and involved in the biosynthesis of arabinogalactans and lipoarabinomannans part of the mycobacterial cell wall (Takayama *et al.*, 1979). In most cases, EMB resistance is due to mutations within the *embCAB* operon, most frequently in the *embB* gene and occasionally in the *embC* gene. Moreover, about 50% of EMB resistance is due to polymorphisms in codon 306 of the *embB* gene (Telenti *et al.*, 1997).

SM is active against a variety of bacterial species, including *M. tuberculosis*. It actively destroys multiplying bacilli but is inactive against dormant or intracellular bacilli. SM inhibits protein synthesis by binding to the 30S ribosomal subunit, resulting in misreading of the mRNA message during translation (Finken *et al.*, 1993). Mutations in the S12 protein encoded by the *rpsL* gene and in the 16S rRNA encoded by the *rrs* gene are the main mechanisms of resistance to SM, in an estimated 50% and 20% of SM-resistant strains, respectively, conferring a high level of resistance (Finken *et al.*, 1993; Nair *et al.*, 1993). To a lower extent, mutations in the *gibB* gene encoding a conserved 7-methylguanosine methyltransferase specific for 16S rRNA, can also confer low levels of SM resistance (Okamoto *et al.*, 2007; Zhang and Yew, 2015). Mutations in the *whiB7* promoter region causes an overexpression of the *eis* and *tap* efflux pump genes leading to cross-resistance to SM and Kanamycin (KM) (Reeves *et al.*, 2013). To note, while SM has been historically used in the first line anti-TB treatment for decades, it is no longer utilized, except in some customized treatments of complex XDR cases, because of its frequent side effects including ototoxicity.

b. Second-line anti-TB drugs

Injectable anti-TB agents (amikacin/ kanamycin, capreomycin). KM and its derivative amikacin (AMK) are also inhibitors of protein synthesis. Mutations in *rrs* such as in position 1401 are associated with a high level of resistance to both KM and AMK (Alangaden *et al.*, 1998; Suzuki *et al.*, 1998). *M. tuberculosis* strains with resistance to capreomycin (CAP) carry mutations in the *tlyA* gene and in the *rrs* gene (A1401G, G1484T) (Maus *et al.*, 2005a). Mutants with A1401G in *rrs* gene have cross-resistance to AMK, CAP and resistance to KM (Georghiou *et al.*, 2012). C1402T *rrs* mutations confer a high level of resistance to CPM and a low level of resistance to KM and Viomycin resistance. Finally, the G1484T *rrs* mutation confers a high level of resistance to

each of the four drugs (Maus *et al.*, 2005b). As indicated above, mutations in the *whiB7* promoter region causes an overexpression of the *eis* and *tap* efflux pump genes leading to cross-resistance to SM and KM (Reeves *et al.*, 2013).

FQ inhibit the DNA gyrase resulting in microbial death. Mutations in *gyrA* and *gyrB* genes respectively encoding the subunits A and B of DNA gyrase are responsible for FQ resistance. Essentially all known resistance conferring mutations are found within the conserved quinolone resistance determining region (QRDR), mostly in *gyrA* (320 bp), and to a lesser extent in *gyrB* (375 bp) (Maruri *et al.*, 2012).

ETH has a bactericidal activity only against *M. tuberculosis* (Zhang and Yew, 2015). It is also a prodrug, which needs to be activated by the mono-oxygenase EthA. Mutations in the EthA activating enzyme of the drug are dominant among *M. tuberculosis* clinical isolates that are resistant to ETH and other thioamides (Baulard *et al.*, 2000). EthR, the EthA repressor, is a more secondary target of mutations that confer resistance to these drugs (Baulard *et al.*, 2000; Engohang-Ndong *et al.*, 2004). Furthermore, a single mutation in *mshA* involved in mycothiol biosynthesis confer resistance to ETH (Ang *et al.*, 2017). In addition, ETH shares the same final target InhA as INH. Therefore mutations in this target confer resistance to both ETH and INH (DeBarber *et al.*, 2000).

LIN binds to domain V of the 23S rRNA peptidyl transferase of the ribosomal 50S subunits in order to inhibit the very first step in protein synthesis (Barrett, 2000). A high level of linezolid resistance is conferred by G2061T and G2576T mutations in the 23S rRNA (*rrl*) sequence of *M. tuberculosis*. Moreover, mutations in the *rplC* gene encoding ribosomal protein L3 confer a low level of LIN resistance (Hillemann *et al.*, 2008; Beckert *et al.*, 2012).

BDQ, a major, newly available drug in the treatment of MDR and XDR TB cases, is active against growing and non-growing *M. tuberculosis*. It works by inhibiting the mycobacterial adenosine triphosphate (ATP) synthase, resulting in ATP depletion. Initial studies performed in vitro first identified mutations in the subunit C encoded by *atpE* in the F₀ moiety of the mycobacterial F₁F₀ ATP synthase, as conferring resistance to BDQ (Andries *et al.*, 2005). It was more recently shown that the upregulation of the efflux pump MmpL5 due to mutations in the transcriptional regulator Rv0678 (alias MmpR5) also leads to cross-resistance to both BDQ and

CFZ (Hartkoorn *et al.*, 2014). In clinical isolates, BDQ resistance appears to be much more frequently associated with mutations in *rv0678* than in *atpE* (Bloemberg *et al.*, 2015; Hoffmann *et al.*, 2016; Zimenkov *et al.*, 2017) although the impact of different individual mutations in *rv0678* on drug resistance levels is unclear (Villemas *et al.*, 2017).

CFZ is a drug that was mostly used for treating leprosy, but is now also recommended in the short-term regimen of MDR TB cases (WHO, 2016c). The exact mechanism of action of CFZ is not well understood. It has been suggested that reactive oxygen species such as superoxide and H₂O₂ are produced by oxidation of reduced CFZ (Van Deun *et al.*, 2010; Cholo *et al.*, 2012). Mutations in *Rv0678* confer resistance to both CFZ and BDQ by indirectly increasing drug efflux (Hartkoorn *et al.*, 2014; Zhang and Yew, 2015). However, mutations in *Rv1979c* and *Rv2535c* have been also described as conferring resistance to CFZ (Zhang and Yew, 2015). In addition, mutations in *PepQ* (*Rv2535c*) were also linked to low level of cross-resistance to CFZ and BDQ (Almeida *et al.*, 2016).

Para-aminosalicylic acid (PAS), along with INH and SM, was one of the first antibiotics used in the treatment of TB (Dookie *et al.*, 2018). Being an analogue of para-amino benzoic acid, PAS competes with this compound for the enzyme dihydropteroate synthase, thereby inhibiting folate synthesis. The T202A mutation in *thyA* gene, initially considered to be associated with PAS resistance, was demonstrated to actually be a phylogenetic marker of Latin American Mediterranean strains (Dookie *et al.*, 2018). Mutations in other genes, *floC* encoding dihydrofolate synthase and *ribD* encoding riboflavin biosynthesis enzyme, have alternatively been associated with PAS resistance in clinical isolates (Dookie *et al.*, 2018).

Cs, a bacteriostatic anti-TB drug and a D-alanine analogue, inhibits peptidoglycan synthesis by blocking the D-alanine- D-alanine ligase (Ddl) action. In addition, it prevents the interconversion of L-alanine to D-alanine (substrate to Ddl) by inhibiting D-alanine racemase (Alr) (Bruning *et al.*, 2011). Alr has been designated the main target of Cs (Cáceres *et al.*, 1997; Feng and Barletta, 2003). However, contradictory evidence exists on the association of mutations in this gene and *ddl* with resistance to Cs in clinical isolates (Halouska *et al.*, 2014; Nakatani *et al.*, 2017).

Delamanid (DLM) is a pro-drug that needs to be activated by deazaflavin dependent nitroreductase enzyme encoded by the *ddn* gene. Its major role is to inhibit mycolic acid biosynthesis (Palomino and Martin, 2014). Mutations in *ddn* and *fgdI*, the genes associated with

the prodrug activation or mutations in the genes associated with the F420 biosynthetic pathway (*fbiA*, *fbiB* and *fbiC*) confer resistance to DLM in *M. tuberculosis* (Manjunatha *et al.*, 2006; Shimokawa *et al.*, 2014).

G. Epidemiology of MDR and XDR TB

The worldwide emergence of anti-TB drug resistance has become a major public health problem and an enormous hurdle for the global control and eradication of the disease. However, despite the global importance of the problem, the incidence of MDR TB considerably varies among world regions (Lange *et al.*, 2018).

Globally, in 2017, there were an estimated 558 000 new cases of RR TB of which an estimated 82% had MDR TB. The highest numbers, representing 47% of the total MDR/RR TB cases are found in India (24%), China (13%), and the Russian Federation (10%) (Figure 8) (WHO, 2018a).



Figure 8: Estimated MDR/RIF TB case-incidence, in countries with more than 1000 reported cases, 2017 (WHO, 2018a).

However and importantly, these estimations are largely imprecise. Indeed, only about 25% of the estimated MDR and XDR TB cases are actually diagnosed and treated as such, especially in low- and middle-income countries where there is a lack of diagnostic capacities for testing susceptibility or resistance to anti-TB drugs (WHO, 2018).

The proportion of MDR TB cases is higher in patients who have previously received anti-TB treatment in the past (18%) (WHO, 2018a). In countries with low MDR TB incidence, such cases are more common among foreign-born patients, such as migrants, often corresponding to imported cases (Dahle *et al.*, 2003). As human travel and migration are now widespread, virtually any country is at risk to have MDR TB cases (Lange *et al.*, 2018). As a prominent example of the impact of migration, a recent international investigation identified a cross-border outbreak of multidrug resistant TB among migrants arriving in Europe (Walker *et al.*, 2018).

H. TB situation in Lebanon and impact of the Syrian crisis

Lebanon is an upper/middle-income and a low-TB burden country. According to WHO estimates in 2017, there were 710 new cases (632 notified) with an incidence of 12/100,000 and an average mortality rate of 1/100,000 (WHO, 2017c).

Although these figures are relatively low, the TB situation has deteriorated over the recent years (Figure 9) and significant risks exist for further degradation, in relation with the crisis prevailing in nearby Syria since 2011. As per the Lebanese government, Lebanon hosts approximately 1.5 million Syrians who have fled the war in Syria. Those include 991,917 million United Nations High Commissioner for Refugees (UNHCR) registered Syrian Refugees (SR), plus hundreds of thousands of unregistered refugees. These refugees are scattered in hundreds of informal sites across the nation, however mostly concentrated in Akkar, Baalbek El Hermel, and Bekaa governorates (Figure 10) (UNHCR, 2018). In addition, the country hosts 34,000 Palestinian Refugees from Syria (PRS), 35,000 Lebanese returnees and a pre-existing population of more than 277,985 Palestinian Refugees in Lebanon (PRL). As a result, Lebanon is the country hosting the largest refugee population proportionally to its national population worldwide (Cousins, 2014; Ismail *et al.*, 2018).

Such populations living in or originating from areas with armed conflicts are particularly vulnerable to TB. The risk of TB disease in these populations is up to 20 times higher than in general populations, linked to malnutrition, overcrowding and discontinuity in health services (Kimbrough *et al.*, 2012). Difficulties in accessing diagnosis, starting and/or completing appropriate treatment can promote TB transmission and the emergence and spread of drug resistance. Such unfavorable conditions are known to prevail in Syria. Indeed, while the officially reported prevalence of TB in the country was 23/100,000 in 2011, the year of crisis onset, the war that followed has led to disruption in drug supplies and treatment of TB patients and to miserable living conditions in multiple places on a national level (Sahloul *et al.*, 2016).

Moreover, Lebanon also hosts large numbers of migrant workers originating from high TB burden settings, thus at risk of reactivating latent TB infection. At present, there are approximately 250,000 women migrant domestic workers in Lebanon with the highest proportion coming from Ethiopia, Bangladesh and the Philippines respectively (ILO, 2016).

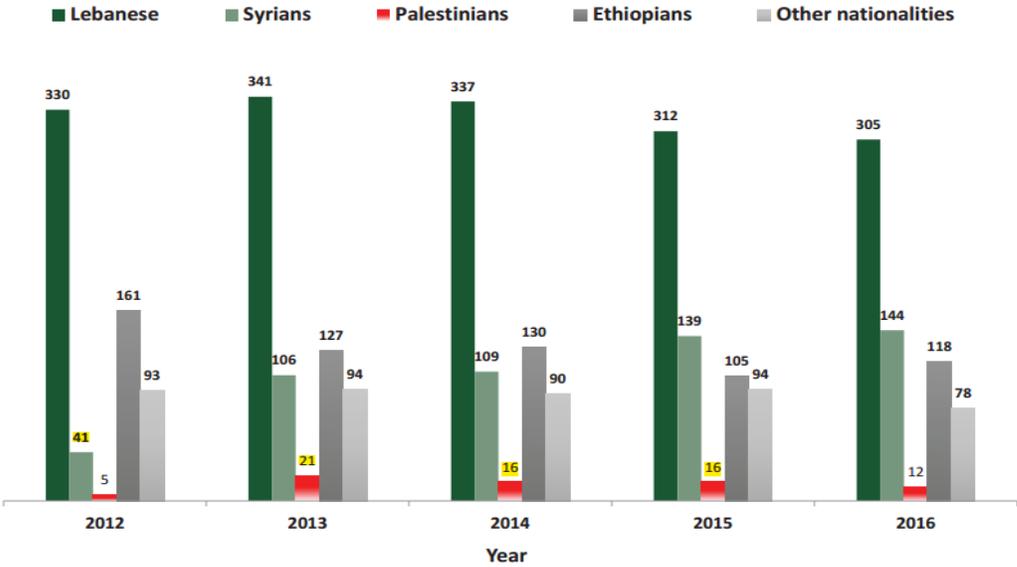


Figure 9: Trend over time of notified TB cases among the national and non-national population, 2012-2016 (MoPH, 2016).

As a reflection of this situation, while the TB incidence decreased in Lebanon until 2011, the National TB Program (NTP) reported a 27-percent increase in notified cases in 2012 compared to the previous year (Cousins, 2014). According to WHO and the Lebanese Ministry of Public Health (MoPH), the number of cases among the non-Lebanese raised from 200 in 2011 to 300 in 2012, which was attributed to SR influx after the beginning of the Syrian war in 2011. In addition, the number of notified cases among Lebanese citizens also increased from 298 in 2011 to 330 in 2012 (WHO and MoPH, 2016). This trend was subsequently confirmed, with a dramatic increase in notified TB cases in Lebanon in 2014, 2015 and 2016, peaking at 682, 666 and 657 respectively. An increase was especially seen among the SR with 109, 139 and 144 notified cases over the same years respectively (Figure 9) (MoPH, 2016). To note, some substantial divergences are noticeable in numbers of reported TB cases between some sources, with e.g. only 424 TB cases reported in 2014 according to an epi monitor bulletin (WHO and MoPH, 2016), as opposed to 682 reported TB cases in the same year according to (MoPH, 2016) (Figure 9).

Before the war in Syria, in Lebanon, the proportion of non-Lebanese over Lebanese among TB patients was 33% (Ismail *et al.*, 2018). However, in 2015, 53% of all the notified cases were among the non-Lebanese in 2015 (MoPH, 2016). Referring to local information from NTP, over half the people currently affected by TB and treated against the disease are not Lebanese nationals.

NTP reported a treatment success rate much higher in the Lebanese population (around 90%) than among the non-Lebanese patients, of which 50% leave the country before the completion of their treatment (MoPH, 2017). Cases of Syrian patients with treatment interruption due to insecurity in Syria were identified. These conditions favor the risk of emergence of drug resistance strains.

Importantly, substantial uncertainty long prevailed on the true burden of MDR TB cases in Lebanon. A sole national survey was made over 2002 to 2004, identifying 15 MDR TB patients out of a total of 206 cases by DST, resulting in an estimated incidence of 7,3%. For all other years, testing for such resistance was done only upon a request from the physician (Araj *et al.*, 2016), and NTP evaluation of the MDR TB situation thus largely relied on estimates. Data from different sources were based on non-representative patient populations (with probable selection bias towards e.g. failed treatment) and were therefore difficult to compare (Table 2).

Importantly as well, DST for second line anti-TB drugs was not available in the country before the beginning of my PhD, making it impossible to detect, and appropriately treat, potential XDR TB cases (MoPH, 2017).

Table 2: MDR TB cases in Lebanon based on different sources from 1994 -2014 (WHO, 2010; Araj *et al.*, 2016; Saabiyeh *et al.*, 2017).

	MDR TB cases (WHO, 2010)	MDR TB cases (Saabiyeh <i>et al.</i> , 2017)	MDR TB cases (Araj <i>et al.</i> , 2016)
1994	NA	NA	11
1995	NA	NA	
1996	NA	NA	
1997	NA	NA	11
1998	NA	NA	
2002	NA	NA	
2003	NA	NA	15
2004	NA	NA	
2005	3	8	NA
2006	3	2	4
2007	2	2	
2008	3	2	NA
2009	4	6	NA
2010	7	7	NA
2011	3	2	NA
2012	6	8	15
2013	9	8	
2014	10	6	NA

(NA: Not available DST data)

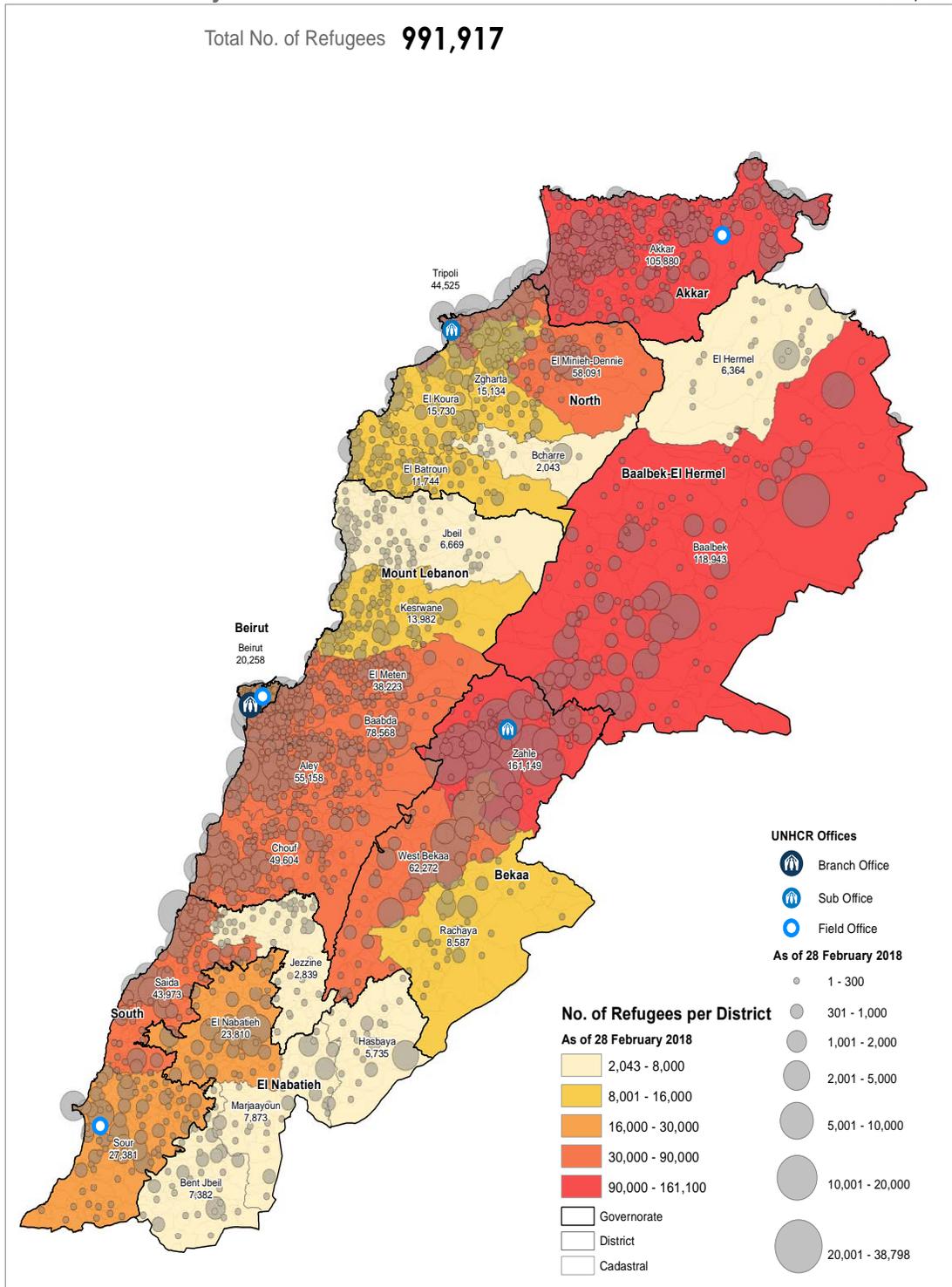
The above uncertainties around the estimated incidence and notified cases largely reflect existing diagnostic gaps in the country. Diagnostic capabilities for TB disease are present in the country but are very often limited to smear microscopy. There are nine TB centers across the country, each receiving samples from patients living in the area. In principle, almost all the samples collected from the different TB centers are transported to the reference TB center located in the capital Beirut known by Karentina TB center, for being subjected to microscopic examination and nucleic acid amplification-based GeneXpert tests. As indicated above, before the onset of my PhD, cultures and DST were however not systematically performed, except upon request e.g. for special cases such as treatment failure or HIV patients. Thus, there was no precise knowledge of the actual epidemiological situation and the prevalence of drug resistant TB in resident and displaced populations in Lebanon. This has driven the definition of the objectives of this PhD work, presented on pages 52-54.

SYRIA REFUGEE RESPONSE

LEBANON Syrian Refugees Registered

28 February 2018

UNHCR Lebanon - Beirut
Country Office



This map has been produced by the Inter-Agency Information Management Unit of UNHCR based on maps and material provided by the Government of Lebanon for operational purposes. It does not constitute an official United Nations map. The designations employed and the presentation of material on this map do not imply the expression of any opinion whatsoever on the part of the Secretariat of the United Nations concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

Figure 10: Distribution of registered Syrian refugees (SR) in different Lebanese governorates, 2017 (UNHCR, 2017)

For further information on map, contact
Jad Ghosn at ghosn@unhcr.org or Maroun Sader at sader@unhcr.org

I. Diagnosis

1. Immunological tests: TST, IGRA

Immunological tests are primarily used for recognition of individuals with LTBI, although they do not accurately differentiate LTBI from active TB. Two main types of tests are used:

Tuberculin Skin test (TST), also known as the Mantoux Intradermal test since 1910, is the oldest test used for the detection of LTBI. It consists of an intradermal test based on the injection of 0.1 mL of a solution containing tuberculin Purified Protein Derivative (PPD). The tuberculin solution contains a complex mixture of more than 200 antigens, common to *M. bovis* BCG and most NTMs. Results are interpreted by measuring the induration diameter 72 hours after the injection (Herrmann *et al.*, 2007). As a limitation, a previous vaccination with BCG or infection with NTM can result in a false positive interpretation. For interpreting an intradermal reaction result and concluding to a probable or more unlikely infection, the observed induration diameter is correlated with possible, specific risk factors of the concerned individual.

Interferon-Gamma Release Assays (IGRA) are somehow an upgrading of TST, which avoids the possible interference of previous BCG vaccination. Two commercially available blood assays exist: QuantiFERON-TB Gold (QFT-G, Cellestis, Carnegie, Australia) and T-SPOT.TB (Oxford Immunotec, Oxford, United Kingdom). The two tests are based on the measurement of the interferon gamma (IFN- γ) response expressed by T cells in reaction to the exposure to major proteins, ESAT-6 and filtrate protein 10 culture (CFP-10) that are present in MTBC and absent in BCG vaccine strains. The TB-SPOT.TB and QFT-G assays quantify the amount of IFN- γ secreted in the patient's blood respectively. In addition to obtaining a quick result, available within a maximum of 24 hours, these tests can be performed in a single consultation since only one blood sample is sufficient (Herrmann *et al.*, 2007). As a limitation, the IGRA tests are unable to accurately differentiate between a latent infection and a disease. Therefore, a physician should base more on the clinical symptoms, imaging and/or culture-based or molecular tests to distinguish between both

options. In addition, in order to accurately measure IFN- γ response by IGRAs, a fresh blood sampling is needed with sustainable white blood cells. In order to overcome this limitation, a new QuantiFERON-TB Gold In tube test (QFT-GIT) (Cellestis Limited, Carnegie, Victoria, Australia) was developed, by tolerating incubation of blood in collection tubes. In addition to ESAT-6 and CFP-10 proteins, QFT-GIT antigens include a third *M. tuberculosis* protein, TB7.7 (Lempp *et al.*, 2017).

2. Imaging

In a highly suspicious case of active TB, imaging plays a valuable role in the patient's first evaluation, especially as a screening tool for pulmonary TB (Nachiappan *et al.*, 2017). Chest X-ray provides better insights into thoracic abnormalities that can be of variable morphologies. Radiological findings in primary TB include lymphadenopathy, pulmonary consolidation, and pleural effusion. Post-primary TB fallouts from reactivation of latent foci. There are three types of radiological lesions in post-primary TB: centrilobular nodule, cavitation, and consolidation mainly located in the upper lobes of the lungs (Nachiappan *et al.*, 2017). It is very hard to differentiate between recent lesions and tubercle sequelae. It is an inescapable but non-specific examination test. A poor reproducibility might be observed between different chest physicians reading (WHO, 2016a). Although it is the first-line imaging examination, a chest X-ray examination may not be sufficient. A complementary imaging modality can be used, namely computed tomography (CT scan) that allows a better analysis of the lesions and is able to differentiate between the active and inactive form of the disease (Piccazzo *et al.*, 2014).

Based on chest radiography results, further laboratory tests should follow (Figure 11).

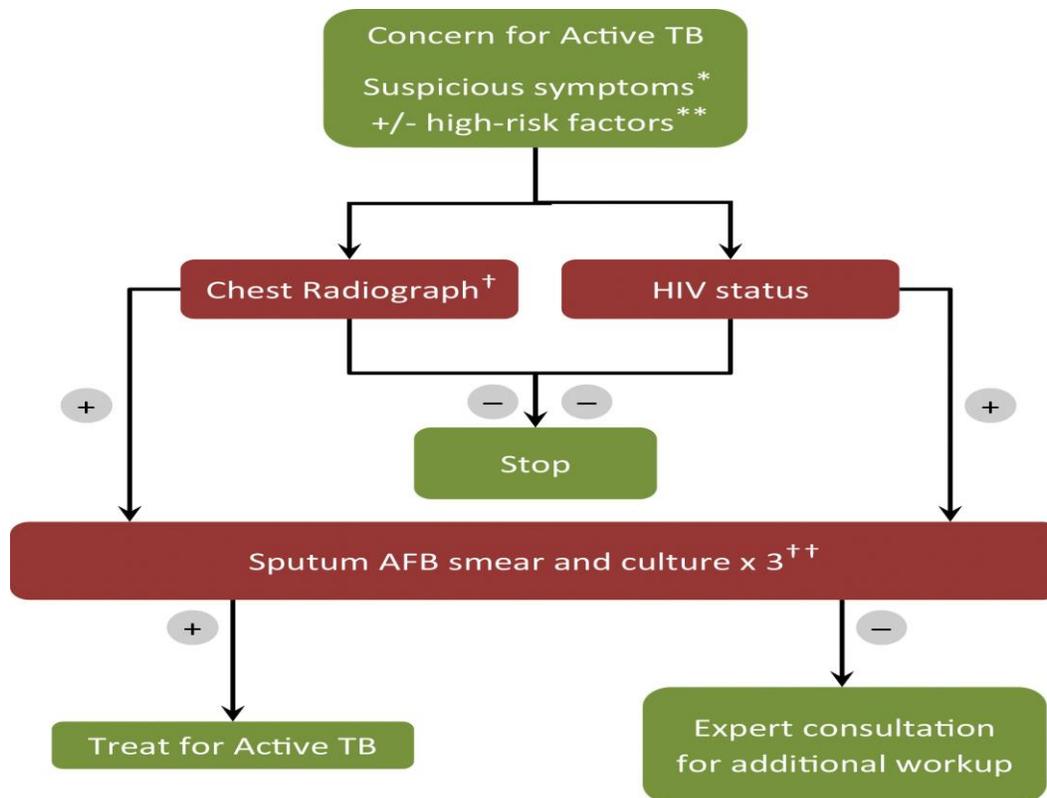


Figure 11: Algorithm for TB patient evaluation (Nachiappan *et al.*, 2017)

*= fever, cough, night sweats, weight loss, hemoptysis; ** = high-risk factors for tuberculosis exposure or reactivation (eg, immigration from endemic area, recent exposure and conversion within the past 2 years, HIV Human Immunodeficiency Virus-positive status, and immunosuppression); † = positive chest radiograph refers to findings that may represent active tuberculosis; †† = send one of the sputum specimens for a nucleic acid amplification test, where available. AFB = Acid-Fast Bacilli.

