CUKUROVA UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Identification Of Efflux Pump Mutants And Genetic Variants Conferring Cross-Resistance Between Bedaquiline And Clofazimine In *Mycobacterium tuberculosis* Clinical Strains

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August, 2023

CUKUROVA UNIVERSITY
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PhD THESIS APPROVAL

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This Thesis was written in the Department of Biotechnology, Institute of Natural and Applied Sciences.
Thesis Number:

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This thesis was supported by the Scientific Research Office of Çukurova University
Project ID: FDK-2020-14008

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CONTENTS

ABSTRACT I
ÖZ II
GENİŞLETİLMİŞ ÖZET II
ACKNOWLEDGEMENT IX
LIST OF TABLES X
LIST OF FIGURES XI
LIST OF SYMBOLS AND ABBREVIATONS XIII
1. INTRODUCTION 1
2. LITERATURE REVIEW 7
2.1. Infectious Diseases 7
2.2. History of tuberculosis (TB) 9
2.2.1. Brief TB History: The five countries of Maghreb 12
2.2.2. Brief TB History: Turkey Between 1923-1950 13
2.3. *Mycobacteria* 13
2.3.1. Taxonomy 13
2.3.2. Salient features of Mycobacterium genus 14
2.3.3. Cell wall 15
2.3.4. The genome of *M. tuberculosis* 16
2.3.5. Virulence factors 17
2.4. TB disease: 18
2.5. Epidemiology 20
2.6. Tuberculosis Diagnosis 23
2.6.1. Collection and Processing of Clinical Specimens 23
2.6.2. Microscopic Examination 23
2.6.3. TB Identification Methods: 24
2.7. TB Treatment: 34
2.7.1. Chronology of the discovery of anti-tuberculosis drugs 34
2.7.2. The different phases of treatment for multidrug-resistant tuberculosis and dosages ofanti-tuberculosis drugs 35
2.7.3. Therapeutic strategies and current treatments for tuberculosis 36
2.7.4. First-line anti-tuberculosis drugs 37
2.7.5. Second-line anti-tuberculosis drugs 41
2.7.6. Bedaquiline and Clofazimine: 46
2.8. Drug resistance 52
3. MATERIAL AND METHODS 55

3.1. Phenotype diagnosis 55
3.1.1. Collection of Samples 55
3.1.2. Drug susceptibility Testing (DST) : 55
3.1.3. Minimum Inhibitory Concentration (MIC): 56
3.2. Molecular diagnosis: 59
3.2.1. Extraction of DNA 60
3.2.2. PCR amplification: 60
3.2.3. Sanger Sequencing: 62
3.2.4. Epidemiology diagnosisSpoligotyping: 64
4. RESULT AND DISCUSSION 69
5. CONCLUSION 91
REFERENCES 93
CURRICULUM VITAE 105

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ABSTRACT

TB is a contagious, endemic infectious disease with polymorphic clinical and radiological features. The prognosis of this disease is usually favorable under well-conducted treatment. The treatment is based on anti-tuberculosis drugs whose success requires compliance with treatment; Bedaquiline and Clofazimine are included in the use of drugs in WHO category 5 antibiotics. That's why the genetic bibliography on resistance mechanisms for the two antibiotics is lacking. This study is the first in Turkey, using 100 local MDR-TB strains to show the mechanisms of resistance in relation to MIC values. Our work is based genetically on the isolation of spontaneous mutants in the genes PepQ, RV0678, and Rv1666 through sanger sequencing and the effect on the expression of the efflux pump, phenotypically on the determination of MIC values, and, in the end, epidemiologically on the building of a table that will improve the bibliography of resistance by using spoligotype methods. MIC values of bedaquiline and clofazimine for our TB strains were determined as 0.25 g/mL:>6.4 g/mL and 0.016 g/mL:>3 g/mL, respectively. The mutations responsible for cross-resistance to bedaquiline and clofazimine in our Turkish strains were not detected by Sanger sequencing, but we did find the mutation C415A in the PepQ gene. Among the clinical samples of Turkish patients, according to the result obtained by the Spoligotyping method, Ghana families with 45.9% members and LAM families with 28.6% members were determined. In conclusion, the mutations we observed reduce the antituberculous effect but do not create a complete resistance for cross-resistance; It has been determined that the high MIC values have a significant bactericidal activity, especially when combined with two antibiotics.

Keywords: Mutations, Drugs, Resistance, MIC, Tuberculosis

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

DOKTORA TEZİ

Mycobacterium tuberculosis Klinik Suşlarında Efluks Pompa Gen Mutasyonlarının, Bedaquiline ve Clofazimin Arasında Çapraz Direnç Bulunan Genetik Varyantların Tespit Edilmesi

Khaoula BALGOUTHI

Danışman: Prof. Dr. Işıl VAR

Biyoteknoloji Anabilim Dalı

ÖZ

Tüberküloz (TB) polimorfik klinik ve radyolojik özellikleri olan bulaşıcı, endemik bir enfeksiyon hastalığıdır. Bu hastalık, uygun tedavi koşullarında genellikle hastalığa yakalanmış bireylerin sağlığına tekrardan kavuşması ile sonuçlanmaktadır. Hastalığın tedavisi, Dünya Sağlık Örgütü'nün 5. Kategori'de sınıflandırmış olduğu Bedaquiline ve Clofazimin adlı antibiyotiklere dayanmasına rağmen, bu ilaçlara dirençli suşlarda tedavi başarısı sınırlı olmaktadır.

Bu çalışma, Çoklu İlaca Dirençli Tüberküloz'a neden olan 100 adet yerel suşda, Minimum İnhibisyon Konsantrasyonu (MİK) değerleri ile ilişkili direnç mekanizmalarını ortaya çıkarmak için kurgulanan Türkiye'deki ilk çalışmadır. Çalışmada genetik olarak PepQ, RV0678 ve Rv1666 genlerindeki spontan mutantların sanger dizileme yoluyla izolasyonu ve efflux pompasının ekspresyonu üzerindeki etkisi, fenotipik olarak MİK değerlerinin belirlenmesi ve sonunda epidemiyolojik olarak spoligotype yöntemleri kullanılarak direniş bibliyografyasını geliştirecek bir tablonun oluşturulması amaçlanmıştır. Çalışmada, bedakuilin ve klofazimin MİK değerleri TB suşlarımız için sırasıyla 0,25 g/mL:>6,4 g/mL ve 0,016 g/mL:>3 g/mL olarak kullanılmıştır. Çalışma sonucunda, Türk suşlarımızda bedakuilin ve klofazimin çapraz direncinden sorumlu mutasyonlar Sanger dizilimi ile saptanmadı, ancak PepQ geninde C415A mutasyonu tespit edilmiştir. Türk hastalarının klinik örnekleri arasında, Spoligotyping metodu ile elde edilen sonuca göre %45,9 üye ile Gana aileleri ve %28,6 üye ile LAM aileleri tespit edilmiştir. Sonuç olarak gözlemlediğimiz mutasyonlar antitüberküloz etkiyi azaltmakta ancak çapraz direnç için tam bir direnç oluşturmamakta; Yüksek MİK değerlerinin özellikle iki antibiyotik ile kombine edildiğinde önemli bakterisidal aktiviteye sahip olduğu belirlenmiştir.

Anahtar Kelimeler: Mutasyonlar, İlaçlar, Direnç, MİK, Tüberküloz

GENİŞLETİLMİŞ ÖZET

Tüberküloz, ilk olarak 2500 yıldan uzun bir süre önce Peru ve Mısır mumyalarından izole edilen, insalık tarihi kadar eski bir hastalıktır. Robert Koch, 1882 yılında Berlin'de bir bakteri olan *Mycobacterium tuberculosis* basilini tüberkülozun kesin nedeni olarak keşfetmiştir. (Barberis et al 2017).

Anti-TB ilaçların keşfi, 1943'te Selman Waxman, Elizabeth Bugie ve Albert Shatz tarafından Andes Dağları'nın topraklarından izole edilen *Streptomyces griseus* isimli bakteriden saflaştırıldığında başlamıştır. Ancak streptomisinin, işitme sorunlarına ve hatta sağırlığa sebep olduğu ve tedaviyi takip eden ilk yılı içinde, bakterilerin streptomisine hızla direnç geliştirdiği tespit edilmiştir. Bu sebeple, 1940'ların sonunda para-aminosalisilik asit (PAS) streptomisin ile beraber tedavide kullanılmış ve pulmoner TBC'li hastalarda iyileşme oranını yaklaşık % 80'e çıkardığı gözlemlenmiştir. TB tredavisinde yüksek etkinliğe sahip olan izoniazid (INH), 1952 yılında üç ilaç firması tarafından keşfedilmiştir. Streptomisin ve PAS için hasta başı 3500 dolar olan maliyet yalnızca 100 dolara düşmüştür. Ancak streptomisinde olduğu gibi INH tedavide tek başına kullan kullanıldığında direnç geliştiği görülmüştür. Hızla gelişen dirence çözüm olarak 1955 yılında üçlü tedaviye geçilmiş ve streptomisin, PAS ve INH kombinasyonunun iki yıl boyunca devam edilmesi ile %100'e yaklaşan tedavi oranlarına ulaşılmıştır (Danielet al. 2006).

Pirazinamid çalışmaları, 1948 yılında nikotinamid modifikasyonu olan tiyasetazonın toksik etki göstemesi sebebi ile başlamış, 1956 yılında ethionamide ve prothionamide keşfedilmiştir. Ancak asıl ilerleme 1959 yılında günümüzde de son derece etkili bir anti-TB ilaç olan rifampisinin keşfi ile gerçekleşmiştir. Rifampisin ve isoniazid beraber kullanıldığında 9 aylık tedavi rejimi sonunda, 2 yıllık üçlü tedavi kadar etkili olduğu görülmüştür.

Etambutol, 1961'de bulunan sentetik, bakteriostatik etkili bir ilaçtır. Etambutolun keşfinden sonra 1962 yılında pirazinamid daha düşük ve toksik olmayan dozlarda tedavide tekrar denenmiş ve izoniazid, rifampisin ve etambutole pirazinamid eklendiğinde tedavi sadece 6 aya kadar kısaltılmıştır. Salvador Luria ve Max Delbruck 1943 yılında, anti-TB ilaçlarına karşı bakteride direncin rastgele mutasyonlar sonucu ortaya çıktığını göstermişlerdir. (Barberis et al. 2017).

İngiltere'de 1955'te yürütülmüş ilk ulusal ilaç direnç araştırmasında, streptomisin, PAS ve izoniazide karşı direnç geliştirmiş TB suşlarının varlığı gösterilmiştir. ABD'de, 1960'ların başında, izoniazid direnci % 6,3 iken, birkaç yıl sonra, 1960'ların sonunda % 9,7'ye yükseldiği ve dirençli türlerin duyarlı hale dönmediği de tespit edilmiştir. Örneğin bir suş streptomisine dirençli ise, hastaya streptomisin verilmemiş olsa bile, suş streptomisine dirençli kalmıştır.

M. tuberculosis'in direnci rastgele spontan mutasyonlardan kaynaklanmakta ve plazmidlere bağlı direnç tanımlanmamaktadır. Yaklaşık 10^6 bakteriden 1'i rastgele mutasyonlarla herhangi bir antibiyotiğe kendiliğinden direnç kazanabilir. Bir kavitede 10^9-10^{10} bakteri bulunabilir, dolayısıyla

verilen herhangi bir antibiyotik tedavisine karşı dirençli olan bir bakteri içerecektir. Kromozomlar üzerinde dirence sebep olan gen bölgelerinin birbirleriyle bağlantılı olmamaları sebebi ile bakterinin spontan olarak iki ilaca birden direnç geliştirme oranı olasılıkların birbirleriyle olan çarpımlarına eşittir (10^{-6} x 10^{-6} =10^{-12}). Tedavide dört ilaç rutin olarak kullanılmaz veya emilim tamamen gerçekleşmez ise, üç ilacın konsantrasyonu düşük olacak ve dördüncü ilaca dirençli basillerin hayatta kalmasına ve çoğalması ile direnç gelişecektir. (Arbex MA, et al 2010).

Primer direnç, dirençli suşla ilk enfeksiyondur. Sekonder direnç ise daha önceden duyarlı suşta tedavi sırasında gelişen dirençtir. Dirençli suşlar ya da primer direnç salgınları 1970'lerin başlarında görülmüştür. (Nachega JB et al 2003).

Ancak 1990'larda, çoğu HIV pozitif hastalarda olmak üzere birçok çoklu ilaca dirençli salgın da görülmüştür. HIV için etkili bir tedavinin bulunmaması, hastaların tüberküloz ve diğer enfeksiyonlara karşı duyarlı hale gelmesine sebep olmuştur. Hastane ve sağlık tesislerinde HIV pozitif hastalar arasında çoklu ilaç dirençli tüberküloz (MDR-TB) suşlarının bulaştığı tespit edilmiş ve antibiyotik duyarlılık testlerinin sonuçlanması 2-3 ay sürmüş, bu süreç içerisinden HIV pozitif MDR hastalarının yarısından fazlası ölmüştür. MDR-TB, en az rifampisin ve izoniazide dirençli tüberküloz suşlarıdır. İsoniazid, mikobakteri hücre duvarındaki mikolik asitler yapında gerekli olan *inhA* enzimini inhibe eder (Zhang Yet al 1992).

XDR-TB tedavi rejimi farklı ilaçlar içermektedir ve tedavisi çok zordur. İrrasyonel XDR-TB tedavisi suşların toplum içerisinde bulaşına sebep olmaktadır. MDR-TB tedavisinde yeni anti-TB ilaçlar olan bedaquilin ve delamanid 2012'de piyasaya sürüldü (Nguyen et al 2018).

Bedaquiline, antitüberküloz ilaçlar arasında yeni bir etki mekanizması olan bir ATP sentaz inhibitörüdür.

Bedaquiline'in aktivitesini doğrulayan randomize faz 3 çalışmalarında, MDR-TB tedavi rejimine eklendiğinde, iyileştirme oranını % 32'den % 58'e yükselttiği gösterilmiş ve 50'den fazla ülkede kullanım alanı bulmuş ve bu durum TB ilaçları için yeni bir hedef olarak elektron transport zinciriyle ilgili araştırmaların artmasına sebep olmuştur (Ismail et al 2019) .

Klofazimine, ilk olarak 1954'te anti-TB antibiyotik olarak sentezlenen hidrofobik bir riminofenazindir. Ancak 1959'da Chang, cüzzamlara karşı etkinliğini göstermiştir.

Klofazimin tedavideki etkinliğinin kanıtlanması ile DSÖ tarafından kısaltılmış dirençli tüberküloz (DR-TB) tedavi rejimlerine dahil edilmiştir (Mirnejad ve ark. 2018). Rifampine dirençli (RR) veya MDR-TB hastalar, klofazimin içeren kısaltılmış DR-TB rejimi veya hem Bedaquiline (WHO grup A) hem de klofaziminden oluşan rejim ile tedavi edilmektedir. (WHO group B) (Wares F et al 2011).

Tüberküloz, yaklaşık 70 yılı aşkın süredir hayatı tehdit eden en eski enfeksiyonlardan birisidir. Tüm dünyada mortalitenin en önemli sebepleri arasında yer almaktadır. Yaklaşık olarak dünya nüfusunun 1/4'nin M. tuberculosis basili ile infekte olduğu, her yıl 10 milyondan fazla kişide aktif tüberküloz geliştiği en az 1 milyon ve günde 4000 kişinin de tüberküloz sebebi ile hayatını

kaybettiği tahmin edilmektedir. Dünya Sağlık Örgütü 2020 raporuna göre, 2018 yılında 186.883 olarak tespit edilen MDR / RR-TB vaka sayısı, 2019 yılında %10'luk artış ile 20.030 olarak bildirilmiş ve tedavi rejimine dahil edilen hasta sayısı 156.205'den 17.099 kişiye yükselmiştir.

Tüberküloz tedavisinde 1970'li yılların ortalarından itibaren tüberküloz tedavisi 6-9 aya indirilebilmiş ve günümüzde ise 2 ay pirazinamid, etambutol, izoniazid ve rifampisin, takip eden 4 ay ise izoniazid ve rifampisin ile toplam 6 aylık HRZE majör anti-tüberküloz ilaçların kullanıldığı tedavi rejimlerine geçilebilmiştir.

Bedaquilin, antitüberküloz ilaçlar için yeni bir etki mekanizması olan bir ATP sentez inhibitörüdür. Bedaquiline'in aktivitesini doğrulayan faz 3 randomize çalışmaları, MDR-TB'nin standart tedavi rejimine eklendiğinde, tedavi oranını % 32'den % 58'e yükseltiğini göstermiştir. Antibiyotik direnci, bir mikroorganizmanın antibiyotiğin zararlı etkilerini yok eden, hücre dışına atılımını sağlayan veya etkilerini bloke eden değişikliklere uğratan mekanizmadır. Britanya'da 1955'te yapılan ilk ulusal ilaç direnci araştırmasında Streptomisin direnci keşfedilmiştir. Son yıllarda yapılan çalışmalarda ise, düşük doz Bedaquiline (BDQ) direnci ve klofazimine (CLF) çapraz direnç kazandıran, hedefe dayalı olmayan yeni bir mekanizma tanımlanmıştır.

Plazmit aracılığı ile direnç kazanan diğer bakterilerin aksine, TB basili farklı olarak, antibiyotiğin modifikasyonu, antibiyotik hedefinin modifikasyonu veya antibiyotiklerin dışa atımı ile antibiyotiklere karşı direnç kazanır. Bedaquiline direnci, *mmpS5-mmpL5* dışa atım pompasını kodlayan genlerin transkripsiyonel bir baskılayıcısı olan *Rv0678*'deki mutasyonlardan kaynaklanmıştır ve bunun sonucunda *mmpL5-mmpS5* dışa atım pompasının aşırı ekspresyonun sonucu olarak, *atpE* ve *pepQ* genlerinde mutasyon ortaya çıkmıştır (Ismail et al 2019). *Rv0678* gen mutasyonunun ve klofazimin MİK değerlerinin artmasının neden olduğu *Rv0678* düzenleyici genindeki (2 T>C- M1A) başka bir mutasyonun da yakın zamanda bir MDR-TB hastasında BDQ ve CLF direnci arasında ilişki olduğunu bildirilmiştir (Almeida et al 2016, Nguyen et al 2018).

Klinik örneklerden izole edilen suşlar arasında MDR ve XDR-TB oranlarındaki artış ve bir anlamda XDR-TB'nin karakteristik suşu olan *Beijing* suşlarının dünya genelinde yayılma eğilimi göstermesi dünya genelinde tüberküloz kontrol programlarının yeniden değerlendirilmesini, yeni stratejiler geliştirilmesini ve özellikle daha hızlı daha duyarlı, daha özgül ve ulaşılabilir tanı yöntemlerinin geliştirilerek etkili sürveyans çalışmalarının yapılmasını mecbur kılmıştır. Bu bağlamda klinik izolatların fenotipik özelliklerini baz alan düşük ayrım gücüne sahip tekrarlanabilirliği düşük fenotipe dayalı sürveyans yerine, MTBK suşlarının evrim özelliklerinin belirlenmesine, kaynak ve portör analizine uygun olup toplum içerisindeki MDR ve XDR-TB suşlarının hareketinin izlenmesine, bulaşta ve hastalıkta rol oynayan konak-patojen risk faktörlerinin belirlenmesine, tekrar eden tüberküloz olgularında tedavi rejiminin düzenlenmesinde, endojen reaktivasyon veya eksojen reenfeksiyon ayırımının yapılabilmesine imkan veren, klonal düzeyde yüksek ayırım gücüne sahip tekrarlanabilikleri ve sonuçlarının standardize edilmesi mümkün kılan genotipik sürveyansı yöntemlerinin kullanılması önemlidir. *M. tuberculosis*

kompleksinin tanımlanması ve genetik çeşitliliğinin belirlenmesi amacı ile birçok yöntem geliştirilmiş ancak bunlardan; Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) tanımlanması yani "spoligotipleme" hızlı ve güvenilir bir yöntem olması sebebi ile dünya genelinde *M. tuberculosis* kompleks suşlarının genotiplendirilmesinde sıklıkla kullanılan bir yöntemdir.

M. tuberculosis kompleksi genomunda farklı sayıda 36 baz çiftlik DR lokusu bulunur. DR lokusu CRISPR, Clustered regularly interspaced short palindromic repeats, düzenli kümelenmiş spacer arası kısa palindromik tekrarlar, ailesi üyesidir. Genomda DR lokuslarının aralarında 35-41 baz çifti uzunluğunda aralık oluşturan spacer olarak adlandırılan diziler bulunmaktadır. DR'lerin kopya sayıları ve spacer dizilerin varlığı suşlar arası farklılıklar gösterir. Yöntem DR lokuslarının genomda oluşturduğu bu multiple direct variable repeat'lerin (DVR) fragmentlerinin polimorfizmin tespiti esasına. Her DVR, 36 baz çift içeren DR lokusu ve DR bölgelerinin arasında bulunan tekrarlanmayan kısa diziler olan spacer bölgelerinden oluşur (Pang et al 2012).