3. Specimen collection

Relying on the clinical manifestations, any sample type may in principle be treated for detecting the possible presence of mycobacteria (Garcia and Isenberg, 2010). In order to be able to perform subsequent tests under good conditions, collection of the appropriate clinical specimen with adequate quantity and quality is required. Analyzed samples are most often of respiratory origin, such as sputum, bronchial aspirates or bronchoalveolar lavage fluid (Caulfield and Wengenack, 2016). Sputum is the most common specimen type. For initial TB suspect screening, two

consecutive specimen collections, best in the morning, are sufficient for the identification of 95-98% of smear-positive TB patients (WHO, 2015a). To note, the presence of saliva might reflect a sample that will produce a false negative result.

Depending on suspicion on potential infection sites or dissemination, extra-pulmonary specimens can be also processed for analysis such as tissues, sterile body fluids, blood, and urine. During transport to the laboratory, specimens must be refrigerated in order to ensure the viability of mycobacteria and prevent the overgrowth of contaminating microorganisms (Baron and Thomson, 2011).

4. Microscopy

For more than 125 years, microscopic examination of stained smears was considered as a primary, simple, inexpensive and rapid screening method for mycobacterial detection within a clinical sample (Small and Pai, 2010). Conventional light microscopy or fluorescent Light-Emitting Diode (LED) microscopy can be used. The latter one is favored by WHO due to its higher sensitivity and rapidity (WHO, 2015a). As a result of its higher cost, fluorescent staining is however less accessible to resource-limited settings or laboratories, unless it is subsidized. Smear sensitivity depends on the bacterial load, often correlated with the severity of the disease. A reduced sensitivity is observed in children and patient co-infected with HIV (WHO, 2015a). A sputum with a minimum of 1000-10,000 Colony-Forming unit (CFU)/mL is required for reliable bacillus detection (Caulfield and Wengenack, 2016). The overall sensitivity for confirming TB varies according to the form of disease and sample, being lower for extra-pulmonary forms (10-20%) than for pulmonary forms (65%) (Guillet-Caruba *et al.*, 2014). It also depends on the type of staining used, AFB concentration within the sample and technician experience (Caulfield and Wengenack, 2016). Because of this low sensitivity, microscopic examination has a low negative predictive value and should therefore ideally be followed by molecular testing and/or by culture, which have higher sensitivities (for molecular testing, when using the latest Ultra version of the Xpert MTB/RIF assay). In contrast, positive AFB smear has a high positive predictive value, but because of its low specificity, it should always be combined with culture and/or molecular testing for species identification.

5. Primary culture

Mycobacterium-containing specimens can be cultured on both solid and liquid media. A liquefaction and decontamination phase, followed by neutralization and concentration, should always precede the inoculation phase, in order to minimize contamination by commensal rapid-growing bacteria. A non-sterile specimen is usually treated with a mucolytic agent (N-Acetyl-L-Cysteine (NALC)) and sodium hydroxide (NaOH) for liquefaction and decontamination, respectively (Pai *et al.*, 2016). NALC major role is to disrupt the mucous present in the specimens and to allow the mycobacteria to access nutrients from culture media to further promote their growth.

A cocktail of antibiotics, inactive on mycobacteria, is usually added to suppress the growth of remaining bacterial and fungal contaminants. This is especially needed for liquid media, which are more prone to contamination by normal flora bacteria. Therefore, PANTA, a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin, is often added prior to broth culture. To some extent, an excess of decontamination might affect the growth of mycobacteria (Pfyffer, 2015). A contamination rate of 6-8% on liquid media and 3-5% on solid media are said to be acceptable (Cruciani *et al.*, 2004; Caulfield and Wengenack, 2016).

Pellet inoculation is most often done on solid, egg-based media known by the LJ and/or liquid broth-based known by Mycobacterial Growth Indicator Tube (MGIT- Becton Dickinson Microbiology Systems, USA), followed by incubation at 35-37°C. In liquid media, positive primary cultures are usually detected after 1-4 weeks, by fluorescence detection as the growth of mycobacteria consumes oxygen that quenches fluorescence within the tube. On solid media, positivity is detected by visual screening for dry and rough colonies with cream color, and may take up to 6-10 weeks (Caulfield and Wengenack, 2016). Compared to AFB smear, the culture of mycobacteria is 100 fold more sensitive, with only 10–100 CFU/mL of the specimen being sufficient for growth (Pfyffer, 2015).

Other commercially available, semi-automated broth-based culturing systems of mycobacteria include the VersaTREK (Trek Diagnostic Systems, Cleveland, Ohio, USA) and the MB/ BacT Alert 3D (bioMérieux Inc., Durham, NC) systems. They detect mycobacterial growth by either measuring the change of gaseous pressure in the bottle headspace above the broth medium or

detecting the color change using a colorimetric carbon dioxide sensor, respectively (Caulfield and Wengenack, 2016).

Even though culture is considered the gold standard test for MTBC detection, it is a long procedure and costly (especially for liquid culture). Moreover, further tests are required to differentiate between MTBC and NTM species. There are several methods for the identification of mycobacterial species, ranging from biochemical tests (e.g. Niacin accumulation, nitrate reduction, heat stable catalase (68°C) reaction and para-nitrobenzoic acid test) to various molecular tests, such as the Seegene MTB/NTM test (page 40). Finally, appropriate biosafety conditions and infrastructure, special equipment and technicians with adequate skills are needed (WHO, 2015a).

6. Phenotypic drug susceptibility testing

Especially in the era of worldwide emergence and spread of multi- and extensively drug resistant TB, DST is the key tool for selecting appropriate and possibly individualized, effective regimens to successfully treat a TB patient (Kim, 2005).

After identification of MTBC either by growth rate, colony pigmentation, and/or biochemical or molecular tests, phenotypic DST can be performed on both solid and liquid media. The so-called proportion method tested on solid agar is still considered as the standard method. It is based on counting MTBC colonies growth on agar plates with and without antibiotics incorporation at critical or different concentrations. If the ratio of colonies on antibiotics-containing media over antibiotic-free media is over 1%, the isolate is considered resistant. Phenotypic DST can be done more rapidly and more simply by using liquid culture systems, by comparing growth in antibiotics-containing media versus appropriate positive growth controls (Gilpin *et al.*, 2016).

Phenotypic DST is considered generally reliable and reproducible for the main first-line anti-TB agents INH and RIF and selected second-line anti-TB drugs, such as KM, AMK, ofloxacin (OFX), and levofloxacin (LFX), with relatively well established critical concentrations (Table 3 and 4). However, other anti-TB drugs available on the market, such as EMB, do not (yet) have a well-identified critical concentration that allows clear-cut discrimination between resistant and susceptible strains. Phenotypic DST for EMB is therefore no more recommended as a reliable and

reproducible test (WHO, 2018c). Moreover, detection of *Mycobacterium* susceptibility to pyrazinamide is non-reliable with both frequent false-sensitivity due to the need of using acidic pH that inhibits growth and false-resistance due to the use of excessively large inoculum that reduce the apparent activity of the drug (Chang *et al.*, 2011).

Table 3: Critical concentration of 1st line anti-TB drugs for the treatment of susceptible cases (WHO, 2018c).

Medicine	Abbreviation	Critical concentrations (µg/ml) for DST by medium			
		Löwenstein-Jensen ^a	Middlebrook 7H10 ^a	Middlebrook 7H11 ^a	BACTEC MGIT liquid culture ^a
Rifampicin	RIF	40.0	1.0	1.0	1.0 ^b
Isoniazid ^c	INH	0.2	0.2	0.2	0.1
Ethambutol ^d	EMB	2.0	5.0	7.5	5.0
Pyrazinamide ^e	PZA	-	-	-	100

Table 4: Critical concentration of 2nd line anti-TB drugs recommended for the treatment of RR/MDR TB cases (WHO, 2018c).

Group	Medicine	Abbreviation	Critical concentrations (µg/ml) for DST by medium			
			Löwenstein-Jensen ¹	Middlebrook 7H10 ¹	Middlebrook 7H11 ¹	BACTEC MGIT liquid culture ¹
Group A	Levofloxacin (CC)	LFX ^{2,3}	2.0	1.0	-	1.0
	Moxifloxacin (CC)	MFX ^{2,3}	1.0	0.5	0.5	0.25
	Moxifloxacin (CB) ⁴			2.0	-	1.0
	Bedaquiline ⁵	BDQ	-	-	0.25	1.0
	Linezolid ⁶	LZD	-	1.0	1.0	1.0
Group B	Clofazimine	CFZ	-	-	-	1.0
	Cycloserine	CS	-	-	-	-
	Terizidone	Terizidone	-	-	-	-
Group C	Ethambutol ⁷	E	2.0	5.0	7.5	5.0
	Delamanid ⁸	DIM	-	-	0.016	0.06
	Pyrazinamide ⁹	PZA	-	-	-	100.0
	Imipenem-cilastatin	IMP/CLN MPM	-	-	-	-
	Meropenem		-	-	-	-
	Amikacin ¹⁰ (Or Streptomycin)	AMK (S)	30.0 4.0	2.0 2.0	- 2.0	1.0 1.0
	Ethionamide	ETO	40.0	5.0	10.0	5.0
	Prothionamide	PTO	40.0	-	-	2.5
Para-aminosalicylic acid	PAS	-	-	-	-	

Red highlight: provisional critical concentrations.

7. Classical molecular diagnostics: Nucleic acid amplification tests (NAAT)

As mycobacterial diagnostics based on culture is slow and cumbersome, considerable efforts have been dedicated to the development of molecular techniques for more rapid identification and drug susceptibility testing of MTBC, from primary culture or even directly from a clinical sample. By potentially providing results in just 1-2 days or just a few hours, the use of such molecular tests can greatly improve patient management, permitting much earlier determination of an appropriate TB treatment when compared to conventional DST methods.

Various molecular tests have been developed over the two last decades. The following presentation will focus on three “classical” molecular tests, consisting of NAAT followed by hybridization to specific nucleotidic probes. The Gene Xpert MTB/RIF and some Hain tests have been endorsed by WHO for detection of MTBC and/or of MTBC drug resistance. As such, these tests are being internationally deployed and used for routine diagnosis. The newer Seegene Anyplex test is much less widespread, but has certain advantages compared to the two former assays and I utilized it in Lebanon for my thesis work.

To note, two most advanced methods, including whole genome sequencing (WGS) and a targeted next generation sequencing (NGS) assay called Deeplex-MycTB, which both combine molecular diagnostics and genotyping for epidemiological tracing purposes, will be presented in section 9 below.

a. Gene Xpert MTB/RIF

The GeneXpert MTB/RIF system was launched by Cepheid in 2010. It consists of an automated, cartridge-based system utilizing a real-time PCR (RT-PCR) technology to simultaneously detect the presence of MTBC along with RIF resistance in less than 2 hours, starting from the clinical specimen. The latter is spotted by screening for the presence of mutations in the 81 bp RRDR region of the *rpoB* gene (see point F.3.a above), through hybridization of an array of different nucleotidic probes to the obtained amplicon (Caulfield and Wengenack, 2016).

In addition to its rapidity, the main advantages of this system include the automation and simplicity, making it a user-friendly test that can be implemented outside centralized laboratories. In addition, a so-called Ultra version has been recently released that augments the sensitivity with a limit of detection claimed to be close to 16 CFU/ml, with however a somewhat increased risk of false positivity (Chakravorty *et al.*, 2017).

On the other hand, the test is limited to the single detection of resistance to RIF, caused by mutations in the RRDR only (WHO, 2015a). As indicated above, the RRDR accounts for >95% of RIF resistance associated mutations in MTBC strains, but RIF resistance associated mutations are also known outside this region, which can be highly prevalent in some world regions (Sanchez-Padilla *et al.*, 2015). Moreover, as a reflection of the fact that such test is not a direct sequencing-based method, some synonymous mutations occurring in the RRDR can also cause false-positive detection of RIF resistance (Mathys *et al.*, 2014).

b. Line probe assays

Line probe assays (LPAs) correspond to so-called reverse dot blot-based methods, where amplicons obtained from targets of interest are hybridized on different nucleotidic probes immobilized on membrane strips. LPAs have been principally developed by the Hain company. GenoType Mycobacterium CM, AS are for detecting and differentiating MTBC and NTM from primary culture, while GenoType CMdirect is detecting MTBC and NTM directly from clinical specimens.

GenoType MTBDRplus and GenoType MTBDRsl are for detection of MTBC, along with mutations associated with resistance to INH (high and low level of resistance) and RIF (Figure 12), or with mutations associated with resistance to FQ, injectables and EMB, directly from a smear-positive pulmonary specimen or cultured isolates of MTBC (Pai *et al.*, 2016; Gilpin *et al.*, 2016). The GenoType MTBDRplus and MTBDRsl test have been endorsed by WHO in 2008 and 2016 respectively (WHO, 2016b; Global laboratory initiative, 2018).

Although they are relatively slower with more hands-on time than the Xpert tests, LPAs are more rapid techniques with lower biosafety risks compared to conventional DST methods (Pai *et*

al., 2016). As another advantage, they allow to test resistance to several drugs that are key to first and second-line treatments, beyond the single rifampicin as with the Xpert test. This assay can thus have a good utility as a screening tool, especially in high MDR TB burden countries.

Among the disadvantages, different tests are needed for precise species identification in the MTBC or differentiation among NTM and profiling of mutation targets associated with the different concerned drugs. Moreover, this method uses a combination of a limited number of probes that are specific to few common mutations only or to the corresponding wild-type alleles. As for the Xpert test, this method is indirect, and prone to false-positivity due to incorrect conclusions based on the lack of hybridization to the wild-type probe, when linked to occurrence of synonymous mutations in the same sequence positions. Like with the Xpert test as well, the sensitivity to reliably detect potential resistant subpopulations (causing so-called hetero-resistance) is quite far from the 1% threshold considered for phenotypic tests. Risk of cross-contamination is also a major drawback (Hain Lifescience, 2018a; Hain Lifescience, 2018b).

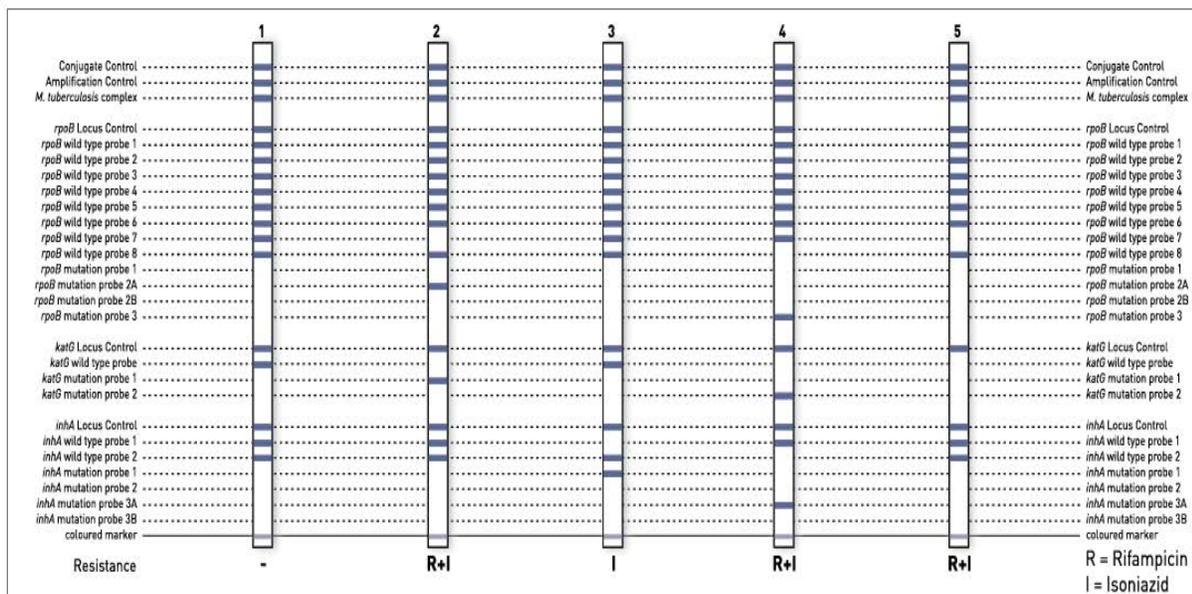


Figure 12: MTBC identification and susceptibility to rifampicin and isoniazid using GenoType MTBDRplus (Hain Lifescience, 2018b).

c. Seegene Anyplex MTB/NTM, MTB/MDR, MTB/XDR

The Anyplex™ MTB/NTM real time detection assay is another rapid TB diagnostic tool. It is a RT-PCR based assay that identifies MTBC and NTM isolates directly from clinical samples or from primary cultures (Anyplex MTB/NTM, Seegene, Korea).

The Anyplex II MTB/MDR and MTB/XDR detection assays were designed to rapidly diagnose MDR or XDR cases through simultaneous detection of MTBC along with resistance to both RIF and INH or resistance to FQ and injectable drugs respectively. These tests thus permit more extended drug resistance profiling than the Xpert test, while being easier than the Hain tests. However, it shares similar limitations with these two other tests. As a foremost example, it only interrogates a limited number of common mutations of a reduced set of main gene targets: *rpoB* (18 mutations), *katG* and *inhA* (7 mutations), *gyrA* (7 mutations), *rrs/eis* (6 mutations) (Table 5) (Igarashi *et al.*, 2017). In a recent evaluation in South Korea, only 61,8% of MDR TB cases and 64,7% of XDR-TB cases were correctly identified using Anyplex II MTB/MDR and MTB/XDR detection assays (Igarashi *et al.*, 2017). Because of their relatively limited sensitivity however combined with a high specificity, these assays can thus be used as rule-in tests for detecting resistance, similarly to the Hain and Xpert (for RIF resistance) tests.

Table 5: Anyplex II MTB/MDR/XDR drug resistance associated mutations (Seegene, ND).

Resistencia a Droga	Gen Relacionado	Mutaciones Blanco			
Isoniazid resistance (INH-R)	<i>katG</i>	S315I (AGC→ATC)	S315N (AGC→AAC)	S315T (AGC→ACC)	S315T (AGC→ACA)
	<i>inhA</i> promoter	-15 (C→T)	-8 (T→A)	-8 (T→C)	
Rifampicin resistance ¹ (RIF-R)	<i>rpoB</i>	L511P (CTG→CCG)	Q513K (CAA→AAA)	Q513L (CAA→CTA)	Q513P (CAA→CCA)
		3 a.a. deletion in 513~516	D516V (GAC→GTC)	D516Y (GAC→TAC)	S522L (TCG→TTG)
		S522Q (TCG→CAG)	H526C (CAC→TGC)	H526D (CAC→GAC)	H526L (CAC→CTC)
		H526N (CAC→AAC)	H526R (CAC→CGC)	H526Y (CAC→TAC)	S531L (TCG→TTG)
		S531W (TCG→TGG)	L533P (CTG→CCG)		
Fluoroquinolone resistance (FQ-R)	<i>gyrA</i>	A90V (GCG→GTG)	S91P (TCG→CCG)	D94A (GAC→GCC)	D94G (GAC→GGC)
		D94H (GAC→CAC)	D94N (GAC→AAC)	D94Y (GAC→TAC)	
Injectable drug resistance (Inj. drug-R)	<i>rrs</i>	1401 (A→G)	1402 (C→T)	1484 (G→T)	
	<i>eis</i> promoter	-37 (G→T)	-14 (C→T)	-10 (G→A)	

8. Molecular surveillance by conventional genotyping

The tests described above are for primary diagnostic and clinical purposes, namely for mycobacterial species identification and drug resistance detection, to confirm the disease and guide treatment definition. However, other types of tests can/must be used in second line to address more public health-related questions, such as identifying potential source cases or confirming or ruling out an outbreak when TB cases are found to aggregate over a limited time period in specific places, such as schools, hospitals, shelters, or tracing potential transmission chains in the general population.

Identifying such source cases and tracing of TB transmission is often difficult, without the help of molecular tools. Indeed, as a reflection of the chronic nature of the infection, transmission of TB in populations is particularly insidious. In many countries, the disease is typically more focused in vulnerable and difficult-to-reach high-risk groups, such as homeless and migrants. This complicates classical contact investigation around TB cases, representing a central component of outbreak control and prevention done in many middle/high income countries. Analysis of genetic clustering between *M. tuberculosis* isolates by conventional genotyping methods has been/is therefore widely used for epidemiological tracing of MTBC strains and to guide investigation towards probable transmission links (Supply *et al.*, 2006; Erkens *et al.*, 2010; Supply, 2018).

a. IS6110 RFLP

IS6110 Restriction Fragment Length Polymorphism (RFLP) was the very first method of MTBC genotyping that was largely used and was standardized in 1993 (van Embden *et al.*, 1993). The method targets genetic polymorphisms associated with the IS6110 insertion sequence. This element is part of the large family of insertion sequences (IS), representing mobile genetic elements present in many bacteria. IS6110 is the most studied IS present in the MTBC genomes and was reported to be restricted to MTBC (Thierry *et al.*, 1990). IS6110 RFLP is used to estimate the number and positions of IS6110 copies in an MTBC genome to generate a strain-related DNA fingerprint.

In such analysis, after extraction from a positive mycobacterial culture, the genomic DNA was digested with PvuII endonuclease and the restriction fragments were separated by gel electrophoresis. Afterward, Southern blot hybridization was performed with an IS6110 probe to generate specific DNA fingerprints that could be analyzed with image analysis software such as GelCompare (Applied Maths, Belgium), BioImage Whole Band Analyzer or Advanced Quantifier (Bio Image System, MI, USA) (Pouseele and Supply, 2015).

For more than a decade, IS6110-RFLP was considered as the best genotyping technique with a high discriminatory power and a relatively high reproducibility. In diverse studies, the majority of isolates from epidemiologically unrelated TB cases clearly showed divergent DNA fingerprints (Van Soolingen, 2001). Nevertheless, isolates from closely related patients from a same chain of transmission generally displayed identical or highly similar DNA fingerprints, forming the base for defining clusters of recent transmission (Yeh *et al.*, 1998; Niemann *et al.*, 2000).

IS6110 fingerprinting was therefore widely used for public health-related questions. This approach allowed to confirm or disclose large MDR TB outbreaks in the general populations (Bifani *et al.*, 1996), nosocomial infections (Michele *et al.*, 1997; Allix *et al.*, 2004), reveal laboratory cross-contamination (Van Soolingen, 2001) and distinguish between exogenous reinfection and initial treatment failure in relapse cases (Small *et al.*, 1993).

However, IS6110-RFLP was labor-intensive and slow, requiring long mycobacterial culturing and DNA purification before subsequent multi-step RFLP analysis. Furthermore, despite the standardization of the method, the obtained banding patterns from tested strains were complex to compare between experiments and laboratories, hampering the construction of international/centralized reference databases (Merker *et al.*, 2017).

b. Spoligotyping

The limitations encountered with IS6110-RFLP stimulated the development of PCR-based methods targeting different markers. The first that was widely used is spoligotyping (Kamerbeek *et al.*, 1997). This method targets the so-called direct repeat (DR) locus that is present in virtually all MTBC strains (Hermans *et al.*, 1991). This locus belongs to

clustered regularly interspaced short palindromic repeats (CRISPR) locus family. It is made up of many conserved repeated sequences (36 bp) named direct repeats, separated from each other by unique and variable 35 to 41bp sequences called spacers (Makarova *et al.*, 2011). Spacers are amplified by PCR using primers targeting the direct repeats that flank each spacer. The amplicons are hybridized on a membrane with immobilized probes that are specific to each spacer of a reference set of 43 spacers obtained from *M. tuberculosis* H37Rv and *M. bovis* BCG. Results are obtained as a portable 43-bit barcode reflecting the presence or absence of spacers in the tested strain (Kamerbeek *et al.*, 1997; Barnes and Cave, 2003).

Spoligotyping is a relatively cheap genotyping assay that however requires a number of manual operations due to the hybridization steps. In order to ease and accelerate the analysis, a more automated system, based on the use of spacers grafted on multiple microbeads analyzed with the Luminex multi-analyte profiling system, was developed by Cowan *et al.*, in 2004 (Cowan *et al.*, 2004). Later on, systems for combined interrogation of the reference spoligotyping spacers or spacer subsets and few common drug resistance-associated mutations have been developed, by additional grafting of nucleotidic probes corresponding to a limited set of drug resistance-associated regions in *rpoB*, *katG* and *inhA* (Gomgnimbou *et al.*, 2013). However, the necessary bead sets are quite expensive, limiting the use of such assays.

The resolution power of spoligotyping is also limited, being e.g. much lower than IS6110 RFLP. Hence, it does generally not provide information at strain level and should always be used in combination with another genotyping method for epidemiological tracing (Kremer *et al.*, 1999; Barnes and Cave, 2003).

However, so-called spoligotype signatures, characterized by the presence and/or the absence of certain spacers, are relatively well conserved at strain lineage and sub-lineage levels (Brosch *et al.*, 2002; Brudey *et al.*, 2006), even if they are not necessarily fully specific of (sub) lineages due to homoplasmy (Comas *et al.*, 2009). In order to exploit this approximate phylogenetic information, different databases, called SpolDB and then SITVIT, were set up to compile spoligotypes identified worldwide and provide centralized information on their phylogeographic distribution. The latest published version of SITVIT, includes more than 8000 spoligotypes collected from more than 50,000 isolates distributed over more than 100 countries (Brudey *et al.*, 2006; Demay *et al.*, 2012).

c. MIRU-VNTR typing

To overcome the limited resolution power offered by spoligotyping, another major PCR-based method was developed, named MIRU-VNTR (Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeat) typing. This method indexes the polymorphisms in repeat numbers in loci containing MIRU-VNTR sequences, with individual unit lengths of 50-100 bp (Frothingham and Meeker-O'Connell, 1998; Supply *et al.*, 2000). After initial systems based on e.g. 5 (Frothingham and Meeker-O'Connell, 1998) or 12 markers (Supply *et al.*, 2000; Mazars *et al.*, 2001), a 24-locus MIRU-VNTR typing was internationally standardized by Supply *et al.* in 2006 (Supply *et al.*, 2006). This 24-locus set was selected based on the analysis of a global set of more than 800 strains to represent a best compromise between high discriminatory power across different strain lineages and clonal stability during infection and transmission, as well as technical robustness (Supply *et al.*, 2006). A complementary consensus set of 4 so-called hypervariable MIRU-VNTR markers has subsequently proposed for improved resolution of remaining clusters based on the 24 standard markers, especially when involving Beijing strains.

Commercially available kits have been developed for robust analysis with both kit sets by Genoscreen, on the campus of the Institut Pasteur de Lille. With these kits, typing with the standard system is performed with 6 quadruplexes PCRs, using primers against the flanking regions of the 24 VNTR markers. The sizes of the amplified fragments and the number of repeats are determined after capillary electrophoresis-based separation on an ABI 3730 XL DNA Analyzer (Applied BioSystems), using a customized software (GeneMapper v. 5.0, Applied BioSystems). Several studies have shown the use of these kits along with automated DNA analyzers offers the highest reproducibility when using MIRU-VNTR typing (de Beer *et al.*, 2012).

As a result, each isolate is characterized by a standardized numerical genotype, representing the combination of the repeat numbers (alleles) determined for each of the 24 loci. This considerably eases the interpretation and sharing information between different experiments and laboratories (Supply *et al.*, 2006).

Several population-based studies performed in low TB incidence settings have shown that in comparison with IS6110-RFLP typing, MIRU-VNTR typing, alone or in combination with spoligotyping, had a similar discriminatory power to determine clustering between isolates as a

proxy to TB transmission among TB cases (Oelemann *et al.*, 2007; Allix-Béguet *et al.*, 2008a; de Beer *et al.*, 2013).

Moreover, especially when based on the 24 standard markers, MIRU-VNTR typing is less prone to homoplasmy than spoligotyping and can be used for phylogenetic inferences such as identification of phylogenetic (sub) lineages, based on the analysis of allelic similarities between strain types (Supply *et al.*, 2006; Allix-Béguet *et al.*, 2008a; Wirth *et al.*, 2008; Comas *et al.*, 2009). For this purpose, a multi-functional database, called MIRU-VNTRplus database, has been developed which allows the users to determine the phylogenetic lineages of their strains by comparison with well characterized reference strains, with lineages identified by reference phylogenetic markers such as RDs and SNPs (Allix-Béguet *et al.*, 2008b; Weniger *et al.*, 2010).

As a reflection of these advantages, this newer PCR-based assay has replaced the previous gold standard IS6110 RFLP for international epidemiological surveillance of TB over the last decade, and is/has been used as such by e.g. US CDC (CDC, 2012) the ECDC (ECDC, 2017), and many reference laboratories worldwide. As an illustration, this standard 24-locus system has been used by a large international consortium to screen a population of 5,000 isolates from 50 some countries part of the Beijing lineage. This screening allowed to identify major clonal branches and select representative for subsequent WGS and reconstitution of the evolutionary history and spread of this major lineage of the MTBC (Merker *et al.*, 2015).

9. Combined molecular diagnostics and surveillance by next-generation sequencing (NGS)

Despite their advantages, the classical molecular diagnostics described above only cover limited sets of common mutations in one (Xpert MTB/RIF) or few gene targets associated with anti-TB drug resistance. Likewise, classical typing techniques like those presented above only target a selected set of known polymorphic regions as a surrogate for determining strain types. As epidemiologically/phylogenetically relevant variation can occur elsewhere in the genome, their discriminatory power is inherently limited. In addition, on a clinical level, they provide no information about gene mutations associated with drug resistance.

These limitations can be overcome by the use of WGS, which has been greatly facilitated by the enormous technical progresses made for NGS over the last decade. By definition, WGS offers near comprehensive genetic information on a clinical isolate, allowing to simultaneously determine strain relatedness and screen for (known) drug resistance associated mutations at genome-wide level. However, for reasons indicated below, WGS directly from clinical specimens is not yet part of routine clinical use, and analysis of WGS data remain complex. Therefore, targeted NGS approaches also directly applicable on clinical specimens have been developed. In particular, the Deeplex-MycTB assay permits detection of drug resistance associated mutations in multiple gene targets, as well as simultaneous genotyping based on spoligotyping combined with phylogenetic mutations (alias phylogenetic single nucleotide polymorphisms (SNPs)). These approaches are described below.

a. Whole genome sequencing (WGS)

WGS represents the ultimate “all-in-one” approach, offering the capability to deliver the most comprehensive molecular information for public health, clinical, and research questions (Merker *et al.*, 2017).

For clinical management of TB infection, WGS can provide complete characterization of an isolate including mycobacterial species identification and detection of all known mutations in the genome associated with drug resistance, in addition to identification of potential genomic clusters suggestive of ongoing transmission (see below). According to a prospective evaluation done by an international team on the use of WGS on newly positive MGIT cultures, such diagnostic information can be delivered faster and even with a slightly lower cost than the classical diagnostic schemes currently in place in most laboratories in high resource settings, including phenotypic DST combined with classical molecular tests used for rapid screening (e.g. Gene Xpert MTB/RIF) and genotyping (MIRU-VNTR). Thus, the perspective of using WGS for routine diagnostics offers the prospect to avoid inappropriate empirical treatments in many cases (Pankhurst *et al.*, 2016).

The degree of knowledge of MTBC genome mutations associated or not with drug resistance obviously is a central aspect for accurate prediction of drug susceptibility and drug resistance. Different studies have established catalogs of such mutations by matching genetic polymorphisms including SNPs and indels in candidate genes as identified by targeted Sanger

sequencing or by WGS with the phenotypic profiles of the corresponding MTBC isolates (e.g. (Miotto *et al.*, 2014; Feuerriegel *et al.*, 2015; Walker *et al.*, 2015; Farhat *et al.*, 2016b; Miotto *et al.*, 2017; CRyPTIC Consortium and the 100,000 Genomes Project *et al.*, 2018). Studies by (Walker *et al.*, 2015) and the (CRyPTIC Consortium and the 100,000 Genomes Project *et al.*, 2018), respectively based on the analysis of 3651 and 10290 MTBC genomes were among the most comprehensive and elaborated approaches to establish and test such mutations catalogs for predicting drug susceptibility and drug resistance. In these studies, extensive sets of mutations identified in gene targets with known association with drug resistance were classified into three main categories, namely benign (i.e. non synonymous mutations not associated with drug resistance, synonymous and phylogenetic mutations), resistance determining, and uncharacterized mutations (i.e. with as yet unknown association or non-association with drug resistance). By using these benign and resistance determining mutations (setting thus apart the uncharacterized mutations), 89% or more phenotypes could be predicted for first (Walker *et al.*, 2015; CRyPTIC Consortium and the 100,000 Genomes Project *et al.*, 2018) and second line drugs (Walker *et al.*, 2015) with an overall accuracy (combined sensitivity and specificity) of around 90%, surpassing the performances of the classical, commercial molecular tests.

As it covers the near totality of the target genome, WGS analysis also allows to detect and delineate outbreaks more precisely, compared to conventional genotyping methods described above. For example, analysis of genome-wide SNP profiles has been shown to provide a finer resolution than 24-locus MIRU-VNTR typing to distinguish distinct transmission chains within a large longitudinal outbreak (Roetzer *et al.*, 2013). This superior resolution power over classical genotyping has been repeatedly demonstrated by other studies (e.g. (Walker *et al.*, 2013; Walker *et al.*, 2014; Wyllie *et al.*, 2018).

However, despite these undisputable advantages, a major challenge for the use of WGS in clinical routine remains the need of a (newly positive) primary culture, usually taking about a week or more, for extraction of sufficient amounts of DNA. In studies that evaluated WGS from DNA extracted directly from sputum, insufficient extraction yield and/or contamination by human and commensal microbial reads frequently prevented sufficient coverage depth of the *M. tuberculosis* reference and thus reliable SNP detection at genome-wide level, even in samples with high bacterial loads (i.e. with high smear positivity grades) (Doughty *et al.*, 2014; Lee and Pai, 2017; Votintseva

et al., 2017; Doyle *et al.*, 2018). Procedures of selective enrichment with RNA baits on beads to deplete contaminating sequences (Brown *et al.*, 2015; Doyle *et al.*, 2018) were shown to improve the results to a certain extent, but because of their technical complexity and cost, they are not routinely used.

As a result of the need for a culture, a great part of the advantage in terms of rapidity of WGS compared to phenotypic DST can be lost, especially if the result is obtained substantially after the patient already started his treatment (Lee and Behr, 2016). Moreover, although some analysis tools are available online (e.g. PhyResSE, (Feuerriegel *et al.*, 2015)), the amount and complexity of WGS data to be analyzed requires specialized bioinformatics skills to interpret the results.

b. Next generation sequencing assays: Next Gen-RDST and Deeplex-MycTB

Early detection of drug resistance is key for rapidly defining appropriate treatment and favoring a positive patient outcome. To overcome some of the main limitations faced with WGS described above, alternative approaches using targeted deep sequencing of amplicons have been developed (Colman *et al.*, 2015; Tagliani *et al.*, 2017; Makhado *et al.*, 2018). Crucially, these methods using target amplification before sequencing are more efficiently applicable directly on clinical samples (as well as on primary cultures), resulting in a very fast turnaround time and limiting the need of biocontainment equipment required for culture. Moreover, the deeper coverage depths that are technically and economically more achievable by targeted sequencing instead WGS allow more sensitive detection of minority populations, potentially causing hetero-resistance (Rinder *et al.*, 2001; Colman *et al.*, 2015).

The Next Gen-RDST assay (Translational Genomics Research Institute, Phoenix, Arizona, USA) (Colman *et al.*, 2015) and the Deeplex®-MycTB assay (Genoscreen, Lille, France) developed in collaboration with my hosting laboratory (Tagliani *et al.*, 2017; Makhado *et al.*, 2018) are currently the only two amplicon-based technologies for drug resistant TB diagnosis directly from clinical sample (WHO, 2018d).

The Next Gen-RDST assay identifies anti-TB drug resistance by detecting mutations in regions of 6 gene associated with resistance, namely *rpoB*, *katG*, *inhA*, *gyrA*, *eis*, and *rrs*. In comparison, Deeplex-MycTB uses a single 24-plex assay to simultaneously interrogate 18 main gene targets associated with resistance to first and second line drug resistance of *M. tuberculosis*, plus targets for mycobacterial species identification (*hsp65*) and MTBC genotyping (spoligotyping and phylogenetic SNPs) (Table 6, and Figure 13). Distinctively as well for the latter assay, a dedicated application with integrated variant databases, hosted on isolated servers in private cloud servers fully compliant with genetic data safety guidelines, is used for rapid and automated analysis, user-friendly interpretation and reporting. Variant detection is done with calibrated filters and a coverage depth-dependent threshold for calling minority variants down to 3%, enabling sensitive detection of heteroresistance, mixed infection by MTBC strains, or concurrent infection or colonization by MTBC and/or (NTM) strains. From an in silico analysis of the 3,551 MTBC genomes included in the study of Walker et al. (Walker *et al.*, 2015), Deeplex-MycTB captures 2068/2112 (97.9%) resistance phenotypes that were predicted by WGS (Supply, 2018).

Starting from direct specimens, up to 16, 48, 96 or 384 tests can be performed in parallel in a single iSeq100, MiSeq, MiniSeq or NextSeq (Illumina) run, respectively, with a total turnaround time of 24h (iSeq100) to 2-3 days (other platforms).

This assay is already routinely deployed in the WHO Supranational Reference Laboratories (NRLs) in Milano and Antwerp, as well as in the University of Leuven, where it has been/is used for WHO-supervised drug resistance surveys organized in Djibouti (Tagliani *et al.*, 2017), Erythrea and Democratic Republic of Congo (DRC), as well as for early diagnostics of (suspected) MDR or XDR TB cases. Moreover, this assay has been used in combination with other molecular tests, including WGS, to characterize MDR TB strains bearing an *rpoB* I491F mutation undetected by the Gene Xpert MTB/RIF and Hain tests (Makhado *et al.*, 2018).

Table 6: Comparison between Next Gen-RDST and Deeplex-MycTB.

		Next Gen-RDST	Deeplex-MycTB
Species identification		X	✓
Genotyping (SNP)		X	✓
Spoligotyping prediction		X	✓
Prediction of mixed infections		✓	✓
Genomic targets	<i>rpoB</i>	✓	✓
	<i>inhA</i>	✓	✓
	<i>fabG1</i>	X	✓
	<i>katG</i>	✓	✓
	<i>ahpC</i>	X	✓
	<i>pncA</i>	X	✓
	<i>embB</i>	X	✓
	<i>gidB</i>	X	✓
	<i>rpsL</i>	X	✓
	<i>rrs</i>	✓	✓
	<i>eis</i>	✓	✓
	<i>tlyA</i>	X	✓
	<i>gyrA</i>	✓	✓
	<i>gyrB</i>	X	✓
	<i>ethA</i>	X	✓
<i>rrl</i>	X	✓	
<i>rplC</i>	X	✓	
<i>Rv0678</i>	X	✓	
Cloud based analysis		✓	✓

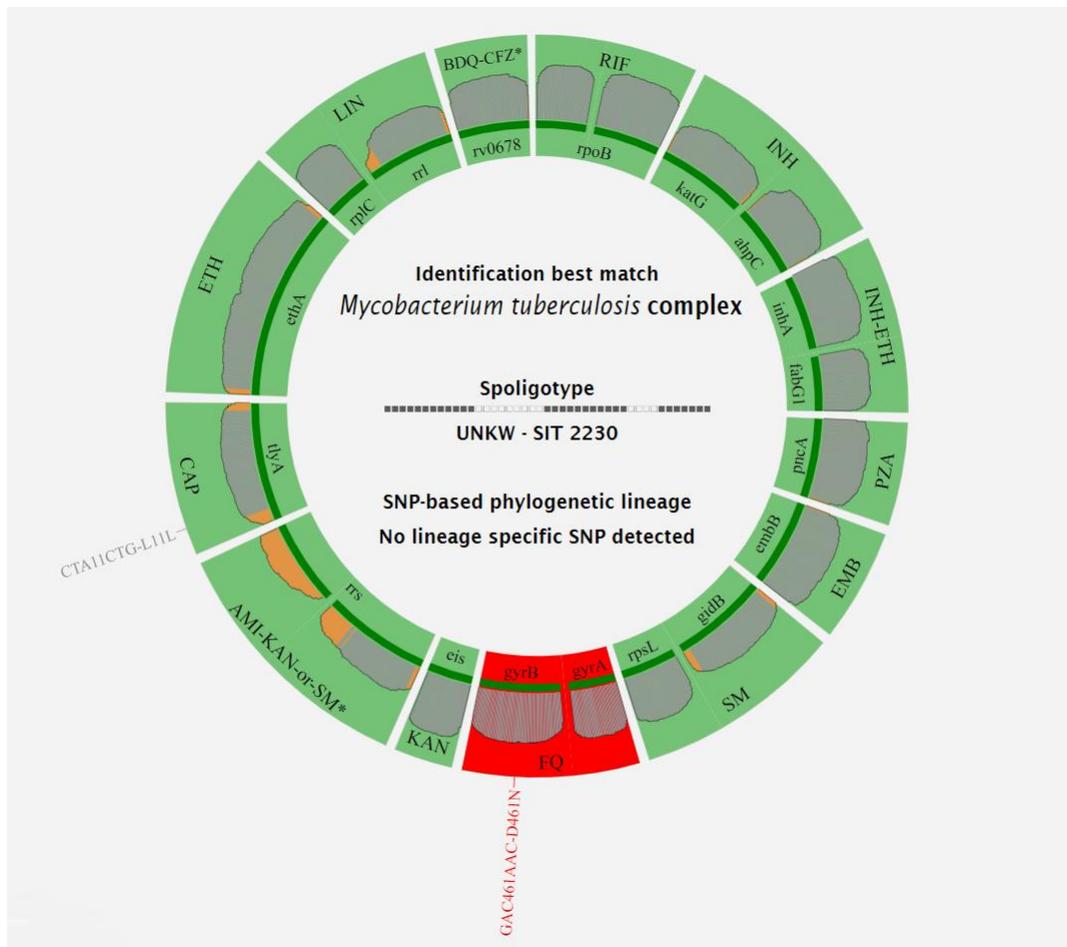


Figure 13: Deeplex-MycTB results identifying TB case with mono-resistance to fluoroquinolone in Lebanon. Target gene regions are grouped within sectors in a circular map according to the antituberculous drug resistance they are associated with. Sectors in red refer to target regions in which drug resistance associated mutations are detected (shown in red around the circular map), whereas sectors in green refer to regions where no mutation or only mutations not associated with resistance (shown in grey around the map) are detected. Green lines above gene names represent the reference sequences with coverage breadth above 95%. Limits of detection (LOD) of potential heteroresistance (reflected by subpopulations of reads bearing a mutation), depending on the coverage depths over individual sequence positions, are indicated by gray (LOD 3%) and orange (variable LOD >3%–80%) above the reference sequences. Information on mycobacterial species identification (based on *hsp65* sequence best match) and genotype of *M. tuberculosis* complex strain (based on spoligotype and lineage-defining, phylogenetic single nucleotide polymorphism (SNP)) are shown in the center of the circle.

AMI, amikacin; BDQ, bedaquiline; CAP, capreomycin; CFZ, clofazimine; EMB, ethambutol; ETH, ethionamide; FQ, fluoroquinolones; KAN, kanamycin; LIN, linezolid; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin; SM, streptomycin; SNP, single-nucleotide polymorphism.

Project objectives and design

Before the onset of my PhD, there was no precise knowledge of the actual epidemiological situation and the prevalence of drug resistant TB in Lebanon, similarly to many other world places outside the Western world. Classical TB diagnostic capacities were present in the country but routine diagnostics was often limited to chest X-ray and smear microscopy, while cultures and DST were mostly performed for special cases (relapse or HIV patient) upon the physician's request. This knowledge gap was especially concerning as the country had recently experienced a massive influx of refugees from Syria, representing a vulnerable population particularly at risk of TB and TB drug resistance. As a reflection of a deterioration of the TB situation, data from WHO and MoPH indicated a substantial increase of TB incidence, especially among non-Lebanese individuals. Moreover, the country hosts as well as numerous migrant workers from high TB incidence countries, which also represent an important risk of importation of TB cases. The existence of this knowledge gap in such a context has driven the definition of the objectives of this PhD work.

The main aim of this PhD project was thus to gain a first more comprehensive overview of the epidemiological situation of TB and TB drug resistance in Lebanon. More specifically, the main objectives were:

- i) to contribute to the setup and running of a system for nationwide collection and routine diagnostics of samples with clinical-epidemiological data from TB patients covered by the NTP over a total time period of 18 months, from both residents and displaced populations. Due to its nature, this organizational and logistic part of the work will be described in a specific section of Material and Methods.
- ii) to evaluate on this nationwide basis the prevalence of drug resistance in the country, by using classical phenotypic and molecular DST, complemented by the selected use of Deeplex-MycTB for confirmation of drug resistance profiles in MDR and XDR cases. This work was initially performed on the first period of 12 months.
- iii) to retrospectively evaluate Deeplex-MycTB versus routine diagnostic tools for prediction and more extensive assessment of drug resistance. This part of the work, as well as the following ones, was done over the total period of 18 months, with Deeplex-MycTB testing done on virtually all culture-positive TB cases.

- iv) to define the precise etiology of TB in Lebanon and determine in particular the prevalence of *M. bovis*-caused versus *M. tuberculosis*-caused and *M. africanum*-caused disease.
- v) to evaluate levels of genetic diversity and molecular clustering of MTBC isolates, based on a combination of standard 24-locus MIRU-VNTR typing, as well as spoligotyping and SNP typing obtained by Deeplex-MycTB testing, as a proxy for identification of potential epidemic strains.

The following Methods section regroups methods that were collectively used to achieve all these objectives over the complete study period.

Methods

A. Nationwide study setup and design

In order to have a complete image on the epidemiological situation of TB and the prevalence of drug resistance TB in Lebanon at a nationwide level, a project proposal including the present study objectives, intended sampling design and approaches jointly defined by my hosting laboratory at the Center for Infection and Immunity of Lille (CIIL) and Laboratoire Microbiologie Santé et Environnement (LMSE) in Tripoli, was submitted to the MoPH in Beirut with the help of the NTP. An agreement was obtained from the MoPH for a nationwide collection of clinical samples from suspected TB cases in May 2016. According to this agreement, from June 1st, 2016 to November 30th, 2017, clinical samples from all national TB centers located in different governorates except those in the Northern region (already received by LMSE) were centralized in the TB reference center in Karentina-Beirut, and then transferred twice a week to LMSE at the AZM Center for Biotechnology at the Lebanese University in Tripoli with the help of the International Organization for Migration (IOM).

A standardized form jointly defined by CIIL and LMSE teams and collecting patient data including e.g. gender, year of birth, nationality, TB history, time of sample collection and culture (Annex 1), was distributed to all 9 TB centers (Karentina, Halba, Tripoli, Zahle, Saida, Nabatiye, Tyre, Hermel, Azouniyeh) part of the NTP. Data were filled by technicians from each TB center and anonymized for subsequent analysis. A written informed consent was signed from all subjects included in the study. The study was approved by the ethics committee of the Azm Center for Research in Biotechnology and Its Applications, Lebanese University (document no. CE-EDST-3-2016), authorized by the Lebanese MoPH.

Initial microscopic examination was completed in each TB center, before shipment to the TB reference center (Karentina). Before sending the samples to LMSE, the TB reference center ensured a significant proportion of TB laboratory services including microscopic examination and GeneXpert testing for *M. tuberculosis* identification and detection of rifampicin resistance. Regardless of the molecular results, samples were packed in a cool bag and transported to LMSE for subsequent testing by a driver. Samples from the northern regions collected at the TB centers in Tripoli and Halba were transported to LMSE either by me or by some staff people from LMSE or TB centers Tripoli and Halba. These samples were subjected to microscopic (re-) examination

and additional molecular tests. Secondary cultures of the isolates were performed and then heat inactivated at 95°C for 30 minutes at LMSE Tripoli, Lebanon and sent to Pasteur Institute Lille, France, after authorization from the Lebanese MoPH, for advanced molecular analysis using Deeplex-MycTB and MIRU-VNTR (see below).

I managed the collection of patient data at LMSE. In cases of missing data, an approach was made toward the TB center to collect missing information. If needed and when possible, I contacted patients by phone to get specific information. It has to be noted that in the case of Syrian refugees, it was harder to obtain some information such as the period of entry to Lebanon, as not all the refugees were registered and considered as legal residents in the country. For the probably few patients coming from the private sector, the LMSE laboratory chief had direct contact with the physician or hospital to obtain information.

During my working periods in Lille, this work of sample collection, processing and culturing was performed by the LMSE TB team for samples received, to ensure continuous inclusion over the complete study period.

Financial support of this nationwide collection was obtained from WHO-TDR, IOM, Lebanese university, NTP, Special Program for Research and Training in Tropical Diseases - WHO, and Hamidi Medical Center.

B. Phenotypic and classical molecular testing

1. Microscopic examination

AFB staining was first done in the local TB centers using Ziehl-Neelsen coloration with a fast cold staining kit (Cold Kinyoun Stain Kit (Methylene Blue Counterstain, Atom Scientific, UK)), according to the manufacturer recommendations. All samples transferred to LMSE were subjected to microscopic (re-) examination (Kit Quick-TB, RAL diagnostics, France) (Figure 14).



Figure 14: Microscopic examination of sputum specimens from two different patients, revealing the presence of *M. tuberculosis* bacilli using Ziehl - Neelsen coloration (photograph taken during my work at LMSE).

2. GenXpert MTB/RIF

GeneXpert MTB/RIF testing (Cepheid, USA) was performed on all samples regardless of the staining results. The test was done either at the Karentina TB center in Beirut or at LMSE in Tripoli, as per the manufacturer's instructions.

3. Anyplex MTB/NTM, MTB/MDR, MTB/XDR Real-time detection Kit

Anyplex MTB/NTM testing (Seegene, Korea) was used as a substitution to GeneXpert in the case of samples contaminated with blood or samples from paraffin-embedded biopsies. This real time PCR test was also used to confirm MTBC in GeneXpert negative and culture positive results. In addition, in urgent cases, such as treatment relapse or RIF resistance detection by GeneXpert MTB/RIF, Anyplex II MTB/MDR and Anyplex II MTB/XDR detection Kits (Seegene, Korea) were used for detection of mutations associated with resistance to INH, RIF, and FQ and injectable drugs respectively (see Introduction, section I.7.c).

4. Primary culture

Culture was performed for all microscopy and GeneXpert positive and negative samples at LMSE using solid (BBL Lowenstein-Jensen LJ, Beckton-Dickinson, MD, USA) and liquid media (BD BBL Mycobacteria Growth Indicator Tube (MGIT), Beckton-Dickinson, MD, USA) following manufacturer's instructions. The specimen was first decontaminated using standard NaOH/N-acetyl-cysteine treatment followed by a neutralization using sterile phosphate buffer and a concentration step. Before pellet inoculation, the liquid media were supplemented with BD BBL MGIT OADC and a lyophilized antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA), vancomycin and cycloheximide. For the solid media, cycloheximide, colistine, vancomycin, pipemidic acid and nyastatin were added prior to the inoculation phase, in order to avoid contamination by fungal and bacterial contaminants. After inoculation, tubes were incubated at 35.5°C for up to 12 weeks. The MGIT tubes were visually read every day using BD BACTEC™ MicroMGIT Reader (Becton Dickson) and for solid media, positivity was detected by visual observation of colony growth (Figure 15).



Figure 15: Bacteriologically confirmed *M. tuberculosis* using Lowenstein-Jensen media (photograph taken during my work at the clinical laboratory at LMSE).

5. Phenotypic DST

Phenotypic DST was performed on MTBC-confirmed cultures 48 hours after positivity, using liquid media with the BD BBL MGIT AST SIRE kit (Becton Dickson) with INH, RIF, SM or EMB at critical concentrations of 0.1 mg/L, 1.0 mg/L, 1.0 mg/L and 5 mg/L respectively, by following the manufacturer's recommendations. For each MTBC-confirmed primary culture, 5 MGIT tubes were thus used in total including the positive control. Each tube was supplemented with OADC, the tested antibiotic, and was inoculated with organism suspension diluted at 1:5. In cases of resistance to RIF and INH, additional testing was performed for AMK, KAN and levofloxacin by using the same liquid culture system, with critical concentrations of 1 mg/L, 2.5 mg/L and 1.5 mg/L, respectively.

C. Advanced molecular testing

1. WGS

As per formal collaboration agreements for technical assistance with the MoPH, WGS of 2 XDR TB cases was performed at the WHO Collaborating/supranational TB reference Centre at the San Raffaele Scientific Institute in Milano, Italy. Genomic DNA was extracted from cultured isolates using the cetyl-trimethylammonium bromide (CTAB) method (van Soolingen *et al.*, 1991), and quantified using the Qubit dsDNA BR assay (Life Technologies, Paisley, UK). Paired-end libraries of 100 bp read length were prepared using the Nextera XT DNA Sample Preparation kit (Illumina Inc., San Diego, CA, USA) and sequenced on an Illumina HiSeq 2500 platform according to the manufacturer's instructions. DNA sequence files were deposited in the BioProject database under project/accession code PRJNA488372. Downstream analysis was performed using a dedicated in-house bioinformatics pipeline in Milano, including quality control check, alignment to H37Rv reference genome, recalibration and variant calling as described in (Tagliani *et al.*, 2017). A mean read coverage depth of at least >30x, with at least 4 reads on forward and reverse strand, at least 4 allele calls with base quality ≥ 20 and allele frequency $\geq 50\%$ were considered acceptable to call variants. The association of mutations with drug resistance was determined based on

available scientific literature (Miotto *et al.*, 2014; Whitfield *et al.*, 2015; Feuerriegel *et al.*, 2015; Ramirez-Busby and Valafar, 2015; Farhat *et al.*, 2016; Miotto *et al.*, 2017).

2. Targeted deep sequencing using Deeplex-MycTB

Following transportation under suboptimal conditions of heat-inactivated clinical specimens and cultured isolates from Lebanon to Lille, a targeted sequencing approach using Deeplex-MycTB kits was employed in Lille, as this method is more tolerant than WGS to smaller amounts and lower integrity of mycobacterial DNA. DNA was extracted using MasterPure™ DNA Purification Kit (Epicentre, Illumina, Madison, WI, USA) as per the manufacturer's recommendations. Multiplex PCR amplification of the concerned gene targets was then performed by using a beta version of Deeplex®-MycTB kit. Amplicons were purified using AMPure® XP magnetic beads (Agencourt®, Beckman Coulter, Indianapolis, IN, USA) and quantified by Qubit dsDNA BR assay (Life Technologies, Paisley, UK). Paired-end libraries of 150 bp read length NGS were prepared as described above using the Nextera XT DNA Sample Preparation kit (Illumina Inc., San Diego, CA, USA). The obtained libraries were sequenced on an Illumina MiSeq platform for an initial subset of samples comprising the MDR and XDR samples collected over the first 12-month period, and in a single NextSeq run for 348 samples from the total 18-month period, according to the manufacturer's instructions. The library preparation and sequencing steps were performed by Genoscreen, as required under sequencing service conditions.

I performed variant calling and genotypic analysis using dedicated, parameterized software developed by GenoScreen. Main sequencing data processing and analysis steps were as follows.