Tüberküloz (TB), Mycobacterium tuberculosis kompleks (MTBK) grubuna ait türlerin sebep olduğu yüksek morbidite ve mortalite ile seyreden kronik ve nekrozitan seyirli ancak tedavi edilebilir bir hastalıktır . Günümüzde dünya nüfusunun dörtte birinin TB basili ile enfekte olduğu tahmin edilmektedir. Yaklaşık olarak 50 yıldır sürdürülen yaygın aşı uygulamaları ve etkinliği ispatlanmış antitüberküloz tedavi rejimlerine rağmen, Dünya Sağlık Örgütü (DSÖ2019) verilerine göre dünya genelinde her yıl 10 milyon yeni tüberküloz vakası görülmekte ve yaklaşık 1.5 milyon hasta tüberküloz sebebi ile kaybedilmektedir. Bu verilere göre tüberküloz ölümleri enfeksiyon hastalıklarına bağlı ölümler arasında ilk sırada yer almaktadır . Özetle tanı, tedavi ve immunisazyon çalışmalarında sağlanan ilerlemeler sayesinde son 10 yılda tüberküloz mortalitesinde %3'lük bir düşüş kaydedilmiş olmasına rağmen, hala artan insidans ve daha önemlisi klinik örneklerden artan oranlarda dirençli suşların izole edilmesi tüberkülozun halk sağlığını tehdit eden önemli bir tehtid olma özelliğini sürdürmesine sebep olmuştur . Doğrudan Gözetimli Tedavi (DGT) çabaların yaygınlaştırılması çabalarına rağmen klinik örneklerden giderek artan sıklıkta TB tedavisinde kullanılan majör ilaçlardan en az isoniyazid ve rifampisine dirençli olan çoklu ilaç dirençli (ÇİD-MDR) ve bu iki ilaca ek olarak ikinci seçenek ilaçlardan bir kinolon ve amikasin, kanamisin veya kapreomisin gibi aminoglikozit grubu bir enjektabl ilaca direnci bulunan yaygın ilaç dirençli (YİD-XDR) M.tuberculosis suşları izole edilmeye başlanmıştır. İlk defa tanı almış ve tedavi başlanmış hastaların % 3.4'ünde ÇİD-MTB suşları izole edilirken, sekonder tüberküloz tanılı yani daha önce tanı almış ve tedavi başlanmış, klinik kür sağlanmış ancak bir süre sonra reenfeksiyon veya re-aktivasyon sebebi ile akut alevlenmeler görülen hastalardan bu oran % 18'e kadar çıkmaktadır. Tüberküloz tedavisinde 1960'lı yılların sonlarına doğru yer alan ve teorik olarak direnç gelişme ihtimali en düşük olan, en etkili birinci seçenek ilaç "Rifampisin'e" dirençli tüberküloz (RD-MTB) suşları arasında ÇİD-MTB oranı ise %78'lere kadar çıkmaktadır . Tüberkülozun az gelişmiş ve gelişmekte olan ülkelerdeki yüksek insidansına ek

olarak, klinik örneklerden izole edilen suşlar arasında ÇİD ve YİD-MTB oranlarındaki artış ve bir anlamda YİD-MTB'nin karakteristik suşu olan Beijing suşlarının dünya 2 genelinde yayılma eğilimi göstermesi dünya genelinde tüberküloz kontrol programlarının yeniden değerlendirilmesini, yeni stratejiler geliştirilmesini ve özellikle eğitim, daha hızlı daha duyarlı, daha özgül ve ulaşılabilir tanı yöntemlerinin geliştirilmesini ve etkili sürveyans çalışmalarının yapılmasını mecbur kılmıştır . Bu bağlamda klinik izolatların fenotipik özelliklerini baz alan düşük ayrım gücüne sahip tekrarlanabilirliği düşük fenotipe dayalı sürveyans yerine, MTBK suşlarının evrim özelliklerinin belirlenmesine, kaynak ve portör analizine uygun olup toplum içerisindeki ÇİD-MTB ve YİD-MTB suşlarının hareketinin izlenmesine, bulaşta ve hastalıkta rol oynayan konak-patojen risk faktörlerinin belirlenmesine, tekrar eden tüberküloz olgularında tedavi rejiminin düzenlenmesinde, endojen reaktivasyon veya eksojen reenfeksiyon ayırımının yapılabilmesine imkan veren, klonal düzeyde yüksek ayırım gücüne sahip tekrarlanabilikleri ve sonuçlarının standardize edilmesi mümkün olan genotipik sürveyans yöntemlerinin kullanılması , DSÖ ve tüberküloz ile ilişkili uluslararası kuruluşların öngördüğü ve planladığı, 2020 yılından 2030'lu yıllara ertelenen "End TB" yani global eradikasyon stratejisine ulaşmada uygulanacak kontrol programlarının başarısının ön şartıdır . Moleküler sürveyans yani genom düzeyinde izlem günümüzde artan insan hareketleri ve TB hastası kadar suşların da toplum içerisindeki hareketinin (dinamiklerinin) klonal düzeyde izlenmesi ve takibini sağlayacak, TB epidemiyolojisi ve kontrol programları ile dirençli suşların moleküler değişimlerinin analizi, dominant genotiplerin orijini, direnç ile olan ilişkileri ve sirkülasyonu hakkında da doğruya en yakın bilgileri sağlayacaktır. Tüberküloz tüm dünyada özellikle düşük sosyo kültürel özelliklere sahip yoksul ve kapalı toplumlarda hala ciddi bir halk sağlığı problemidir. TB insidansı 8185/100.000 olan bir güneydoğu Brezilya cezaevindeki iki blokta mahkumlardan izole edilen Mycobacterium tuberculosis izolatlarının genotiplerinin tespit edildiği bir çalışmada, farklı hastalardan izole edilen suşların marker spesifik moleküler epidemiyolojik yöntemler ile filogenetik ilişkisinin kurulması, kapalı alan gibi toplu yaşanılan yerler veya bölgelerde aynı suşun farklı hastalardan izole edildiğini yani toplum içi bulaş olduğunu göstermiş ve suşların hareketinin izlenmesi ve TB artışını en aza indirebilmek için halk sağlığı ölçümleri hayati önem taşıdığını, moleküler epidemiyolojik yöntemler kullanılarak yapılan aile ve alt dal tanımlamaları M. tuberculosis genotip değişimlerinin ölçümlenebilmesi için son derece önemlidir . 3 M. tuberculosis kompleksinin tanımlanması ve genetik çeşitliliğinin belirlenmesi amacı ile birçok yöntem geliştirilmiş ancak bunlardan; Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) tanımlanması yani "spoligotipleme", insersiyon sekans 6110 Restriction Fragment Length Polymorphism (IS6110-RFLP), Region of Difference (RD) tekrarları ve Mycobacterial Interspersed Repetitive Units –Variable Number of Tandem Repeats (MIRU-VNTR) polimorfizmini baz alan yöntemler yaygın olarak kullanılmaktadır . MTBK tiplendirilmesinde ve epidemiyolojik araştırmalarında altın standart olan IS6110-RFLP metodu, zaman alıcı olması, yüksek standardizasyon gerektirmesi, laboratuvarlar arası suş profillerinin

karşılaştırılmasındaki zorluklar ve düşük sayılı veya hiç kopya içermeyen suşların ayrımındaki yetersizlikler sebebi ile yerini daha hızlı ve kolay uygulanabilen spoligotipleme ve MIRU-VNTR yöntemlerine bırakmıştır . Ülkemiz ve özellikle Çukurova bölgesi 2013 yılından beri komşu ülkelerde yaşanan iç karışıklıklar sebebi ile büyük ölçekli insan göç hareketlerine sahne olmuştur. Bu hareketlilik ile bölgeye mülteci olarak gelen insanlar, uzun süren karışıklık sebebi ile ülkelerindeki sağlık network'ünden ayrılmış, sağlık adına kontrolsüz bireylerden oluşmaktadır ve ülke ve bölgemize alışkın olmadığımız sağlık problemlerini taşıma potansiyeline sahiptir. Bu riskler arasında çeşitli ekzo ve endoparazitler, viruslar ve özellikle düşük hijyen ve beslenme şartlarında insanlar arasında kolaylıkla bulaşarak yayılan M. tuberculosis gibi bakteriyel kökenli enfeksiyonlar ilk akla gelenlerdir. Türkiye, coğrafik konumu itibariyle ÇİD-TB oranı yüksek olan ülkelerle komşuluk yapmaktadır. Dirençli tüberküloz sürveyans verilerine göre komşu ülkelerden gelenler arasında ÇİD-TB ve YİD-TB tanı ve tedavisi alan hastalar bulunmaktadır .

Klofazimin ve Bedaquiline arasında çapraz dirence sebep olan mutasyonların tespit edilmesi önemlidir. MDR-TB tedavi rejimininin kısaltılmasını hedefleyen STREAM çalışmalarında klofazimin, önemli bir antibiyotiktir ve bedaquiline ile birlikte kullanılmaktadır.

Biz bu çalışmada, Ç.Ü. Rektörlüğü THAUM bünyesinde yer alan T.C. Sağlık Bakanlığı Halk Sağlığı Kurumu Adana Bölge Tüberküloz Laboratuvarında akciğer tüberkülozlu hastaların klinik materyallerinden izole ettiğimiz Mycobacterium tuberculosis complex suşlarında Bedaquiline ve klofaziminin çapraz direncinden sorumlu, mmpl5, mmps5, pepQ ve Rv0678 genlerindeki mutasyonların varlığının araştırılmasını, suşlar arasında yaygın genotiplerin ve ilaç direnci ile arasındaki muhtemel ilişkinin tespitini amaçladık.

ACKNOWLEDGEMENT

Bu çalışmanın gerçekleştirilmesinde, 3 yıl boyunca değerli bilgilerini bizlerle paylaşan, tez çalışmamda planlanmasında, araştırılmasında, yürütülmesinde ve oluşumunda ilgi ve desteğini esirgemeyen, tecrübelerinden yararlandığım, yönlendirme ve bilgilendirmeleriyle çalışmamı bilimsel temeller ışığında şekillendiren, her sorun yaşadığımda yanına çekinmeden gidebildiğim, güler yüzünü ve samimiyetini benden esirgemeyen kullandığı her kelimenin hayatıma kattığı önemini asla unutmayacağım saygıdeğer hocam Prof. Dr. Fatih Köksal'a ve büyük bir ilgiyle bana faydalı olabilmek için elinden gelenden fazlasını sunan, tezime zaman ayırıp değerlendiren ve üniversite hayatımın bu son döneminde bana kattığı her bilgi için danışmanım Prof. Dr. Işıl VAR 'ya teşekkürü borç bilirim.

Teşekkürlerin az kalacağı diğer hocalarım başta Prof. Dr. Burhan ARIKAN olmak üzere bana 6 yıllık üniversite hayatım boyunca kazandırdıkları her şey için hepsine teşekkür ederim. Ayrıca sekanslama işlemleri sırasında yardımcı olan Prof. Dr. Ayşe SERİN'e teşekkürlerimi sunarım.

Çalışmam boyunca benden bir an olsun yardımlarını esirgemeyen abim ve ablam Hamdi ve Huda; çalışma süresince tüm zorlukları benimle göğüsleyen değerli arkadaşlarım Ali'ye ve Moc'a sonsuz teşekkürlerimi sunarım.

Beni bu günlere sevgi ve saygı kelimelerinin anlamlarını bilecek şekilde yetiştirerek getiren ve benden hiçbir zaman desteğini esirgemeyen bu hayattaki en büyük şansım olan Aileme sonsuz teşekkürler.

LIST OF TABLES

Table 3.1. Preparation of Bedaquiline stock solutions....58
Table 3.2. Preparation of Clofozamine stock solutions 59
Table 3.3. Primers....61
Table 3.4. PCR mix of genes. 61
Table 3.5. Rv0678 gene PCR conditions. 61
Table 3.6. PepQ gene PCR conditions. 62
Table 3.7. Rv1979c gene PCR conditions. 62
Table 3.8. PCR Amplification components 63
Table 3.9. Cycle Sequence Amplification Stages 63
Table 4.1. Clinical samples from which M. tuberculosis strains are isolated....70
Table 4.2. Sample Types....70
Table 4.3. MIC values of bedaquiline and clofazimine 73
Table 4.4. PepQ mutations....76
Table 4.5. Efflux Pump Gene mutations....81
Table 4.6. Spoligotyping results 85

LIST OF FIGURES

Figure 2.1. The causative agents of infectious diseases. 9
Figure 2.2. Detection of Mtb-specific DNA in a Peruvian mummy, approx. 1000A.C.....10
Figure 2.3. Robert Koch 10
Figure 2.4. Children vaccinated without serious side effects.....11
Figure 2.5. The five countries of Maghreb 13
Figure 2.6. *Mycobacterium* Phylogenetic Tree 14
Figure 2.7. Salient features of *Mycobacterium* genus.....15
Figure 2.8. Cell wall structures 16
Figure 2.9. Chromosomes of *M. tuberculosis* H37Rv. 17
Figure 2.10. Pathogenic Life Cycle of *M. tuberculosis* 20
Figure 2.11. Estimated incidence of MDR/RR-TB in 2021.....22
Figure 2.12. A positive microscopy results.....24
Figure 2.13. A- Mycobacterium Growth Indicator Tube of MGIT 960 system 26
Figure 2.14. B-The procedure of MGIT 960 system.....27
Figure 2.15. The 41 different species in the M. tuberculosis genome 29
Figure 2.16. The structure of the DR locus.....29
Figure 2.17. The DR locus containing 36 base pairs and spacer regions.....30
Figure 2.18. Overview of the spoligotyping method.....32
Figure 2.19. Spoligotyping results 33
Figure 2.20. Chronology of the discovery of anti-tuberculosis drugs.....35
Figure 2.21. Isoniazid chemical structure 37
Figure 2.22. Mode of activation and action of isoniazid.....38
Figure 2.23. Rifampin chemical structure.....39
Figure 2.24. Activation of PSA in POA.....40
Figure 2.25. Ethambutol chemical structure 40
Figure 2.26. Aminoglycosides chemical structures 42
Figure 2.27. Fluoroquinolones chemical structures 43
Figure 2.28. Photograph by Koen Andries and Jérôme Guillemont. 46
Figure 2.29. A-structure chimique de la bédaquiline , B- fumarate de bédaquiline.....47
Figure 2.30. Structure of ATP-synthase of a *Mycobacterium*.....48
Figure 2.31. MIC of bedaquiline for different mycobacterial species 49
Figure 2.32. The efflux pump MmpL5 50
Figure 2.33. 2022 Global New TB Drug Pipeline 51
Figure 4.1. Bedaquiline-Resistance in MDR-TB strain with software detection. 71
Figure 4.2. Clofazimine-Resistance in MDR-TB strain with software detection.....71

Figure 4.3. Correlation between CLZ and BED....72
Figure 4.4. PCR products of amplified Rv1979c gene....74
Figure 4.5. PCR products of amplifiedRv0678 gene....74
Figure 4.6. PCR products of amplified PepQ gene....75
Figure 4.7. Lollipop figure with all observed variants across the PepQ gene....79
Figure 4.8. Spoligotyping profiles....84
Figure 4.9. Distribution of Spoligotype Families....88

LIST OF SYMBOLS AND ABBREVIATONS

AIDS	: Acquiert Immune Deficient Syndrome
BCG	: Bacille Calmette-Guerin
Bp	: Base pair
°C	: Celsius Degree
DMSO	: Dimethyl sulfoxide
DNA	: Deoxy Ribonucleic Acid
DOTS	: Directly Observed Therapy Short course
EMB	: Ethambutol
ETH	: Ethionamide
G+C	: Guanine + Cytosine
HIV	: Human immunodeficiency
INH	: Isoniazid
LJ	: Lowenstein Jensen
MDR-TB	: Multidrug-resistant tuberculosis
MGIT	: *Mycobacteria* Growth Indicator Tube
MTB	: Mycobacterium tuberculosis
MTBC	: *Mycobacterium tuberculosis* complex
PCR	: Polymerase Chain Reaction
PZA	: Pyrazinamide
qPCR	: Real-time quantitative PCR
RIF	: Rifampin/Rifampicin
RR-TB	: Rifampicin-resistant tuberculosis
SNP	: Single nucleotide polymorphism
TB	: Tuberculosis
TE	: Tris/EDTA

1. INTRODUCTION

The first known cases of tuberculosis date back to the end of prehistory around -3500 BC. History teaches us that the oldest manifestations of the disease are due to bone tuberculosis observed on Egyptian mummies (Namouchi et al. ,2005). In addition, signs of pleural and parenchymal damage to organs such as the lungs were found. Indeed, the lungs were often removed before the mummifications to be preserved in canopic jars. It is a period during which the disease did not spare any class of the company; it affected the priests as well as the pharaohs as well as the people of ancient Egypt. Tuberculosis will continue to affect humans without interruption over the centuries. It is rampant in ancient Greece, where it is called "phthisis" (wasting away) to signify the long-term weight loss that characterizesthe disease (Brickner et al. ,2008).

The origin of the disease and its mode of transmission have not ceased to be discussed over the centuries and especially in the 19th century by various doctors and scientists. The hypothesis that tuberculosis was due to the presence of a tubercle was widely held until the implementation of evidence of the tubercle bacillus by Robert Koch in 1812. Thus, in 1803 Gaspard Laurent Bayle discovers that the fundamental lesion of tuberculosis is: the tuberculum (small round nodule located on the bones or the skin or, in the lungs). While René Laennec rather offers a unicast conception of tuberculosis, namely that the basic anatomical lesion goes through different stages: miliary, cru and encysted in 1812. This conception will be rigorously refuted by Rudolf Virchow, a German physician of the same period (Smith et al.,2015). The tubercle (solid initial lesion) and the cesium (pasty secondary lesion which will be eliminated by a natural conduit) are of a different nature. At that time, there were also other theories, such as the theory of a hereditary predisposition, reinforced by the observation of a large majority of family cases of the disease. In 1865, Jean-Antoine Villemin showed that tuberculosis was a disease infectious, contagious and therefore preventable. But he will be strongly opposed by powerful "anti-contagionist" lobbies who will go so far as to force him to renewhis experiences in his Val-de-Grace laboratory. It was therefore necessary to wait until 1882 for Robert Koch to demonstrate that the presence of the tubercle bacillus was at the origin of the development of the disease and not the tubercle (Van et al., 2022).

Robert Koch, to demonstrate the existence of the tubercle bacillus, first postulated a bacterial transmission disease, based on the work of colleagues and former students such as Loeffler and Gaffky who successively discovered the diphtheria bacillus for the first and the typhoid bacillus for the second. They had succeeded in culturing these bacteria and observing their development, thus proving their existence. Only, for tuberculosis, which was a much more dreaded and enigmatic infectious disease, Koch realized that the bacterium was resistant to conventional microbiological cultures (Dover et al.,2004). It was therefore necessary quite quickly to experiment with new cultivation methods. For this, Koch will first use Ehrlich methylene blue staining which will allow him to observe a small quantity of bacteria from tuberculous tissue (Badawy et al. ,2021). Then; he

will add a brown counterstain to it, in the hope of obtaining a better topographic contrast, but this rather allows him to observe a greater quantity of bacteria in the form of rods. He will eventually alkalize the methylene blue stain with potash in order to observe countless bacteria. This work by Koch will later influence the work of Hans Christian Gram, Franz Ziehl and Friedrich Nelsen, who will give their names to the corresponding microbiological stains, allowing the identification of mycobacteria (Zhang et al., 2013).

The observation of the bacillus now allows Robert Koch to validate the origin of the disease, since he always manages to highlight the presence of the bacillus in tuberculosis patients, unlike healthy subjects. Moreover, the presence of bacteria always precedes the formation of the tuber, which itself always precedes the classification (Lewis et al., 2008). For Koch, it is then time to move on to the ultimate stage, namely to inoculate the bacteria into humanbeings live namely animals to demonstrate that it is indeed the cause of the disease.

But this will prove more difficult than expected, since he still does not know that the culture of this bacterium is more difficult than that of many other bacteria. He had to test several culture media, different temperatures to find that the colonies grew after two weeks on clotted blood serum at 37-38°C (Zurenko et al. ,2001). Koch will then inoculate the bacteria from pure culture strain, to more than 217 healthyanimals, all of which will develop the disease. It's strong of these experiments that Robert Koch was able to present, on March 24, 1882 at the meeting of the society physiology of Berlin his work on tuberculosis, with the support of more than two hundred preparations microscopic, which then earnedhim international fame. Koch's bacillus belongs to the kingdom of bacteria, of the order of actinomycetal, to the family of mycobacteria, which includes the only genus Mycobacterium. Discovered by the German Robert Koch in 1882, its genome was fully sequenced in 1998 (Fortun et al. ,2005).

The incidence of tuberculosis is estimated at more than 8 million cases per year, with a lethality without treatment close to 25%. Over 95% of cases and deaths occur in the poorest countries, mostly among young adults. In France, tuberculosis is responsible for approximately 6,000 new cases and 700 preventable deaths each year (Koul et al. ,2004). Among these new cases, 3% worldwide and 0.6% in Francepresent a common initial resistance to rifampicin and isoniazid, associated with significant mortality. The treatment of tuberculosis disease (to be differentiated from latent tuberculosis infection, see physiopathology chapter) is based on the combination of anti-tuberculosis drugs (at least 3 initially in order to avoid the selection of resistant mutants) for at least 6 months. Rigorous adherence to treatment is necessary to ensure its effectiveness (Baykan et al. ,2022).

Insufficient duration or irregular treatment intake increases the risk of relapses and the appearance of resistance or multi-resistance to anti-tuberculosis drugs (Brown, 2017). The contagiousness of the condition, which only affects patients with pulmonary tuberculosis, decreases rapidly at the start of treatment but may require, in some cases, respiratory isolation measures

(hospitalization in a room only, wearing a mask, etc.). Declaration is mandatory with the DDASS for any tuberculosis disease and for tuberculosis infection before the age of 15. The diseaseis 100% covered by health insurance as part of long-term illnesses (ALD) (Brickner et al. ,2008).

Tuberculosis, largely controlled by vaccination in Western countries, is a particular problem in immunocompromised subjects and in subjects with AIDS in particular, with an increased susceptibility to the development of tuberculosis disease in a field of latent tuberculosis infection (Nam et al., 2009).

Similarly, the existence of a history of tuberculosis poses problems for the management of certain chronic rheumatological conditions, in particular inflammatory rheumatism, where it constitutes acontraindication to the use of immunomodulatory or immunosuppressive drugs, cases tuberculosis disease having in particular been observed in patients treated with anti-TNFa monoclonal antibodies (Pang et al. 2012). Tuberculosis results from an infection with a mycobacterium of the tuberculosis complex or Koch's bacillus, acquired through contact with an infectious subject. The contagiousness of tuberculosis is limited: transmission usually requires prolonged contact and depends on the contagiousness of the affected subject and the duration of contact. Only 10 to 30% of people in contact with a contagious subject will be infected. Once in the body, in 90% of cases, the tubercle bacillus remains in a quiescent state. It is the latent tuberculosis infection (ITL), non-contagious andresulting from a balance with the immune system, during which the infected subject is completely asymptomatic (Özer et al., 2020).

Tuberculosis disease only develops in about 10% of cases. The risk of development is major during the 2 years following infection (development period of 50% of cases of tuberculosis disease). It is increased in children and people with weakened immunity (Zurenko et al., 2001). Tuberculosis disease preferentially affects the lungs (pulmonary form) but can spread to other organs in its extra-pulmonary forms (lymph node, bone/joint, adrenal or meningeal involvement, for example). The general symptoms oftuberculosis are not specific. They can associate among others fever, weight loss, night sweats (Zhang et al., 2013). A cough, chest pain and sometimes homotopic sputum are classically found in pulmonary tuberculosis.Other symptoms may be encountered depending on the organs affected. Untreated, tuberculosis disease progresses and can lead to death. The solid culture medium is mainly represented by the Löwenstein-Jensen medium which is then no longer used for M. tuberculosis (Shetye and Franzblau, 2020). It is a medium with egg, potato starch, asparagine and other mineral elements that ensure the growth of mycobacteria to the requirements complex. The addition of malachite green serves to inhibit the cultivation of most other bacteria. The major drawback of this medium is the slow growth of bacterial colonies, which only appear after three to four weeks in the form of cauliflower, a yellow-beige coloring. In the liquid medium MGIT (Mycobacterial Growth Indicator Tube), a method is used whose manipulations can be manual or automated. In this case, the method used is called BacTec 960TB (this method replacesthe BacTec 460 in which radioactive products were used). The principle is based on the use of ruthenium salts

which fluoresce in violet light. Fluorescence increases with decreasing oxygen in the medium (Wright et al., 2017). The decrease in oxygen being itself a sign of multiplication bacteria in the medium. The obstacle to the expansion of this method is its high cost which limits its use in many countries. *M.tuberculosis* is a human pathogen. Human-to-human contamination occurs through the air, via theinhalation of aerosols of infective Plugged droplets (1 to 5 bacilli/droplet, knowing that a cough produces around 3,500 droplets and a sneeze produces around 40,000) (Witek et al. ,2017).