The obtained fastq files including the sequencing data were uploaded on the web application (<https://deeplex.bluebee.com/deeplex/#!/login>) by a GenoScreen personnel using a dedicated connector system installed on a local computer. All samples were selected and automatically analyzed by using the proprietary pipeline implemented in the web application (red arrows number 1 and 2, Figure 16). After inspection for successful analysis (red arrow number 3, Figure 16), on the main dashboard, recapitulated results of mycobacterial species identification and predicted resistotypes were examined for all samples. This dashboard contains the following information (Figure 16):

- *Sample filtering*: sample selection
- *Sample*: sample ID
- *Date of submission*: the period of uploaded data
- *Analysis mode*: the version of the pipeline used for analysis
- *Species identification*:
 - Red: *Mycobacterium tuberculosis complex*
 - Purple: other mycobacteria
- *Resistotype*: predicted drug resistance (only when MTBC is identified). The color scheme used:
 - Green: Absence of variants or variant known not to be associated with resistance.
 - Yellow to Red: One or multiple variants identified to be linked with resistance. Colors may vary based on percent subpopulation bearing the variant(s), from fixed (red) to very minor (yellow) in the bacterial population within the sample.
 - Blue: One or multiple uncharacterized (or non-synonymous) variants identified. Colors may vary based on percent subpopulation bearing the variant(s), from fixed (dark blue) to very minor (light blue) in the bacterial population within the sample.
 - Grey: when more than 5% of the reference target is not covered by sequence reads, prompting for further verification, which was done as follows. For all samples with genes with low coverage, the known drug resistance positions were verified in the concerned target by using the individual target view mode (see below). If at least one drug resistance associated position was not covered by a minimum of 5 reads in a gene, predictions for the associated anti-TB drug were not done (either as susceptible or resistant) and scored as unknown, because of suboptimal target coverage.
- *Activation code*: the kit code used for analysis of the uploaded sample sequencing data.

The overall coverage depths achieved on all samples were additionally inspected by using the map function (on the top right in the menu bar, Figure 16), generating circular maps including the same color coding for visualizing the gene coverages and drug resistance predictions (Figure 17).

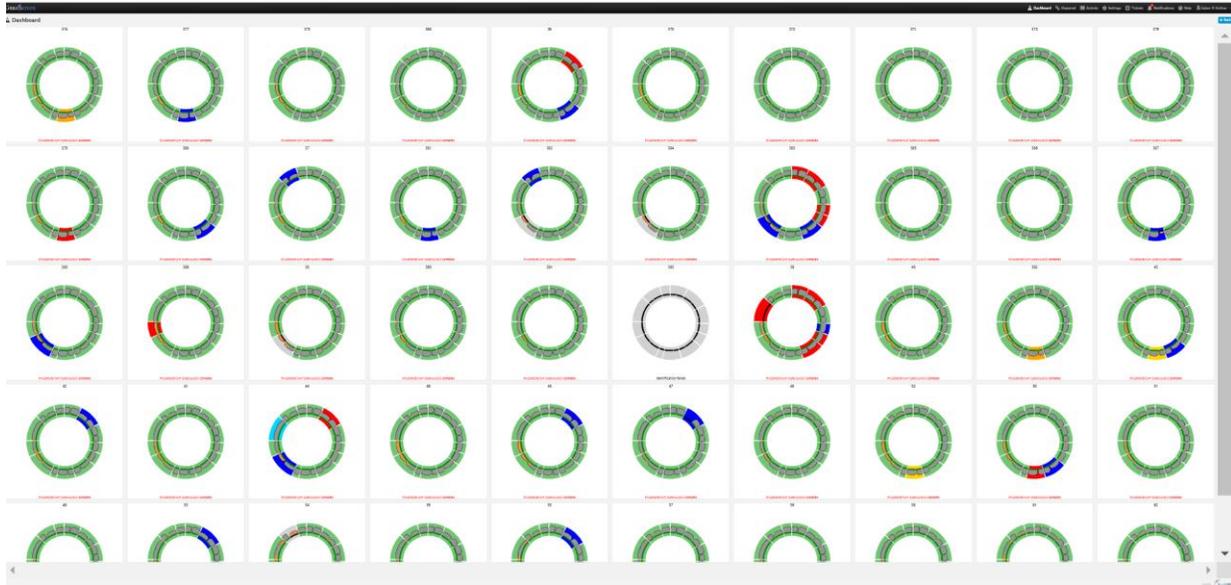


Figure 17: Visualization of Deeplex-MycTB results for multiple samples by using the map function. The figure shows an overall visualization of results for 50 samples. Green sectors indicate the absence of any variant associated with resistance in the corresponding gene targets, red to yellow sectors indicate the detection of drug resistance–associated mutations based on detected percent subpopulation, blue sectors indicate the identification of uncharacterized non-synonymous variants, and grey sectors indicate situations where more than 5% of a reference gene target is not covered by sequence reads.

Individual samples were then selected and inspected for detailed analysis of mutation profiles and genotypes, by using the sample view mode, showing a Deeplex circular map and detailed information on the results (Figure 18). Information shown in the Deeplex map was verified, including mycobacterial species identification by best match based on *hsp65* sequence, spoligotype binary code, spoligotype family name and SIT code of *M. tuberculosis* complex strain, and MTBC lineage identification based on phylogenetic SNPs (Coll *et al.*, 2014; Feuerriegel *et al.*, 2015). Additional detailed information that was verified included sample result metrics, control results, resistotype, detailed species identification results (e.g. percent identity vs *hsp65* reference sequence), variants associated with drug-resistance and uncharacterized variants, additional information on spoligotype and potential mixed infection (as identified by the presence of mixed nucleotide calls on phylogenetic SNP positions). Spoligotyping spacer coverage was especially in case of the detection of different spoligotypes detected in a same MIRU-VNTR cluster, to detect

potential spacers with a borderline coverage depth, i.e. close to the minimal threshold of 10 reads to call a spacer.



Figure 18: Detailed result of a selected sample. A Deeplex circular map is shown on the left side. In this map, target gene regions are grouped within sectors according to the antituberculous drug resistance they are associated with. Sectors in red refer to target regions in which drug resistance associated mutations are detected (shown in red around the circular map), whereas sectors in green refer to regions where no mutation or only mutations not associated with resistance (shown in grey around the map) are detected. Green lines above gene names represent the reference sequences with coverage breadth above 95%. Regions with coverage depths over individual sequence positions above 200 x or below 200 x are indicated by grey and orange zones above the reference sequences, respectively. Information on mycobacterial species identification (based on hsp65 sequence best match) and genotype of *M. tuberculosis* complex strain (based on spoligotype and lineage-defining, phylogenetic single nucleotide polymorphism (SNP)) are shown in the center of the circle. On the right side, detailed results are indicated including sample information, resistotype, sample controls and metrics, plus (not visible on the figure) *hsp65*-based species identification, drug resistance associated variants, uncharacterized variants, spoligotype, SNP-based phylogenetic lineage and potential mixed infection.

RIF, rifampin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol; SM, streptomycin; FQ, fluoroquinolones; KAN, kanamycin; AMI, amikacin; CAP, capreomycin; ETH, ethionamide; LIN, linezolid; BDQ, bedaquiline; CFZ, clofazimine.

Coverage depths of gene targets with mutations of interest within a sample were inspected in details by using the individual target view mode, showing coverage information and quantitative metrics on nucleotide calls across individual reference sequences (Figure 19).



Figure 19: Individual target view mode, showing detailed sequencing data obtained for the *katG* gene in a sample. Coverage breadth (i.e. the fraction of the reference covered by a minimum of 5 reads) and the average coverage depth are shown at the top. Red boxes on the dotted line indicate known positions of resistance mutations included in the built-in reference databases. Orange and grey zones in the histogram indicate coverage depths below and above 200 x (note that the scale on the right is logarithmic). The bottom part shows detailed information for the position in which a drug resistance associated S315T mutation was detected in the sample.

Finally, sample results including all the obtained information with e.g. identified resistotype, detected variants and genotyping information, were collectively exported as excel tables for subsequent global analysis and comparisons e.g. of mutation and genotype distributions.

D. Conventional genotyping using MIRU-VNTR typing

Standard 24-locus MIRU-VNTR typing was performed as described in (Supply *et al.*, 2006) using kits for amplification with 6 quadruplex PCRs (Genoscreen, Lille). The sizes of the amplified fragments and the numbers of repeats in the target loci were determined after capillary

electrophoresis-based separation on an ABI 3730 XL DNA Analyzer (Applied BioSystems, USA), using a customized software (GeneMapper v. 5.0, Applied BioSystems, USA). In order to perform cluster analysis and prediction of genetic lineage of isolates, the genotypes were analyzed and compared with reference strain genotypes using tools implemented in the MIRU-VNTRplus database (<http://www.miru-vntrplus.org>), as described in (Allix-Béguec *et al.*, 2008b; Weniger *et al.*, 2010). Genotyping analysis was made blinded from microbiological data and patient data. The classification of MTBC strains into different lineages and sub-lineages was performed based on MIRU-VNTRPlus database functionalities pooled with spoligotyping signatures for visual confirmation. Molecular clusters of MTBC isolates, indicative of endemic strains and/or possible transmission links when taken in conjunction with information on patient residency and familial links, were defined based on a combination of identical 24-locus MIRU-VNTR types, spoligotypes and identical SNP profiles (not associated with drug resistance) as obtained by Deeplex-MycTB. Drug resistance associated SNPs were subsequently considered in the identified clusters to screen for potential transmission of drug resistance.

E. Statistical analysis

Statistical analysis was conducted on a collaborative basis by Claire Pinçon (Lecturer in applied statistics, Lille university) using SAS software (SAS v9.4, SAS Institute Inc., Cary, NC, USA) in part I and part III in results. Age was expressed as mean \pm SD, categorical variables were expressed as absolute numbers and percentages. Multivariate logistic regression was used to test TB history as an independent predictor of drug resistance, after adjustment for age, gender and nationality. The log-linearity assumption was checked for the continuous covariate (patient age). The multivariate model was built by first including all predictors and then using a manual backward selection to reduce the model, minimizing Schwarz's Bayesian Criterion and maximizing the c-statistics and the p-value of Hosmer-Lemeshow test. A two-tailed type I error rate of 5% was considered for statistical significance.

Results and Discussion

Part I: Prevalence of drug resistant tuberculosis in Lebanon: A 12-month nationwide study

Note: This part of the work has been published in the Emerging Infectious Diseases issue of March 2019 (DOI: [10.3201/eid2503.181375](https://doi.org/10.3201/eid2503.181375)), concomitantly with the World TB Day.

Drug-Resistant Tuberculosis, Lebanon, 2016–2017

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In a 12-month nationwide study on the prevalence of drug-resistant tuberculosis (TB) in Lebanon, we identified 3 multidrug-resistant cases and 3 extensively drug-resistant TB cases in refugees, migrants, and 1 Lebanon resident. Enhanced diagnostics, particularly in major destinations for refugees, asylum seekers, and migrant workers, can inform treatment decisions and may help prevent the spread of drug-resistant TB.

Populations in crisis-affected areas are particularly vulnerable to tuberculosis (TB) linked to malnutrition, overcrowding, and discontinuity in health services (1,2). Difficulties accessing diagnosis and starting or completing appropriate treatment can promote the emergence and spread of multidrug-resistant (MDR) TB (resistant to at least rifampin and isoniazid) and extensively drug-resistant (XDR) TB (additionally resistant to ≥ 1 second-line injectable drug and 1 fluoroquinolone) in the countries of origin or in countries of transit or refuge after migration (3).

Lebanon hosts the largest per capita refugee population in the world. In addition to 450,000 refugees from Palestine, ≈ 1.5 million refugees from Syria are scattered in hundreds of informal sites across the nation (2,4). Moreover, the country hosts $>250,000$ migrant domestic workers, mostly originating from regions with high TB incidence rates, such as Ethiopia, Bangladesh, the Philippines, and Sri Lanka (5).

The last national survey on the prevalence of drug-resistant TB in Lebanon was performed 15 years ago (6), well

before the beginning of the Syria crisis in 2011. Even most recent reported MDR TB rates largely relied on estimates rather than on systematic laboratory confirmation (6). Second-line drug susceptibility testing (DST) and individualized XDR TB treatments were not available. We report results from a June 2016–May 2017 nationwide study combining extensive phenotypic and molecular testing. This national survey was approved by the ethics committee of the Azm Center for Research in Biotechnology and Its Applications, Lebanese University (document no. CE-EDST-3-2016), authorized by the Lebanese Ministry of Public Health. Informed consent was obtained from the study patients.

The Study

The study included 720 cases of suspected TB, corresponding to all suspected cases reported from June 1, 2016, through May 31, 2017, to the TB centers from the 9 governorates that make up Lebanon's national TB program. After testing of all corresponding microscopy-positive and microscopy-negative samples, 284 were considered confirmed TB cases on the basis of solid (Lowenstein-Jensen [LJ]) or liquid (BBL MGIT Mycobacteria Growth Indicator, BD Diagnostics, <http://www.bd.com>) culture results or molecular testing results (Xpert MTB/RIF, Cepheid, <http://www.cepheid.com>). For samples contaminated with blood, Anyplex MTB/NTM Real-time Detection (Seegene, <http://www.seegene.com>) (Appendix 1, <https://wwwnc.cdc.gov/EID/article/25/3/18-1375-App1.pdf>) was used. Thirty-four cases could not be subjected to DST because of culture negativity ($n = 28$), contamination ($n = 3$), insufficient sample amount for culture ($n = 2$), or reagent contingencies ($n = 1$).

Of the 250 remaining patients, 51% (128/250) were men; the mean age was 34 years (Table 1; Appendix 2, <https://wwwnc.cdc.gov/EID/article/25/3/18-1375-App2.xlsx>). Patients were from Syria (74/250, 29.6%), Lebanon (70/250, 28%), Ethiopia (57/250, 22.8%), Bangladesh (13/250, 5.2%), Palestine (7/250, 2.8%), or other nations (29/250, 11.6%).

Rifampin resistance was detected among 7/250 (2.8%) patients, concordantly with Xpert testing results for all cases (Table 1). We used multivariate logistic regression to test TB history as an independent predictor of rifampin resistance, after adjusting for age, sex, and nationality (Appendix 1). Log-linearity was checked for age. A 2-tailed type I error rate was set at 5%. TB history information was available for 246 (98.4%) patients. The proportion of

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Table 1. Details of 250 TB cases with available phenotypic drug susceptibility profiles, Lebanon, 2016–2017*

Characteristic	No. (%) patients			
	Total, n = 250	New cases, n = 228	Previously treated or relapsed, n = 18	Missing data, n = 4
Sex				
M	128 (51.2)	112 (49.1)	14 (77.8)	2 (50)
F	122 (48.8)	116 (50.9)	4 (22.2)	2 (50)
Country of origin				
Lebanon	70 (28)	64 (28.1)	4 (22.2)	2 (50)
Syria	74 (29.6)	65 (28.5)	9 (50)	0
Ethiopia	57 (22.8)	54 (23.7)	1 (5.6)	2 (50)
Bangladesh	13 (5.2)	13 (5.7)	0	0
Palestine	7 (2.8)	7 (3.1)	0	0
Other	29 (11.6)	25 (11)	4 (22.2)	0
Age, y	34 ± 14	34 ± 14	38 ± 13	23 ± 5
Drug resistance				
RIF	7 (2.8)	3 (1.3)	4 (22.2)	0
Mono	1 (0.4)	1 (0.4)	0	
MDR	3 (1.2)	1† (0.4)	2‡ (11.1)	
XDR	3§ (1.2)	1§ (0.4)	2§ (11.1)	
INH	16 (6.4)	15 (6.6)	1 (5.6)	0
Mono	9 (3.6)	8 (3.5)	1 (5.6)	
INH + SM	7 (2.8)	7 (3.1)	0	
EMB only	1 (0.4)	1 (0.4)	0	0
SM only	23 (9.2)	21 (9.2)	2 (11.1)	0
Susceptible to all first-line drugs	203 (81.2)	188 (82.4)	11 (61.1)	4 (100)

*Age is expressed as mean ±SD; categorical variables are presented as absolute numbers and percentages. EMB, ethambutol; INH, isoniazid; MDR, multidrug resistant; mono, monoresistant; RIF, rifampin; SM, streptomycin; XDR, extensively drug resistant.

†Resistant to RIF and INH.

‡Resistant to RIF, INH, EMB, and SM.

§Resistant to RIF, INH, EMB, SM, amikacin and kanamycin, and levofloxacin (representing all tested drugs for MDR and XDR isolates).

rifampin resistance was 22.2% (4/18) among previously treated patients and patients with relapse and 1.3% (3/228) among patients with new TB cases (adjusted OR 21.4, 95% CI 4.4–105.2; $p < 0.01$). One case in a patient without previous TB history was confirmed by liquid culturing DST as monoresistant to rifampin; 3 other cases, including 1 in a patient without previous TB history, were MDR TB, 2 of which showed resistance to all 4 first-line drugs tested (i.e., ethambutol and streptomycin in addition to rifampin and isoniazid). Moreover, 3 XDR TB cases were detected, including 1 in a patient without previous TB history, showing phenotypic resistance to amikacin, kanamycin, and levofloxacin in addition to all 4 first-line drugs tested. Among all 250 cases, 203 (81.2%) were susceptible to all 4 first-line drugs, 9 (3.6%) were resistant to isoniazid only, 1 (0.4%) to ethambutol only, 23 (9.2%) to streptomycin only, and 7 (2.8%) to isoniazid and streptomycin (Table 1).

To assess their extensive drug-resistance profiles, we subjected isolates from the 3 patients with XDR TB to targeted sequencing by use of a new assay, Deeplex-MycTB (GenoScreen, <https://www.genoscreen.fr>), which covers 18 drug resistance-associated gene targets (7) (Figure; Appendix 1). Two of these cases were confirmed by whole-genome sequencing. In 1 case (patient identification no. 74), no mutation was found to explain phenotypic resistance to amikacin and kanamycin. For the other drugs for this isolate, and for the 2 isolates analyzed by both tests, we detected drug resistance-associated mutations (8–10)

in *ipoB*, *katG* or *inhA*, *gyrA*, *rrs* or *tlpA*, and *embB*, confirming the resistance phenotypes (Table 2). Moreover, we detected different drug resistance-associated deletions in *ethA* in all 3 XDR TB isolates and drug resistance-associated mutations in *pncA* in 2 XDR TB isolates. These mutations predict additional resistance to ethionamide and pyrazinamide, which are not phenotypically tested in Lebanon or in many other countries.

Results of genotypic analysis of the 3 MDR TB isolates by Deeplex-MycTB also were consistent with phenotypic profiling overall, considering that a rare F129S mutation in *katG* was previously described in association with isoniazid resistance (11), along with other well-established mutations. An ethambutol resistance-associated M306V mutation in *embB* in 1 isolate was phenotypically undetected, probably reflecting known poor phenotypic reproducibility for this mutation (10). Of note, in the same isolate, Deeplex-MycTB testing detected a *gyrA* S91P mutation, which generally confers low levels of levofloxacin resistance (12), as a minority population (5.2%). This detection was confirmed by Anyplex results but was not correlated with phenotypic resistance to levofloxacin tested at a standard critical concentration of 1.5 µg/mL. As with the XDR TB isolates, nonsense insertion or deletion mutations additionally detected in *pncA* or *ethA* predicted supplementary pyrazinamide and ethionamide resistance in some isolates.

None of the MDR or XDR TB cases clustered with any other case in the study population tested by standard

Table 2. Genotypic and phenotypic drug susceptibility profiles of drug-resistant TB cases, Lebanon*

Category	Drug resistance classification						
	MDR			XDR			
Patient ID	14	48	125†	74	168	185	
TB drug							
RIF	Gene <i>ipoB</i>	S450L	S450L	S450L	S450L	S450L	S450L
	Phenotypic						
INH	Genes <i>katG</i>	S315T	F129S‡	S315T	S315T		S315T
	<i>inhA</i>				C-15T		
	Phenotypic						
PZA	Gene <i>pncA</i>		Y103Stop	InserA192	A46P		H71Q
	Phenotypic	ND	ND	ND	ND	ND	ND
EMB	Gene <i>embB</i>	Q497R	M306V	M306V	Q497R	Q497R	M306V
	Phenotypic						
SM	Genes <i>ipsL</i>	K43R		K88R	K43R		K43R
	<i>rrs</i>				A908C		
	Phenotypic						
AMI/KAN	Gene <i>rrs</i>				A1401G		A1401G
	Phenotypic						
FQ	Gene <i>gyrA</i>	S91P§			D94A	S91P	D94Y
	Phenotypic						
CAP	Genes <i>tlyA</i>				InserC313		
	<i>rrs</i>						
	Phenotypic	ND	ND	ND	ND	A1401G	A1401G
ETH	Genes <i>ethA</i>	Deleted¶		141 nt del#	Deleted¶	DelG632	DelG1338
	<i>inhA</i>				C-15T		
	Phenotypic	ND	ND	ND	ND	ND	ND
MIRU-VNTR type**		100-32	19431-157	21404-32	10156-32	21416-15	?-15
<i>M. tuberculosis</i> complex lineage††		2 (Beijing)	4 (Euro-American)	3 (Delhi-CAS)	2 (Beijing)	4 (H37Rv-like)	4 (Haarlem)

*Only genes with detected resistance-associated mutations are shown. No mutation was detected in targets associated with linezolid or bedaquiline and dofazimine resistance. Mutations are shown as amino acid changes with the corresponding codon position, nucleotide changes in promoter regions, or inserted or deleted base (inset or del with position in coding sequence) resulting in a frameshift. Bold text shows mutations concordantly detected by whole-genome sequencing and Deeplex-MycTB (GenoScreen, <https://www.genoscreen.fr>) in samples subjected to both assays. Other mutations are those detected in samples analyzed by Deeplex-MycTB only. Drug resistance predictions are based on reference data from available scientific literature (8–10), and for *pncA* also on data from Yadon et al. (14). Black represents phenotypic resistance to the different drugs and gray represents phenotypic susceptibility. For phenotypic testing, levofloxacin was the only fluoroquinolone tested. AMI, amikacin; CAP, capreomycin; EMB, ethambutol; ETH, ethionamide; FQ, fluoroquinolones; KAN, kanamycin; INH, isoniazid; ND, not done; PZA, pyrazinamide; RIF, rifampin; SM, streptomycin.

†Deeplex-MycTB result obtained on a primary specimen (sputum). The other results were obtained on indirect samples (primary cultures).

‡Mutation described in association with isoniazid resistance once before by Wang et al. (11). This mutation is not detectable by Anyplex testing.

§Detected as a minority variant, at 5.2% in this sample (see text). Percentages of fixation of other mutations within individual samples range from 80.6% to 100%.

¶Putative deletion, as inferred by absence of reads mapped specifically on the corresponding gene target, in contrast to all other, well covered targets.

#Internal deletion, resulting in a frameshift, from gene position 859 to 999.

**According to MIRU-VNTRPlus website (<http://www.miru-vntrplus.org>) nomenclature (15). For patient 185, a question mark in the genotype reflects the absence of a detectable allele in locus 4052.

††According to Deeplex-MycTB (spoligotyping and phylogenetic SNPs) and MIRU-VNTRPlus identification, confirmed by whole-genome sequencing results, when done.

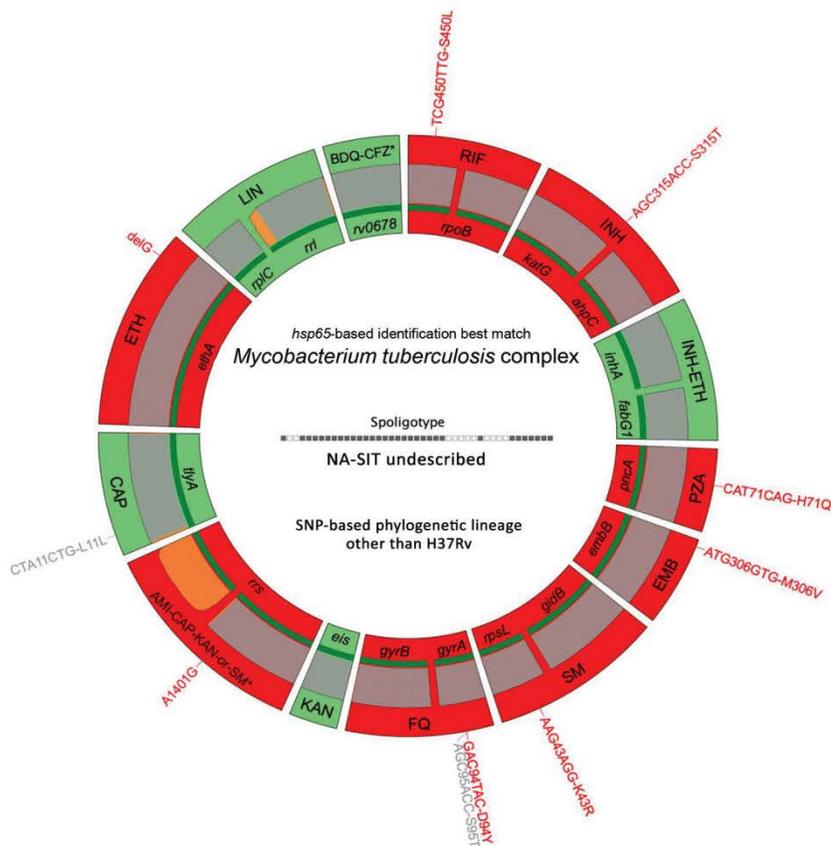
24-locus mycobacterial interspersed repetitive unit–variable-number tandem-repeat (MIRU-VNTR) typing of isolates, showing no support for drug-resistance transmission (Appendix 2). Consistently, 4 of the 6 cases involved were previously treated, and the 2 new cases were in migrant workers, presumably representing imported cases. Two cases were in Syria refugees; 1 patient with MDR TB had repeated failed treatment in Syria, and 1 XDR TB case was a relapse after patient arrival in Lebanon. Of the other previously treated cases, 2 had Beijing strain genotypes; the isolate from an XDR TB case in a patient originating from eastern Europe differed by a single allele from the 100-32 MIRU-VNTR haplotype and the isolate from an MDR TB case in a patient from Lebanon fully matched the 100-32 MIRU-VNTR haplotype (Table 2). This haplotype represents a major, presumably highly transmissible MDR-associated clonal complex epidemically spreading across

Eurasia (13). Although an XDR TB patient of foreign origin returned to his country after diagnosis because of initial unavailability of proper treatment in Lebanon, the 2 other XDR TB patients received treatment and, as of January 2019, responded positively to ongoing treatments, as were the patients treated for MDR TB.

Conclusions

Although the prevalence of rifampin-resistant TB estimated in Lebanon is relatively low (2.8%), identification of XDR TB and MDR TB cases, including TB strains with strong epidemic potential and complex resistance patterns, calls for sustained diagnosis of MDR TB. We recommend that Lebanon test all TB-positive isolates for resistance to first- and second-line drugs, to inform treatment decisions and prevent the spread of drug resistance. Other major destinations for refugees, asylum seekers, and migrant

Figure. Deeplex-MycTB (GenoScreen, <https://www.genoscreen.fr>) results identifying an extensively drug-resistant genotypic profile in an isolate from a tuberculosis (TB) patient in Lebanon. Results correspond to TB patient no. 185 in Table 2. Target gene regions are grouped within sectors in a circular map according to the drug resistance with which they are associated. Red indicates target regions in which drug resistance-associated mutations are detected (red text around the map), whereas green indicates regions where no mutation or only mutations not associated with resistance (gray text around the map) are detected. Dark green lines above gene names represent the reference sequences with coverage breadth above 95%. Limits of detection (LOD) of potential heteroresistance (reflected by subpopulations of reads bearing a mutation), depending on the coverage depths over individual sequence positions, are indicated by gray (LOD 3%) and orange (variable LOD >3%–80%) above the reference sequences. Information on mycobacterial species identification, based on *hsp65* sequence best match, and genotype of *Mycobacterium tuberculosis* complex strain, based on spoligotype and lineage-defining phylogenetic SNP, are shown in the center of the circle. AMI, amikacin; BDQ, bedaquiline; CAP, capreomycin; CFZ, clofazimine; EMB, ethambutol; ETH, ethionamide; FQ, fluoroquinolones; KAN, kanamycin; LIN, linezolid; INH, isoniazid; PZA, pyrazinamide; RIF, rifampin; SM, streptomycin; SNP, single-nucleotide polymorphism.



workers should also consider using enhanced diagnostics to help prevent the spread of drug-resistant TB.

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P.S. is a consultant for Genoscreen; S.D., F.D.M., and C.G. are employees of Genoscreen.

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Ms. El Achkar is a PhD student with supervision shared between Université de Lille and Université Libanaise. Her work focuses on TB drug resistance and transmission in Lebanon.

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EMERGING INFECTIOUS DISEASES

Supplementary discussion:

This retrospective survey represents the most comprehensive tuberculosis drug resistance survey done in Lebanon to date. The single previous nationwide study that was made dated from more than a decade ago, with a recruitment period from July 2002 to April 2004 (WHO, 2005; Araj *et al.*, 2016). In contrast to our study which also included smear negative pulmonary TB and extra pulmonary TB cases, this study was restricted to smear positive pulmonary TB cases. Moreover, it was limited to phenotypic testing of susceptibility to the first-line drugs (i.e. RIF, INH, EMB and SM). Out of the total of 206 patients enrolled, 15 (7%) had MDR TB. Susceptibility to second-line drugs was not tested. Over the subsequent period, the evaluation of MDR TB prevalence was largely based on estimates and partial data, which showed relatively wide fluctuations over the years, as testing for resistance was mostly done upon physician's request for special situations such as HIV or relapse cases (Araj *et al.*, 2016). Moreover, as a consequence of the continuous absence of second-line drug susceptibility testing, the prevalence of XDR TB remained unknown.

Questions related to these uncertainties increased in the context of the increase of TB incidence seen from 2012 on, largely attributed to the influx of refugees fleeing the war in Syria. Although there were also large degrees of uncertainty around the estimates, some data tended to suggest a significant problem of drug resistance in this country before the onset of the war. According to a national survey done in 2003, MDR/RR TB cases in Syria represented 8% of the new TB cases (WHO, 2015b). A previous study showed a MDR TB rate as high as 62.5% among cases with a failed treatment or with a relapse from Syria, from 2003 to 2005 (Rahmo and Hamze, 2010). Irrespectively of the exact prevalence prevailing before the conflict, the situation is thought to have deteriorated after the beginning of the war in 2011, leading to disruption in drug supplies and treatment of TB patients, and difficulties to access diagnosis and treatment in hosting countries for those who fled (Ismail *et al.*, 2018). As indicated in Introduction (point H), an important increase in the number of notified TB cases among Syrian refugees in Lebanon was observed over the recent years.

Our study provides valuable, new representative data in this respect. During a 12-month period over 2016 and 2017, we detected 7 RR/MDR TB cases among 250 cases with available DST (2.8%), from an initial total of 284 confirmed TB cases countrywide. While this prevalence can be considered relatively low, the use of phenotypic and molecular testing newly extended to second-

line drugs allowed us to reveal the existence of 3 XDR TB cases among them. These cases represent the first XDR TB cases identified in the country. Lebanon thus further adds to the long list of world countries that reported XDR TB cases (77 reported a total of 10800 XDR TB cases in 2017 (WHO, 2018a)). Furthermore, Deeplex-MycTB testing detected as well in several MDR and XDR TB cases genotypic resistance to PZA and/or ETH, which are not phenotypically tested. The majority of the 6 MDR TB cases had thus more/far more complex resistance patterns than what could be presumed based on the diagnostic system in place, which was mostly used for special cases. These diagnostic gaps will be discussed more globally in the General Discussion.

The history of individual MDR and XDR TB patients can be somewhat more detailed in this Supplementary Discussion. One MDR patient was a Syrian refugee whose treatment failed twice in his country before entering in Lebanon in 2014, two years before being diagnosed in this final destination. Another Syrian refugee with XDR TB who arrived in Lebanon in 2012 reported onset of symptoms as a result of relapse at least one month before diagnosis of the new disease episode. The first identified XDR case was a Ukrainian patient who reportedly interrupted his treatment in his country because of shortage in anti-TB drug supply, before traveling to Lebanon. The corresponding isolate, as well as the isolate from a Lebanese MDR patient, had Beijing genotypes differing by a single allele from, or fully matching the so-called 100-32 MIRU-VNTR haplotype, respectively. This haplotype represents a major, presumably highly transmissible MDR-associated clonal complex, epidemically spreading across Russia and Eastern Europe (Merker *et al.*, 2015). The circumstances that preceded the onset of care for these patients, as well as the strains carried by some of these patients, raise thus concerns about potential secondary infections with MDR or XDR strains. This represents a supplementary reason for recommending sustainable testing of all TB-positive isolates for resistance to first- and second-line drugs in the country.

In the following parts, we extended our investigations by using phenotypic and molecular testing comprising Deeplex-MycTB to virtually all confirmed TB cases over the complete period of 18 months.

Part II: Prevalence of drug resistant tuberculosis using Deeplex-MycTB and phenotypic DST, June 2016 – November 2017

Introduction:

The data obtained over the first 12-month period, by using phenotypic DST complemented by selected Deeplex-MycTB testing and WGS analysis of isolates from (selected) MDR and XDR TB patients, revealed the existence of XDR and MDR with complex resistance profiles on a national level, disclosing thus unsuspected extra layers of drug resistance in the country. Given these findings, as well as the uncertainties around the estimated annual incidence of MDR TB cases as well as the constant flux of populations including Syrian refugees and migrant domestic workers from high TB burden country over the recent years, we extended the collection of clinical samples and analysis of the drug resistance prevalence to a total period of 18 months. We retrospectively tested all culture-positive samples with available DST over this complete period with Deeplex-MycTB, in order to obtain extensive drug susceptibility profiles and detect other potential layers of TB drug resistance undetected by phenotypic DST and molecular testing with GeneXpert and Anyplex routinely performed in the country.

In addition to evaluate the performances of this novel assay on a nationally representative sample versus available reference data for the drugs tested by phenotypic DST, our objective was to evaluate and improve the coverage and quality of diagnosis, to prevent potentially inappropriate empirical treatment of the patient and ensure further successful outcomes.

Furthermore, we used a combination of standard 24-locus MIRU-VNTR typing, spoligotyping and SNP typing obtained by Deeplex-MycTB testing, to evaluate the genetic diversity of the strain population, and the molecular clustering of isolates, suggestive of potential endemic strains and/or epidemic transmission. The study workflow that was followed is depicted in figure 20.

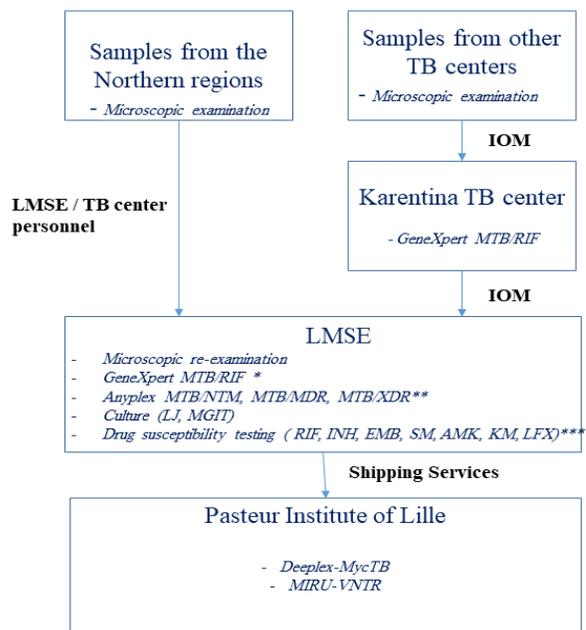


Figure 20: Study workflow for a period of 18 months.

*: Restricted to samples from the Northern regions, **: Only under physician's request, ***: AMK, KAN, LFX are tested only in cases of resistance to RIF and INH. RIF: Rifampicin, INH: Isoniazid, EMB: Ethambutol, SM: Streptomycin, AMK: Amikacin, KM: Kanamycin, LFX: Levofloxacin, IOM: International Organization for Migrations.

Results and discussion

I. Study population

A total of 1104 TB samples all obtained from different presumptive patients were reported to 9 TB centers and centralized in LMSE with the help of IOM for classical phenotypic analysis between 1st of June 2016 and 30 November 2017. After testing of all received samples by GeneXpert Cepheid, Anyplex MTB/NTM or culture on liquid and solid media, 417 patients were diagnosed with TB. Of these, sixty-three cases could not be subjected to DST, due to culture negativity (n=54), primary or secondary culture contamination (n=5), culture not completed (insufficient quantity of sample or biopsy in formol) (n=3) or reagent contingencies (n=1).

Among the 354 confirmed TB cases with available DST, 51.7% (183/354) were female and the majority (48.6%; 172/354) aged between 25-34 years old. Of these 95.8% (339/354) had pulmonary TB disease.

In terms of patient residence, among the eight Lebanese governorates (Akkar, North Lebanon, Mount Lebanon, Beirut, South governorate, Baalbek-El Hermel, Bekaa, and Nabatieh), Mount Lebanon was the most represented, including 35% (124/354) of the TB patients. To note, 2.3% (8/354) of TB patients were in jail (Table 7).

Lebanese nationals represented 28% (99/354) of the total TB cases. Among the foreign-born patients, 26.6% (94/354) were Syrians, 3.1% (11/354) Palestinians, and the remaining represent 42.3% (150/354) (including 26.5% (94/354) Ethiopians) of the total TB cases (Table 7).

II. Phenotypic drug susceptibility

DST was performed on all culture-confirmed MTBC. Out of the 354 confirmed MTBC cases, 81.9% (290/354) of total TB patients were susceptible to all 4 first-line drugs, 3.4% (12/354; i.e. 3 more compared to the initial 12-month period) have mono-resistance to isoniazid, 0.3% (1/354) with mono-resistance to rifampicin (already identified in the first period), 0.3% (1/354) with mono-resistance to ethambutol (already identified in the first period), and 8.5% (30/354) mono-resistance

to streptomycin (7 more relatively to the first period). Twelve cases were resistant to both isoniazid and streptomycin (5 more relatively to the first period).

A total of 8 MDR TB cases (2 more relatively to the first period) were diagnosed in Lebanon during our study period. One case of MDR was resistant to rifampicin and isoniazid only; another MDR was resistant to rifampicin, isoniazid, and streptomycin only while an additional 6 were resistant to all 4 first-line drugs. Of the 8 MDR cases, 3 were already detected as XDR-TB cases in the first period (Table 7).

Table 7: Sample description including demographic patient characteristics and drug susceptibility test profiles.

		Total (n=354)	
		N	%
Sex	Male	171	48.3
	Female	183	51.7
Age	0-14	7	2
	15-24	59	16.7
	25-34	172	48.6
	35-54	77	21.7
	55-64	25	7.1
	≥ 65	14	3.9
Nationality	Lebanese	99	28
	Syrians	94	26.5
	Palestinian	11	3.1
	Ethiopian	94	26.5
	Others	56	15.8
	Akkar	26	7.3

Residency by governorates	North governorates	43	12.1
	Mount Lebanon	124	35
	Beirut	86	24.3
	Nabatieh	1	0.3
	South governorate	29	8.2
	Bekaa	29	8.2
	Baalbek-El Hermel	8	2.3
	Jail	8	2.3
Sample type	Pulmonary	339	95.8
	Extra-pulmonary	15	4.2
DST	SSSS	290	81.9
	RSSS	1	0.3
	SRSS	12	3.4
	SSRS	1	0.3
	SSSR	30	8.5
	SRSR	12	3.4
	MDR	5	1.4
	XDR	3	0.8

n = number of patients with characteristics, S= susceptible, R= resistant, DST: drug susceptibility testing. Order of antibiotics for DST: rifampicin, isoniazid, ethambutol, and streptomycin.

III. Deeplex-MycTB DST predictions versus phenotypic DST

A total of 348 *M. tuberculosis* isolates with available DST results were subjected to Deeplex-MycTB testing, while the remaining 6 samples were discarded due to insufficient quality or amount of extracted DNA. In our study collection, to characterize a mutation, we considered that synonymous and lineage-defining mutations do not cause resistance except for the H57D mutation in *pncA* (Scorpio and Zhang, 1996) and the N236K mutation in *tlyA* (Walker *et al.*, 2018), both representing phylogenetic mutations (presumptive in the case of the second mutation), respectively associated with PZA and CAP resistance. We categorized mutations as uncharacterized if they were not identified as resistance- or susceptibility- associated determinants as per the reference databases implemented in the Deeplex-MycTB web app and/or other previous literature.

Nine out of the 348 (2.6%) samples could not be analyzed due to insufficient coverage depths over multiple gene targets, and no identification as *M. tuberculosis* complex due to insufficient coverage of the *hsp65* reference. For the remaining 339/348 (97.4%) analyzable samples, a total of 4184 out of 4407 (94.9%) possible phenotypes could be predicted for the 13 drugs/drug classes covered by Deeplex-MycTB. The 223/4407 phenotypes (5.1%) that could not be predicted resulted from uncharacterized mutations (50.7%) or the lack of minimal coverage of one or more known drug resistance associated positions in the corresponding individual targets (49.7%).

Of these predicted phenotypes, 1282/1380 (92.9%) matched the phenotypic results available for the first-line drugs and SM tested for all samples, and for the second-line drugs tested for the MDR and XDR samples. Based on detectable resistance genetic determinants, INH, RIF, EMB, and SM resistance were concordantly predicted with 90.3%, 100%, 100%, 52.8% sensitivity, respectively, and susceptibility with 99.6%, 100%, 99.4%, 99.6% specificity, respectively (Figure 21). Importantly, Deeplex-MycTB also predicted susceptible or resistant profiles for the second line drugs not tested by phenotypic DST for most or all of the isolates, i.e. FQ, AMK, KAN, PZA, CAP, ETH, LIN, and BDQ/CFZ (Figure 21).

Results of resistance and susceptible phenotype predictions for the exploitable 339 samples are described in more details below for the different individual drugs. For the sake of simplicity, the

results of the 3 MDR and 3 XDR TB cases described in the first result chapter are considered jointly with the newly obtained results.

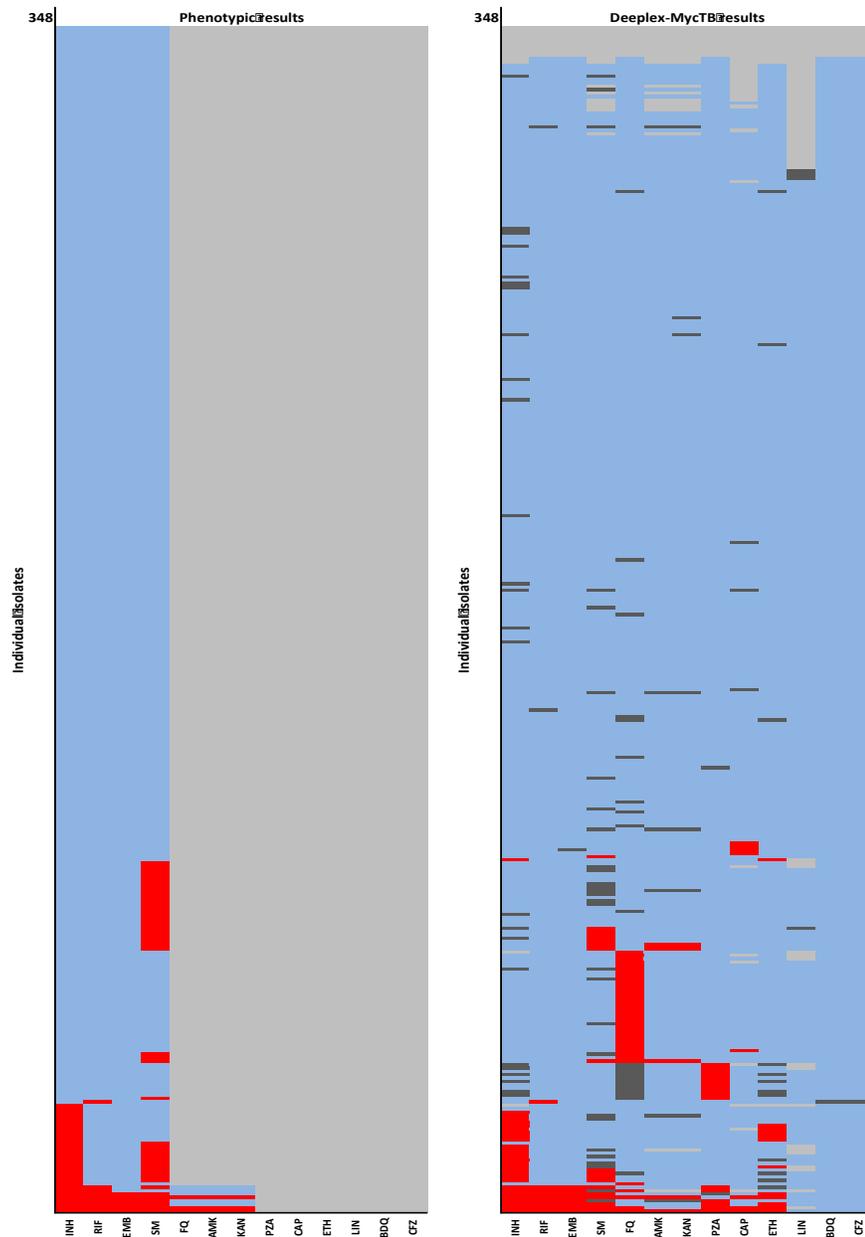


Figure 21: Phenotypic and genotypic DST for all 348 tested isolates.

The left panel shows the phenotypes of tested drugs. The right panel shows the genotypic predictions based on the presence or absence of detected drug resistance associated mutations by Deeplex-MycTB. Color coding of individual phenotypes or predictions are as follows: Red: resistant, blue: susceptible, light grey: not tested or not predicted, dark grey: uncharacterized mutation (right panel, Deeplex-MycTB).

INH

INH resistant phenotypes were detected for 32 isolates. Genotypic analysis using Deeplex-MycTB was concordant in 87.5% (28/32) of the resistant phenotypes. Most of the concordantly predicted INH resistant isolates harbored either a S315T mutation in *katG* gene (59% (19/32)) or a C-15T mutation in *fabG1* (22% (7/32)) associated with high and low levels of INH resistance respectively (Feuerriegel *et al.*, 2015; Walker *et al.*, 2015; Miotto *et al.*, 2017) (Figure 23). Such dominance of the S315T mutation in *katG* and the C-15T mutation in *fabG1* is consistent with well-established data on INH resistance determinants (Walker *et al.*, 2015).

Interestingly, we also identified two additional, more exceptional INH resistance-determining mutations. In one case, a F129S mutation was detected in *katG* in one MDR TB isolate. This mutation had been reportedly associated only once previously with INH resistance (Wang *et al.*, 2010). Even more interesting was the other case, for which INH resistance was predicted on the basis of the selective absence of any coverage of the *katG* gene target, while all other targets were very well covered, which was suggestive of a genomic deletion affecting this region (Figure 22). This genomic region is known to be vulnerable to deletion events (Tsolaki *et al.*, 2004). This conclusion was independently supported by the observation of missing alleles from both MIRU-VNTR markers 2163b and 2165, according to the MIRU-VNTR analysis of the same sample. As these two markers are localized in the *katG* genomic region, their concordant absence of PCR amplification thus represent additional (negative) evidence for the loss of this region. Such deletion in this *katG* region was previously confirmed by WGS analysis in a *M. bovis* BCG derivative with missing 2163b-2165 MIRU-VNTR alleles (Supply *et al.*, 2014).

Deeplex-MycTB failed to predict INH resistance phenotypes for 3 isolates, with no drug resistance associated mutations detected despite high coverages in *katG*, *inhA*, *fabG1* or *ahpC*. These false negative results relatively to phenotypic DST may be due to the presence of other INH resistance mechanisms outside these main genes targets. The absence of resistance determinants in these genes has also been documented by WGS analysis of other phenotypically INH resistant isolates (Walker *et al.*, 2015). Finally, a single case of INH resistant phenotype had an unpredictable INH result by Deeplex-MycTB due to low coverage of corresponding gene targets.

Of the 307 INH susceptible phenotypes, 90.6% (278/307) had no mutation associated with INH resistance and 8.1% (25/307) had uncharacterized mutations, while 1% (3/307) showed no

minimal coverage in the genes targeted by Deeplex-MycTB. In a single case only, a INH resistance associated C-15T mutation was detected in *fabG1* (Figure 23). This mutation is known to cause low levels of INH resistance phenotypically. Therefore, this discordance between the Deeplex-MycTB prediction and the phenotypic result might reflect a borderline resistance level for this specific strain, i.e. close to the critical concentration, given the partially overlapping MIC distributions of wild type strains and of strains bearing this mutation (Lempens *et al.*, 2018).

Of note, in our total study population, 24 TB patients had mono-resistance to INH (not considering additional resistance to SM, now only used as a potential surrogate of CAP in MDR-TB treatment). According to the WHO, 8% of TB patients worldwide have mono-resistance to INH, which, together with RIF, represents one of the two major drugs used for the first-line treatment of TB (WHO, 2018a). The emergence INH resistance reduces the effectiveness of the standard first-line treatment regimen, even in a RIF susceptible context. In the cases of low level resistance, linked to *inhA* mutations, a first-line regimen with an elevated INH dose is recommended. In the cases of high level resistance, linked to *katG* mutations, treatment with RIF, PZA, EMB, and LVX for 6 months is recommended after ruling out resistance to FQ (WHO, 2018c). However, all patients with monoresistance to INH in our study received the standard treatment regimen including INH at a normal dose, due to the absence of rapid molecular susceptibility tests for INH at the time of diagnosis.

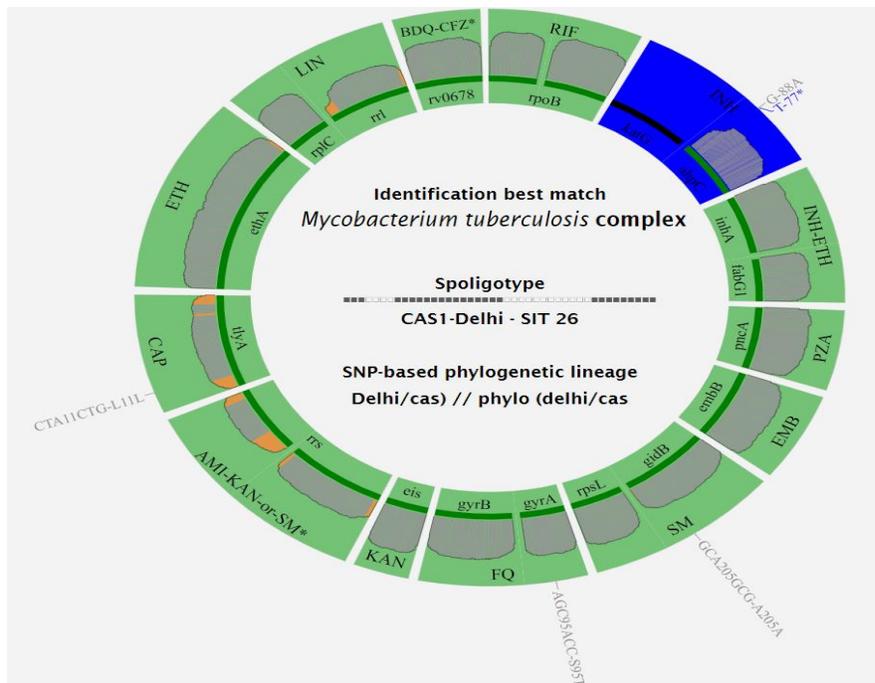


Figure 22: Deeplex-MycTB results identifying monoresistance to isoniazid in a TB patient in Lebanon, characterized by a genomic deletion in the *katG* gene. Note that the initial prediction of this deletion as an uncharacterized mutation (represented by a blue color) was overruled manually into a resistance prediction. Such gene deletion is now automatically called as associated with resistance in a new version of the Deeplex-MycTB analysis pipeline to be deployed soon (P. Supply, personal communication).

RIF

Phenotypic RIF resistance was detected among 9 TB cases. According to WHO recommendations, Xpert MTB/RIF is widely used as the reference rapid molecular diagnostic test for RIF susceptibility testing (WHO, 2018c). In our study population, consistency between phenotypic testing and GeneXpert was observed in all cases for RIF DST.

Results from genotypic analysis using Deeplex-MycTB were also 100% concordant with phenotypic DST of RIF. Of the 9 RIF resistant isolates, 88.9 % (8/9) harbored the S450L mutation in *rpoB*, which also represents the globally dominant *rpoB* mutation associated with RIF resistance (Walker *et al.*, 2015). The remaining case had a deletion of 6 nucleotides in *rpoB*, known to be linked to RIF resistance according to ReSeq TB data (Miotto *et al.*, 2017) (Figure 23). Unlike MDR/RR TB strains circulating in Southern Africa and in some other world regions (Makhado *et*

al., 2018), none of the strains from our study population harbored mutations associated with RIF resistance outside the RRDR region, undetected by the Xpert MTB/RIF.

Of the 330 RIF susceptible phenotypes, 99.4% (328/330) also concordantly showed no mutation in *rpoB* by Deeplex-MycTB, even as minor variants, and 0.6% (2/330) isolates had uncharacterized mutations in *rpoB* (Figure 23).

EMB

Phenotypic DST revealed a total of 6 TB patients with EMB resistance. All were concordantly predicted as resistant by Deeplex-MycTB, with isolates carrying the Q497R (3/6), M306V (2/6), and G406A (1/6) mutations in the *embB* gene (Figure 23). These mutations are all amongst the most frequently *embB* mutations associated with EMB resistance (Walker *et al.*, 2015).

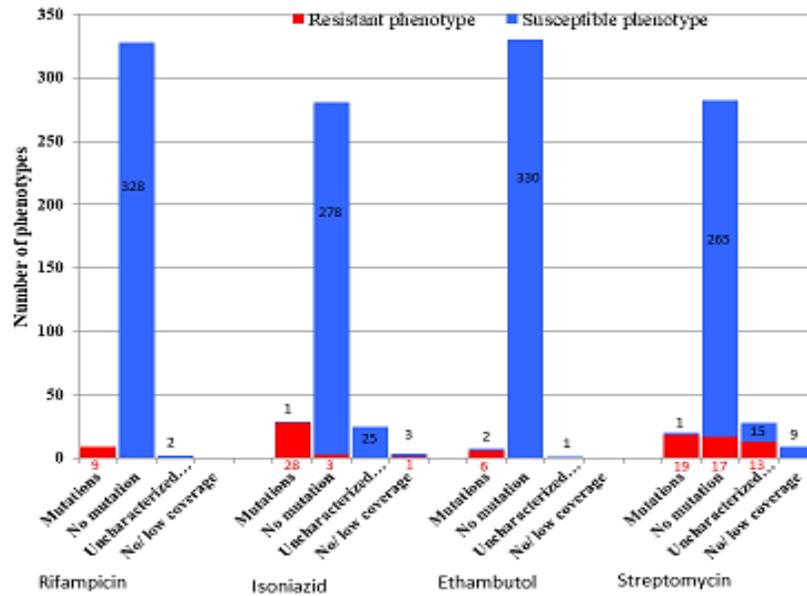
Out of 333 susceptible phenotypes, 99.1% (330/333) concordantly had no mutation associated with EMB resistance detected by Deeplex-MycTB, even as a minor population, and 1 (0.3%) harbored an uncharacterized A313V mutation in *embB* gene. Only two (0.6%) known resistance-determining mutations were detected in *embB* in 2 susceptible phenotypes, namely the M306V and G406D mutations (Figure 23). As indicated in Part I (results and discussion), these discordant cases likely reflect the known unreliability and irreproducibility of the phenotypic test for EMB, due to the largely overlapping distributions of MICs of wild-type and mutant strains for this drug (Walker *et al.*, 2015). DNA sequencing is therefore now considered to be the most reliable method for the detection of EMB resistance (WHO, 2018c).