The droplets are able to hold for several hours in the atmosphere, which enhances contagiousness, the infectious dose being 10 bacilli. The greater the exposure to the source of contamination (intensity of cough, duration of exposure, etc.) the risk of contamination is great 16 (Witney et al., 2015). The droplets that carry the bacilli, once they are inhaled by the individual, will lodge in the pulmonaryalveoli, which therefore constitute the primary focus of the infection. In effect, it is a bacterium whose multiplication takes place in a very oxygenated environment, which therefore explains this preferential localization in the lungs. Once in the lungs, the bacilli tuberculous, will be phagocytosedby alveolar macrophages (Kaczmark et al., 2021).

Once phagocytosed, the bacilli will continue to multiply inside the macrophages themselves. This phenomenon will be responsible for the establishment of a pro-inflammatory reaction. Thereby, the body begins to recruit monocytes from nearby blood vessels, resulting in thus to the formation of the early granuloma which constitutes the main characteristic of the disease (Van et al. 2022). The granuloma consists of a nucleus of infected macrophages, surrounded by lymphocytes as well as other monocyte phagocytes. Over the following weeks, the more the granuloma matures, the more it develops aroundit a fibrous capsule which, envelops the nucleus of macrophages, excluding central lymphocytes. On the other hand, there are almost no more blood vessels near the macrophage nucleus, which thereforenow forms a necrosis called caseous, which contains quiescent bacilli (Vija et al., 2013).

Tuberculosis (TB) is a chronic and necrotic but treatable disease with high morbidity and mortality caused by species belonging to the Mycobacterium tuberculosis complex (MTBC) group. It is estimated that one quarter of the world population is infected with TB bacillus. Despite widespread vaccination practices and antituberculosis treatment regimens with proven effectiveness for nearly 50 years, according to the World Health Organization data, 10 million new tuberculosis cases are seen worldwide every year and approximately 1.5 million patients die due to tuberculosis (Beckert al. ,2012). According to these data, tuberculosis deaths are the first among the deaths due to infectious diseases ranks. In summary, although there has been a 3% decrease in tuberculosis mortality in the last 10 years, thanks to the advances in diagnosis, treatment and immunization studies, there is still an increasing incidence and, more importantly, clinical samples (Tiberi al. ,2017). The isolation of resistant strains at increasing rates has caused tuberculosis to continue to be an important threat to public health. Despite efforts to expand Directly Observed Treatment (DOT) efforts, clinicalexamples multi-drug resistant (MDR-MDR), which is at least resistant to isoniazid

and rifampicin among the major drugs used in the treatment of TB with increasing frequency; resistant (YID-XDR) M. tuberculosis strains have been started to be isolated (Thee et al. ,2016). MDR-MTB strains were isolated in 3.4% ofpatients diagnosed and treated for the first time, while secondary tuberculosis from patients with a diagnosis, that is, previously diagnosed and treated, clinical cure was achieved, but after a while, acute exacerbations due to reinfection or reactivation were observed (Tiberi et al. ,2017). This rate goes up to 18%. MDR-MTB among tuberculosis (RD-MTB) strains resistant to the most effective first-choice drug "Rifampicin", which was theoretically the least likely to develop resistance in the treatment of tuberculosis towards the end of the 1960s. The rate reaches up to 78% (Tao et al. ,2012).

The most important factor influencing TB's re-entry into the world's agenda is the increase in the rate of multidrug-resistant strains. The determination that the Beijing-W genotype, which is estimated to originate from the Beijing region of China and then spread to the whole world, has multi-drug resistance has led epidemiological studies to shift in this direction (Tahar et al. ,2010).

HIV infection has caused significant changes in the epidemiology of tuberculosis, such as the incidence of tuberculosis in younger patients in developed countries such as the USA, the increase in primary tuberculosis cases compared to reactivation, and the increase in the number of multi-drug resistant tuberculosis. After America and Africa, South East Asia and Ancient the coexistence of these two diseases has increased in the countries of the Soviet Union. It is estimated that approximately 1/3 of the world population is infected with tuberculosis bacillus as a result of PPD test positivity. It is expected that the number of patients will reach 50 million by 2015, three million of them will be associated with HIV infection and 30 million people will die because of this (Stanley, et al. ,2010).

The estimated number of patients with tuberculosis in the world in 2011 is millions. While the number of patients who died is 1.4 million, 430 thousand of them are also HIV positive (Stanley et al. 2010). According to the 2012 Global Tuberculosis Report of the World Health Organization, 17 thousand 388 in Turkey in 2009; While there were 16 thousand 551 registered patients in 2010, it is reported that 15,679 tuberculosis patients were included in the records in 2015. WHO uses the technical term "case finding rate" when estimating the number of patients with tuberculosis in countries, and this rate has been calculated as 85 percent for Turkey (Pym, 2016). In other words, WHO thinks that 15 percent of thepatients in Turkey in 2011 were not included in the registry. The diagnosis was made by seeing microbes in the sputum of 56 percent of patients with pulmonary tuberculosis. 8 of them are patientswho have been treated before. This rate has been decreasing over the years. There is a 92 percent treatment success rate in 2008, 91 percent in 2009 and 92 percent in 2010 in registered patients. Although the success of treatment in previously treated patients is lower than in new patients, it wasreported as 74 percent in 2008, 73 percent in 2009 and 68 percent in 2010 (Smith et al., 2015).

Our first objective is to improve the genetic bibliography on the mechanisms of resistance to antibiotics. This is why our work was based on the search for the mutations responsible for the cross-resistance between bedaquiline and clofazimine, and more particularly the gene variants of pump efflux. one cannot exclude the importance of phenotypic diagnosis through mics, and also the importance of diagnostic epidemiology. through the three parts we could build a table which develops the bibliography (Singh et al. ,2020).

2. LITERATURE REVIEW

2.1. Infectious Diseases

Infectious diseases include diseases caused by the penetration into the body of an infectious agent: bacteria, virus, parasite, pathogenic prion or fungus (Almeida et al.,2016; Gler , 2016; Smith et al. 2015). Infectious diseases include diseases caused by the penetration into the body of an infectious agent: bacteria, virus, parasite, pathogenic prion or fungus.

Infectious agents, commonly called "germs", are pathogenic microorganisms that contaminate the body and cause disease. These are most often bacteria, viruses, parasites or fungi. A special case: prions (Cohen , 2000).

Our body is made up of at least as many bacteria as human cells. Between 17,000 and 400,000 viruses enter our lungs every minute! Some viruses and bacteria are beneficial for our organism, such as the microorganisms of our intestinal flora, also called microbiota (Bruchfeld et al.,2015), which help us during digestion and increase our defense capacities. Most of the time, our cells coexist in perfect harmony with all these "microbes" (Nam et al., 2009).

2.1.1. Infectious agents:

Bacteria

Bacteria are microscopic organisms, a few thousandths of a millimeter (about 50 times thinner than a hair), composed of a single cell. They are present in all environments, from the bottom of the oceans to the tops of mountains. Bacteria have even survived 3 years in space, attached to the International Space Station (ISS)! In a favorable environment, bacteria proliferate very quickly. We have been using it since prehistoric times to make fermented foods like cheese and alcoholic beverages. The pharmaceutical and food industries also use bacteria, as well as certain pollution control techniques (Almeida et al.,2016).

Virus

Viruses, much smaller than bacteria, are "obligatory intracellular parasites": they lack the functioning of a cell they have infected to their advantage, where they will be able to multiply and produce viral proteins. As such, they are considered both inert outside a cell and alive inside an infected cell. Viruses are specific to a host, that is to say that they are specialized in the infection of either animal, plant or bacterial cells (viruses are then called bacteriophages). Crossing the species barrier (transition from animal to human) remains very rare. In humans, viruses are even generally specific to a tissue: thus, the influenza virus targets respiratory cells, HIV certain immune cells (Cohen, 2000).

Parasite (excluding fungi)

Parasites are living beings (animal or fungus) that live at the expense of another living being (host organism) to take their food from it. They can be microscopic, like the parasite that causes malaria, or much larger like worms and ticks. Some parasites live in harmony with their host (Bruchfeld et al.,2015). Others can cause disease: this is called parasitosis. Depending on the parasite, different tissues or organs can be affected: skin, intestines, liver, lungs, nervous system, etc. The parasites are transmitted by contact, with contaminated food, or through vectors (flies, mosquitoes) (Cohen et al. ,2000).

Fungi

Fungi are micro-organisms of plant origin which can cause different types of "mycoses", by multiplying on the skin, mucous membranes or skin appendages (nails, hair). The fungi responsible for mycosis are widespread in the environment and in the body (Bruchfeld et al.,2015). They are generally not harmful. A change in conditions (humidity, maceration, taking antibiotics or corticosteroids, weakening of the immune system, etc.) can promote their development and make them pathogenic, causing superficial or deep infections. The "black fungus" disease, a fataland very violent opportunistic disease particularly affects convalescents of Covid-19 in India (Dover et al. ,2004).

Fungi multiply by producing and releasing spores into the environment, which cause infections. Transmission is due to inoculation in the event of a skin lesion, inhalation or contact with an infected person or animal (Cohen, 2000).

Prion

Initially considered "unconventional viruses", prions are actually proteins devoid of genetic material. The prion protein, whose role is still poorly understood, is naturally present in the body. In neurons, the prion can become pathogenic by changing its three-dimensional structure: it is then able to cause the same structural change in neighboring prion proteins, with which it aggregates to form deposits which multiply inside and outside brain cells (Gler ,2016).

Prions are responsible for degenerative brain diseases such as Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy (mad cow disease) in cattle and humans, scrapie in sheep and goats, and chronic wasting disease in deer. Prion diseases are rare and fatal: there is currently no treatment. The challenges of research are to understand the behavior and impact of prions in order to develop specific treatments (Cohen et al. ,2000).

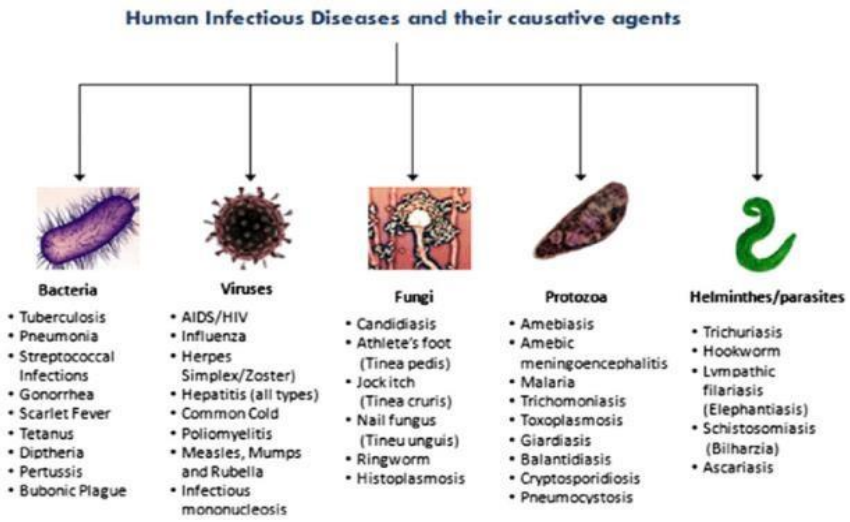


Figure 2.1. The causative agents of infectious diseases (Bruchfeld et al., 2015).

2.2. History of tuberculosis (TB)

The earliest discoveries of tuberculosis, which is believed to have existed throughout human history, date back to British Columbia. It was obtained by molecular microbiological examinations on mummies belonging to the Pharaoh period between 3300-2300 BC. Lesions due to Pott's disease It has been seen in mummies dating back to the 2400s (Daniel, 2006). Tuberculosis disease, it has been named variously throughout history due to the dramatic death tables he created on people. While tuberculosis, which Hippocrates defined as "melting, exhaustion", causes people to die by fading their colors, in various societies names such as "white death", "white plague", or "captain of death" are given, while in the Middle East and later in Europe it is called "King's Evil" (Elbek ,2015).



Figure 2.2. Detection of Mtb-specific DNA in a Peruvian mummy, approx. 1000 A.C (Campos et al., 2003)

Sanitariums were first established in the early 19th century for the treatment of disease. Patients were tempted to recover with good care and fresh air, but it was understood that only mild cases were treated with this method (Daniel, 2006). During these years, it is known that infectious diseases are caused by microorganisms.

Dr. Robert Koch, who believed that tuberculosis was not an inherited disease, showed the bacillus which he isolated from the lung lesion of a patient who died of tuberculosis in 1882, and gave the bacterium the name that we use today. the bacillus in culture, develops staining techniques to examine it under the microscope, infects laboratory animals and observes that the image of tuberculosis is formed. In the same year, the acid-fast staining method, which is still used today, was developed by Franz Ziehl and Friedrich Nelsen (Baykan et al., 2022). Robert Koch (by courtesy of the Wellcome Trustees). In 1880 he was appointed to theKaiserliche Gesundheitsamt (Imperial Health Office) in Berlin (Figure 2.3).



Fig 1 *Robert Koch (by courtesy of the Wellcome Trustees).*

Figure 2.3. Robert Koch (Almeida et al., 2016).

The tuberculin skin test (PPD: Purified Protein Derivative = purified protein derivative), was developed by Von Purged in 1907 to determine whether a person is infected with the TB bacillus. With this test, the development of delayed-type hypersensitivity reaction in infected individuals is detected by subcutaneous administration of antigenic components of TB bacillus (Baykan et al. ,2022).

In the 1920s, two researchers named Albert Calmette and Camille Guerin BCG (bacilli Calmette Guérin), a licensed TB vaccine still in use today, was developed by attenuating the *Mycobacterium bovis* strain with serial passages (Daniel, 2006). While this vaccine is not applied in the routine vaccination calendar in the United States (USA) and some European countries, it is also available in Turkey. It is included in the infancy vaccination program in most countries around the world (Brown, 2017). Between 1924-1928, 114.000 children vaccinated without serious side effects (Figure 2.4.)



Figure 2.5. Children vaccinated without serious side effects.

Streptomycin (STR) was the first drug used in the treatment of disease used in 1944 discovered (Tiberi et al., 2017). In the same year, with the detection of high oxygen consumption by the tubercle bacillus, it was thought that para-aminosalicylic acid (PAS) would be effective against mycobacteria. Successful treatment began to be achieved with its use together. In 1952, isoniazid (INH) with its bactericidal activity and pyrazinamide acting on a dormant bacillus (PZA), were discovered and putted into use (Stanley et al., 2010).

In the 1960s, ethambutol (EMB) was discovered, replaced PAS because it was better tolerated by patients. With the discovery of rifampicin (RIF), which is still used today as the most effective drug in the treatment of tuberculosis, in 1970 the important successes have been achieved. While the duration of treatment with multidrug therapy was reduced from 24 months to 9 months, the discovery of this drug also caused the closure of most sanatoriums in the world (Dover, 2004). A decrease in cases of tuberculosis, during these periods, was reported. But the prevalence of HIV cases after 1985, the increase in immunosuppressive treatments and the disruption of health screening programs, these reasons make the number of TB cases increased again (Mdluli et al., 2015).

2.2.1. Brief TB History: The five countries of Maghreb

The Maghreb, baptized "Jazirat El Maghreb", literally the sunset island by conquerors from nearby East in the 7th century, is bordered by the Mediterranean Sea to the east and north (90% of the coastline), to the west by the ocean Atlantic, and to the south by the Sahelian strip (Tao et al.,2012). Five countries constitute it, bound by the dominant culture and religion (Islam for almost all populations), integrated since 1989 within a regional union.It spans a territory of 5.6 million km2 of which the Sahara occupies the major part. The population,100 million inhabitants including 80 for Algeria and Morocco, is concentrated in the coastal areas. Mauritania is one of the least densely populated in the world (3 inhabitantsper km^2) (Singh et al. ,2020).

In Tunisia, the revival of the fight against tuberculosis, in 1959, was piloted by the National Institute of Pneumonia photobiology, Professor B. El Gharbi, director of the establishment from 1964 to 1985, was, with doctor L. Azzouz, the engine. The relay was gradually taken by the relevant departments (departments in charge of prevention) of the Ministry of Health. The institute, which became in the meantime specialized university hospital, was responsible for supervise the care and follow-up of patients, within the framework a mission of expertise, advice and training (Tahar and Rachid ,2010).

In Algeria, independent from 1966, the program, whose development about tuberculosis has been hampered by instability and troubled situation that characterized the 1990s, was relaunched in 2001, monitoring is provided by management of prevention jointly with the National Institute of public health, the in-depth assessment process and review of the strategy, to which are associated experts (in 2011 for example, P. Chaulet, D. Larbaoui, F.Boulahbal, pioneers in the fight against tuberculosis) is renewed every 5 or 6 years (Aslan and Yapıcı , 2022).

In Morocco, the first program dates from the end of the 1970s. Forty years later, progress having deemed unsatisfactory, a so-called “acceleration of reducing the incidence”, covering the period 2013-2016 was set up. In 2017, the procedure for the development of a national strategic plan has been initiated, the goal being, in the long term, the elimination oftuberculosis by as a health problem (1 case per million inhabitants) by 2050. In Mauritania,the 2017-2020 health plan lists the targets in the fight against tuberculosis, the objective wasin 2012, to lower the prevalence to 25 per 100,000 at maturity of 2020 (Tahar and Rachid, 2010; Haas and Belknap, 2019).



Figure 2.6. The five countries of Maghreb

2.2.2. Brief TB History: Turkey Between 1923-1950

Tuberculosis, which is one of the disasters that devastates mankind, is also known as a "ince hastalık" among people (Almatar et al., 2019). In the early years of the Republic, the fight against tuberculosis as part of the fight against epidemics continued for a long time with the activities of voluntary associations due to the limited financial possibilities. The İzmir Tuberculosis Society, founded in 1923, and the Istanbul Tuberculosis Association, founded in 1927, have assumed great responsibility in guiding and helping the public. Dispensaries and sanatoria are at the forefront in the diagnosis and treatment of tuberculosis patients. Although the limited bed capacities of sanatoriums fell far short of the needs, especially at the point of treatment, in the early period attempts were made to increase bed capacities over time. In 1930, tuberculosis was included on the list of compulsory diseases with the public health law II. After the rate of spread of tuberculosis increased during World War II, a period of systematic tuberculosis control began in 1945. The B.C.G, a vaccine began to be applied more as of 1947 (Cohen ,2000). Again in 1948, the Turkish National Tuberculosis Association was established. In 1949, the 1st Tuberculosis Advisory Committee met and determined the plan to be carried out in the fight against tuberculosis, and then the Tuberculosis War Law No. 5368 was passed in 1949 (Elbek ,2015). With the aforementioned law, the allocation by the State of an allocation of 30 million Turkish lira for the fight against tuberculosis over a period of ten years was a sign that the fight against tuberculosis would make great progress. Propaganda activities aimed at raising public awareness continued with the efforts of tuberculosis societies until 1948, after which the organization was undertaken by the Turkish National Tuberculosis Association (Baykan, et al., 2022).

2.3. *Mycobacteria*

2.3.1. Taxonomy

Mycobacteria are the only genus in the family *Mycobacteriaceae*, which is part of the *Actinomycetales* team (Parvez et al., 2017). As of 2019, more than 200 species have been defined in the genus *Mycobacterium* (Eluk et al., 2021).

Among these species, pathogens, opportunists, there are pathogenic and non- pathogenic mycobacteria (saprophytes). Pathogenic mycobacteria are divided into two as agents of tuberculosis and leprosy. The mycobacteria responsible for tuberculosis are classified in the *M. tuberculosis* complex. The species included in this group are *M.tuberculosis, M.bovis, M.bovis BCG, M.caprae, M.canetti, M.microti, M.africanum and M.pinnipe* (Fogel , 2015). *M. leprosy*, which causes leprosy, is another type of pathogenic mycobacterium. Mycobacteria that can be opportunistic pathogens or saprophytes are called nontuberculous mycobacteria (TBDM) or atypical mycobacteria (Fortun et al. ,2005).

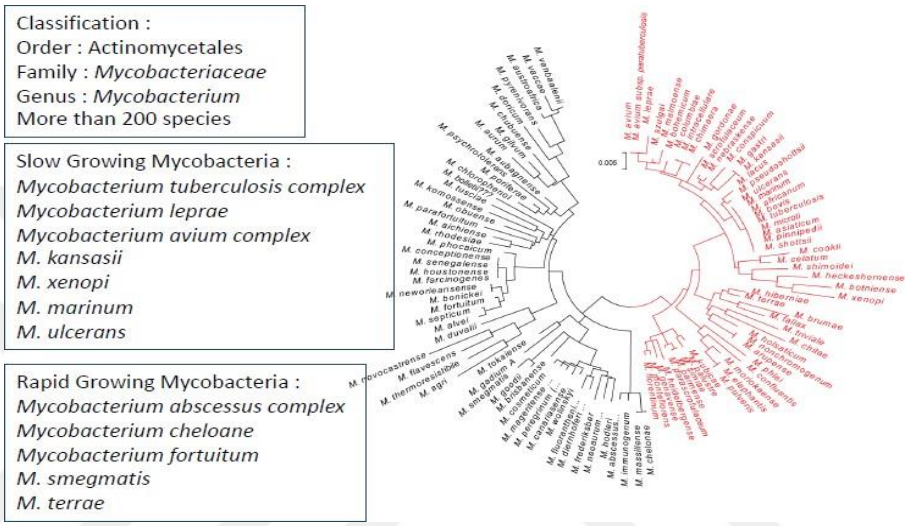


Figure 2.7. *Mycobacterium* Phylogenetic Tree

2.3.2. Salient features of *Mycobacterium* genus

Mycobacteria are widely present in nature almost everywhere, abundant in water, soil and animals. Obligate aerobes are intracellular bacteria in the form of nonmotile, nonencapsulated, spore less, thin, weakly gram-positive, flat or slightly curved bacilli (Özer et al., 2020). The bacilli are 0.2–0.6 µm wide, 1–10 µm long and reproduce slowly dividing every 18 hours on average. Therefore, they can be grown for upto 6-8 weeks (Smith et al., 2015).

Cell walls are rich in lipids. For this reason, they are not colored with aniline dyes like gram stains, and they also show resistance to alcohol, acid, alkali and dry environment. Acid-resistant Rhod coccus and Nocardia, they are more intensely colored (Thee et al. ,2016). Mycobacteria grow in Löwenstein-Jensen (LJ) medium, which is a selective medium, at 37°C, pH 7.0 ± 0.2 and 3 to 11% CO2 on average for 4 to 6 weeks (Figure 2.2.a) (Özer et al., 2020). In MGIT 960 broth, they reproduce on average 7 to 42. Tuberculosis bacilli are very resistant to adverse conditions and can survive for weeks at +4°C and for many years at -70°C. they can be examined under a light microscopeby staining with Ehrlich Ziehl-Nelsen (EZN) acid-fast staining methods or Kenyon'smethod, or by staining with fluorescent dyes with a fluorescence microscope. In the EZN method, mycobacteria cannot be destined with 3% acid alcohol. For this reason, they are also called

acid-fast bacilli (ARB) (Pang et al. ,2012).