SM

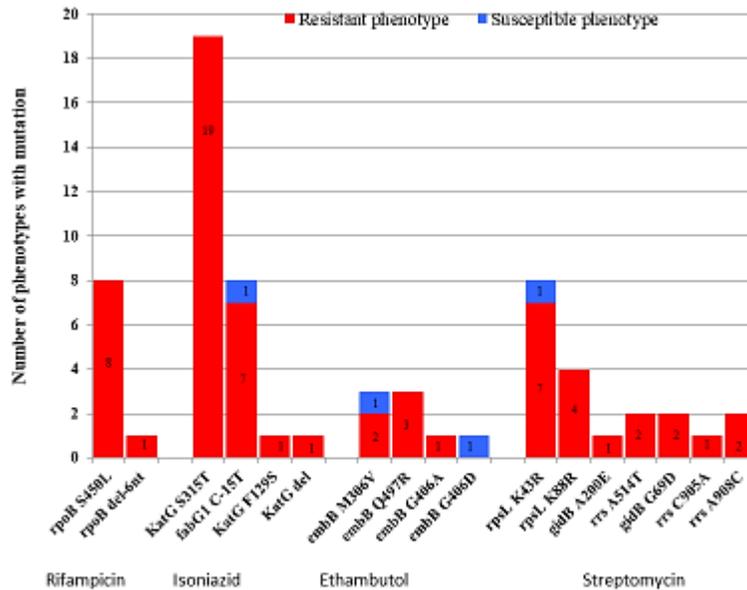
Although SM is generally not included in the treatment regimen of MDR TB cases, it can be used as a surrogate to AMK when the latter drug cannot be used (WHO, 2018b). Of the 49 SM resistant phenotypes, only 19 (38.8%) cases were detected as harboring SM resistance mutations in *rpsL* (K43R, K88R), *gidB* (A200E, G69D), or *rrs* (A514T, C905A, A908C) (Jnawali *et al.*, 2013; Walker *et al.*, 2015; Miotto *et al.*, 2017). Among the remaining cases, 34.7% (17/49) of the resistant SM phenotypes had no mutation in the targeted genes, while 26.5% (13/49) of the remaining

resistant SM phenotypes harbored uncharacterized mutations (Figure 23). While these findings might reflect the existence of other resistance mechanisms outside the genes targeted Deeplex-MycTB in some cases, they probably reflect in a large part the known high variability in the performance of SM DST, linked to the uncertain critical concentration to be used, as shown by a meta-analysis (Horne *et al.*, 2013) and as confirmed by experts at the WHO supranational reference laboratory in Antwerp (L. Rigouts, personal communication).

Of the 290 SM susceptible phenotypes, only 1 (0.3%) was found to harbor a K43R mutation in *rpsL*, known to be a highly confident SM resistance mutation (Miotto *et al.*, 2017). Of the remaining cases, 91.4 % (265/290) consistently had no mutation in *rrs*, *gidB*, and *rpsL*, and 5.2% (15/290) had uncharacterized mutations, while 3.1% (9/290) had no sufficient coverage in the genomic targets, resulting in unpredictable results (Figure 23).



a.



b.

Figure 23: Prediction of drug-resistance and drug-susceptible using Deeplex-MycTB versus phenotypic DST. a. Deeplex-MycTB versus DST by phenotype b. resistance determining mutations detected by Deeplex-MycTB versus phenotypes with mutation.

PZA

As per the diagnostic means available in Lebanon and like in many other countries, TB cases were not subjected to PZA testing phenotypically. Phenotyping testing is considered as unreliable, linked to high-levels of false positivity and false negativity, due to the use of large inocula that reduce the PZA activity and the acidic pH used in the test that can prevent growth (Scorpio and Zhang, 1996; Chang *et al.*, 2011). Consequently, DNA sequencing is now considered to be the new reference for PZA resistance prediction (WHO, 2018c).

Deep sequencing by Deeplex-MycTB predicted a total of 17 isolates with resistance to PZA, including 4 MDR and 2 XDR TB cases. Noteworthy, 64.7% (11/17) of these PZA resistant TB isolates harbored the phylogenetic SNP H57D in *pncA*, causing natural resistance to PZA in *M. bovis* strains. The latter cases were specifically studied as described in Results and discussion Part III of this work.

AMK, KAN and CAP

According to the algorithm in place in Lebanon, also shared with many other countries, susceptibility to AMK and KAN (and to FQ; see below) was phenotypically tested only in MDR TB cases, as they represent situations for which the presumption of resistance to these drugs become higher. Of note, KAN and CAP have been recommended to be no more used in the treatment of MDR TB cases, as a result of poor treatment outcomes (WHO, 2018b).

Among the 8 MDR or XDR isolates identified herein, 3 had resistance to AMK and KAN phenotypically. Deeplex-MycTB concordantly predicted 2 of these 3 isolates as AMK- and KAN-resistant isolates, as they harbored a A1401G mutation identified as a highly confident AMK, KAN, and CAP resistance mutation (Miotto *et al.*, 2017). In the remaining case, no mutation was found to explain the phenotypic resistance.

Apart from the MDR and XDR-TB cases, only 5 isolates were predicted as resistant to an injectable drug, namely CAP, as they carried the N236K mutation in *tlyA* associated with CAP resistance (Maus *et al.*, 2005b; Miotto *et al.*, 2017). Interestingly, on the basis of a WGS study of a large cross-border TB outbreak in Europe (Walker *et al.*, 2018), this mutation has recently been proposed to represent the sole other example, in addition to the H57D mutation in *pncA*, of a mutation being both a resistance determinant and a phylogenetic marker, namely of L4/sublineage Cameroon. Our

Deeplex-MycTB results bring additional support to this hypothesis, as they indicated that the 5 isolates bearing this mutation in our strain population shared a same spoligotype signature, characterized by the absence of spacer 13, typical of lineage 4.

ETH

Like for PZA, susceptibility to ETH is not phenotypically tested in many countries, including in Lebanon. Due to problems in phenotypic DST of ETH, according to recent WHO recommendations, DNA sequencing of the *ethA*, *ethR* genes, and *inhA* promoter region has been proposed as the reference method for the prediction of ETH resistance (WHO, 2018c).

Aside from MDR and XDR-TB cases (see results and discussion part I), the only other isolates for which ETH resistance was predicted by Deeplex-MycTB were the 7 isolates that harbored the C-15T mutation in *fabG1*, determining cross-resistance to INH and ETH.

FQ

FQ is considered the key drug in the treatment of MDR TB (short and long term regimen), as well as in the recently recommended treatment of high level INH mono-resistant TB. Moreover, FQ is an important component of new, recently or currently clinically evaluated TB treatments, such as REMox (with MFX in substitution of an individual drug of the standard first-line regimen for shorter treatment of drug sensitive TB) (Gillespie *et al.*, 2014) or BPAmZ (MFX with PZA, BDQ and pretomanid) (TB ALLIANCE, 2019).

WHO recommended recently systematic DST for FQ when faced with RIF resistance or INH monoresistance (WHO, 2018c). However, although RIF and INH susceptibility testing is routinely performed in most TB endemic countries, it is not the case for FQ (WHO, 2017d). Hence, population-based data about FQ resistance levels are scarce (Zignol *et al.*, 2016). In our study, FQ was likewise not systematically tested phenotypically and/or by Anyplex except for the MTB/XDR patients (see section methods).

The use of Deeplex-MycTB allowed the systematic search for FQ resistance beyond the MDR and XDR isolates, by interrogating *gyrA* and *gyrB* mutations associated with FQ resistance in all the samples. Quite unexpectedly at first glance, out of the 339 isolates with exploitable

Deeplex results, 10% (34/339) of the non-MDR cases were detected with high confidence FQ resistance associated mutations in *gyrA* and/or *gyrB* (Figure 24). The vast majority (88.2%, 30/34) were diagnosed as new TB cases, thus excluding any recognized previous history of TB. Among the 34 patients, 53% (18/34) were Syrians, 38.2% (13/34) were Lebanese while the remaining 8.8% (3/34) were migrant workers, from Ethiopia and Nepal. As a consistent reflection of these patient geographic origins, the majority of these FQ resistant isolates belonged to the L4 (Euro-American) lineage (91.2% (31/34)) with the most common L4 sublineages, H37Rv-like and undefined L4 (14), TUR (11) and LAM (2). The remaining (8.8%) were part of the L2 (Beijing, n=2) and L3 (Delhi/CAS, n=1) lineages.

Even more remarkable was the observation that 22 of these 34 non-MDR isolates (65%) showed heteroresistant populations in *gyrA* and/or in *gyrB*, as defined by frequencies of a resistant variant below 97% within a sample (Table 8). The proportions of the 43 instances of such minor FQ resistance-associated variants that were detected in total within individual samples ranged from an upper limit of 84.9% to 3.1%, when using the standard 3% threshold for calling minor variants, or 2.0% when using an exceptional 2% threshold for the purpose of this specific analysis (a total of 7 variants detected among the 22 samples had a proportion of representation between 2 and 3%). Crucially, not a single instance of such a minor variant not associated with FQ resistance was detected in *gyrA* or *gyrB* in these samples, nor in the remaining 305 isolates with exploitable deep sequencing results. Likewise, only a single example of detection of minor variant - associated with resistance or not - was observed in only one of the 16 other gene targets with the 3% limit, in a single isolate out of the 339 samples, namely with two synonymous mutations represented at 10 and 12% in *tlyA*. Thus, these observations of minority populations virtually exclusively confined to FQ resistance associated variants in *gyrA* or *gyrB* strongly suggest that these minor variants do not represent false positive calls of minor variants, i.e. due to technical noise, but represent instead the result of FQ selection pressure.

Further interestingly, 12 individual isolates harbored multiple different unfixed FQ mutations, in addition to 10 isolates with single unfixed mutations. Out of the 12 individual isolates with multiple unfixed FQ resistance mutations, 4 had a mixture of 2 different mutant subpopulations, 7 samples a mixture of 3 mutant subpopulations, and one even showed a mixture with 4 mutant subpopulations. These data thus strongly suggest incomplete or ongoing selection

for FQ resistance, with competition between subpopulations for selection of the mutant with the best balance between (highest) level of resistance and (lowest) fitness cost. This hypothesis was compatible with the differences in representation of the different *gyrA* and *gyrB* variants in the different heteroresistant subpopulations versus populations where such variants were fixed (i.e. with a proportion above 97% within a sample) (Table 8 and Figure 24). For instance, the A90V *gyrA* mutation, which is associated with a low level of MFX resistance, occurred as unfixed in 11 isolates, but only in a single isolate as fixed. Likewise, D94Y and D94H *gyrA* mutations and A504V, R446C and T500P mutations in *gyrB* were exclusively identified as unfixed mutations. Although D94Y and D94H *gyrA* both are high confidence, high resistance levels mutations, they are very infrequently represented among strains that are resistant to FQ in global datasets (Rodwell *et al.*, 2014; Farhat *et al.*, 2016a; Manson *et al.*, 2017) suggesting that they might be associated with a high fitness cost. Similarly, *gyrB* mutations, presumably associated with lower FQ resistance levels, are very infrequent relatively to *gyrA* mutations, among strains that are FQ resistant. In contrast, D94G in *gyrA*, associated with a high resistance level and that is the most frequently fixed mutation in our sample set, is very frequently among FQ resistant strains globally (Manson *et al.*, 2017), suggesting an optimal balance between a high resistance level and a low fitness cost. Similar differential patterns of distributions of *gyrA* mutations in heteroresistant or fixed populations have been described in a smaller, selected set of cultured isolates from South Africa (Metcalf *et al.*, 2017).

Out of the 34 non-MDR isolates with FQ resistance, 24 were grouped in 12 different clusters by MIRU-VNTR and spoligotyping. However, mutations associated with FQ resistance were conserved only between 2 patients in a single cluster, thus more in favor of independent acquisition of FQ resistance by individual patients in most of the cases. Of note, a brother-sister link was observed between 2 patients that belong to the same MIRU-VNTR and spoligotype cluster, but their respective isolates also had different *gyrA* mutations (A90V and D94G) associated with FQ resistance. They were both Syrian refugees that entered Lebanon in 2012 and were diagnosed as new TB patients. The sister reportedly had symptoms 1 year earlier compared to her brother, suggesting the direction of transmission in accordance. While acquisition of FQ resistance might have occurred independently in these patients, resistance transmission to the second patient cannot be excluded, as a result of possibly undetected or unrepresented minority populations in the expectorated samples specifically used for diagnosis. Indeed, laboratory results can vary between

different samples of a same individual, consequently, and analysis of more than one sample might give more representative results (Datta *et al.*, 2017).

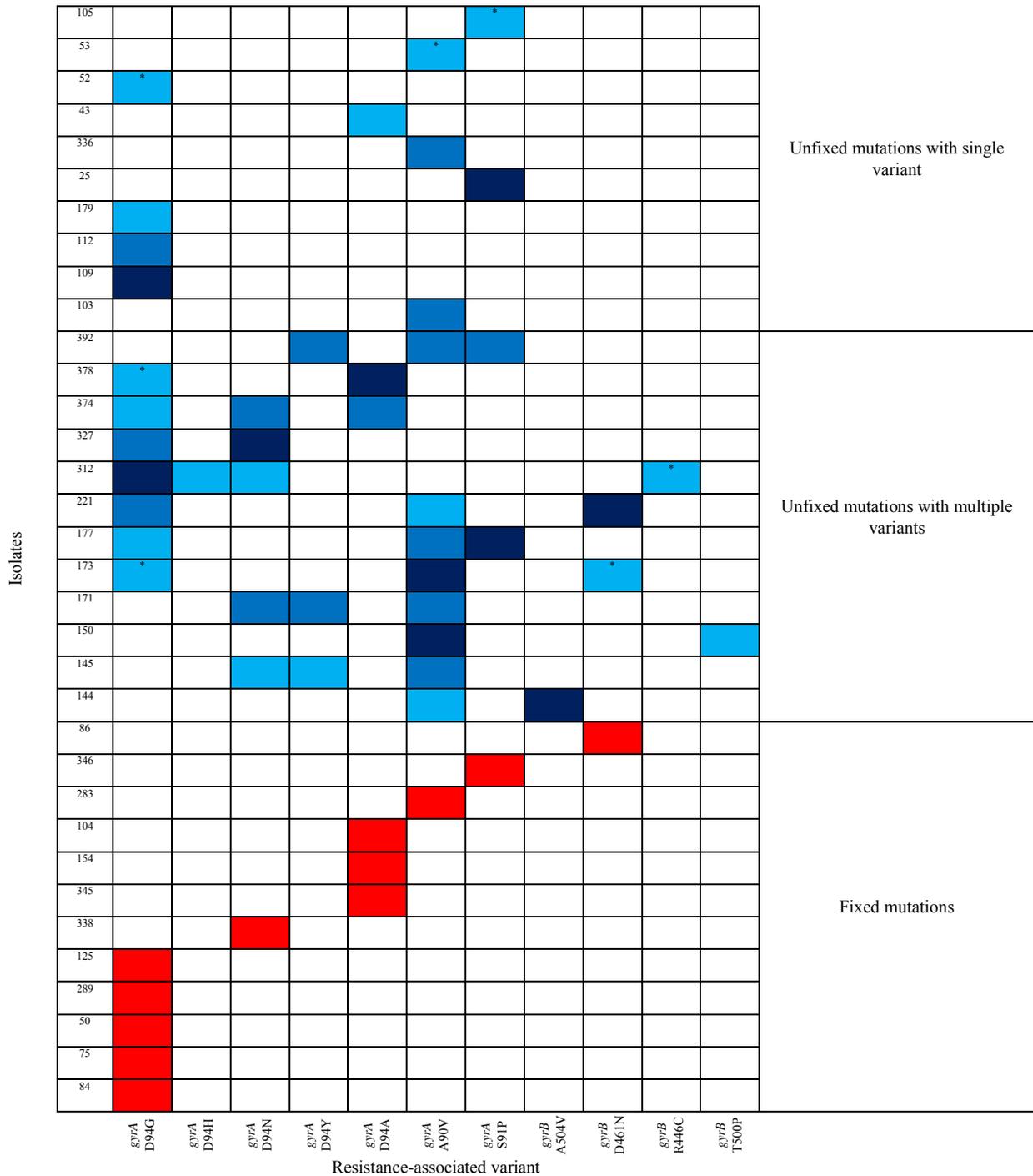
This detection of 10% FQ resistant cases that are not MDR represent an alarming finding, likely reflecting the non-rational or unregulated, monotherapeutic use of FQ for treating respiratory-tract infections generally or urinary tract infections (UTI) in many world regions (Oliphant and Green, 2002; Zignol *et al.*, 2016). The frequent occurrence of heteroresistance is consistent with this hypothesis, as such treatments are often taken over relatively short periods of time, presumably in response to the manifestation of first TB symptoms as well, resulting in relatively short periods of selection. In Lebanon, there is an inappropriate FQ prescription, dosing, and duration of treatment both at the community and hospital setting levels (Kabbara *et al.*, 2015). Self-medication without any specialist consultation is a major problem in many countries especially in, but not restricted to Asia (Ayalew, 2017) (Nepal and Bhatta, 2018). In Lebanon, almost 42% of sold antibiotics are delivered without prescription at the community settings (Cheaito *et al.*, 2014). The major reason for dispensing antibiotics without a medical prescription is the inability of the patient to afford a physician visit (Farah *et al.*, 2015). The present findings thus represent further strong incentives to tackle this problem of antibiotic misuse.

Currently available diagnostic technologies based on Xpert MTB/RIF, WGS and SL-LPA are able to identify resistant genotypes making up >65%, >10% and 5% of a population, respectively (Folkvardsen *et al.*, 2013; WHO, 2018d). Despite the fact that DST using MGIT can detect down to (about) 1% of a mutant population that is generally considered for clinical significance, MGIT testing is not able to accurately quantify proportions of heteroresistance (Folkvardsen *et al.*, 2013). Moreover, SL-LPA, which is the currently recommended commercial rapid test for initial FQ resistance testing in case of RIF resistance, is capable to detect only 85% of FQ resistant isolates, bearing the most common *gyrA* and *gyrB* mutations (WHO, 2018d). In comparison, Deeplex-MycTB provides direct sequence information over the complete QRDR regions of *gyrA* and *gyrB*. Moreover, our results indicate that it offers a very high specificity for detecting heteroresistant population down to 3% frequency, as a result of deep coverage depths achieved.

However, as a limitation to our study, we used cultured isolates for deep sequencing due to suboptimal transportation of clinical samples from Lebanon to France. This might have resulted in

underestimation of heteroresistance levels in the original specimens due to a selection bias of only more fit mutants by culture (Eilertson *et al.*, 2014).

Table 8: Table showing the distribution of percent variant of mutations in *gyrA* and *gyrB* associated with FQ resistance. Percent variant: > 97% → Red (fixed mutation), 50%-97% → Dark Blue, 10%-49% → Blue, <10% → Light blue, *: < 3%



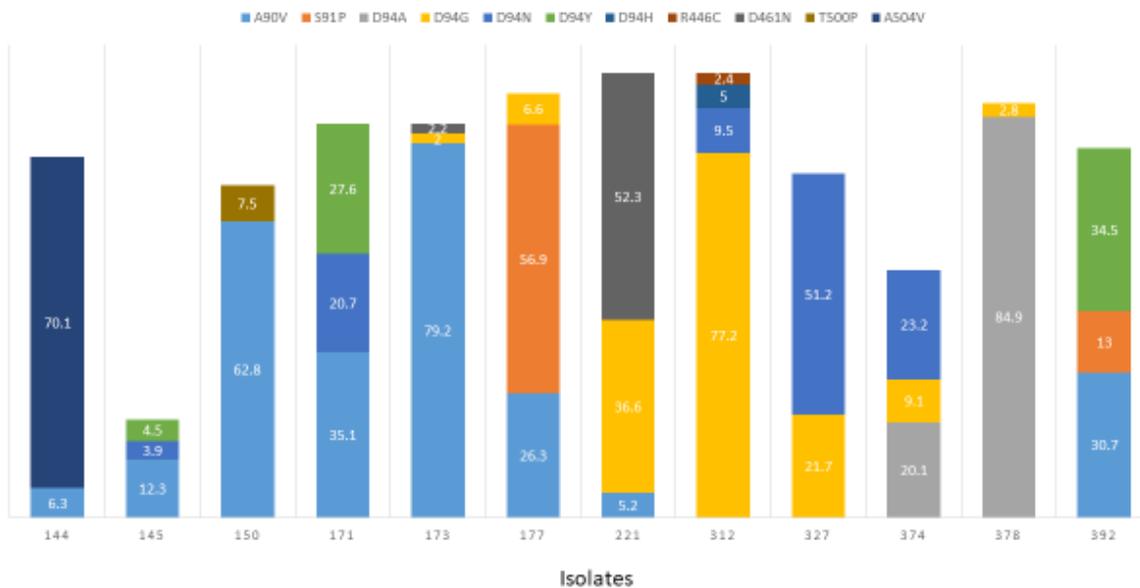


Figure 24: Variable mixtures with unfixed fluoroquinolone resistance associated mutations within different individual isolates.

Uncharacterized mutations

In our study collection, only 223 of the 4407 (5.1%) phenotypes could not be predicted due to uncharacterized mutations (50.7%) or low coverage (49.3%) in some genes. The vast majority of uncharacterized mutations identified were present in single isolates, or in two or three isolates only. As a notable exception, the G142A mutation in *ahpC* occurred in all of the 14 isolates that belonged to the L1 lineage (alias EAI spoligotype family), all with INH susceptible phenotypes and unique 24-locus genotypes, thus indicating that this is a specific phylogenetic SNP (Table 9). As another major exception, a A403S mutation in *gyrB* was shared all 11 *M. bovis* cases with analyzable sequencing data and the 3 isolates belonging to L5 and L6 lineages (alias West Africa I and II), all with a FQ susceptible phenotype and unique 24-locus genotypes (Table 9). These results are thus also highly suggestive of a phylogenetic mutation, shared by early branching lineages in the MTBC phylogeny.

Table 9: Uncharacterized mutations found in the study collection.

Anti-TB drugs	Gene	Amino Acid Mutations	Number of isolates			SNP-based phylogenetic lineage
			R	S	NA	
Rifampicin	<i>rpoB</i>	E183D		1		Delhi/CAS
		S195I		1		Other than H37Rv
Isoniazid	<i>katG</i>	A122T + M126T		1		L4.3
		R253Q		1		Delhi/CAS
		A264V		1		Other than H37Rv
	<i>fabG1</i>	G27C		2		Bovis
	<i>ahpC</i>	C150T		1		Other than H37Rv
		T82C		1		Delhi/CAS
		G-52T	1*			H37Rv-like
		T-31C	1*			Delhi/CAS
		T89G		1		Bovis
		G142A	1*	13		EAI
		KatG / <i>ahpC</i>	P280S / G142A		1	
	<i>ahpC</i> / <i>fabG1</i>	T44C / G27C		3		Bovis
	Pyrazinamide	<i>pncA</i>	S67W			1
D145H					1	Other than H37Rv
Ethambutol	<i>embB</i>	A313V		1		Other than H37Rv
Streptomycin	<i>gidB</i>	G773A		3		Other than H37Rv
		K163T		1		Delhi/CAS
		D85E		1		Other than H37Rv
		R197C		1		EAI
		A180T		2		Other than H37Rv
		V115M		1		L4.3
		CGG347*G		1		Other than H37Rv
		Stop225C		1		Other than H37Rv
		H48R	1			Other than H37Rv

		P29L + L49P	1			H37Rv like
		G157V	1			H37Rv like
		D85H	1			Other than H37Rv
		K163E	1			Delhi/CAS
		A183V	1			L4.3
		R21P	1			L4.3
		DelG97	2			Delhi/CAS
		DelC111	1			Delhi/CAS
		inserG97	1			H37Rv like
		inserG346	1			Delhi/CAS
	<i>rrs</i>	C1314T		1		EAI
		A1012G		1		Delhi/CAS
		G891A		2		Cluster Delhi/CAS
		T943C	1			Other than H37Rv
Fluoroquinolones	<i>gyrA</i>	A126P			1	H37Rv like
	<i>gyrB</i>	A403S			14	Bovis (11) + L5 (2) + L6 (1)
		R550G			1	Other than H37Rv
		S447F			2	Other than H37Rv + H37Rv
		V387F			1	Delhi/CAS
		K526Q			2	H37Rv like
		D434G			1	Other than H37Rv
Amikacin	<i>rrs</i>	G891A			2	Delhi/CAS cluster
		C1314T			1	Other than H37Rv
		T943C			1	Other than H37Rv
		A1012G			1	Delhi/CAS
Kanamycin	<i>rrs</i>	G891A			2	Delhi/CAS cluster
		C1314T			1	Other than H37Rv
		T943C			1	Other than H37Rv
		A1012G			1	Delhi/CAS
	<i>eis</i>	C100T			1	Delhi/CAS
		Indel			1	EAI

Capreomycin	<i>tlyA</i>	G28V			1	Other than H37Rv
		L251V			1	Other than H37Rv
		R137H			1	Other than H37Rv
Ethionamide	<i>ethA</i>	G124D			2	L5
		D396G			1	Other than H37Rv
		S266R			1	Beijing
		I337V			1	L6
		M1R			1	Other than H37Rv
		DelT1238			1	Delhi/CAS
	<i>fabG1</i>	G27C			5	M.bovis
Linezolid	<i>rrl</i>	G2489T			1	Delhi/CAS
		G2472T			1	L1,5,6,7 or animal
	<i>rplC</i>	T115M			1	H37Rv like
		G674A			1	EAI
Bedaquiline	<i>rv0678</i>	D5N			1	L4.3
Clofazimin	<i>rv0678</i>	D5N			1	L4.3

Abbreviations: NA: Not available, EAI: East African Indian, L: lineage, *: Genotypically resistant isolates. Mutations in red are probable newly identified phylogenetic mutations.

IV. Genetic diversity of MTBC isolates

a. Phylogenetic classification based on SNPs versus MIRU/VNTR with/without spoligotyping

Phylogenetic SNPs are considered to be the gold standard for MTBC lineage identification. For this purpose, Deeplex-MycTB implements the broadly accepted reference SNP set published by Coll et al., among different reference SNP sets available (Coll *et al.*, 2014). The classification of MTBC strains into different lineages and sub-lineages was thus first performed based on these SNPs in the 339 samples with exploitable sequencing results (Table 10). A total of 44% (149/339) of the isolates could be classified in a specific (sub)lineage by detection of a phylogenetic SNPs with Deeplex-MycTB. Among these, 4.4% (15/339) of isolates belonged to L1 (lineage-specific mutation in *embB* E378A), 3.5% (12/339) to L2 (E92D mutation in *gidB*), 15% (51/339) to L3 (G-88A mutation in *ahpC*), 14.2% (48/339) to L4/H37Rv-like sublineage (absence of S95T mutation in *gyrA*), 2.7% (9/339) to L4/LAM sublineage (L16R mutation in *gidB*), 0.6% (2/339) to L5 (E378A mutation in *embB*), 0.3% (1/339) to L6 (E378A mutation in *embB*), 3.2% (11/339) belong to *bovis* lineage (H57D mutation in *pncA*). Among the remaining 190 isolates, 2 strains belonged to either L1, L5, L6 or animal lineages, while 55.5% (188/339) could be only generically classified as belonging to a lineage other than that of H37Rv (S95T mutation in *gyrA*).

We then compared the results obtained with these phylogenetic SNPs with lineage identification results based on MIRU-VNTR typing data, as obtained by using the tools implemented in the MIRU-VNTRPlus database as described in Methods. MIRU-VNTR results were obtained for the 348 samples from our collection, indicating that MIRU-VNTR is slightly more tolerant to low DNA quality compared to Deeplex-MycTB. The additional 9 isolates recognized by MIRU-VNTR were classified to *M. bovis* (1/9), *M. africanum* (L6, 1/9), EAI (L1, 1/9), Delhi/CAS (L3, 1/9), URAL (L4, 1/9), and Euro-american (L4, 4/9) (sub)lineages.

Table 10 illustrates lineage identification based on phylogenetic SNPs compared to MIRU-VNTR for the 339 isolates analyzed in common, by taking into account the known correspondences between respective lineage nomenclatures (Niemann *et al.*, 2016). A complete

concordance was seen in L1, L4.3 (LAM), L5, and *bovis* lineages. In contrast, discordances were e.g. observed for all isolates that belonged to the L4.8/H37Rv-like lineage identified by phylogenetic SNPs, as they were assigned as an unknown sublineage of lineage 4 by MIRU-VNTR typing alone. In order to increase the phylogenetic information and identify sublineages based on known spoligotype signatures (Niemann *et al.*, 2016), strain spoligotypes were added in correspondence of the different branches of the phylogenetic tree based on MIRU-VNTR data alone. As could be expected from previous work (Allix-Béguec *et al.*, 2008b), more precise/complete lineage identifications were obtained (Table 11), as e.g. the number of isolates with unknown lineage was reduced from 137 to 4.