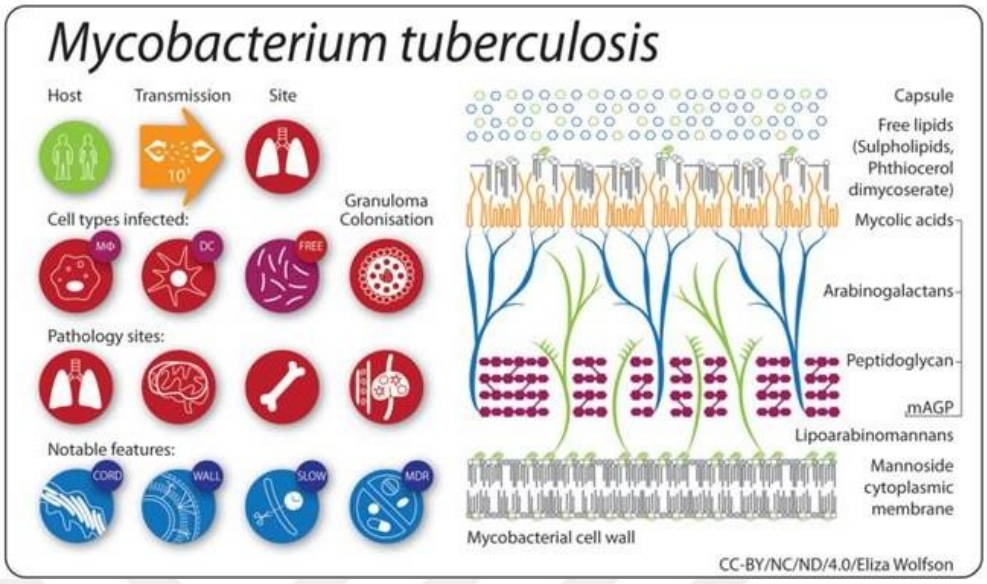


Figure 2.8. Salient features of *Mycobacterium* genus

2.3.3. Cell wall

Except for the lipid layer, the cell wall structures of mycobacteria are similar to those of gram-positive bacteria. They have a thick layer of peptidoglycan above the cell membrane and lack an outer membrane. The peptidoglycan layer gives shape and strength to bacteria. On this layer, there is another polysaccharide layer called arabinogalactan. Arabinogalactan allows mycobacteria to survive in macrophages (Nguyen et al. ,2016). Mycolic acid, which accounts for 11% of the total amount of lipids in the cell wall, is attached to the arabinogalactan layer and is a structure consisting of long branched fatty acids with 70-90 carbons extending towards the exterior (Nam et al., 2019). Mycolic acids give mycobacteria most of their characteristics such as acid and dye resistance, slow growth, resistance to detergents, most antibiotics and host immune response, and antigenicity (Rocha et al. ,2021). The outer cell wall contains proteins such as lipoarabinomannan, lipomannan, sulfolipids, glycolipids and peptidoglycolipids and phosphatidylinositol mannoside. The 15% transport proteins and porins of the cell wall, are biologically important antigens that stimulate the human cellular immune response (Stanley et al. 2010).

Antigens 5, 6, and 60, which are soluble antigens found in tuberculosis, are used in the serological diagnosis of tuberculosis (Pang et al. ,2012).

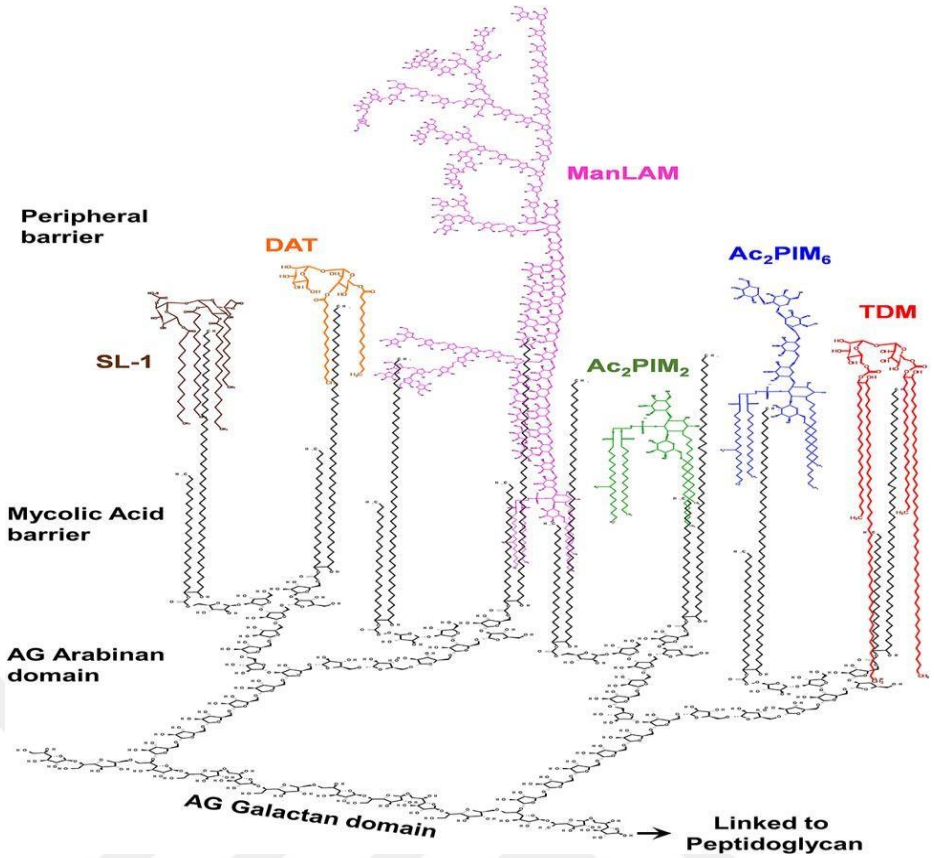


Figure 2.9. Cell wall structures

2.3.4. The genome of *M. tuberculosis*

The genome of *M. tuberculosis* consists of 4,411,532 base pairs (Figure 2.9). Its genome encoding approximately 4,000 genes contains 61 to 71% guanine and cytosine. With the high gene coding capacity of *M. tuberculosis,* various enzymes used in lipogenesis and lipolysis are produced. This free-radical property distinguishes M. tuberculosis from other bacteria (Prasad et al., 2021).

In the *M. tuberculosis* genome, drug resistance develops through spontaneous point mutations. Lateral gene transfer is not observed in mycobacteria. Therefore, the genes responsible for drug resistance can be fully identified (Donald , 2010). Thus, resistance to most anti-tuberculosis drugscan be determined by various PCR (Polymerase Chain Reaction) methods, it is not possible to detecta large gene region with a large number of scattered point mutations, such as in pyrazinamide resistance, with a single PCR. Another important issue concerns the unknown mutations that strains of M. tuberculosis can develop when exposed to newly discovered drugs. With WGS analyses, all these mutations are easily detected. Additionally, phylogenetic analyzes of *M.tuberculosis* strains can be performed with NGS technology and their origins can be determined (Nagiec , 2005). According to phylogenetic analyses, the bacteria of the TB complex are classified into 6 different lineages according to the geographical regions from which they originate. *M.canetti* isolates are classified into a single distinct group (Mirnejad et al., 2018).

Although the data on the analysis of the origin of the *M. tuberculosis* strains isolated in Turkey is limited, the *M. tuberculosis* strains isolated in the studies are the 4th lineage that spread from Africa to Europe then to America because Turkey is on the migration route. NGS technology and WGS analysis are used as the most advanced technology today to illuminate curious questions about phylogenetic lineages. WGS analyzes of the rather large *M. tuberculosis* genome can only be studied on the Novaseq 6000 NGS device (Rocha et al., 2021).

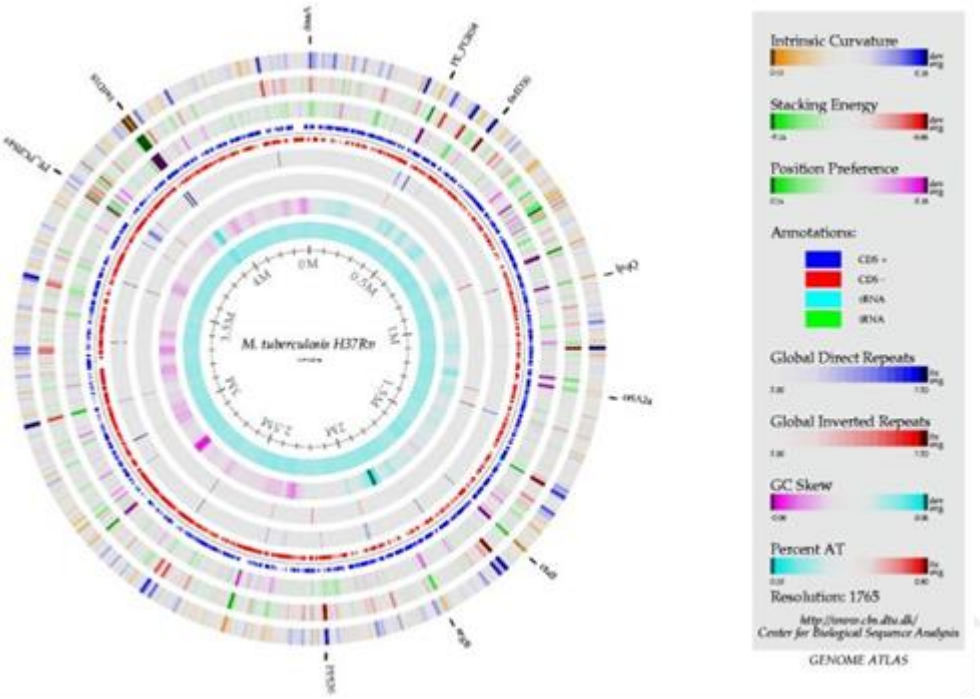


Figure 2.10. Chromosomes of *M. tuberculosis* H37Rv.

2.3.5. Virulence factors

Mycolic acids, which are responsible for cell wall thickness, bind to trehalose sugars (trehalose-6,6′-dimicolate) to form cord factor, which is the most important virulence factor in mycobacteria. On microscopic examination, the bacilli appear as parallel cords (Pang et al. ,2012). Cord factor is formed by virulent strains. stumps cannot form. Cord factor prevents leukocyte migration, causes the formation of chronic granulomas and may act as an immunological adjuvant. Mycobacteria do not contain endotoxins in their cell walls and they do not have exotoxins (Eluk et al., 2021). In the cytoplasm by inhibiting phago-lysosome fusion within macrophages, they multiply. Depending on the intense immune response, they cause local tissue damage and cause cavities in the lungs. They show systemic effects mediated by cytokines in the host body. As a result, the patient experiences fever and weight loss. Certain mycobacterial antigensare recognized as virulence factors (Singh et al., 2020). Lipoarabinomannan stimulates cytokines and superoxide

dismutase keeps them alive in macrophages. Other structures responsible for the virulence of the bacterium are arabinogalactan, phosphatidyl inositol mannoside, muramildipeptide, sulfolipids and phosphatide (Ndjeka et al., 2015).

2.4. TB disease:

Tuberculosis is a droplet caused by coughing or sneezing from person to person.

It is transmitted by inhalation or ingestion of the seeds from the air, by contact with the patient or simply by talking to the patient (Eluk et al., 2021). It is a systemic disease that can affect all organs and systems of the human body, especially the lungs (Fogel et al., 2015). The infecting dose of the bacterium is very low and 1 to 3 bacilli carried by a few droplet nuclei can cause tuberculosis. Smear-positive patients, patients with a bacilli load of at least 104 bacilli/ml in sputum, adult patients with cavitary lesions, and crowded and poorly ventilated rooms are factors that increase the risk of transmission (Jia et al., 2022). The pathogenesis of tuberculosis includes 4 stages (Rodriguez et al., 2002)

Stage 1: encounter with the tuberculosis bacillus

Stage 2: Proliferation and spread

Stage 3: Development of cellular immunity

Stage 4: Liquefaction, cavitation, proliferation of bacilli and endobronchial dissemination

Stage 1: After exposure to the tuberculosis bacillus, 30% of the bacilli are eliminated mechanically from the respiratory tract or by immune cells. Of the 70% of tubercle bacilli that can reach the lungs by inhalation, some are phagocytosed by alveolar macrophages in the lower and middle areas of the lungs, near the pleura. By inhibiting the early endosomal autoantigen 1 (EEA1) of virulent macrophage bacilli, it prevents phagosome- lysosome fusion (Loxton and Van Rensburg, 2021). Macrophages cannot kill the bacilli continue to multiply inside the macrophages. The destruction of mycobacteria by macrophages during this perioddepends on the virulence of the ingested mycobacteria and the microbicidal capacity of the host macrophages. such as cord factor and sulfolipids. When bacilli with virulence-increasing properties encounter inactivated macrophages, macrophage destruction occurs more rapidly. First, mycobacteria, which can escape intracellular destruction, proliferate and cause macrophage degradation (Singh et al., 2020).

Stage 2: In this stage, which covers the first 2-3 weeks after bacillus ingestion, monocytes and lymphocytes are drawn from the circulation by the host chemotactic factors to the region where the bacilli are located. Monocytes arriving in the area turn into macrophages; they phagocytize the bacilli more easily but cannot destroy them. bacilli at this stage proliferate by increasing logarithmically within macrophages. As a result of overgrowth of bacilli, macrophages are disintegrated and the bacilli enter the systemic circulation via the lymphatics (Gopalaswamy et al., 2020). By lymphohematogenous route, the bacilli spread to the kidneys, epiphyses, vertebrae and

meninges by mixing with both the adjacent lymph nodes and the blood in the lower parts of the lungs and regionally. Inflammation occurs in the lymph nodes and granulomas surrounded by fibrin form. Thus, primary infection develops (Gler et al., 2016).

Stage 3: At this stage, the immunological protection mechanism is activated. 2-4 weeks after the onset of infection, macrophages begin to synthesize and secrete interleukin-1 (IL-1). IL-1 plays a role in the activation of T lymphocytes, which are determinants of TB immunity. Activated T lymphocytes secrete IL-2 when they encounter TB bacillus antigen, allowing CD4+ cells to proliferate. CD4+ T helper 1 cells activate macrophage by secreting interferon-γ. Cytokines secreted from activated macrophages and lymphocytes provide the formation of cellular immune response (See Figure 2.4.1) (Haas et al., 2019). Thus, T helper cells gain the ability to activate macrophages and kill bacilli, and logarithmic proliferation of bacilli stops. Extracellular growth of mycobacteria is inhibited and bacilli becomes dormant. At this stage, granuloma formation develops under the influence of TNF-α, IL-6, IL-8, IL-12 and a specific lesion called 'tubercle' occurs. The purpose of tuberculin, which consists of activated macrophages, epithelioid histiocytes and lymphocytes, is to limit the bacilli and prevent their proliferation and spread. At the end of 3-8 weeks after the bacillus enters the host, cellular immunity and late hypersensitivity reaction develops (Bruchfeld et al., 2015).

At this stage, the tuberculin test, PPD, is positive. This is the most important diagnostic finding of primary TB infection (Brown et al., 2017; Faksri et al., 2019).

Stage 4: Secondary infection, TB reactivation (relapse), or TB

Although it is called reinfection, it is a form of TB that is seen again in individuals who have had TB before. It is also called reactivation tuberculosis because the most common form is the reactivation of endogenous bacilli (See Figure 2.10) (Hameed et al., 2018).

It occurs as a result of the re-activation of dormant bacilli that settle in the lungs during the primary infection period. Secondary infection occurs in the upper lobes of the lungs, where oxygen is abundant. Typical in secondary tuberculosis liquefaction and cavity formation are observed. Here, the bacilli proliferate in the cavity (Bruchfeld et al., 2015). If the lesions, also called classification necrosis, coincide with a bronchus, the bronchial wall is ruptured and all the bacilli in the cavity are transferred to the bronchus. Thus, the patient exhales billions of bacilli into the air. The patient develops difficulty in breathing due to the cavity. When the capillaries and blood vessels in the area where the cavity is formed are damaged, the patient begins to produce bloody sputum. At this stage, the PPD test may be negative in 20% of the patients (Gigli et al., 2017). Exogenous reinfection such as endogenous reactivation of TB may occur in the presence of diabetes, renal failure, malignancy, corticosteroid use and immunosuppressive treatment, silicosis, and HIV- lowering conditions. There may be exogenous transmission with resistant bacilli (Lewis , 2008).

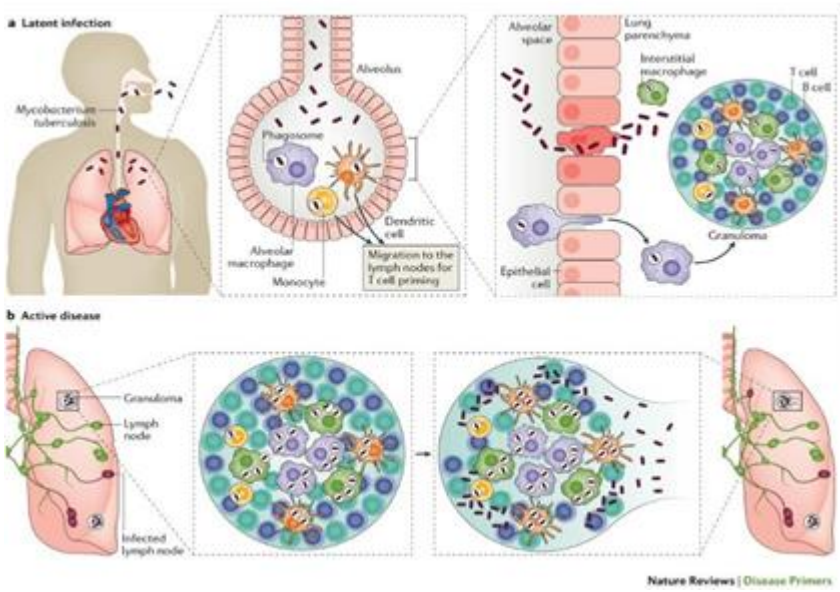


Figure 2.11. Pathogenic Life Cycle of *M. tuberculosis*

2.5. Epidemiology

All treatments against tuberculosis known to exist throughout human history. Despite diagnosis, treatment and prevention programs, TB still kills people today, seen as an important infectious disease. In the list of deaths caused by a single infectious agent worldwide, TB ranks first above HIV/AIDS and is among the top 10 most common causes of death (Bagchi et al., 2023). To control the spread of tuberculosis and ensure its eradication, despite the TB control programs (End TB, Stop TB) applied for the purpose of TB, ¼ of the world's population is still infected with TB (De Majumdar et al., 2019). TB is most common in Africa (72%), India (27%) and China (9%). The main reasons for the increase in TB incidence in Asia and Africa are poverty, increase in the number of homeless people, HIV positivity and TB drug resistance (Daniel et al., 2006; Bagchi et al., 2023).

Western Europe and North America have seen a dramatic decrease in the incidence of TB over the past 30 years. TB cases had decreased thanks to good nutrition and living conditions and preventive practices such as vaccination in developed countries (Witney et al., 2015). However, recently this decrease has reached a plateau stage and TB cases have started to increase again in some developed countries. TB continues to be an important problem in developed countries, especially due to the increase in the number of African and Asian immigrants, drug and alcohol addicts, and HIV-infected individuals (Mardassi et al., 2005). As a matter of fact, according to the WHO 2022 Global TB report, there were approximately 10 million TB patients in total, with 6.4 million newly diagnosed worldwide in 2017, 5.8 million of the patients were men, 3.2 million of them were women and 1 million were children (Parvez et al., 2017).

It is stated that 1.3 million of HIV-negative patients and 300,000 of HIV-positive patients lost their lives due to TB. While the activation rate of tuberculosis is 10% per year in HIV-infected individuals, the activation rate in normal individuals is 10% throughout life. The main reason for this situation is that cellular immunity is the most effective mechanism in defense against tuberculosis. HIV is active as it increases the likelihood of latent tuberculosis being reactivated causes severe tuberculosis. For this reason, it is reported that one out of 4 people infected withHIV died due to TB. 72% of HIV-positive TB cases are African (Bagchi et al., 2023). HIV positivity among TB patients in Turkey, its incidence is 0.12 per 100,000 (Elbek et al., 2015). According to current data on TB, which continues to be an important public health problem in Turkeyas well as all over the world, a population of 80 million in 2016 (Hsu et al., 2020).

While 12,417 TB patients were registered in Turkey, a total of 12,046 TB patients were registered in 2017. In 2016 and 2017, the incidence of TB in Turkey was 15.3 and 14.6 per hundred thousand, respectively. Although this rate has decreased by 14.8 per 100,000 since 2005, the spread of the disease, especially among the young population (Jia et al., 2022).

One of the most important problems encountered in the treatment of TB is the development of drug resistance. RD or MDR-TB (resistant to rifampicin and isoniazid) is seen in 3.5% of new TB cases and 18% of patients who have received previous TB treatment worldwide. Among TB patients in Turkey in 2019, 191 cases MDR-TB is seen. Today, epidemiological data is needed in order to implement more effective prevention and control programs against tuberculosis (Loxton et al., 2021).

The frequency of occurrence and transmission routes of different species and strains found in the M. tuberculosis complex in different geographical regions can only be determined by molecular studies. Prevention and prevention with epidemiological data obtained from molecular analyzes on their own *M. tuberculosis* strains from each country success of control programs can be measured (Gopalaswamy et al., 2020).