Table 10: Phylogenetic classification based on phylogenetic SNPs versus MIRU-VNTR

		MIRU-VNTR								
		L1 EAI (n=15)	L2 Beijing (n=11)	L3 Delhi/CAS (n=39)	L4.3 LAM (n=8)	L5 West Africa I (n=2)	L6 West Africa II (n=0)	<i>M. bovis</i> (n=11)	L4 Euro- american (n=116)	Unknown (n=137)
Deeplex- MycTB Phylo- Genetic SNPs	L1 EAI (n=15)	15								
	L2 Beijing (n=12)		11	1						
	L3 Delhi/CAS (n=51)			37						14
	L4.3 LAM (n=9)				8					1
	L5 West Africa I (n=2)					2				
	L6 West Africa II (n=1)						0			1
	<i>M. bovis</i> (n=11)							11		
	L 1,5,6 or animal (n=2)									2
	H37Rv-like (n=48)									48
	Other than H37Rv (n=188)			1					116	71

Table 11: Phylogenetic classification based on phylogenetic SNPs versus MIRU-VNTR and spoligotyping

		MIRU-VNTR + Spoligotyping								
Deeplex-MycoTB		L1 EAI (n=15)	L2 Beijing (n=12)	L3 Delhi/CAS (n=50)	L4.3 LAM (n=9)	L5 West Africa I (n=2)	L6 West Africa II (n=1)	<i>M. bovis</i> (n=11)	L4 Euro-american* (n=235)	Unknown (n=4)
	L1 EAI (n=15)	15								
	L2 Beijing (n=12)		12							
	L3 Delhi/CAS (n=51)			50						1
	L4.3 LAM (n=9)				9					
	L5 West Africa I (n=2)					2				
	L6 West Africa II (n=1)						1			
	<i>M. bovis</i> (n=11)							11		
	Lineage 1,5,6 or animal (n=2)									2
	H37Rv-like (n=48)								48	
	Other than H37Rv (n=188)								187	1

b. Phylogenetic diversity based on MIRU-VNTR typing with spoligotyping

Based on the validation vs phylogenetic SNPs described above, and as MIRU-VNTR combined to spoligotyping provides a better resolution power to identify different sublineages, we used this combination (as described above) to analyze the phylogenetic diversity of our nationwide strain set from Lebanon. As expected, this analysis revealed that 72% (244/339) of the isolates in Lebanon belong to the L4 lineage, alias the Euro-American lineage (including LAM). This MTBC lineage is the most prevalent cause of human TB worldwide and comprises various multiple sublineages (Coll *et al.*, 2014; Stucki *et al.*, 2016).

Our MTBC L4 strain set was predominated by a group of strains 34.2% (116/339) with no sufficient similarity with any reference strain in the MIRU-VNTRplus genotype set for a more specific identification than undefined L4 strains. Consistently, the following predominating group in this lineage belonged to the so-called TUR lineage, typical of the Turkish region (Durmaz *et al.*, 2007), represented by 18% (61/339) of the total isolates in our dataset.

However, a variety of other minor lineages and/or sublineages detected in our analysis according to the MIRU-VNTR*Plus* nomenclature were as follows: Delhi/CAS (14.7%), Haarlem (9.4%), EAI (4.4%), Beijing (3.5%), *bovis* (3.2%), LAM (2.7%), URAL (2.4%), X (1.8%), S (1.8%), Unknown (1.2%), Uganda I (0.9%), Cameroon (0.6%), West Africa I (0.6%), West Africa II (0.3%), and Ghana (0.3%) (Figure 25).

Also consistently, many patients of this study population were infected by a strain genotype highly associated with their country of origin. For instance, almost all of the 61 isolates with TUR genotypes were obtained from Lebanese and Syrian patients except for a single patient from Ethiopia and a single patient from Bangladesh. Likewise, isolates of East African Indian (EAI), most prevalent in regions around the Indian Ocean (Gagneux, 2018), were almost exclusively found among patients from Bangladesh and the Philippines, and only in two patients from Syria.

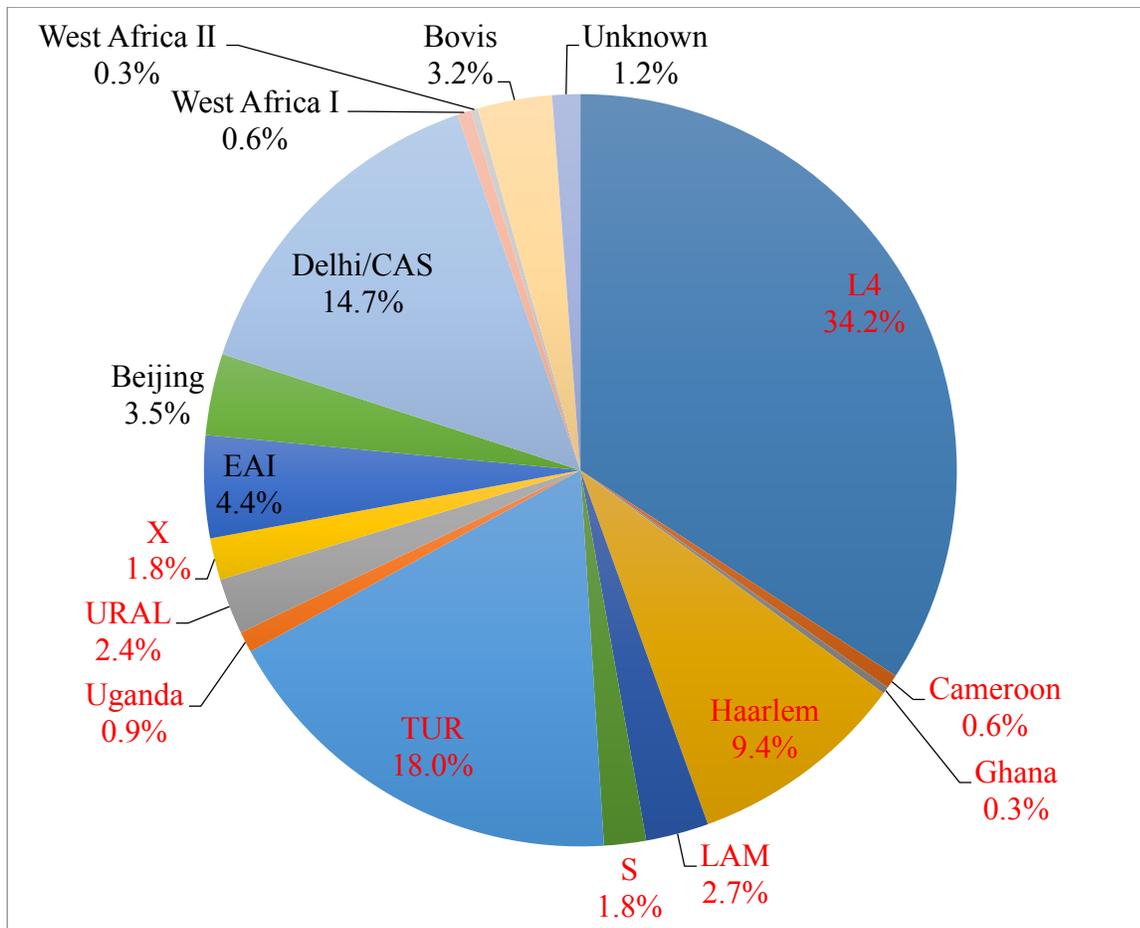


Figure 25: Distribution of MTBC strains lineages based on MIRU-VNTR and spoligotyping signatures in Lebanon. This pie chart shows the distribution of strain lineage in Lebanon. LAM: Latin American-Mediterranean, CAS: Central Asian Strain, EAI: East African Indian, TUR: Turkish. L4: lineage 4 (strains that belong to Euro-American lineages with undefined sub-lineage). Red highlight: sub-lineages of Euro-American lineage

V. Molecular cluster analysis of MTBC strains from different patients

Cluster analysis was performed based on MIRU-VNTR typing in combination with spoligotyping and SNPs not known as associated with drug resistance, in order to search for potential epidemic spread of clones and/or active transmission chains in the study population.

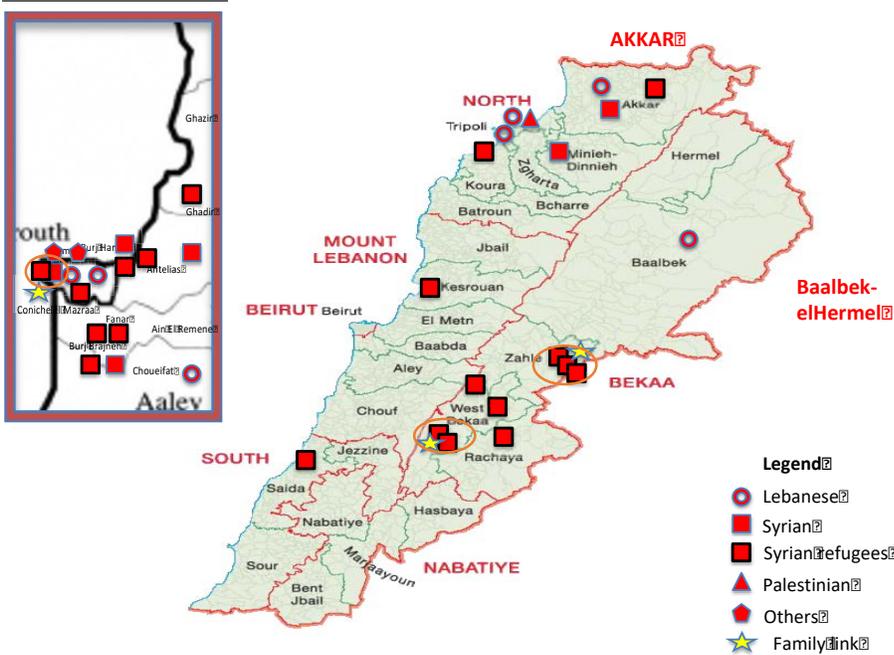
In our survey, 199 patient isolates displayed unique 24-locus genotypes, indicating absence of epidemiological links among the corresponding patients. In contrast, thirty-five strain clusters were identified. Of note, dates of isolation of the clustered isolates were sufficiently different to rule out laboratory contamination. In addition, a reproducibility test of MIRU-VNTR typing was performed with a strain subset for verification and to rule out technical errors.

Each cluster was composed of 2-9 isolates, except for the largest strain cluster (n° 13) composed of 36 isolates with the same TUR genotype. This cluster involved 26 Syrian, 7 Lebanese, 1 Palestinian, 1 Ethiopian and 1 Bangladeshi patients, residing in seven different places in the country according to information received from clinicians (Figure 26). This large cluster suggests the circulation or active transmission of a predominant regional clone. Geographical clustering of at least part of the patients in other molecular clusters (Figure 26) was also suggestive of potential recent transmission. However, WGS analysis would be needed to further test this hypothesis. Nevertheless, very little doubt prevails about active transmission in the 7 instances of familial links that were identified between patients within the largest cluster (n° 13).

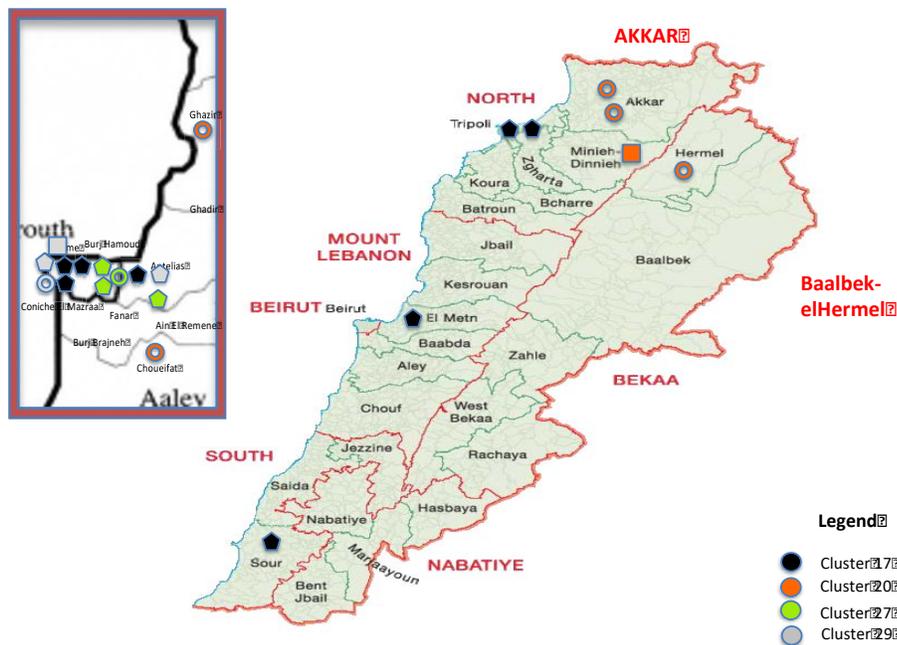
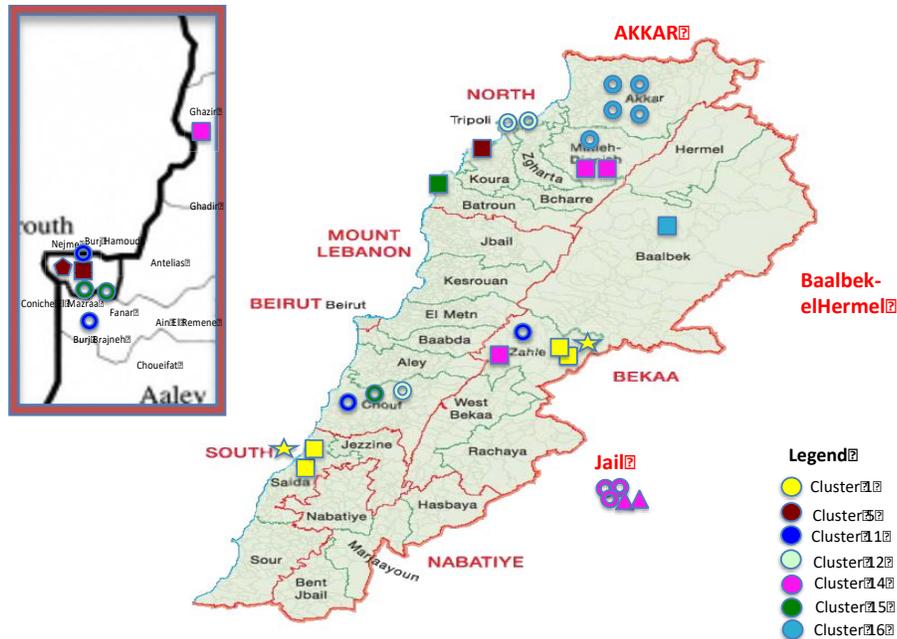
Setting apart the big cluster described above, strain clusters analysis against patient's geographic origin showed that 10 clusters were exclusively composed of patients originating from a same foreign country. These clusters presumably reflect the importation of an endemic genotype from the country of origin rather than in-country TB transmission. In addition, 18 other strain clusters were composed of patients with "local" nationalities only, i.e. Lebanese, Syrian and Palestinian (LSP) patients, which probably also reflect the circulation and/or perhaps some ongoing local transmission of local strains, given not only the contemporaneous contacts but also the common historical roots and open boundaries between Lebanon, Syria, and Palestine.

Finally, 6 clusters were composed of strains belonging to patients with different foreign origins, some of which included LPS patients. As mentioned above, Lebanon hosts a large number

of migrant workers with the highest proportion being domestic workers. As a consequence, clusters including both migrants and LPS might reflect high social/household daily contacts between both patient groups in some cases, as previously proposed for other Arab countries (Varghese *et al.*, 2013).



a.



b.

Figure 26: Maps of Lebanon showing the geographical distribution of strains within clusters. Each TB case is indicated by a symbol (based on nationality) on the map according to the corresponding patient residency. Symbols are colored according to clusters, with cluster numbers according to MIRU-VNTR, spoligotyping and SNP **a.** TB cases being part of the large cluster number 13. **b.** Top and bottom. Clusters composed of 4 or more cases.

☉ Lebanese □ Syrian △ Palestinian ◊ Others ★ Family link

Conclusions:

The first evaluation and utilization of Deeplex-MycTB, a novel targeted deep sequencing assay, in a nationwide study demonstrates important benefits that could be expected with implementation of rapid NGS tools, to improve TB diagnosis and subsequent patient care. Based on detectable resistance genetic determinants, this molecular tool predicted RIF, INH, and EMB resistance with 100%, 90.3%, and 100% sensitivity, respectively, and susceptibility with 100%, 99.6%, and 99.4, respectively, compared to reference phenotypic DST routinely performed on all isolates. By expanding the sample collection to an 18-month period, we confirmed 2 additional MDR TB cases, including one with PZA resistance not covered by the diagnostics in place, corroborating the relatively low prevalence of MDR in Lebanon.

Deeplex-MycTB also allowed the prediction of genotypic susceptibility or resistance to 10 other, second line anti-TB drugs not routinely tested for non-MDR TB patients, composing the vast majority of the study population. This enabled us to uncover that 10% of the patients had TB genotypically resistant to FQ independently of MDR. A majority of the samples had FQ heteroresistant populations, including some undetectable by other commercially available molecular tested. In addition to further indicating the consequences of antibiotics misuse, these findings support the relevance of systematic testing of FQ resistance before starting treatment of INH mono-resistant and MDR TB patients as recently recommended (WHO, 2018b; WHO, 2018c).

Finally, the use of Deeplex-MycTB, complemented by MIRU-VNTR typing, allowed us to detect and determine the prevalence of *M. bovis*-induced zoonotic TB in the country, which is the subject of the next chapter.

Part III: Prevalence of zoonotic TB in **Lebanon**

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Mycobacterium bovis-induced zoonotic tuberculosis in humans in Lebanon, June 2016-November 2017, assessed by next-generation sequencing: a nationwide observational study

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To the Editor:

The World Health Organization (WHO) and other supranational bodies recently called for formally assessing and (re)prioritising the burden of zoonotic tuberculosis in people, due to *Mycobacterium bovis* [1, 2]. Its global contribution to human tuberculosis, otherwise principally caused by *Mycobacterium tuberculosis*, might be underestimated [2]. Nationally representative prevalence data are virtually non-existent on continents with the highest presumed burdens, i.e. in Africa and Asia [3].

In addition to more frequently cause hard-to-diagnose extrapulmonary tuberculosis, *M. bovis* is naturally resistant to pyrazinamide [4], a crucial drug for the standard short course anti-tuberculosis therapy. Due to reliability issues, phenotypic susceptibility to pyrazinamide is often not tested [5]. Globally deployed, WHO-endorsed phenotypic and molecular diagnostics do not differentiate *M. bovis* from *M. tuberculosis* [1]. *M. bovis*-infected patients may thus receive inadequate treatment, risking poorer outcome [6]. Underdiagnosis in people also entails undetected animal and food sources and zoonotic risks escaping common tuberculosis control measures [1, 2].

In Europe, patients with *M. bovis* infection are often African- or Southern Mediterranean-born, suggesting regional endemicity [6, 7]. We determined the *M. bovis*-caused tuberculosis prevalence in an 18-month nationwide survey in Lebanon. In addition to its national population and more than 1.5 million Syrian and Palestinian refugees, this Mediterranean country hosts large numbers of migrant workers from Africa and Asia [8]. Many are from Ethiopia [8], where proportions of extrapulmonary tuberculosis and *M. bovis*-caused disease apparently culminate, reaching ~30% [9] and 15-30% (in focal studies; [10]), respectively. We used a novel targeted next-generation sequencing-based assay for extensive drug resistance detection, including to pyrazinamide, and genotypic differentiation between *M. bovis* and *M. tuberculosis* [11].

This survey was approved by the Azm Center/Lebanese University ethical committee (document CE-EDST-3-2016). Clinical samples were collected from all 1104 different suspected TB patients reported to all national anti-TB centres between June 1, 2016 and November 31, 2017. From all microscopy positive and negative samples, 417 tuberculosis cases were molecularly and/or bacteriologically confirmed by solid (Lowenstein-Jensen) and/or liquid (BBL MGIT, Beckton Dickinson, USA) culturing, and/or GeneXpert MTB/RIF (Cepheid, USA) or Anyplex MTB/NTM Real-time (Seegene, Korea) molecular testing. Of these, 54 were culture negative, 5 had culture contamination, and 4 no culture performed (e.g. biopsies).

Available DNA extracts, obtained from 348/354 primary cultures by using MasterPure™ DNA Purification Kit (Epicentre, Illumina, WI, USA), were subjected to targeted sequencing, more tolerant to low DNA integrity (following suboptimal sample transportation from Lebanon to France) than whole genome sequencing. Briefly, the Deeplex-MycTB assay (Genoscreen, France) uses a 24-plexed amplification of mycobacterial species identification (*hsp65*), genotyping (spoligotyping and phylogenetic single nucleotide polymorphisms (SNPs)) and 18 *M. tuberculosis* complex drug resistance-associated gene targets, including the main pyrazinamide resistance-associated gene *pncA* (Fig. 1). Paired-end amplicon libraries of 150-bp read length (Nextera XT kit, Illumina, CA, USA) were sequenced in a single Illumina NextSeq run. Variant calling and genotypic analysis were performed using a parameterized software (GenoScreen).

Of 339/348 (97.4%) samples with exploitable sequencing data, 11 were concordantly identified as *M. bovis* strains by typical spoligotype signatures (missing spacers 39-43) and the canonical phylogenetic SNP *pncA* H57D causing natural pyrazinamide resistance [5] (Fig. 1). No other known drug resistance-associated mutations were detected. Standard 24-locus MIRU-VNTR typing [12] followed by MIRU-VNTR*Plus* database identification [13] confirmed *M. bovis*

classification in all cases, and identified one additional *M. bovis* infection among the 9 cases without sequencing data. A total of 12/348 (3.4%) patients were thus infected with *M. bovis*, while the remaining had human-associated tuberculosis strains (i.e. *M. tuberculosis* or *M. africanum*).

Logistic regression analysis was used to identify independent predictors of *M. bovis* infection. Potential covariates were patient age, gender, treatment outcome, new case vs case with previous tuberculosis history, nationality, extrapulmonary tuberculosis. The log-linearity assumption being violated for patient age, a piecewise log-linear regression model was considered. The multivariate model was built by minimizing Schwarz's Bayesian Criterion and maximizing the c-statistics. The final model was assessed with Pearson goodness of fit test. The two-sided type I error was set at 5%. Mean patient age was 40 years [22; 62] with *M. bovis* vs 29 [25; 38] with human-associated strains (per year adjusted OR if age ≥ 55 1.14, 95% CI 1.01-1.28, $p=0.04$). Of the 12 patients with *M. bovis*, 5 (41.6%) had extrapulmonary tuberculosis (including 1 gastric for a two-year baby), versus 8/336 (2.4%) patients with human-associated strains (adjusted OR 16.9, 95% CI 3.5-80.7, $p=0.0004$). While Lebanese nationals represented only 86/336 (25.6%) of the patients with human-associated strains, they composed 10/12 (83.3%) of the *M. bovis* cases (adjusted OR 6.9, 95% CI 1.4-35.5, $p=0.02$). The two remaining patients were a Syrian refugee and a Syrian resident. Other covariates were not statistically associated with risk of *M. bovis* infection.

Patients with *M. bovis* were all from different places disseminated over the country, suggesting absent mutual links. Unique 24-locus MIRU-VNTR types detected for each isolate were also unresponsive of inter-patient transmission. Phylogenetic reconstruction indicated that these different strains represent at least two distinct *M. bovis* sublineages, mutually distinguished e.g. by a *fabG1* SNP and specific spoligotype signatures (Fig. 1). Infections by distinct strains in residents

from disseminated places inform the probable existence of multiple zoonotic sources (e.g. infected cattle, unpasteurized dairy produce) unidentified in the country.

Surprisingly, none of the 148 migrant patients carried *M. bovis*, although they originated mainly from Ethiopia (n=94) and Sudan (n=13) where zoonotic infection is thought to be highly prevalent due to widespread pastoralism [10]. Other migrants were from other high tuberculosis incidence countries (e.g. 18 from the Philippines, 9 from Sri Lanka). Rather than merely implying low or absent *M. bovis* burdens in these countries, we postulate that the lack of *M. bovis* infection among migrant workers may be the combined consequence of i) selective recruitment of candidates who manifest no medical issues (thus unlikely to have active tuberculosis) by overseas employment agencies in the country of origin, ii) much lower risk of progression to disease among patients latently infected with *M. bovis* than with *M. tuberculosis* [14], iii) residency in the country of destination often limited to few years. Absence or scarcity of *M. bovis*-caused cases among migrant workers after their entrance, including in many other countries with similar recruitment systems, might thus further mask the true zoonotic burden in countries of origin.

In a Dutch longitudinal study, no association was found between patient outcome and the use of standard first-line TB treatment (4-month rifampicin/isoniazid/ethambutol/pyrazinamide, 2-month rifampicin/isoniazid) instead of the WHO-recommended regimen for treating *M. bovis* disease (9-month isoniazid/rifampicin, 2-month ethambutol) [6]. Nevertheless, the mortality with *M. bovis* disease was found to be higher relative to *M. tuberculosis*, presumably reflecting more prevalent miliary and central nervous system (CNS) localization of *M. bovis*. While the 12 *M. bovis*-infected patients in our observational study received the standard first-line TB treatment, none of them had a miliary or CNS localization and they were all reported as cured or with a completed treatment

according to WHO classification. Prolonged follow-up will be necessary to assess their risks of relapse.

Although this first extended, nationwide estimation in a non-Western world country revealed a relatively low zoonosis prevalence among tuberculosis patients (3.4%), our findings are aligned with global calls for appropriate diagnostics and treatment of these patients and tuberculosis control measures at the animal/human interface [1, 2].

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Supplementary discussion:

According to WHO, in 2017, the global incidence of human TB caused by *M. bovis* was estimated to be 142, 000 cases, with an estimated mortality of 12,500 deaths(WHO, 2018a). The highest burden of zoonotic TB is thought to be localized in the non-Western world, especially in the Asian and African regions (WHO, 2018a). However, as a result of the lack of specific identification by routine diagnosis and the virtual absence of population-based studies especially in countries with presumably higher endemicity of zoonotic TB, confidence intervals around incidence estimates on different continents span an order of magnitude(WHO, 2018a). This large uncertainty has recently raised concerns about potential underestimation of the problem, and elicited international calls for re-assessment and prioritization (Olea-Popelka *et al.*, 2017).

Our study represents a first step for filling this knowledge gap, as it represents the first extended, nationally representative study on the prevalence of *M. bovis*-caused human TB in a non-Western world country. Of note, a recent work identified 2 *M. bovis* infection cases among TB patients from Lebanon, by WGS analysis (Panossian *et al.*, 2018). However, these 2 cases were identified among a total of just 13 TB isolates conveniently sampled over a period of three years among TB patients in the country, providing no reliable indication on the actual prevalence. Our results show that zoonotic TB actually account for a smaller proportion (3.4%) of all TB cases in the country. Interestingly, albeit not population-based, the single other study with a substantial sample size performed in a nearby country found 13 *M.bovis* isolates by spoligotyping among 482 *M. tuberculosis* complex isolates obtained between 2009 and 2014 in the Izmir region in Turkey (Çavuşoğlu and Yılmaz, 2017), representing a 2.7% proportion thus close to the prevalence measured in Lebanon. More anecdotally, a much smaller study likewise identified 2 *M. bovis* cases among 31 isolates obtained between 2005 and 2010 from TB patients from the West Bank, Palestinian Territories, indicating at least the existence of zoonotic sources also in this region proximal to Lebanon (Ereqat *et al.*, 2012). Such existence was also supported by detection of *M. bovis*-specific DNA (by IS6110 amplification and real-time PCR detection of an *M. bovis*-specific *oxyR* gene allele) among 3.1% of milk and tissue biopsy samples from 254 cows and goats from the same geographic territories (Ereqat *et al.*, 2013).