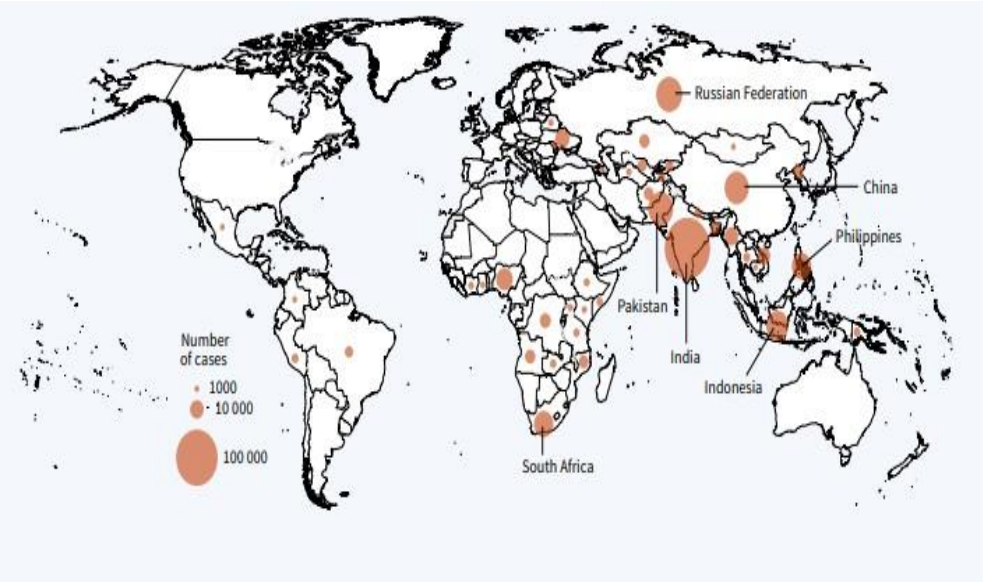


Figure 2.12. Estimated incidence of MDR/RR-TB in 2021

Despite the availability of anti-tuberculosis therapy, the increasing incidence of TB and the emergence of anti-tuberculosis drug-resistant strains; first to the dissemination and triad of DGT and concurrently to MDR-TB patients with TB/HIV co-infection and the Stop TB control strategy targeting patients with XDR-TB, that is, patients in the high-risk group (Haas et al., 2019). The primary goal of Stop TB was to stop the increase in TB incidence in 2015, to reduce the TB prevalence and incidence rates determined in 1990 by 50%, and finally to completely eradicate TB by 2035 (Singh et al., 2020). However, with the success criteria achieved over time remaining below the target, "elimination of tuberculosis", which means reducing tuberculosis to <1 case in a million population, was postponed to 2050, and the name of the program or new target "EndTB" was transformed into a global eradication strategy (Özer et al., 2020).

Intense human movements and socioeconomic problems due to wars, trade, tourism and immigration in the last thirty years have resulted in the diagnosis and diagnosis of tuberculosis. caused disruptions in control programs and especially with the emergence of the HIV/AIDS epidemic, it caused an unusual increase in the incidence of tuberculosis in many countries (Figure 2.11) (Tahar et al.,2010).

In the WHO 2019 Global Tuberculosis report; The estimated incidence of TB disease in our country is 18 per hundred thousand and the estimated mortality rate is 0.62 per hundred thousand. According to the data of the TB Department, the total number of patients diagnosed with TB in 2017 was 12,046, although 92.2% of them were new cases, 7.8% were previously treated cases, 42.3% of the diagnosed patients were women, 57.7% were women. It has also been reported that they are male. In the report, 66.1% of the cases were pulmonary. It was stated that 33.9% of them were patients with tuberculosis and non-pulmonary organ involvement (Loxton et al., 2021). On the other hand, according to the Ministry of Health Public Health Agency report, which shows the success of control programs in our country, the number of registered patients, which was 20,535

(29.4/100,000) in 2005, decreased to 11,786 (16/100,000) in 2018, has been reported. In addition to these results, it was determined that the incidence of diagnosed TB patients decreased by an average of 5% annually in the last 10 years (Figure 2.11) (Li et al., 2021).

In these reports, treatment success was observed in 85.4% of TB cases diagnosed and treated in 2016, while this rate was recorded as 67.2% in previously treated cases and 76.4% in those born in foreign countries. across our country. In 2017, 191 patients were diagnosed with MDR-TB. In line with WHO recommendations, our country also aims to achieve the objectives of the "End TB" Strategy (Lewis et al., 2008).

2.6. Tuberculosis Diagnosis

Although the disease has many symptoms, its definitive diagnosis is bacteriological, and it is diagnosed by culturing M. tuberculosis (Singh et al., 2020). Diagnosis is made when acid- fast bacilli are seen in the microscopic examination of the clinical sample and the bacteria are grown in culture. In addition to microbiological diagnosis, clinical symptoms and radiological imaging also support the diagnosis. In addition, immunology-based tuberculin skin test, interferon gamma release tests and molecular methods are also used in the diagnosis of TB (Prasad, et al. ,2021). It is recommended that TB culture be studied in biosafety level 3 laboratories, in class 1 or 2 biosafety cabinets, wearing a protective apron with N95/FFP2 mask or positive pressure respirator (Zhang et al., 2013).

2.6.1. Collection and Processing of Clinical Specimens

The type of sample taken for the diagnosis of TB depends on the organ affected by the disease. For this purpose, sterile and non-sterile samples are examined. While TB culture is performed by centrifuging sterile clinical samples such as CSF only, it is observed that the microbiota members in non-sterile clinical samples such as sputum are grown in culture during the long incubation period required for the growth of *M. tuberculosis*. Clinical sample should be processed before culture for suppression (Tahar et al., 2010). Although there are many commercial kits designed for this purpose, concentrating TB bacilli with a refrigerated centrifuge recommended by the Clinical and Laboratory Standards Institute (CLSI), which is widely used around the world, N-acetyl-L- cysteine-sodium. It is the process of homogenization of sputum with hydroxide and decontamination of the sample with NaOH and phosphate buffer. The processing of samples is the most important step affecting microscopic examination and culture sensitivity (Zimenkov et al.,2017).

2.6.2. Microscopic Examination

Microscopic examination of clinical specimens in the diagnosis of tuberculosis is the oldest method used in diagnosis and continues to be widely used throughout the world as a simple, rapid,

inexpensive and specific method which still constitutes the first step in diagnosis. The sensitivity of microscopic examination varies between 20 and 80%, depending on the different staining methods applied and the type of sample (Koh et al., 2009). Three sputum samples are collected from patients with suspected miliary TB. The ideal is to examine the first sputum collected in the morning for three days, because the bacilli are better highlighted in the sputum accumulated in the bronchi during the night. The amount of sputum should be 3-5 ml. In patients who cannot produce sputum, induced sputum or fasting gastric juice is recommended and examined three times. If a sample cannot be collected in this manner, bronchoalveolar lavage fluid can be used for any purpose (Manjunatha et al.,2006).

Microscopy plays an important role in detecting new cases of tuberculosis. For ARB to be visible under microscopic examination, there must be at least 5,000 to 10,000 bacteria per milliliter of sample. There are no special laboratory conditions necessary for the application of culture processes, which are the gold standard diagnostic method in the diagnosis of tuberculosis, but tuberculosis is common (Zhang et al., 2013).

In low-income countries, microscopy is the most important diagnostic tool. In addition, a positive microscopy result is very valuable due to the long culture time of *M. tuberculosis*. For evaluation in microscopy, at least 300 microscope fields must be scanned. The absence of bacilli in the evaluated area is considered negative. Presence of a few bacilli is suspicious, 1-9 bacilli in 100 areas (+), 1-9 bacilli in 10 areas (++), 1-9 bacilli in each area (+++), 10 or more bacilli in every area (+) +++) is reported as (Bagcchi et al, 2023).

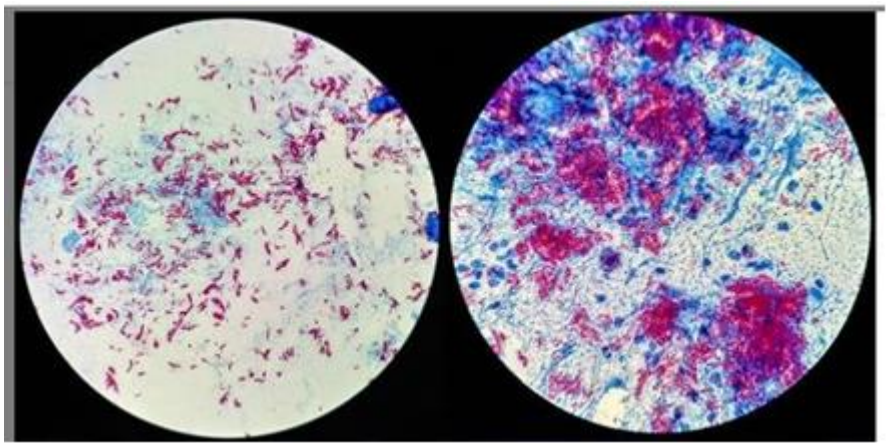


Figure 2.13. A positive microscopy results

2.6.3. TB Identification Methods:

The purpose of TB diagnostic methods is to show the presence of mycobacteria in clinical samples and isolate the disease agent. The diagnosis of TB from clinical specimens is made by

phenotypic, immunological and genotypic methods.

Phenotypic Identification Methods:

Today, the method accepted as the gold standard in the laboratory diagnosis of tuberculosis is the culture method. The advantage of culture methods is the detection of live mycobacteria, the isolation of bacilli, and the ability to study antimicrobial susceptibility tests. Mycobacteria grow on selective media in an aerobic environment at 35-37ºC in an average of 7-21 days. Culture periods are 6-8 weeks. The culture methods used in the production of mycobacteria are divided into two as conventional and automated (fast) culture systems (Badawy et al., 2021).

The media used for the production of mycobacteria by conventional methods are classified into two groups as solid and liquid. Solid media are divided into egg and agar-based. In order to provide faster results in automated culture systems compared to conventional methods, liquid media is used. While solid media are used for passage of mycobacteria and for long-term storage of cultures, liquid media are preferred for rapid diagnosis and for the study of antimicrobial drug susceptibility (Bahraminia et al., 2021).

Mycobacterial culture methods are divided into two as solid and liquid media. Two types of media, egg-based and agar, are used in solid media. Löwenstein-Jensen (LJ) is the most widely used medium today, with the exception of solid media such as Petragami and American Trudeau Society. Thanks to its content, it prevents contamination and provides suitable breeding conditions for mycobacteria. The most preferred agar-based media are; Middlebrook 7H10 and Middlebrook 7H11. Various broths are available, such as Bactec 460TB, Bactec 900 MB, Bactec MGIT 960, ESPII, MB Redox, MB/BacT ALERT 3D. For the isolation of mycobacteria modified Kirchner medium is used (Baykan et al., 2022).

Automated Culture Systems

In the bacteriological diagnosis of tuberculosis, rapid culture systems are widely used in routine diagnosis because they can isolate the causative bacteria much faster and at a higher rate than conventional methods. In most of these systems, O2 usage and CO2 formation in the tube are measured fluorometrically or colorimetrically using 7H9-based broth (Borisov et al., 2019).

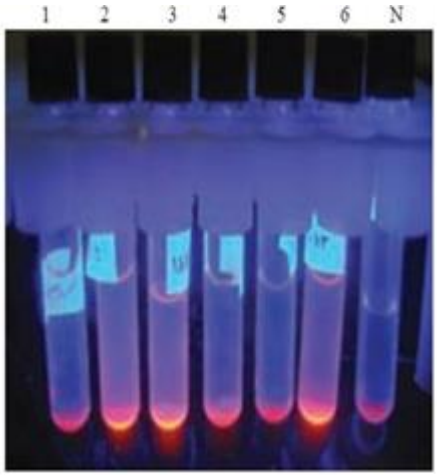


Figure 2.14.A- *Mycobacterium* Growth Indicator Tube of MGIT 960 system

Today, there are many automated liquid culture systems produced for TB culture. These include Myco-ESP (Extra Sensing Power) II, (Trek Diagnostics, Inc., Westlake, Ohio, USA), MB/Bact T (Organon Teknika, Durham, NC, USA), Bactec 9000 MB (BD Biosciences, Sparks, MD, USA) and BACTEC Mycobacterium Growth Indicator Tube (MGIT) 960 (BD Biosciences, Sparks, MD, USA) sample can be given. Among these systems, the MGIT 960 system is the most widely used automated liquid culture system worldwide (Brickner et al.,2008).

BACTEC MGIT 960 System

Rapid culture of mycobacteria of the WHO BACTEC MGIT 960 system recommends its use. There are 7 ml of Middlebrook 7H9 broth in 10 ml special tubes used in this system (Bruchfeld et al., 2015). At the bottom of the tube is silicon containing oxygen sensitive ruthenium metal complex. Before planting clinical specimens, OADC (Oleic acid, Albumin, Dextrose, Catalase and Polyoxymethylene state) solution necessary for the growth of mycobacteria into MGIT tubes and a possible in order to prevent contamination, PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and assoiling) containing 5 different antibiotic mixtures is added to the medium. The MGIT 960 device provides an incubation environment at 37ºC to the seeded tubes and checks for growth in the tubes with UV every hour (Campos et al., 2003).

When there is no growth in the media used, fluorescence does not occur when UV light is sent to the silicon layer by the device due to the presence of oxygen; As the mycobacteria grow, the indicator is released from the bottom of the tube due to the consumption of oxygen in the tube and the amount of fluorescence produced is index (Growth Unit), is measured as (Figure 2.8). This system can be used for the cultivation of all clinical samples other than blood. Mycobacteria reproduce in an average of 4-21 days in the MGIT 960 system (Cohen al., 2000).

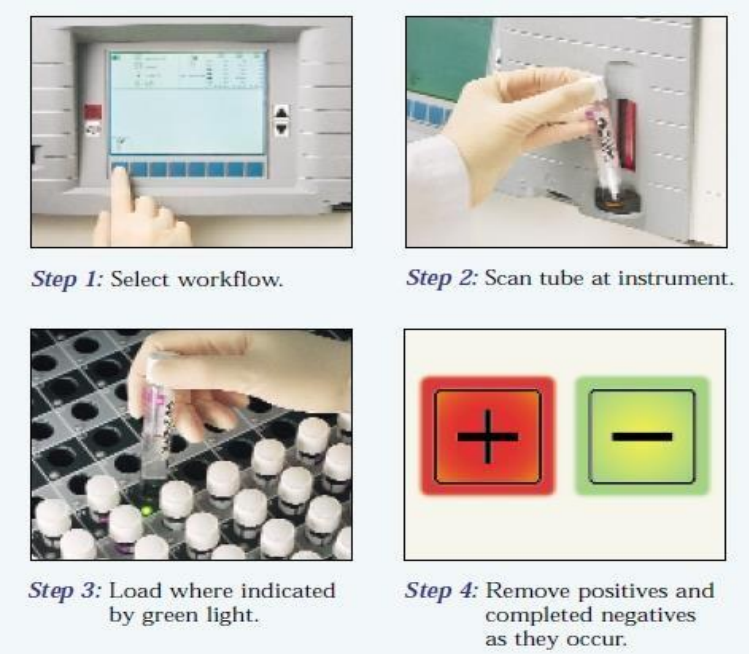


Figure 2.15. B-The procedure of MGIT 960 system

Immunological diagnostic technique

TB infection encompasses infections in both active and latent form. Latent TB infection occurs when a person is exposed to TB bacillus and the body is unable to control and destroy the infection. In up to 10% of latent TB patients, TNF-alpha blocker, etc., which suppresses the immune system. In the presence of drug use or some infections such as HIV, the disease can turn into an active form. Immunological diagnostic methods used in the diagnosis of the disease are tuberculin skin test and interferon gamma release tests (Churchyard et al., 2000).

Epidemiological Methods

In recent years, it is of great importance to understand the epidemiology of TB for the early detection of suspected outbreaks and to indicate possible epidemiological links between molecular typing approaches and TB patients. The use of molecular classification methods together with phenotype-based methods; In addition to the precise separation of strains from each other, a better understanding of the causes of transmission and spread of mycobacteria in the community has provided access to extremely valuable epidemiological information such as identifying the source cases, determining the difference between cross-contaminations in laboratories and reactivation and reinfection (Zurenko, et al., 2001).

Determination of risk factors and regional control programs evaluation may enable more rational and effective control measures to be taken (Zimenkov et al., 2017). If a significant portion of tuberculosis cases in a society have occurred as a result of a newly formed infection, this is the case of tuberculosis in that society. It shows the inadequacy of the preventive measures taken to

prevent it. What is expected from a typing method is that it can reveal a strain-specific genetic profile. This profiler fingerprint is called “fingerprint”. If two or more strains form the same or very similar profile,they are said to be in the same cluster (Zhang et al.,2013).

Isolates isolated from different patients but showing the same genetic profile are most likely epidemiologically related. This situation generally reflects new transmission among patients. With the development of molecular techniques, the phylogenetic characteristics and spreading dynamics of *M. tuberculosis* have become better understood (Zhang et al., 2013). With molecular typing, recurrence of tuberculosis, whether secondary infection is reinfection or reactivation, and the presence of infection with two or more strains at the same time in a patient can be revealed. Thus, tuberculosis control and treatment, the effectiveness of the protocols can be investigated. The genome of mycobacteria is homogeneous and stable. Few silent mutations occur in its genome, and genetic recombination’s usually occur through mobile elements such as transposons or by polymorphism of repetitive sequences (Witek et al., 2017).

IS6110-RFLP

The M. tuberculosis genome contains repetitive mobile localization sequences (IS) with copy numbers ranging from 0 to 25. M tuberculosis strains have different localization sequences defined such as IS6110, IS8110, IS1547. From these sequences, IS6110 is the most widely used in phylogenetic analyses. IS6110-RFLP gold standard in genotype determination is accepted as with this method, IS6110 regions in M tuberculosis DNA are cut with PvuII restriction endonuclease enzyme, then the separation of DNA fragments according to their size is performed on the gel in an electric environment (Witney et al., 2015). DNA fragments in the gel After electrophoresis, the southern blotting technique, SB, is replicated to the nylon membrane. After the DNA hybridizes to the membrane, the bands that are copied to the membrane, namely DNA fragments, are made visible by means of the labeled IS6110 probe and the chemiluminescent. occur the incoming radiation is transferred to the XR film and interpreted (Witek et al., 2017). Although this method has high discrimination power, RFLP has not been widely used due to reasons such as the need for large amounts of DNA, the long and time-consuming processes, the need for intensive labor, and the insufficient discrimination power for strains with a copy number of 5 or less. However, by combining this method with spoligotyping, MIRU-VNTR and SNP methods in experienced centers, results with high discrimination power can be produced (Witney et al., 2015).

MIRU-VNTR

The MIRU-VNTR method has a variable number of repeats in the MTBC genome. It is based on the analysis of sequences (VNTR). It is an effective, sensitive and highly specific method for typing M. tuberculosis, and it is a standardized rapid method due to its reproducibility between laboratories. 41 different species in the *M. tuberculosis* genome (Zurenko et al., 2001).

VNTR sequences with variable sequence repeats were defined and these sequences were named mycobacterial interspersed repetitive units (MIRUs) (Figure 2.15) (Witek et al., 2017).

Based on the band sizes obtained as a result of PCR using primers that recognize the neighboring regions that include these loci, the size of the DNA segments located on both sides is determined bythe number of repetitive sequences (Zhang et al., 2013).

In the typing of MTBK isolates with this method, the 12 locus MIRUVNTR method was used before, and then the standard MIRU-VNTR method was developed by increasing the number of loci to 15 and 24, respectively. When this method, which is sensitive and specific for MTBK, was compared with IS6110-RFLP and spoligotyping, it was seen that the discrimination power was higher (Tiberi et al., 2017).

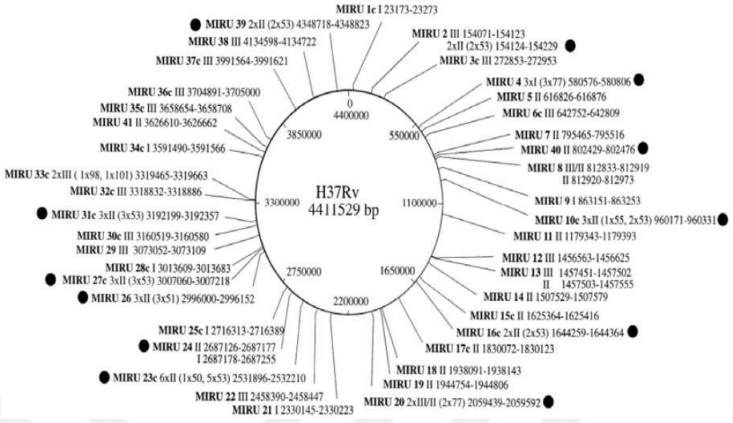


Figure 2.16. The 41 different species in the *M. tuberculosis* genome

Spoligotyping

Spacer Oligotyping (Spoligotyping) is a widely used PCR-based reverse dot blot hybridization method, first described in 1997, and is a fast, simple and reproducible method (Wells et al., 2015). This method is the first genotyping method that allows simultaneous identification and typing of *M. tuberculosis* complex strains in clinical samples. Because spoligotyping is a fast and reliable method, it is a method frequently used in genotyping of M. tuberculosis complex strains around the world (Vija et al.,2013).

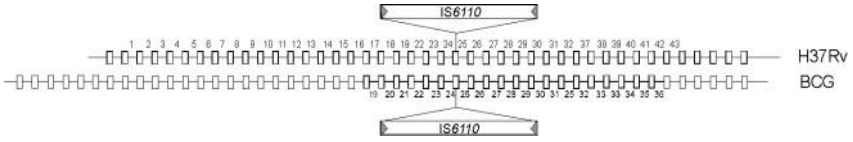


Figure 2.17. The structure of the DR locus

Structure of the DR locus in the genome of 0WXEHUFXORVLV H37Rv and 0ERYLV BCG P3. The rectangles depict the 36 bp Direct Repeat (DR).

There are different numbers of 36 base pair DR loci in the genome of the M. tuberculosis complex. The DR locus is a member of the CRISPR, Clustered family of regularly interspaced short palindromic repeats, regularly clustered interspace short palindromic repeats. As spacer between DR loci in the genome that creates a 35-41 base pair long gap between them, are called series. The copy numbers of DRs and the presence of spacer sequences differ between strains. The method is based on the detection of polymorphism of fragments of these multiple direct variable repeats (DVR) formed by DR loci in the genome. Each DVR consists of the DR locus containing 36 base pairs and spacer regions, which are short non-repeating sequences located between the DR regions (Vija et al.,2013).

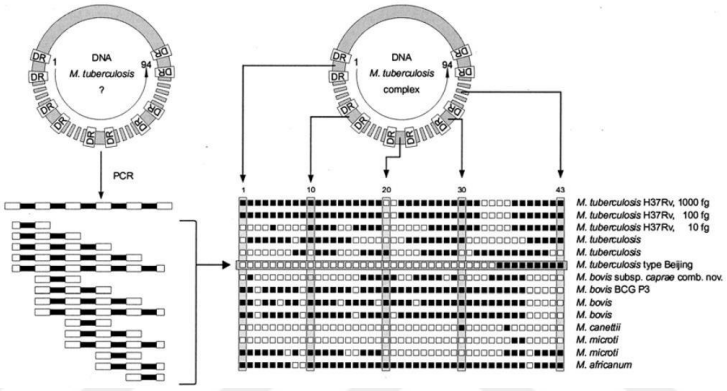


Figure 2.18. The DR locus containing 36 base pairs and spacer regions

Using biotin-labeled primers targeting DRs, these loci different spacers between are amplified. The spoligotype data collected from the studies carried out to date, classification, population genetics and epidemiological studies were combined in a database named fourth international spoligotyping database (SpolDB4) for standardization and collection in a database. This database plays a major role in the construction of the global phylogenetic relationship of MTBC and includes epidemics, including sporadic strains (Van et al., 2022).

It is used to obtain information about the source and distribution routes of strains and their movements in the community. According to the first hypotheses regarding the evolution of mycobacteria, in the horn of Africa Mankind, who domesticated cattle, took *M. bovis* from cattle. Later, it was suggested that human mycobacteria, *M. tuberculosis*, evolved from *M. bovis*, similar to the emergence of other animal mycobacteria such as *M. caprae*, *M. pinnipedii*, as a result of SNPs and deletions (Jia et al., 2022). However, MTBC strains as a result of the phylogenetic analyzes carried out today, it has been shown that mycobacteria, which were animal tuberculosis species before the Neolithic Demographic Transition, differentiated from *M. prototuberculosis*, that is, the ancestral tuberculosis species, as a result of deletional mutations (Tiberi et al., 2017). Indeed, the genome of the *M. tuberculosis* strain is approximately 4.4. The fact that the *M. bovis* genome is 4.3 mDa while it is mDa proves that the delusional mutation is from *M. tuberculosis*to *M. bovis*. In

addition, the human-related MTBC, the species are obligate human pathogens with no known animal or environmental reservoir, suggesting that changes in human demographics influenced the evolution of MTBC (Thee et al., 2016).

As a result of genome sequencing studies of 186 strains representing the global diversity of MTBC and 39 strains belonging to the Beijing family, 34,167 single point mutations were identified that were decisive for establishing phylogenetic relationships between strains excluding repetitive and mobile elements. As a result of this genome-based phylogeny study, 7 major lineages (L1-L7) were determined. These lineages until today; three “ancestral”, that is, rapidly diverging after *Mycobacterium prototuberculosis* or Most Recent Common Ancestor (MRCA); L1: East-African Indian (EAI), containing two strains of *M. africanum* L5 and L6, in addition to the L2, L3 and L4 lineages named as L7, it forms an intermediate lineage originating from Ethiopia (Figure 2.17) (Tahar et al., 2010).

Species belonging to MTBK are characterized by polymorphisms in their chromosomes. Based on the combination of M. tuberculosis complex strains, they were classified into three main genetic groups (PGG) and distributed into these three groups according to their spoligotype patterns. The first group (PGG1); EAI, Beijing and Central Asian Strain (CAS)/ Delhi, second group; (PGG2), Harlem (H), Latin American and Mediterranean (LAM) and X strains. T strain and other strains that are not yet well characterized are classified in the third group (PGG3) (Tao et al., 2012). The Beijing, LAM, Haarlem, T, and X families are classified in the "modern" lineage with the deletion of the TbD1 sequence in the MTBK genome, while the EAI family is classified in the "ancestral" lineage, the TbD1+ (Tao et al., 2012).

It was observed that the Beijing strain in the L2 family still has modern and ancestral subspecies, and MDR-TB is related to the modern Beijing strains, while the L4 family has the greatest genetic diversity (Figure 2.11) (Singh et al. ,2020). Each family is associated with prevalence in a particular geographic continent. For example, the Beijing strain represents about 50%of the strains found in Far East Asia, while CAS is mainly found in the Middle East or South Asia and India; EAI is common in the Central, South-East, Far East-Asia, Middle East and Oceania. The fact that the Haarlem strain is found in Europe as well as in Central America/Caribbean suggests that it is associated with historical European colonialism (Stanley et al., 2010).

LAM is seen in South America, Mediterranean and Caribbean coastal areas. The X family in North America and Central America was associated with Anglo-Saxon ancestry, but is now associated with African-Americans, while the T family has spread to all continents. The TUR (Turkish) subfamily, which was first defined, was observed to represent 10-30% of all clinical isolates, depending on the region, and was named “LAM7-Tur” in 2005 (Shi et al., 2005). The absenceof the synonymous mutation C1212G, which is in the phylogenetic relationship of the LAM subfamilies, has led to the renaming of the LAM7-Tur subfamily as “TUR” (Smith et al., 2015).

The TUR family has been identified in many neighboring countries, such as Bulgaria, and

has recently been observed to spread to the Middle East and European countries, as demonstrated by the SIT41 map created by SITVITWEB (Singh et al.,2020).

Membrane of Spoligotyping:

After amplification with primers targeting the DR loci, the denatured amplicons are hybridized with 43 types of oligonucleotide probes that are complementary to the different spacer regions attached to the membrane and located between the DRs (Smith, 2003). The presence or absence of spacers is shown in the digital environment. The resulting image is in the form of squares on the upper surface of the membrane. Spacers differ between strains and distinguished by their spoligotyping patterns (Stanley et al.,2010).

This method, which is simple, fast and highly reproducible, is the most widely used method after IS6110. Its advantage over IS6110 is all about the high discriminatory power in low copy numberstrains (Singh et al. 2020). During the interpretation of the spoligotype results, the presenceof spacer regions is evaluated by taking into account the blotting that occurs as a result of hybridization of the PCR product with the membrane to which spacer oligonucleotides are covalently attached. The results are given in the table. It was converted to "Octal code" format consisting of 15 numbers between 0-7 using the octal coding key. Clusters and families (clades) were determined using the obtained data SITVIT2 database. Database address:

http://www.pasteur-guadeloupe.fr:8081/SITVIT2/

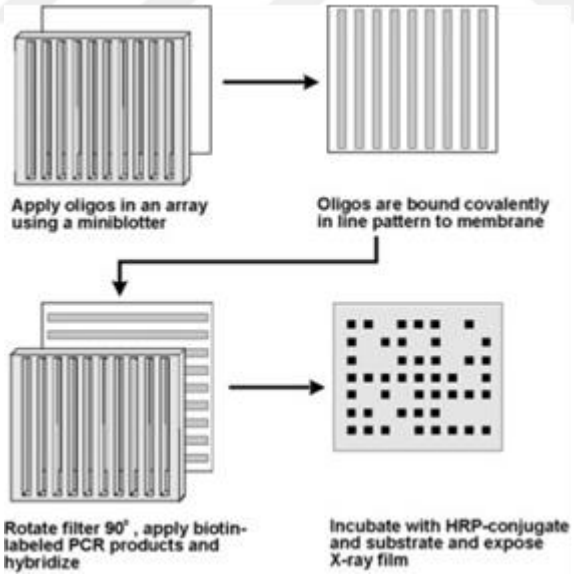


Figure 2.19. Overview of the spoligotyping method.

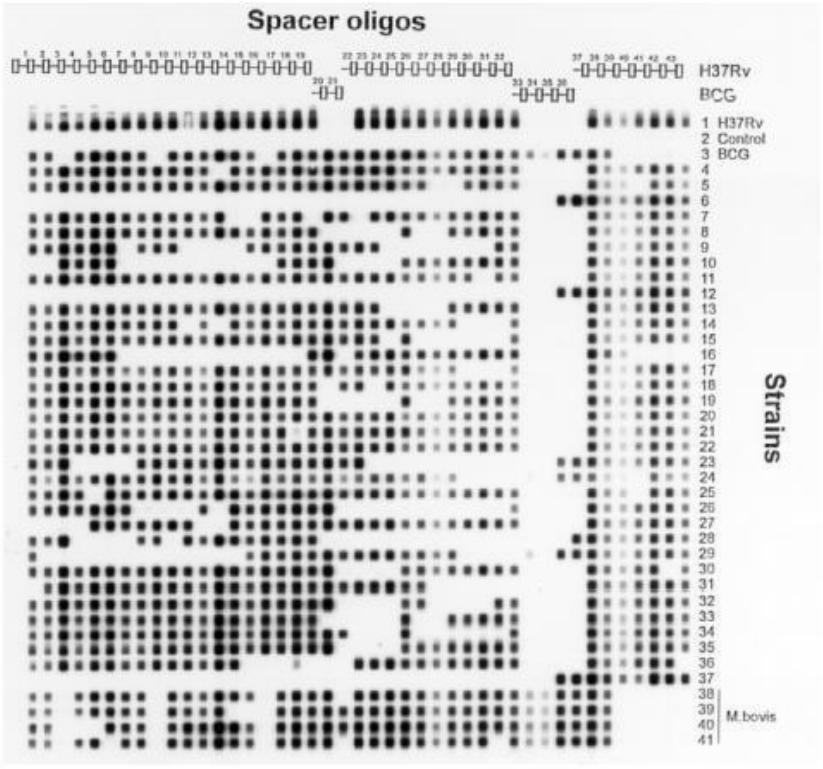


Figure 2.20. Spoligotyping results

Spoligotyping result of 0WXEHUFXORVLV H37Rv, 0ERYLV BCG P3 and 38 different clinical isolates. A membrane with 43 spacer oligonucleotides was used (vertical lines). The spacer oligonucleotides were derived from the spacers of 0ERYLV BCG P3, 0WXEHUFXORVLV H37Rv.

Microbead Based Hybridization (TB-SPOL):

Microbead-based DNA chip systems are a technology that uses flow cytometry or fluorescent imaging, theoretically targeting up to 500 markers in the same tube. These technologies have gained wide acceptance for tissue typing and immunology. Their use for infectious disease control is expanding even in low-resource countries. In parallel, genotyping techniques targeting CRISPR regions are promising for new bacterial models (Shetye et al.,2020).

In our study, DPO (Dual-Priming Oligonucleotide) method; We used CRISPR to develop a 4-Plex multiplex polymerase chain reaction (PCR) that can co-amplify the rpoB hotspot, katG, and inhA genes. It is currently possible to detect more than 68 markers with the maximal version of this method (Shi et al.,2005).

59 of these were routinely used in one-step (Lumineks 200) or two-step methods (magPix). Bead-based Spoligotyping, previously developed separately, is determined by combining magnetic or polystyrene beads with a charge-coupled device (CCD) imager on the same platform with Luminex200 and Magpix instruments with a 532 nm report and a 635 nm classification channel. method (Tahar et al., 2010).

2.7. TB Treatment:

2.7.1. Chronology of the discovery of anti-tuberculosis drugs

From the discovery of Koch's bacillus in 1882 to the present day, tuberculosis has been a major public health problem. Medicine remained at a therapeutic impasse for a long time, before the discovery of numerous molecules from 1943 (figure 14) (Schnippel et al., 2018). Indeed, the first molecule to have been discovered was streptomycin, which was used as monotherapy in the treatment of tuberculosis, as a single agent in 1943. Since the 1950s, the need for a combination in the treatment of tuberculosis has been widely recognized. To this end, many molecules have been able to integrate the therapeutic arsenal for the fight against tuberculosis. More or less long-term drug combinations then followed one another, always to the benefit of therapeutic innovations that were intended to be better than the previous ones, especially in terms of treatment duration (Sakula et al., 2003). Here are someexamples of treatments that have been carried out:

- In 1952, we combined: streptomycin + aminosalicylic acid + isoniazid for 24 months,
- In 1960, we combined: streptomycin + ethambutol + isoniazid for 18 months,
- In 1970, seven years after its discovery, rifampicin + streptomycin + ethambutol + isoniazid for 9-12 months,
- In 1980, we will finally replace streptomycin with pyrazinamide which part of the anti-tuberculosis molecules known since 1953.

This is how it was establishing the association which is still used today and which corresponds to the 1st line anti-tuberculosis drugs: rifampicin + isoniazid + pyrazinamide + ethambutol. For a duration of treatment which varies from 6 to 8 months (Rodriguez et al., 2002).

First-line anti-tuberculosis drugs have shown unfailing efficacy, until the appearance of forms of tuberculosis resistant to them from the 1980s. The birth of TB-MDRs led to the relaunch of fruitless research into new molecules (Rocha al., 2021). It was therefore appropriate to conduct research within the classes of antibiotics already known such as the fluoroquinolones, which hold a central place in the therapeutic strategy of what will be called 2nd line antituberculosis drugs; which are indicated in the treatment of multidrug-resistant tuberculosis (Rao et al., 2013). Second-line anti-tuberculosis drugs are composed for the most part of the molecules which had beenset aside in favor of the use of first-line anti-tuberculosis drugs. Those are:

- Aminosides (kanamycine,amikacine, capréomycine and streptomycine),
- Thionamides (ethionamide),
- Cyclosérine and térizidone,
- Acide para-aminosalicylique (pas),
- Fluoroquinolones (ofloxacine, lévofloxacine and moxifloxacine).

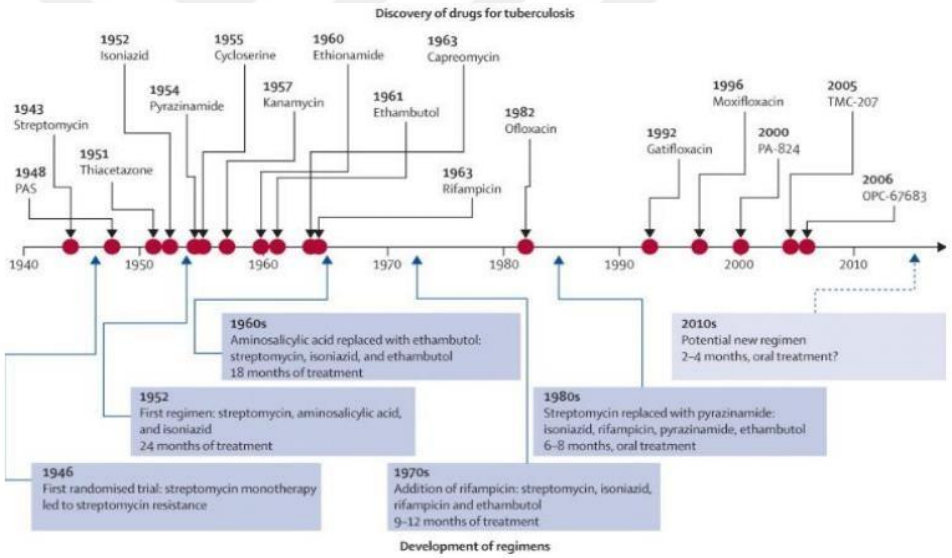


Figure 2.21. Chronology of the discovery of anti-tuberculosis drugs

Unfortunately, resistance to 2nd line anti-tuberculosis treatments is increasingly being encountered. This is called extensively drug-resistant tuberculosis (TB-XDR) (Rao et al., 2013). Recently, a new major anti-tuberculosis drug, bedaquiline, has been marketed to combat these resistance phenomena.

2.7.2. The different phases of treatment for multidrug-resistant tuberculosis and dosages of anti-tuberculosis drugs

The duration of MDR-TB treatment is based on two phases:

An intensive phase during which at least the following 5 drugs are initiated (unless

sensitivity not documented) which will be modified according to the patient profile. Amikacin (or Kanamycin) + levofloxacin (or Moxifloxacin) + ethionamide + cycloserine (or PAS or Protionamide) + pyrazinamide (vit B6) (or E), the intensive phase is defined by the duration of treatment, in the presence of the injectable agent. It lasts from 6 to 8 months knowing that the treatments of the intensive phase must be maintained at least 4 months after the smear of the cultures has returned negative (Pym et al., 2016).

A maintenance phase is marked by stopping the injectable agent (Am or Km). The other treatments being continued, this phase lasts an average of 12 months (Parvez et al., 2017). The average treatment duration of a treatment after culture conversion is 18 months, o the total treatment duration of MDR-TB is between 18 and 24 months. The administration of each drug is done according to a weight range. The evolution of MDR-TB depends above all on the duration of treatment and the quality of care (Vija et al., 2013).

2.7.3. Therapeutic strategies and current treatments for tuberculosis

Current anti-tuberculosis treatments are necessarily based on multi-therapy over several months. Generally, the multi-therapy is a quadruple therapy comprising: rifampicin, isoniazid, pyrazinamide and ethambutol (Prasad et al.,2021). The principle is based on the synergy of action of the various anti-tuberculosis drugs used, in order to bring the patient to complete recovery, to avoid anyrisk of relapse or even the selection of resistant strains. The procedures for initiating anti-tuberculosis treatment are accompanied by a few administrative procedures. In fact, it is first and foremost a notifiable disease in France, the declaration of which must be made both by name to the medical inspector of the ARS (regional health agency) for the purpose of operational (Tiberi et al., 2017).

Anonymous notification must be made for data collection epidemiological. In addition to these mandatory administrative procedures, it is imperative to inform the patient about the modes of transmission of Koch's bacillus (according to the law of March 4, 2002: diseases exposing third parties to a risk of contamination) (Pozniak et al., 2003). Then comes the education of the patient on the duration and the composition of his treatment which are essential notions, given that the therapeutic success is largely based on the good observance of the treatment. Very often, the treatment is spreadover several months (six months at least), which can be very constraining for the patient given the many adverse effects, due to the multiplicity of treatments to be taken on a daily basis (Pym et al., 2016).

Before initiating anti-tuberculosis treatment, it is important to carry out some biological and clinical examinations which will provide information on the patient's condition (Prasad et al., 2021). This information will subsequently help to assess the patient's tolerance to the treatment, as well as to optimize the monitoring of biological constants. The main examinations concern:

- Renal function (serum creatinine),
- Liver function (transaminases),
- The search for pregnancy in a woman of childbearing age (assay of β-HCG),
- Uricemia (when the treatment includes pyrazinamide),
- Fundus, visual fields and color vision (when the treatment includes ethambutol),
- HIV, hepatitis B and C serology, due to their epidemiological association with tuberculosis.

We are now going to see more in detail, 1st line anti-tuberculosis drugs (Pang et al., 2012).

2.7.4. First-line anti-tuberculosis drugs

The classic treatment plan is a two-month quadruple therapy, which consists of combining isoniazid (INH) with rifampicin (RMP), with ethambutol (EMB) and pyrazinamide (PZA). Then continuous treatment with dual therapy followed for four months, which combines isoniazid with rifampicin (Özer et al. ,2020).

Isoniazid (INH):

It is the most important drug used in TB treatment regimens since 1952. It is a prodrug activated by catalase-peroxidase KatG, which forms various radicals and adducts that inhibit the production of mycolic acids that form the cell of Mycobacterium. This activity makes INH a potent bactericidal agent. In addition, it has a synergistic effect with other strains produced by KatG and other drugs used in the treatment of TB (Pym et al., 2016). There are four CDC- recommended multidrug regimens containing INH for drug-susceptible strains. The regimens consist of an initial phase of 2 months followed by a continuation phase of 4 or 7 months (Matsumoto et al., 2003).

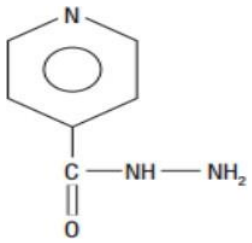


Figure 2.22. İsoniazid chemical structure

Isoniazid is an antibiotic said to be bactericidal on intra- and extracellular bacilli. Isoniazid is a pro-drug, which can only be activated by an enzyme present in *M.tuberculosis*, namely KatG (Figure 15), which exerts a catalase-peroxidase function. Once activated, the active metabolite of isoniazid will target the InhA protein which; is an enoyl-ACP reductase 35 involved in the FAS-II fatty acid elongation system that contributes to the formation of mycolic acids (Mdluli et al.,2015). Thus, inhibited by the active metabolite of isoniazid, InhA loses its activity and can therefore no longer participate in the biosynthesis of mycolic acids, which, it should be remembered, are

essential constituents of the bacterial cell wall (Migliori et al.,2009). The direct consequence is an increase in cell permeability, since we have seen previously that the peptidoglycan which is closely linked to mycolic acids in the bacterial structure, was porous and therefore capable of letting certain substances such as antibiotics diffuse (Mirnejad et al.,2018). This increase in permeability leads inexorably to cell death (Nagiec et al., 2005).

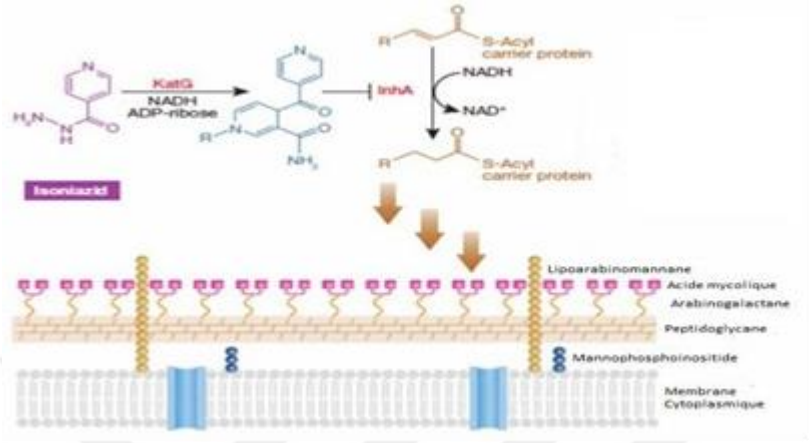


Figure 2.23. Mode of activation and action of isoniazid

The use of isoniazid is contraindicated in people with hypersensitivity to this molecule or to one of the components of the different specialties (Nam et al., 2009). It is also contraindicated in people with severe hepatic impairment. In the long list of adverse effects associated with isoniazid, we frequently find hepatotoxicity which can manifest itself by an elevation of transaminases and more rarely by severe drug-induced hepatitis which leads either to the discontinuation of treatment for the latter, or requires strict monitoring of transaminases, peripheral neuropathies (vitamin B6 deficiency) (Rao et al., 2013).

Rifampicin:

Rifampicin is an antibiotic from the rifamycin family. It consists of a macrocyclic structure, which includes a naphthohydroquinone type chromogen (Mardassi et al., 2005). This antibiotic is obtained by hemi synthesis, from rifamycin B, which has several centers of asymmetry. Rifampicin has good digestive resorption when taken on an empty stomach. The plasma peak is reached two to three hours after the administration of a dose of 600 mg of rifampicin taken away from meals. Diffusion into tissues such as lungs, bones, kidneys and liver are excellent thanks to its lip solubility. There is also good intracellular penetration, especially inside macrophages. Plasma protein binding is 80% (Otsuka et al. 2017). It is mainly metabolized in the liver, in the form of an active deacetylated metabolite, which has the same antibacterial activity. Rifampicin has an inducing effect on its own metabolism. Indeed, it is a potent inducer of hepatic enzymes (CYP3A, 2C9, and 2C19). Its elimination half-life varies between 3-4 hours after administration of 600 mg.

Rifampicin is mainly eliminated via the bile at 80% and up to 18% via the kidneys (Nam et al., 2009).

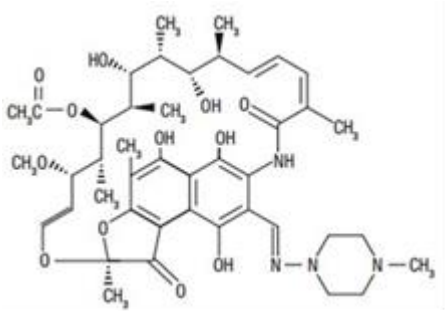


Figure 2.24. Rifampin chemical structure

Rifampicin is a bactericidal antibiotic against intra- and extracellular bacilli. It inhibits the action of RNA polymerase of mycobacteria (Merker et al., 2020). Rifampicin establishes a covalent bond with the β subunit of the enzyme, such that a stable drug-enzyme complex is formed (Figure 17) which prevents the formation of messenger, ribosomal and transfer RNAs which are molecules necessary for the survival of the bacterium. Rifampicin is a potent enzyme inducer, its association with certain classes of drugs should be avoided whenever possible (Mirnejad et al., 2018). When it proves to be essential; it is essential to carry out monitoring with the followingtreatments:

- Oral anticoagulants: combination requiring monitoring of the INR.
- The estrogen-progestogen pill: need to change the method of contraception.
- Antiretrovirals (protease inhibitors) whose combination with rifampicin leads to a very significant decrease in their plasma concentration and is therefore contraindicated (Nguen et al., 2016).