M. bovis-induced TB cases in autochthonous patients from Western world countries often involve elderly people, reflecting probable endogenous reactivation of *M. bovis* infection acquired during times predating general pasteurization of milk products and while *M. bovis* infection in livestock was still highly prevalent (Majoor *et al.*, 2011). Although the mean patient age tended to be higher with *M. bovis* infection vs *M. tuberculosis* infection in our study population, 6 out of the 12 patients with zoonotic TB were below 40 years of age, including e.g. a two-year baby and a 12-year teenager patient. These observations suggest that at least part of the different multiple *M. bovis* strains identified by our Deeplex and MIRU-VNTR data represent independent, contemporaneous zoonotic sources of infection in Lebanon. The genetic tree reconstructed by using MIRU-VNTR data revealed that these multiple strains represent two distinct *M. bovis* sublineages, distinguished by the presence or absence of a G27C mutation in *fabG1* and some distinct spoligotype patterns (Figure 27). All the detected *M. bovis* isolates displayed identical mutations in EMB, FQ, CAP and SM, thus representing probable phylogenetic mutations common to the complete set.

Among the 12 *M. bovis* cases identified by Deeplex-MycTB and MIRU-VNTR, 41.7% had extra-pulmonary TB, including 1 case in the fallopian tube, in addition to 1 gastric TB, 1 peritoneal TB and 2 pus. Genital TB occurs mostly due to infection by *M. tuberculosis* in 90-95% and by *M. bovis* in 5-10% of cases (Gatongi *et al.*, 2005; Kumar *et al.*, 2008). Generally, TB of the genital tract occurs secondarily to a primary TB infection by spread through hematogenous or lymphatic routes with the fallopian tubes being the first target (Aliyu *et al.*, 2004; Rajaratnam *et al.*, 2013). It can occur at any age group but the majority 75% are in the reproductive age group (20-45 years) (Qureshi *et al.*, 2001). The burden of female genital TB is underestimated as the patient generally is asymptomatic and is diagnosed with genital TB during investigation for infertility reasons (Grace *et al.*, 2017). In addition, gynecologist should be aware as it mimics other conditions (cancer) (Gatongi *et al.*, 2005). In our study, the patient infected with *M. bovis* in the fallopian tube had 22 years at the time of diagnosis. As per available information, during the first visit, her gynecologist was expecting granuloma, but biopsy testing was negative. The patient was diagnosed with TB only three months later. Of note, as an old myth in rural areas, yogurt is/was sometimes used in the treatment of urinary tract infection (UTI), as it contains lactobacillus, thus potentially exposing to a risk of zoonotic TB if the milk was unpasteurized. However, according to information obtained in this case, the patient never experienced UTI. Although the patient received standard treatment for TB, she was reported as cured. Unfortunately, we could not obtain any information regarding

the gastric TB case involving a baby, e.g. if he consumed unpasteurized milk. Although the treatment should have been adapted as EMB is excluded from the treatment regimen for babies and *M. bovis* is naturally resistant to PZA, this patient completed her treatment as per the NTP physician (WHO, 2018a).

Although the 3.4% prevalence of zoonotic infections among human TB cases in Lebanon might be considered as a minor public health issue, our finding call for building up or strengthening surveillance and control of animal TB and food quality, to screen for and remove potential sources of infection. Animal health surveillance and food quality control are key to avoid animal-human transmission (WHO *et al.*, 2017a). However, data on the prevalence of animal/bovine TB in Lebanon are not available, and the lack of veterinary health control can result in an increased risk of zoonotic TB infection. Populations mostly at risk to develop the disease are people who consume infected animal products (unpasteurized milk or meat) and population living or working in close contact with infected cattle (inhalation of aerosols) (Adesokan *et al.*, 2018). In the latter category, persons in direct contact with livestock in closed spaces (e.g. abattoir workers, veterinary personnel executing cattle necropsy) are at high risk; while people in direct contact with livestock but in outdoor spaces are at medium risk (Torres-Gonzalez *et al.*, 2013; Vayr *et al.*, 2018). According to FAO, the main source of living of livestock farmers in Lebanon depend largely (60%) on the dairy sector. While the dairy products are vital constituents in the Lebanese diet, the majority of dairy farmers (70%) are classified as poor to very poor groups producing minimal quality and poorly hygienic milk products (FAO, 2016). The war in 2006 also led to undesirable consequences, as this sector was more exposed to insufficient resources, deficiencies in hygiene, experience and skills. To overcome these obstacles, a collaboration between FAO, the government and the ministry of agriculture were settled as an effort for a healthier dairy sector in Lebanon (FAO, 2016).

Surveillance and control efforts should also be undertaken in neighboring countries, where people are also at high risk of infection as they consume unpasteurized milk and raw meat. In those regions, dairy products are frequently produced, stored, and transported below the required sanitary standards. Furthermore, as part of their traditions, populations such as Bedouins tend to sleep around animal sheds (cohabitation with animals), hence with a potentially higher risk of transmission (Erekat *et al.*, 2013). International and local trading between Lebanon and Palestinian territories, Egypt, Jordan, Turkey and along with livestock movement between these territories

increase the risk of cross-border disease transmission (Ereqat *et al.*, 2013; Çavuşoğlu and Yılmaz, 2017). One key to reduce the risk of infection by *M. bovis* would thus to ensure healthy food at a regional scale. Food safety should be ensured not only at the industrial/commercial level but also at a household level, especially in rural areas where people still have their own livestock. In addition, personnel with high and medium occupational risk should be aware of the seriousness of the disease and should take/be enabled to take preventive measures during manipulation by wearing gloves and masks when they are in direct contact with the animals (Vayr *et al.*, 2018). In addition, more generalized milk pasteurization before consumption and slaughterhouse control by routine animal testing will help reduce the risk of both bovine and zoonotic TB as they are highly connected (Ereqat *et al.*, 2013; WHO *et al.*, 2017a; Vayr *et al.*, 2018).

Whenever possible, widespread implementation of diagnostic molecular tools such as those that we used – i.e. Deeplex-MycTB, with the capacity of differentiating between MTBC members and providing extensive resistance profiling, including to pyrazinamide – will be key to resolve the problem of the specific identification of *M. bovis*. Before our study, the laboratory techniques most commonly used in Lebanon to diagnose TB were based on GeneXpert, microscopic examination and culture (occasionally), none of which differentiate between *M. tuberculosis* and *M. bovis* (MoPH, 2017). In addition, phenotypic DST to PZA was not tested due to its unreliability (Scorpio and Zhang, 1996). Implementation of such molecular tools along with implementation of routine surveillance systems will ensure adequate patient treatment and will help to obtain a clear image on the incidence of zoonotic TB especially in countries presumably with a high endemicity. These steps will be necessary to reach the ultimate goal of eradicating tuberculosis by 2050 (WHO *et al.*, 2017a).

General conclusion

Among our main findings, our work revealed the previously unsuspected existence in Lebanon of i) XDR TB cases, ii) MDR cases with complex resistance patterns predicted by Deeplex-MycTB, iii) zoonotic TB cases, with an intrinsic resistance to pyrazinamide, iv) baseline resistance to fluoroquinolones, not associated with MDR/RR TB, in 10% of the study population, v) the presence of a highly prevalent endemic genotype potentially associated with a large cluster of ongoing transmission. The existence of XDR TB cases was disclosed by both second line DST newly implemented in the country and confirmed by Deeplex-MycTB and WGS. In contrast, the other extra layers of TB drug resistance and etiology were detected by Deeplex-MycTB, complemented by MIRU-VNTR typing, but remained undetectable by the routine diagnostics tests currently in place in the country. This situation thus represents an emblematical example of the global diagnostic gaps that exists for the disease.

While TB remains the first infectious disease killer worldwide rivaling thus HIV, the End TB strategy, aims to attain by 2035 a 90% reduction in TB incidence and 95% reduction in TB mortality. For this ambitious goal to be achieved, early and accurate diagnosis including comprehensive determination of susceptibility profiles to both traditional and new anti-tuberculous drugs will be key and should be globally implemented. However, the latest data that are available show that substantial stumbling blocks remain for reaching these ultimate goals. Indeed, of the estimated 10 million new cases in 2017, 3.6 million were either not reported or not diagnosed (WHO, 2018a). Moreover, only 22% of the total number patients estimated to suffer from rifampicin resistant or MDR TB were diagnosed and received the necessary second-line treatment (WHO, 2018a). As examples of national situations prevailing as of 2017, China and India accounted for 39% of the total 75% gap between the estimated incident number of MDR/RR TB and individuals enrolled in the treatment of MDR TB worldwide (WHO, 2018a).

Such global underdiagnosis and suboptimal treatments reflect deficiencies at different stages of the so-called TB care cascade (Subbaraman *et al.*, 2019). Construction of such cascade of care has been used as a useful model for evaluating patient loss or retention throughout consecutive stages of care that are required for a successful outcome. The major benefits expected from this model are thus to determine the quality of health services at the different stages of TB care and their relative contribution to successful patient outcomes. The evaluation of each step in the cascade can consequently provide useful information for monitoring the TB control program in a country and target existing gaps that can and need to be ameliorated. This model was recently evaluated for

India and South Africa (Subbaraman *et al.*, 2019). Starting from the estimated number of any form of active TB in 2013 as the 100% reference, the greatest gaps in terms of patient losses in India were observed at the stages of accessing to TB diagnostic tests and receiving a diagnosis of TB, representing total losses of 43% (i.e. only 57% of the patients were diagnosed with TB) (Figure 28). Comparatively, losses at the subsequent stages of registration for TB treatment (57% to 50%) and treatment success (50% to 43%) were smaller in absolute terms. Although lower than for India, the total loss up to confirmed TB diagnosis in South Africa was still substantial (18%, from 100% to 82%). Importantly, as could perhaps be expected due to enhanced diagnostic means required, losses in the cascade of care of MDR or RR cases were even worse in both countries. The total losses up to confirmed diagnosis amounted to 81% in India and 26% in South Africa (Figure 29) (Subbaraman *et al.*, 2019)

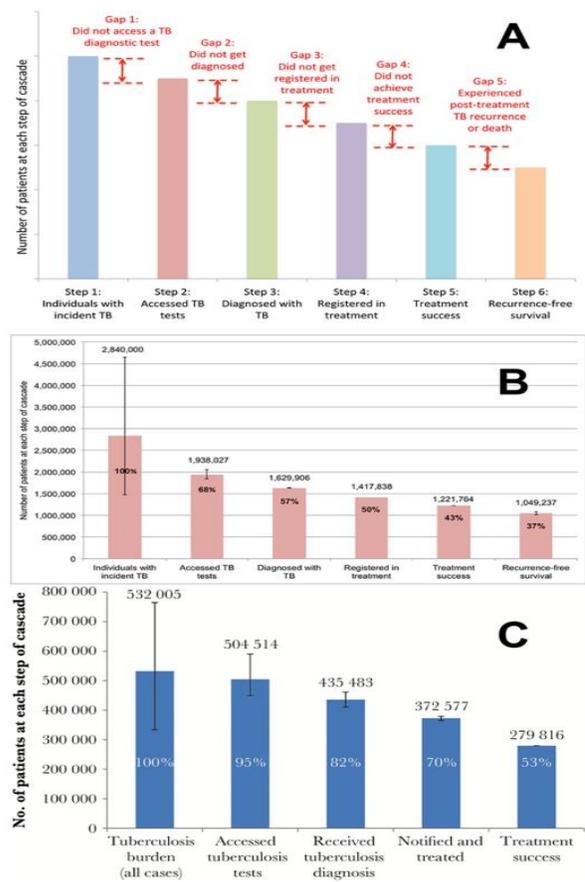


Figure 28: TB care cascade for active TB cases (Subbaraman *et al.*, 2019).

(A) A generic model (B) A care cascade of active TB cases in India in 2013 (C) A care cascade of active TB cases in South Africa in 2013

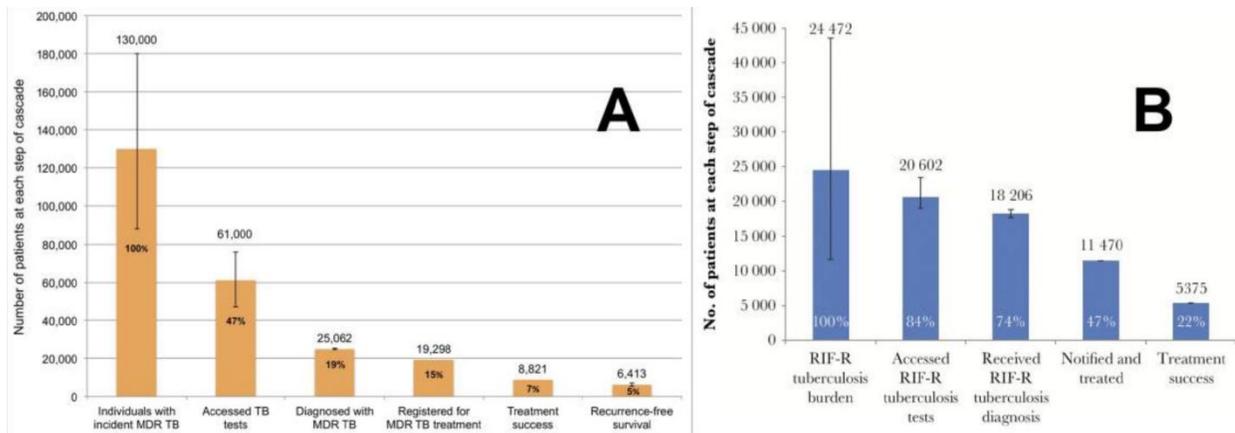


Figure 29: MDR TB care cascade examples (Subbaraman *et al.*, 2019).

(A) Care cascade of MDR TB cases in India in 2013 (B) Care cascade of RR TB cases in South Africa in 2013.

Although the TB care cascade has not been formally evaluated in Lebanon, some previously available estimations as well as observations obtained with our work also indicate the existence of substantial gaps in the country. As of the WHO report, while 710 incident TB cases were estimated to occur in 2017, 618 new and relapse cases were notified, of which only 54% were tested with a rapid diagnostic at the time of diagnosis (WHO, 2017c). It is noteworthy as well that during our 18-month study period, from June 1, 2016 to November 30, 2017, a total of 417 TB cases were molecularly and/or phenotypically confirmed among patients reported to national TB centers in the country, which represents a total substantially smaller compared to what could be expected based on the aforementioned annual estimate in 2017. Part of these differences likely reflects the fact that WHO estimates are partly extrapolated from notified cases to account for the indeterminate diagnostic coverage of patients managed by the private sector, who are usually subject to low notification rates (Subbaraman *et al.*, 2019). Moreover, the diagnostic coverage of the large populations of refugees hosted in the country is also indeterminate. Indeed, wars compound TB transmission and containment in affected zones. In addition, displaced population are at higher risk to develop and transmit TB compared to the general population (Kimbrough *et al.*, 2012). The settlement of refugees in crowded places (e.g. camps), with poor living conditions, enable the spread of airborne diseases including TB (Clark *et al.*, 2002). Refugees living in camps are at higher risk of death due to TB (Bhatia *et al.*, 2002). In addition, displacement of such population can result

in poor access to health facilities, causing delays in diagnosis and in access and start of treatment (Hosten *et al.*, 2018). In this respect, it is noteworthy that in response to the similar influx of SR in Jordan, the Jordanian NTP has recently implemented active contact tracing for detecting active TB (by chest X-rays and verbal screening of TB-related symptoms) and LTBI (by TST) among contacts of all refugees diagnosed with pulmonary TB and registered by the IOM. Such strategy revealed a high prevalence of LTBI and active TB, diagnosed in 24.1% and 2.1% of the contacts, respectively (Hosten *et al.*, 2018). If resources allow, the implementation of such a system, in Lebanon and in other countries hosting refugee populations, could thus help both for further closing detection gaps and for preventing TB morbidity/mortality and transmission in these populations.

With respect to MDR, official data from WHO indicated a total estimate of 10 MDR-TB cases, with an upper limit of 19 for the confidence interval, among notified cases in the country for 2017 (WHO, 2018). Of these, only 6 MDR-TB and 2 XDR-TB cases were laboratory confirmed, including the 3 MDR and 2 XDR cases that were identified through our work from January 1 November 30 of the same year. These data thus likewise indicate the probable existence of substantial gaps at MDR-TB diagnosis level, such as those existing in India and South Africa (see above). Moreover, our Deeplex-MycTB data revealed that most of the MDR and XDR cases that were part of our study exhibited supplementary genotypic resistance to PZA and/or ETH, which are not phenotypically tested in Lebanon and in many other countries.

Of note, genotypic resistance to PZA and ETH is also not tested by the currently used molecular tests in Lebanon. These tests, as well as LPA Hain tests provide only common mutations associated with resistance either to RIF alone (Xpert) or to RIF, INH, EMB, FQ and injectables (Hain) or to RIF, INH, FQ and injectables (Anyplex), respectively. In addition, such assays are also prone to false-positive RIF resistance results, e.g. due to synonymous mutations (Mathys *et al.*, 2014), and false-negative RIF resistance results, due to undetected minority resistant populations heteroresistance (Dheda *et al.*, 2017; Zürcher *et al.*, 2019). While all 9 RIF positive cases detected by GeneXpert in our study population were confirmed by both phenotypic and Deeplex-MycTB testing, the prevalence of RIF resistance in our setting was likely too low to detect the occurrence of such problems.

Rapid molecular DST extensively informative for first and second-line drugs should be implemented locally and used at treatment initiation to improve MDR and XDR TB patients'

diagnosis and treatment outcomes. To reach this goal, WGS, representing the most comprehensive genetic test, is a very promising approach. However, its affordability, as well as the levels of skills needed for data analysis and interpretation remain major obstacles for implementation in low and middle-income countries (Dheda *et al.*, 2017; Zürcher *et al.*, 2019). In addition, WGS is not applicable directly on samples in clinical routine, implying thus still the need for a primary culture. Moreover, minor heteroresistant populations (i.e. below 10%) cannot reliably be captured at usual coverage depths of 50-100x used with WGS (Schön *et al.*, 2017; WHO, 2018d). The use of culture free, targeted deep sequencing such as Deeplex-MycTB can help by-pass a large part of these problems, for improved rapid diagnosis and personalization of treatment regimen. Although less comprehensive than WGS, Deeplex-MycTB simultaneously determines mutations associated with drug resistance in 18 main gene targets associated with 1st and 2nd line drug resistance of *M. tuberculosis*, in addition to providing mycobacterial species identification and information on MTBC genotype (Tagliani *et al.*, 2017). With this test, we identified in MDR and XDR TB cases additional mutations predicting resistance to PZA and ETH, which are not tested by classical phenotypic DST and molecular testing, making diagnosis and individualized treatment more accurate. While these two drugs are part of the recently defined group C of anti-TB drugs to be used in longer treatment of MDR-TB, Deeplex-MycTB also includes the main molecular targets associated with resistance to all drugs part of Group A to be used in priority, i.e. FQ, BDQ and LIN, as well as to CFZ in group B and PZA, AMI and STR, also part of group C (WHO, 2018b). Of further interest in this respect, WHO recently recommended genotypic testing as a reference for determining resistance to PZA and EMB, given the lack of reproducibility and accuracy in EMB and PZA phenotypic DST (WHO, 2018c).

According to the common diagnostic algorithm in place in Lebanon, like in many other countries, non-MDR cases, including the 24 cases with mono-resistance to INH, were not subjected to FQ resistance testing (phenotypically). They received first-line treatment in accordance with recommendations by WHO during the study period. However, recent data indicated generally poorer outcomes in patients with INH monoresistance who receive classical first line treatment (Gegia *et al.*, 2017; van der Heijden *et al.*, 2017; Zürcher *et al.*, 2019). Nevertheless, recent evidence suggests that in the presence of low INH resistance level associated mutations in *inhA*, an increased dose of INH in the first line regimen may still be effective. Our Deeplex-MycTB data detected such *inhA* mutations in 7 (note that 1 of them was phenotypically susceptible to INH) of

the 24 cases identified as INH monoresistant in our study population, thus indicating that a first line regimen with an elevated dose of INH could have been used for these patients under the latest recommendations by WHO (WHO, 2018c). However, in the case of high INH resistance level mutations in *katG*, the use of FQ in substitution to INH is recommended, after ruling out resistance to FQ. Deeplex-MycTB identified INH resistance mutations in *katG* in the 14 remaining cases (with 13 having a S315T mutation and 1 with a probable KatG deletion) identified as INH monoresistant, who should thus have been treated with FQ in substitution of INH in the first line regimen. Although Deeplex-MycTB detected in total 10 % of the non MDR *M. tuberculosis* isolates as exhibiting FQ resistance associated mutations in *gyrA* and/or *gyrB*, only one of these was identified as INH “monoresistant”, by phenotypic testing but not by Deeplex-MycTB. By chance thus, the treatment with FQ in substitution of INH would have probably been inadequate for one patient only in this population.

Importantly, the use of deep sequencing enabled us to show that the majority of the isolates carrying FQ resistance associated mutations in *gyrA* and/or *gyrB* consisted of heteroresistant populations. Among these, a substantial number showed very minor subpopulations (with a representation in the range of 10% or lower) bearing these mutations. These findings most likely reflect a consequence of improper use of FQ. In Lebanon, as elsewhere, self-medication is a major public health problem (Cheaito *et al.*, 2014; Ayalew, 2017; Nepal and Bhatta, 2018). Indeed, FQ is extensively used in an improper manner in patients with respiratory disease or UTI. In this context, we hypothesize that very minor resistant subpopulations putatively result from short periods of FQ monotherapy, sufficient to select for emergence of resistance but often insufficient for substantial representation or complete fixation of the mutations. Of note, commercial molecular DST assays, as well as WGS at classical coverage depths, have suboptimal sensitivity to detect such minor resistant populations, which might result in treatment failure if the concerned drug is important in the prescribed regimen (Metcalf *et al.*, 2017). These observations thus indicate both the existence of an additional important gap with the currently deployed molecular tests, and the potential of deep sequencing approaches to close it.

A final diagnostic gap highlighted by our findings, mostly obtained with the use of Deeplex-MycTB, concerns the detection of zoonotic TB. Many countries rely on GeneXpert as a rapid diagnostic tool for TB identification. Unfortunately, it does not differentiate MTBC into distinct species. Likewise, none of the other WHO endorsed phenotypic and molecular diagnostic tools

differentiate between *M. tuberculosis* and *M. bovis*. Thus, zoonotic TB could not be detected with existing diagnostic tests available in Lebanon, and all cases of zoonotic TB were not diagnosed as such and did not receive an adequately adapted treatment. A similar situation prevails in many other countries, especially in Africa and South-East Asia with a putatively high burden of zoonotic TB, leading to a potential underestimation of the true global burden of zoonotic TB (WHO *et al.*, 2017b). The biggest challenge is that zoonotic TB control needs a cooperative approach where the human and animal health sectors as well as food safety sectors are engaged (WHO *et al.*, 2017a). The importance of identifying the right causative agent is to eventually ensure adequate patient treatment, as *M. bovis* is naturally resistant to PZA, to precisely determine the contribution of *M. bovis* in the human TB epidemiology, and to reveal thereby the potential existence of animal sources that need to be investigated and controlled. Despite the fact that *M. bovis* infection represented only 12 cases out of total TB cases in our study population, the problem of zoonotic TB should therefore not be neglected, and plans should be undertaken to control it.

Further gaps exist beyond diagnostic steps, at the treatment completion/success stage. The ministry of Public Health in Lebanon estimated that out of the migrant workers that were diagnosed with TB over periods that preceded the start of this PhD, about 50% on average left the country before treatment completion (MoPH, 2017). In our study population, we observed a grossly consistent proportion, as 45 out of 150 migrant workers/patients with nationality other than Lebanese, Palestinian or Syrian, were reported as having left the country before treatment completion. Thus, a substantial gap exists at the treatment success stage. Another gap at the treatment stage also existed in the initial part of our study period, as manifested by the fact that the XDR TB patient originating from Ukraine has to return to his country due to treatment unavailability at the time of his diagnosis in Lebanon. Better identifying and eliminating these gaps in the TB care cascade will need efforts by all partners involved in TB control.

Although our study provided new insights into TB diagnostic gaps existing in Lebanon and in many other countries probably, it had several limitations. Deeplex-MycTB testing could have also been directly applied on clinical samples (Tagliani *et al.*, 2017), in order to have results more quickly and to perform comparisons with both Deeplex results obtained from cultured isolates and phenotypic DST results. Unfortunately, due to difficult transport conditions, the DNA extracted from heat inactivated samples received in Lille turned out to be too deteriorated for effective direct application. This obstacle, and the impossibility to organize transportation of samples as soon as

they were obtained in Lebanon in a cost-effective manner, also prevented the evaluation of this test under prospective conditions. Along the same lines, even if it was adequate for targeted deep sequencing, the quality of DNA extracted from heat inactivated primary cultures was deemed insufficient for performing large-scale WGS. This prevented in particular WGS analysis of the few isolates that were phenotypically resistant to some first-line drugs (e.g. INH) but were predicted as susceptible by Deeplex-MycTB, as well as the isolates that were part of the largest MIRU-VNTR cluster in order to verify which fraction of them possibly represented recent transmission.

As another limitation, common to many other TB surveys, we were not able to collect samples from patients managed by the private sector. Thus, the collaboration between the private and public sectors should be strengthened. In addition, as per the NTP medical doctor, a great majority of extra-pulmonary TB samples were not collected as they are directly tested in the hospital, and are usually not transmitted to the NTP centers. As a consequence, and since *M. bovis* is more frequently associated with extra-pulmonary TB as our own data confirm, the 3.4% prevalence of zoonotic TB might actually be an underestimate.

As perspectives for further exploiting the results of this work, sample collection should be continued on a national level in order to see if the relatively low prevalence of drug resistance that we determined maintains or tends to increase over time. Efforts should be dedicated to reinforce the diagnostic algorithm in place in order to better guide TB patient treatments. As elsewhere, for obtaining a complete diagnosis including drug resistance profiling, 4-6 weeks are needed on average in our setting using classical liquid culture based DST. Albeit faster, the available molecular tests (GeneXpert MTB/RIF and Anyplex) provide incomplete information for predicting susceptibility and resistance to first and second anti-TB drugs, as demonstrated by our results obtained with Deeplex-MycTB. In addition, as shown by a recent multicenter study, the accuracy of such routine drug susceptibility algorithms can be moderate, which can have an important impact especially on MDR and XDR TB patient outcomes (Zürcher *et al.*, 2019).

When possible, Deeplex-MycTB assay, preferably directly applied on clinical samples, or rapid WGS on newly positive cultures, should be considered for implementation in the TB reference laboratory in Lebanon. WGS analysis should also be undertaken in order to further confirm or exclude ongoing TB transmission, especially in the large molecular cluster that we identified. Finally, the detection of significant baseline resistance to FQ and zoonotic infections among TB

patients indicate that further efforts should also be dedicated to better control the antibiotics use and implement TB control measures at the human/animal interface and food production levels.

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Annex

Annex - 1



UNIVERSITE LIBANAISE
Ecole Doctorale des Science et Technologie
Plateforme Azm pour la Recherche en Biotechnologie et ses Applications
Laboratoire de Microbiologie Santé et environnement
Information concernant un Tuberculeux



Code CMUL :

Référence :

1) Informations concernant le patient:

Nom:..... P rénom:

Age:..... A dresse:

Sexe:..... Médecin traitant :

Nationalité*:

* Si Syrien : Refugié Oui (date d'entrée :/...../.....)
 Non

Date d'apparition des symptômes : Ambulant / Hospitalisé
Nature de prélèvement :

Contact avec une personne atteinte de tuberculose

Oui → Famille / Hors famille
→ Avant / Simultané

Non

Statut de la maladie

Tuberculose récente
 Rechute
 Suspicion
 Contrôle sous traitement
 Contrôle après arrêt du traitement

Type de tuberculose

Pulmonaire
 Extra – pulmonaire (Préciser :))

2) Analyses effectués au laboratoire

- Test à la tuberculine : IDR : Induration :
- Examen microscopique : Résultat du Ziehl : Positif / Négative (Grade :))