Pyrazinamide (PZA):

Pyrazinamide is a derivative of nicotinamide in the same way as isoniazid. It is a pyrazine carboxyl amine (Nam et al., 2009). It is a prodrug which will be hydrolyzed into paranoic acid under the action of an amidase. The absorption of pyrazinamide is rapid and complete at the gastrointestinal level. There is good tissue diffusion in the lung, kidney and liver (Nagiec et al., 2005). Essentially hepatic metabolism allows the production of its active metabolite: pyrazino acid, which is degraded by xanthine oxidase into 5-hydroxy-pyrazinoic acid. These two metabolites are the main forms of renal elimination of the molecule. 4% of the pyrazinamide will be eliminated unchanged approximately 24 hours after its administration. Binding to plasma proteins is almost zero. Furthermore, an elimination half-life of approximately 9 hours is observed after ingestion of 1.5 g to3 g of pyrazinamide (Mdluli et al., 2015).

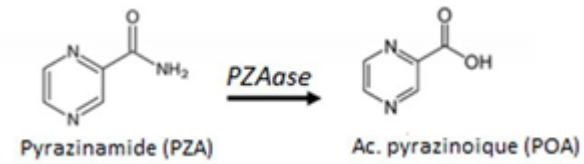


Figure 2.25. Activation of PSA in POA

Pyrazinamide is indicated for the treatment of pulmonary tuberculosis as a short course, in combination with standard quadruple therapy antibiotics (rifampicin, isoniazid and ethambutol) (Matsumoto et al., 2003). It is also indicated in the treatment of pulmonary and extrapulmonary tuberculosis with resistant bacilli, in combination with the standard antibiotics of the quadruple therapy or, with other available antibiotics (Migliori et al., 2009).

Ethambutol:

Ethambutol is a major antituberculosis drug, less active than isoniazid or rifampicin. It is a derivative of ethylene diamine (N, N'-dihydrobutylethylene). It is used therapeutically in the form of a salt (dihydrochloride of the dextrorotatory compound) which corresponds to an enantiomer of configuration (S, S) (Micheni et al., 2021).

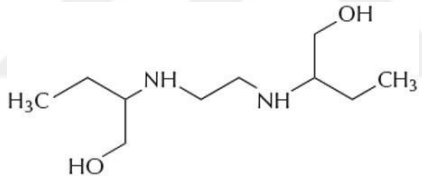


Figure 2.26. Ethambutol chemical structure

Digestive absorption of ethambutol is quite rapid, the plasma peak is reached after about two hours (Merker et al., 2020). This absorption is around 80% and is independent of any food intake even if it can be reduced by taking antacids. Diffusion in the lung parenchyma as well as in other tissues is good. Alveolar macrophages sometimes reach concentrations 7 times higher than theserum peak. Low metabolism takes place in the liver and urinary elimination is 80% via the kidneysin an active non-metabolized form. The elimination half-life varies between 9 and 12 hours (Mdluli et al., 2015).

Ethambutol (EMB) is an antibiotic capable of acting as an anti-metabolite that blocks RNA synthesis. It is both bacteriostatic with activity on extracellular bacilli (Matsumoto et al.,2003). It acts by inhibiting arabinosyltransferases (embA and embB) which are enzymes involved in the synthesis of the bacterial wall of mycobacteria (Figure 21) (Micheni et al., 2021). As a result, it interferes with the synthesis and polymerization of arabinans, since arabinosyltransferases are necessary for the synthesis of the latter (Matsumoto et al.,2006). More particularly, with

arabinogalactans (AG) and lipoarabinomannan (LAM); which are the two arabinans essential for the formation of mycolic acid layers (Mardassi et al., 2005).

2.7.5. Second-line anti-tuberculosis drugs

Second-line anti-tuberculosis drugs are reserve treatments (Mardassi et al., 2005). They are used for so-called multi-resistant tuberculosis to isoniazid and/or rifampicin. According to the WHO, the therapeutic principle which consists in setting up a second-line anti-tuberculosis treatment is based on the following principles (Mantefardo et al., 2021). In the initial intensive phase, which must last at least 6 months, the treatment must consist of a combination of at least five antibiotics, one of which must be a fluoroquinolone and another whose active ingredient is injectable. The maintenance phase that follows lasts between 12 and 18 months after conversion of the cultures. It must consist of at least three antibiotics among the most active and best tolerated of the four in the initial phase (Manjunatha et al., 2006).

The main classes of antibiotic molecules used today are mainly aminoglycosides and fluoroquinolones. Rifabutin, ethionamide, cycloserine, para-amino salicylic acid (PAS) and linezolid will be used secondarily; only on medical advice and, after temporary authorization for use (Merker et al.,2020).

Aminoglycosides:

The four compounds of the aminoglycoside family that are used in multidrug-resistant tuberculosis are: streptomycin, amikacin, kanamycin and capreomycin (Mac et al., 2001).

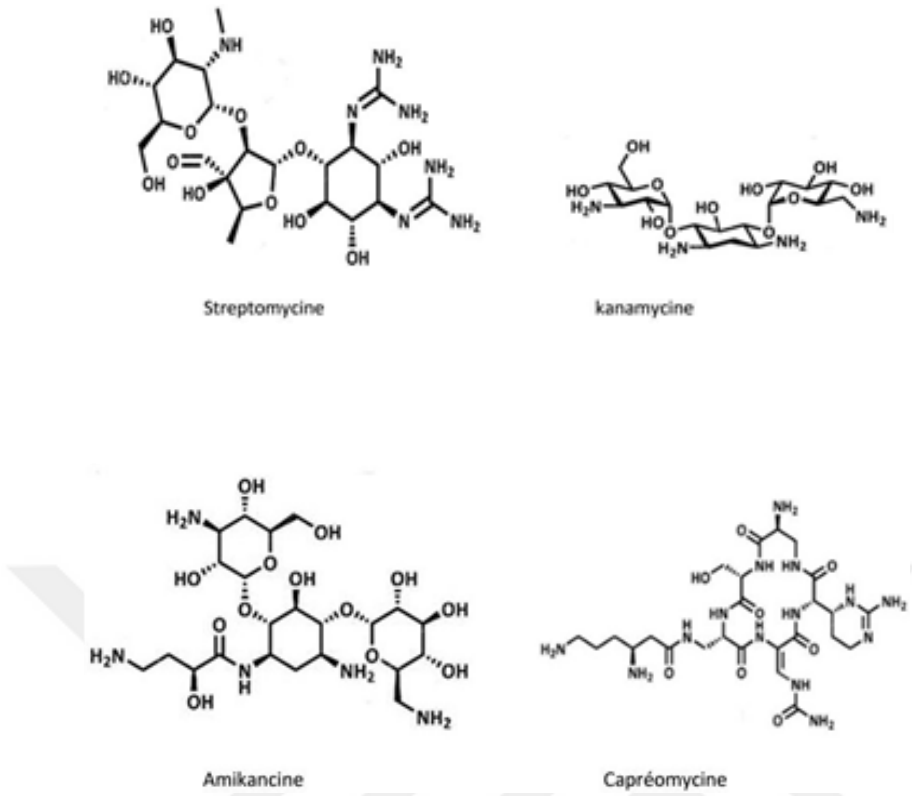


Figure 2.27. Aminoglycosides chemical structures

Aminoglycosides are natural or semi 37 molecules. small synthetics (Luthra et al., 2018). It is the structure of their side chain that will be responsible for the differences in activity from one molecule to another. These are bases, whose antibacterial activity is optimal only at alkaline pH. Aminoglycosides are molecules which are administered intravenously or more rarely intramuscularly (Loxton et al., 2021). There is no enteral absorption for this family. They have excellent diffusion in the pulmonary, renal and pericardial parenchyma, while diffusion in the other tissues is mediocre. Their half-life is two hours on average. Elimination is exclusively renal in unchanged form and active for 85 to 90% of the dose administered o from the first 24 hours that follow (Liu et al., 2019).

Aminoglycosides are bactericidal molecules on extracellular bacilli. They block the 30s subunit of the ribosome and thus prevent the synthesis of bacterial proteins (Koul et al.,2004).
The use of aminoglycosides is contraindicated with the state of myasthenia and pregnancy (Li et al., 2021). In case of renal insufficiency, dosage adjustments are made, guided by serum assays.

Kanamycin, Amikacin, Capreomycin: Many of the drugs in this group shows similar characteristics in the field. Drug NTMs and MDR-TB cases used in the treatment (Lewis et al., 2008). Since the drugs in this group have similar pharmacokinetics and toxicity, theyare only given parenterally. Since the drugs in the group are ototoxic and nephrotoxic, they should not be used

more than once at the same time and should be used in combination with STR. Kanamycin and amikacin is an aminoglycoside (Jeffrey et al., 2017). Capreomycin is an antimycobacterial cyclic peptide formed by Streptococcus capreolus. In stand-alone use bacterial resistance develops. Combined use with other drugs is recommended in patients with drug-resistant TB (Jia et al., 2022).

Ethionamide (ETM): It is used in cases that are among the second-generation anti-TB and first-generation drugs cannot treat or contraindicated (Koh et al., 2009). It is produced from is nicotinic acid and has a bactericidal effect. The exact mechanism of action is unknown. However, as with INH, it is thought to inhibit mycolic acid synthesis. The drug isused only orally (Koul et al., 2004).

Protionamide: The drug is an INH derivative and is similar to ETM in terms of pharmacological properties. Cross-resistance is seen between protionamide and ETM (Nam et al., 2009).

Cycloserine: It is a broad-spectrum drug produced by Streptococcus orchidaceous. It is an analogue of A-alanine that competitively inhibits the action of alanine racemase and D- alanyl-D-alanine synthase. By inhibiting the precursors in the cell wall structure, the enzymecauses death by lysis in the growth and regression of the cell. The drug is especially used in treatment failure of pulmonary and extrapulmonary TB. Currently, no cross-resistance has been observed with other anti-TB drugs (Balgouthi et al., 2021).

Fluoroquinolones: Three representatives of the fluoroquinolone family are used in cases of multi- resistant tuberculosis, these are: levofloxacin, moxifloxacin and ofloxacin (Cohen et al., 2000).

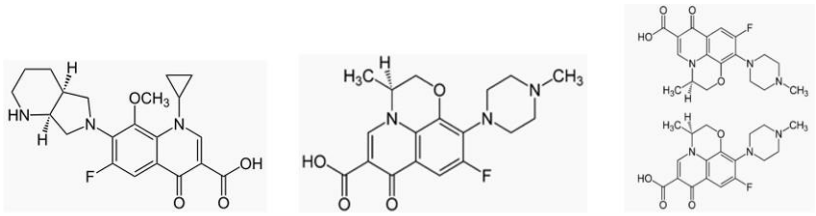


Figure 2.28. Fluoroquinolones chemical structures

Fluoroquinolones are synthetic antibiotics, which all derive from nalidixic acid (1st generation quinolone) which was their leader and it was widely used in the treatment of urinary E. coli infections (Elbek et al., 2015). The spectrum of this family has been broadened, since the addition of a fluorine atom and a piperazine nucleus. Today, 1st generation quinolones areno longer used, only 2nd generation quinolones or fluoroquinolones remain, whose pharmacokinetic characteristics and broad antibacterial spectrum allow use in many cases of systemic infections. Fluoroquinolones have good digestive absorption, very wide tissue and intracellular diffusion, but low blood concentrations. Elimination can be hepatic and/or urinary (Fortun et al., 2005).

Fluoroquinolones are bactericidal against intra- and extracellular bacilli. They work by blocking two enzymes: DNA gyrase (topoisomerase II) and topoisomerase IV (Georghiou et al., 2012). Which results in an inhibition of bacterial DNA elongation and the blocking of bacterial replication.

Levofloxacin and moxifloxacin are the two fluoroquinolones that the WHO recommends for use incases of multidrug-resistant tuberculosis. Ofloxacin has poor efficacy against bacilli compared to the other two (Jeffrey et al., 2017). It is still part of the therapeutic Arsenal. The main contraindicationsof fluoroquinolones are:

- Pregnancy,
- The growing child,
- A history of tendinopathy linked to the administration of fluoroquinolones,
- QT prolongation and liver failure with moxifloxacin.

The other molecules

Rifabutin is a derivative of rifamycin, which has a much longer plasma half-life than rifampicin (Koh et al., 2009). The advantage of its use lies in the possibility of bi-weekly administration. The use of rifabutin is reserved for cases of multi-resistant tuberculosis for which the use of rifampicin is contraindicated (Ismail et al., 2021). This is the example of HIV-positivepatients in whom the metabolism of anti-proteases is strongly disturbed when there is a co- administration of these, at the same time as rifampicin (Ismail et al., 2019).

These are molecules whose use is limited due to their high toxicity or poor tolerance. Cycloserine, for example, does not encounter any cross-resistance problems with other antituberculosis drugs, but is of high toxicity, responsible for its limit of use. Linezolid is often found in combinations of treatments for multidrug-resistant tuberculosis, because of its low maximum inhibitory concentration (MIC), however, because of its high toxicity, it will only be used in half doses, i.e., i.e., 600mg/day. Para-amino salicylic acid will be mainly used as a bacteriostat to preventresistance to isoniazid (Faksri et al., 2019).

Linezolid: It is a synthetic oxazolidinone derivative antibacterial drug developed against gram positive bacteria (Gupta et al., 2015). It binds to peptidyl transferase in the 23s parts of the 50s subunit of the microorganism, suppresses the formation of the 70s initiator complex, and exerts a bacteriostatic effect by inhibiting protein synthesis (Holmes et al., 2016). Resistance to other antimicrobial agents as it has a unique mechanism of action does not show (Haver et al., 2015). Drug resistance occurs through point mutations. The decrease in LZD binding site occurs in 23s rRNA (Hillemann et al., 2008). Recently, it has been used especially in multi-drug resistant Gram-positive bacterial infections. The first report of resistance was reported in 2002 as a gene mutation in 23s rRNA. In long-term use, the effect of the initial dose decreases, and the duration of effect is

shortened (Haver et al., 2015). When used in combination with RIF, linezolid decreased effect of the drug due to the decrease in serum level, is an observable condition. Linezolid resistance is mainly associated with a mutation at nucleotide position 2061 in rrl and a T460C mutation in rplC (Hartkoorn et al., 2014).

Tedizolid: FDA approved in 2014, completed phase llll,it is an axolidinone derivative and is used in sensitive and resistant Gr (+) bacterial infections. It interacts with the 23s rRNA of the bacterial ribosome, preventing translation initiation and inhibiting protein synthesis (Haas et al., 2019).

Delamanid: A new nitroimidazo-oxazole structure, also known as OPC-67683, it is an antituberculosis drug. In April 2014, it was approved by the European Medicines Agency (EMA) to be used in the treatment of MDR-TB. It is produced as 50 mg tablets by the Otsuka company under the name Deltyba (Otsuka Novel Products GmbH, Munich, Germany) (Cohen et al., 2000). As of 2018, it is in phase 3, it is suitable for use inthe treatment of HIV-positive patients with tuberculosis and latent tuberculosis, as well as MDR-TB(Gygli et al., 2017). Although the mechanism of action is not fully understood, it inhibits mycolic acid biosynthesis by inhibiting the synthesis of methoxymicholic acid and ketomycholic acid required for wall synthesis in mycobacterial cells (Gupta et al., 2015).

It is a drug, in order for the drug to show its effectiveness, it must be activated by the mycobacterial F420 system (Haas et al., 2019). This drug, which affects cell wall metabolism, should beused at a dose of 100 mg twice a day for 6 months (Guglielmetti et al., 2015). Delamanid, which has been obtained as a result of great researches over many years,it has been found that resistance develops immediately after its use (Gupta et al., 2015). Delamanid resistance occurs mainly with mutations in mycobacterial F420 genes (ddn, fgd1, fbiA, fbiB and fbiC) (Gopalaswamy et al., 2020). Delamanid resistance occurs by inhibition of prodrug activation. The rate of spontaneous resistance development in delamanid during TB treatment was found to be between 6.44x10-6 and 4.19x10-5. According to the Concentrations Report and EUCAST 2019 MIC Guidelines, the delamanid MIC value was reported to be 0.06 mg/l (Gler et al., 2016). The use of Delamanid has not yet been approved in Turkey; however, as of the end of 2017, it has been used in the treatment of MDR and XDR-TB cases in 42 countries around the world (Georghiou et al., 2012). Delamanid is an option that can be used in patients with tuberculosis if there is no response to existing drugs. Except for asymptomatic QT prolongation, noside effects different from placebo have been observed yet. It shows high binding affinity to human serum albumin proteins such as bedaquiline. It should be used in the right indication and under strict control so that drug resistance does not develop (Fortun et al,2005). Many new TB drugs are being developed, apart from bedaquiline and delamanid (Fogel et al., 2015).

2.7.6. Bedaquiline and Clofazimine:

Bedaquiline

History and discovery of the molecule:

Bedaquiline belongs to the diarylquinolines or DARQs, which are a new class of antituberculosis compounds with an innovative mechanism of action on their targets (Borisov et al., 2019). The discovery of BDQ is the result of a long screening of more than 70,000 molecules, tested on Mycobacterium smegmatis (non-pathogenic for humans and whose in vitro culture is faster than that of Mycobacterium tuberculosis) (Bagcchi et al., 2023). Several clinical trials in humans have confirmed the activity of the molecule on both multi-resistant and ultra-resistant tuberculosis (Beckert et al., 2012)

It was in 2005 that the science journal published a scientific article announcing the discovery of bedaquiline by a team of Franco-Belgian researchers (Baykan et al., 2022). They are Jérôme Guillemot (France) and of Koen Andries (Belgium) and their teams. In June 2014, they received the European Inventor Award in the industry category, awarded by the European Patent Office (EPO) for having found a truly new medicine for 40 years, and which constitutes a powerful weapon (by its completely innovative mechanism of action) in the fight against tuberculosis multi resistant (Bahraminia et al., 2021).



Figure 2.29. Photograph of Koen Andries and Jérôme Guillemot

Koen Andries is a microbiologist, a veterinarian by training who is mainly interested in animal viruses (Beckert et al., 2012). In 2001, reading an alarming WHO report on the growing worrying about tuberculosis, decides him to turn to human medicine. It is in association with Jérôme Guillemot that they will carry out research work against tuberculosis (Elbek et al., 2015).

Jérôme Guillemot is a doctor in chemistry, graduated from the University of Rouen. He leads a research team in anti-infectious medicinal chemistry, based at the center of the Janssen Pharmaceutica laboratory, which today markets BDQ under the name Sir Turo (Dover et al., 2004). Bedaquiline Chemical Structure.

The chemical name of bedaquiline is: (1R, 2S)-1-(6-bromo-2-methoxyquinolin-3-yl)-4-(dimethylamino)-2-(naphthalen-1-yl)-1-phenylbutan- 2-ol (Baykan et al., 2022). The molecular formula of bedaquiline (figure 25 (a)) is: C32H31BrN2O2, its molecular weight is 555.50 g/mol Sirturo® contains bedaquiline fumarate (figure 25(b)) whose molecular formula is C32H31BrN2O2.C4H4O4 and the molecular weight of 671.58 g/mol (555.50+ 116.07) (Brown et al., 2017).

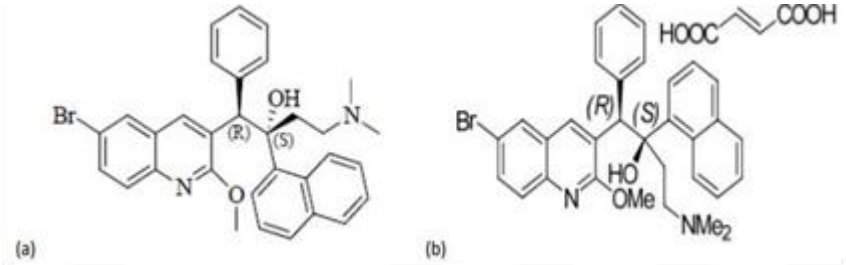


Figure 2.30. A-structure of diarylquinoline bedaquiline, B- Fumarate of bedaquiline

Action mechanism

Action on ATP-synthase of mycobacteria:

Bedaquiline (BDQ) is a diarylquinoline which acts by inhibiting the proton pump of mycobacterial ATP synthase. It thus blocks the production of ATP (adenosine triphosphate) within Mycobacterium tuberculosis. ATP is a molecule known to be the energy support necessary for all metabolic reactions allowing bacterial growth and survival (Fluk et al., 2021).

Structure and function of mycobacterial ATP-synthase:

To better understand the mechanisms governing the interaction between BDQ and ATP-synthase, it is necessary to take stock of the structure of the latter:

ATP synthase (figure 28) is an enzyme composed of two transmembrane domains (F0 and F1), which function in a complementary manner to produce the synthesis of ATP (Gandhi et al., 2014).

The F0 membrane domain is composed of a mobile part called the rotor ring (subunit c) and a fixed part called the stator (subunit a) (Gygli et al., 2017). The interface between these two parts is used for the passage of protons which then cause the rotation of the ring (Holmes et al., 2016). The F1 domain is the extra-membrane extension of the previous domain. Indeed, it includes the γ rod of the rotor ring as well as the three β subunits of the stator (Aslan et al., 2022).

The rotation of the rotor ring caused by the passage of protons, will cause the rotation of the γ rod which in turn will cause the deformation of the β subunits which have the active sites for the synthesis of ATP (Andries et al., 2005).

One full rotation will produce three molecules of ATP by oxidative phosphorylation (i.e., one adenosyl-diphosphate or ADP and one inorganic phosphate or Pi will produce an ATP molecule under the action of an ATP-synthase) (Almeida et al., 2016).

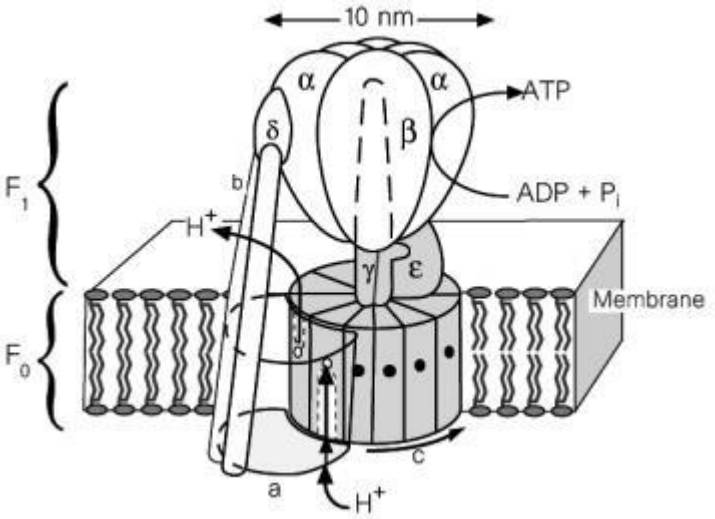


Figure 2.31. Structure of ATP-synthase of *Mycobacterium*

BDQ will therefore target the rotor ring of mycobacterial ATP-synthase. In doing so, she will cover the ionic bond sites present on the rotor ring, preventing it from serving as a shuttle for the passage of protons through the membrane. BDQ blocks the functioning of ATP-synthase mycobacterial, being 20,000 times more selective for the bacteria's ATP-synthase than for ATP synthase in human cells (Dover et al., 2004). It is through this new mechanism of action that it has been possible to observe that bedaquiline caused a powerful inhibition in vitro of the various susceptible and multidrug resistant Mycobacterium tuberculosis strains (Almeida et al.,2016).

The spectrum of BDQ activity is specific and unique to mycobacteria. The minimum inhibitory concentrations (MIC) for Mycobacterium tuberculosis vary between 0.03 µg/ml and 0.12 µg/ml (AlMatar et al., 2019). While the MIC of rifampicin is around 0.5 µg/ml andthat of isoniazid 0.12 µg/ml. BDQ has equally strong activity for strains of mycobacteria sensitive to current anti-tuberculosis drugs than on strains that are resistant to them (Alffenaar et al., 2010).

Espèces mycobactériennes	Nombre de souches	CMI pour les différentes souches (µg/ml)	CMI moyenne (µg/ml)
M.tuberculosis, H37Rv	1	-	0.03
M. tuberculosis,souches cliniques sensibles	6	0.03 -0.12	0.06
M.tuberculosis résistant à l'isoniazide	7	0.003 - 0.006	0.01
M.tuberculosis résistant à la rifampicine	1	-	0.03
M.tuberculosis résistant à l'INH & RMP	2	0.03 – 0.03	0.03
M. tuberculosis résistant à l'INH & streptomycine	1	-	0.01
M. tuberculosis résistant à l'éthambutol	1	-	0.01
M.tuberculosis résistant à la pyrazinamide	1	-	0.03
M.tuberculosis résistant aux fluoroquinolones	2	0.06 – 0.012	0.09
M.bovis	1	-	0.003
M.ulcerans	1	-	0.5
M.avium/M. inntracellulare (MAC)	7	0.007 – 0.01	0.01
M. kansasii	1	-	0.003
M. marinum	1	-	0.003
M. fortuitum	5	0.007 – 0.01	0.01
M. abscessus	1	-	0.25

Figure 2.32. MIC of bedaquiline for different *Mycobacterial* species

Low MICs have been observed for certain mycobacterial species such as*: Mycobacterium bovis*, *Mycobacterium kansasii*, and *Mycobacterium ulcerans.* other species naturally resistant to many anti-tuberculosis agents and which are often implicated in opportunistic infections *Mycobacterium avium complex* (MAC), *Mycobacterium abscessus, Mycobacterium fortuitum* and *Mycbacterium marinum*) also showed low MICs (Borisov et al., 2019). Species of mycobacteria are naturally resistant to BDQ, these are: *M. xenopi, M. novocastrense, M. Shimoidei* (Ahmed et al., 2013). The natural resistance of these species would be explained by the genetic diversity observed on the atpE gene coding for the c subunit of ATP-synthase which modifies the binding possibilities of BDQ on these sites, thus hindering its action (Chen et al., 2022).

Clofazimine

Clofazimine can cause pink or red to black discoloration brownish skin and eyes. The color of your skin and eyes should return to normal when you stop taking clofazimine. But this could take several months and even years. If this effect is bothersome, notify your doctor. Clofazimine can cause

dryness and itching skin and eyes. You should always use a good cream moisturizing to prevent skin dryness skin. Also, your skin might be more sensitive to the sun and sunlamps. Consult a doctor immediately if you have a skin rash (patches red on the skin). Discoloration (red or brownish-black) stools, around the eyelids, spitting, sweat, tears and urine is common. This side effect usually requires no care medical. However, if you wear contact lenses, it is recommended that you wore glasses to prevent discoloration of your contact lenses. Many people present with nausea, vomiting, upset stomach and diarrhea with clofazimine. If these effects are bothersome, tell your doctor or your pharmacy.

Clofazimine is a hydrophobic riminophenazine that was initially synthesized as an anti-TB antibiotic in 1954. But in 1959 Chang demonstrated its effectiveness against leprosy, it was called Clofazimine, r e c e n t l y incorporated into the WHO shortened drug-resistant tuberculosis (DR-TB) regimens due to recent evidence of good efficacy. Patients with rifampin-resistant (RR) or MDR-TB can be placed on the shortened DR-TB regimen containing clofazimine or on a regimen comprising both bedaquiline (WHO group A) and clofazimine (WHO group B). The clofazimine resistance was caused by Rv0678 gene mutation with increased clofazimine MIC values, another mutation in the Rv0678 regulator gene (C leading to M1A)was also recently reported to be associated with bedaquiline and clofazimine resistance in MDR-TB cases. They both cause increased expression of the efflux pump MmpL5, and the efflux pump out the drug out of the bacteria and creates resistance. It also has a problem with prolongation of the QT interval, and it can also cause brown discoloration of the skin and gastrointestinal upset.

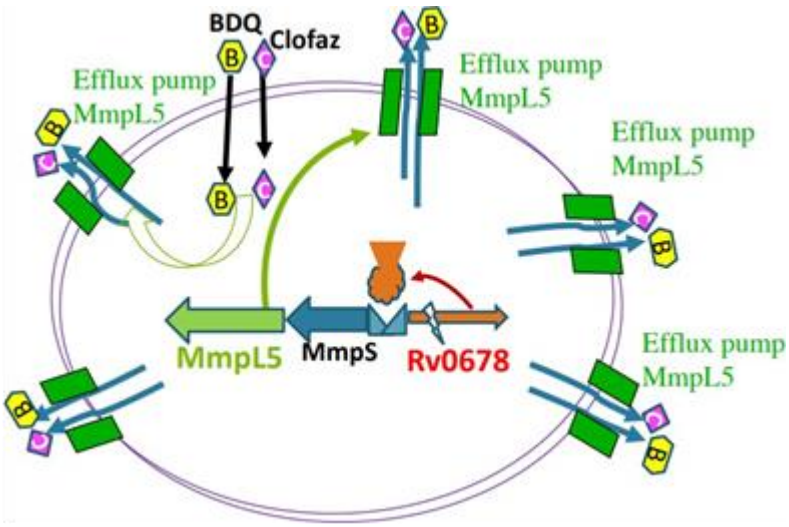


Figure 2.33. The efflux pump MmpL5

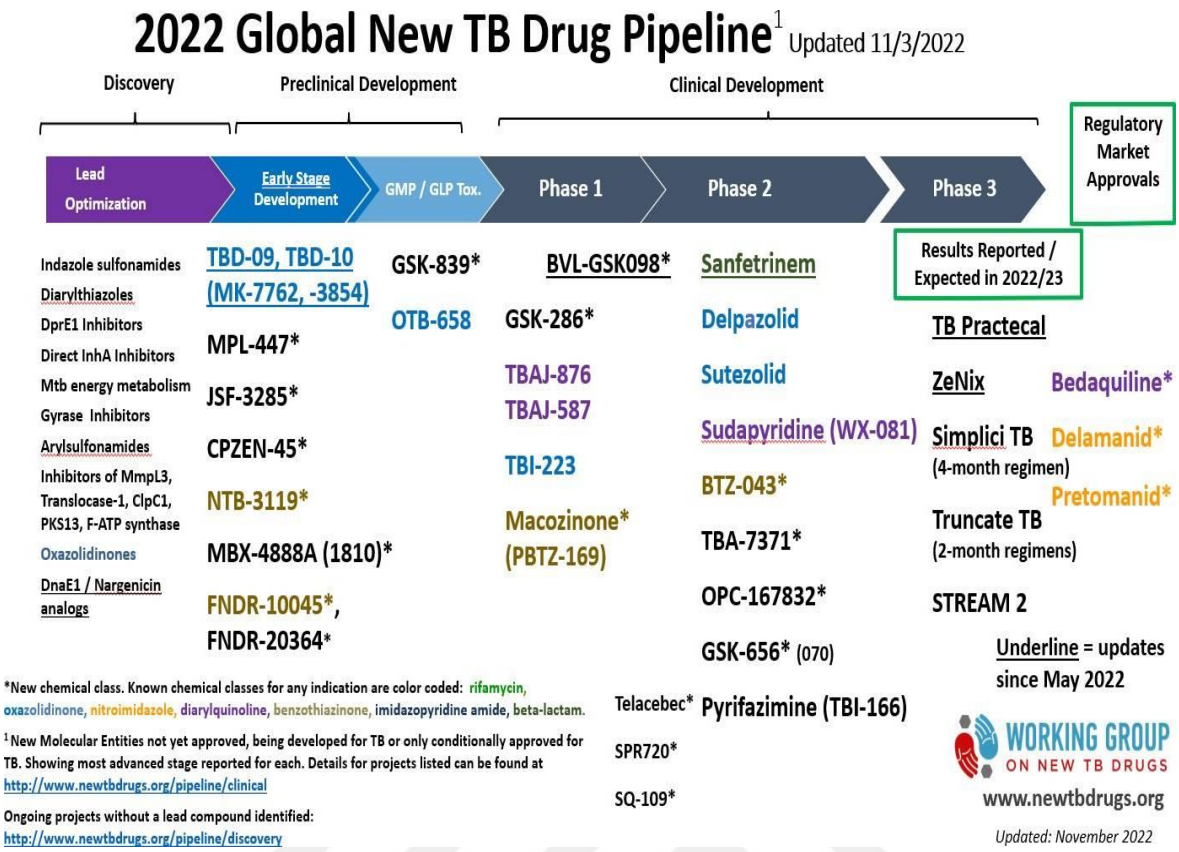


Figure 2.34. 2022 Global New TB Drug Pipeline

2.8. Drug resistance

Unlike other microorganisms, resistance acquisition in mycobacteria usually occurs during the treatment of sub-optimal strains of spontaneous mutations occurring in genes (Faksri et al., 2019). In the phenotypes of MDR, only a single gene mutation is not shown as drug resistance. Resistance to other drugs initially causes classical mutations (Fogel et al., 2015).

It also causes complex MDR phenotypes among the accumulation of mutational changes. Although spontaneous mutations were observed at low levels in prokaryotic cells (Fajardo TT et al., 2006), the mutations that occur are inversely proportional to the binding of the base pair and the size of the genome (EMA, 2013). In the first studies, it was claimed that the selected drugs caused the mutations, but considering the knowledge of the mutations that cause resistance in many of the anti-TB drugs used in the first choice and the possibility of spontaneous mutations occurring in the bacillus; It has been reported that the probability of mutation in each cell division that occurs is 10 times. Considering the calculations; the expected and known risk of mutations in the gene(s); for RIF it has been reported to be $3.32x10^{-9}$, $2.29x10^{-8}$ for INH, and $1x10^{-14}$ for RIF+INH at the same time (Eluk et al., 2021).

It is possible to see bacterial bacillus. Intrinsic resistance among bacilli is also seen alongside resistance acquired by chromosomal mutations (Elbek et al., 2015). Mycolic acids in the cell wall of mycobacteria reduce the cell wall permeability outside of normal structural features and prevent otherantibiotics from entering the cell (Luthra et al., 2018). In addition, thanks to the active efflux pump, the bacilli provide natural resistance against fluoroquinolones, aminoglycosides and tetracyclines, has been reported. Thanks to bioinformatic analysis, it has been reported that the proteins found in the outer membranes of Rv1698 and Rv1973 strains play a role in intrinsic resistance to some antibiotics (Dover et al., 2004). Considering the existence of all these structures, it shows the existence of other mechanisms apart from these mechanisms in intrinsic resistance. As a result, the whiB7 gene found in MTB is a MDR determinant (Daniel et al., 2006).

As a result, it can be said that there is an ancestral MDR determinant. Intrinsic resistance factor is important for the success of TB treatment. It has the potential to be effective against M. tuberculosis in the discovery of new agents that inhibit all these mechanisms (Cohen et al.,2000).

Since the 1950s, when first- and second-line anti-tuberculosis treatments were discovered, the medical world has seen no further innovation in the treatment of tuberculosis (Daniel et al., 2006). This lack of innovation has had repercussions for patients, particularly in terms of the duration of treatment, which has become more or less long, depending on the therapeutic regimen chosen (Brown et al., 2017). On the other hand, the presence of bacteria resistant to first-line antibiotics has forced the medical world to turn to broad-spectrum antibiotics which had been abandoned until then (Conrade et al., 2020). The resistance of M. tuberculosis manifests itself differently depending on the molecular target of the antibiotic used (Cohen et al., 2000). We will say that M. tuberculosis is multi-resistant when it is resistant to at least two major antibiotics in the conventional treatment of tuberculosis, namely rifampicin and isoniazid. How does this multi-resistance manifestitself.

We already know that rifampicin must bind to the β subunit of the *M.tuberculosis* Rna polymerase which is encoded by the rpoB gene. It is by this mechanism that rifampicin blocks the transcription of genes in the bacterium to cause its death. In resistant bacteria, a 27-codon mutation located in the center of the rpoB gene has been observed (Cohen et al., 2000). This mutation is responsible for a defect in the binding between the β subunit and rifampicin, hence its ineffectiveness (Chen et al., 2022).

It is important to remember that: isoniazid undergoes an oxidation reaction by a catalase peroxidase encoded by the KatG gene inside *M.tuberculosis* (Churchyard et al., 2000). This reaction transforms INH into a more active derivative which leads to the death of the bacteria. In the most resistant strains, either a chromosomal deletion of the KatG gene or a mutationof this gene has been observed, resulting in a reduction in its catalytic activity for INH, hence its loss of effectiveness. Furthermore, one of the targets of INH is the InhA protein, encoded by the InhA gene, and which plays a role in the synthesis of mycolic acids in the bacterial cell wall (Campos et al., 2003). When the oxidation of INH does not take place, we notice that a mutation of the InhA gene occurs, causing an overexpression of the InhA41 protein (Conradie et al. 2020). In addition to multi- resistance, there is also the phenomenon of ultra-resistance. We speak of ultra-resistant strains whenthey are resistant to both isoniazid, rifampicin and one of the major antibiotics for the second-line treatment of tuberculosis, either a fluoroquinolone, or an antibiotic by injection, an aminoglycoside. The different variants of ultra-resistance are detailed below (Georghiou et al., 2012).

Fluoroquinolones target DNA-gyrases which are composed of 2 subunits A and B. They are encoded respectively by the gyr A and gyr B genes. Resistance to fluoroquinolones is due to a mutation around codon 90 of gyrA (Jeffrey et al., 2017).

Resistance in aminoglycosides is most often related to modification of their target (Bruchfeld et al., 2015). Thus, resistance to streptomycin will be caused either by themutation of the rpsl gene of the ribosomal protein, or by the mutation of the gene of the 16S RNA. Kanamycin and amikacin are cross-resistant due to the mutation of the gene. However, there arestrains resistant to kanamycin but sensitive to amikacin (Brown et al., 2017).

Bedaquiline and clofazimine are new drugs, used against tuberculosis, so it is very important to discover the mechanisms of bacterial resistance to apply molecular tests in the diagnostic routine and also to find solutions in cases of failure. The changes in antibiotic sensitivities of the population must always be under control.

Our first objective is to improve the genetic bibliography on the mechanisms of resistance to antibiotics. This is why our work was based on the search for the mutations responsible for the cross-resistance between bedaquiline and clofazimine, and more particularly the gene variants of pump efflux. one cannot exclude the importance of phenotypic diagnosis through mics, and also the importance of diagnostic epidemiology. through the three parts we could build a table which develops the bibliography.

3. MATERIAL AND METHODS

3.1. Phenotype diagnosis

3.1.1. Collection of Samples

Our first objective is to improve the genetic bibliography on the mechanisms of resistance to antibiotics. This is why our work was based on the search for the mutations responsible for the cross-resistance between bedaquiline and clofazimine, and more particularly the gene variants of pump efflux. one cannot exclude the importance of phenotypic diagnosis through mics, and also the importance of diagnostic epidemiology. through the three parts we could build a table which develops the bibliography. Between 2021-2022, the Ministry of Health Public Health Institution Adana Regional Tuberculosis Laboratory, which is within the body of Çukurova University Rectorate Tropical Diseases Research and Application Center, Adana, MPT64 card test isolated from clinical materials belonging to patients with suspected pulmonary tuberculosis sent from the provinces of Hatay, Kilis, Osmaniye, Kahramanmaraş, Mersin, Gaziantep and Adıyaman for diagnosis, using MGIT 960 and LJ media. We included a total of 80 MDR isolates, including 20 sensitive strains as a control samples.

3.1.2. Drug susceptibility Testing (DST):

Determination of antibiotic susceptibility with MGIT 960 Systems Preparation of antibiotic solutions

Antibiotics were supplied by the manufacturer in lyophilized form in the MGIT SIRE kit.

These lyophilized drug bottles were diluted and stock solutions were prepared. 4 mL of sterile distilled water was added to each vial of lyophilized antibiotics and mixed until completely dissolved. For each test, 5 MGIT tubes were taken and overwritten. (Control-K, to others as S, I, R and E). 0.8 ml of BACTEC MGIT 960 SIRE Supplement (Oleic acid, dextrose, catalase, bovine albumin, polyethylene stearate) was added to each tube under aseptic conditions. A micropipette aid of 100 µL of reconstituted lyophilized drug solutions was added to each of the four SIRE tubes under aseptic conditions in accordance with company recommendations. No antibiotics were added to the controltube (Thee et al., 2016).

Preparation of inoculum

Positive MGIT tubes were used to prepare the inoculum in the study. According to company recommendations, an MGIT tube can be used up to day 5, including the day (day 1) after being positive on the MGIT 960 device. In the study, if the tube was positive for more than 5 days, a new MGIT tube was sub cultured and followed until positive 1-5. used for antibiogram. If the day the tubes were positive did not exceed 1-2 days, the tubes were mixed directly and used as inoculum. If the tubes were positive for 3-5 days, they were diluted 1/5 with physiological saline

and this suspension was used as inoculum (Tiberi et al., 2017).

Inoculation

0.5 mL of the inoculum suspension was added to each of the four labeled MGIT tubes (S, I, R, E). To prepare a 1/100 control suspension, 0.1 mL of the microorganism suspension was added to 9.9 mL of sterile SF and mixed well.0.5 mL was added to the tube. They were recorded and placed on the MGIT 960 device (Rao et al., 2013).

Interpretation of results

Each medicated tube and control tubes are connected to the MGIT 960 device, the reproductive index (GI) was tracked daily with the "Epicenter Reader". The samples with the results of the tests were interpreted as resistant or susceptible by the MGIT 960 device. Observation of 400 GI growth in the control tube before 4 days was interpreted as contamination or inoculum excess and the antibiogram was repeated. On the other hand, control tubes that did not grow after 13 days (400 GI) were evaluated as insufficient bacterial density and the antibiogram was performed again.

3.1.3. Minimum Inhibitory Concentration (MIC):

Positive signal of culture tube planted with M. tuberculosis in MGIT 960 system, the day it is given is considered as day 0. In order for ADT to be studied, 1-5 days of the culture after the device gives a positive signal. It should be incubated at 37ºC for the next day (Prasad et al., 2021). ADT cannot be studied from positive cultures that have waited more than five days. These tubes should be re-passed, and ADTs should be studied in the first 5 days after the positivity. Therefore, all *M. tuberculosis* cultivated MGIT. On the day when the tubes gave a positive signal, they were removed in a 37ºC oven and kept for 1-5 days, and then ADTs were studied. For each strain, dilutions containing bacteria at 3 different concentrations, which we named 'A', 'B' and 'C', were prepared (Rocha et al., 2021).

Dilution A) 1:5 Pre-Dilution of *M. tuberculosis*

1-5 Days: A 1:5 dilution in 0.9% NaCl (0.9% saline solution containing sodium chloride) was prepared from the MGIT culture. This step is a pre-dilution.

For this purpose, 8.0 ml of 0.9% NaCl solution was placed in a sterile 50 ml centrifuge tube; 2.0 ml of vortexed bacterial culture was added to it.

Dilution B) 1:100 for Growth tubes

Dilution B dilution is done to prepare the inoculum to be added to tubes containing different drug concentrations. For this purpose, 9.9 ml 0.9% NaCl was added to a new sterile 50 ml centrifuge tube. Solution was placed. 0.1 ml of A dilution was added on it. Thus, the bacterial concentration was diluted 1:100.

Dilution C) 1:10,000 Dilution for Growth Control Tube (GC)

The C dilution is made to prepare the inoculum to be transplanted into the growth control tube (GC tube). For this purpose, 9.9 ml 0.9% NaCl was added to a new sterile 50 ml centrifuge

tube. Solution was placed. It was added to it by taking 0.1 ml of B dilution. Thus, the dilution was prepared as 1:100 x 1:100 = 1:10.000 for the inoculum concentration to be seeded into the GC tube.

Preparation of Drug Solutions:

Pure powders of bedaquiline and clofazimine were requested from the manufacturers to be used in phenotypic susceptibility tests; however, the manufacturers of these two drugs, which are in phase 3, refused to give the powder formulations of the drugs to be used for experimental purposes. For this reason, bedaquiline (100 mg) and clofazimine (100mg) tablet forms were used in the study (Liu et al., 2019). Drugs in tablet form were dissolved in 100% pure dimethyl sulfoxide (DMSO, Merk, USA). The applied method is one of the investigators' methods, which is also shown in the WHO Critical Drug Concentrations Guidelines (Zurenko et al., 2001). The calculations for the preparation of drug concentrations are as follows:

Bedaquiline tablet weighs 470 mg and contains 120 mg of bedaquiline fumarate. This is equivalent to 100 mg of bedaquiline.

Total Volume = MGIT Medium (7.0 ml) + MGIT Growth Supplement (0.8 ml) + drug (0.2 ml) + inoculum (0.5) = 8.5 ml

Calculation of dilution factor = Total volume / drug volume = 8.5 / 0.2 = 42. **Preparation of drugstock solution =** Highest concentration x dilution factor = 6.4 x 42.5 = 272 mg/1000 ml **for 4 strains** = 200 µl drug x 4 = 800 µl + 200 µl (pipet error margin) = 1000 µl.

In this study, drug solutions were prepared for 100 strains with possible repetitions. Up to 2.35% DMSO in the MGIT tube can be tolerated by *M. tuberculosis* bacilli (9). Similar calculation of different bedaquiline clofazimine concentrations in Table 3.1.

Table 3.1. Preparation of Bedaquiline stock solutions

	Concentrations (mg/L)	Preparation of Pharmaceutical Stock Solutions	Volume (ml)
Bedaquiline	1.6	Stock 1: 13.6 mg of bedaquiline is dissolved in 50 ml of 100% DMSO.	25
	1	Stock 2: 8.5 mg of bedaquiline is dissolved in 50 ml of 100% DMSO.	25
	0.6	Stock 3: 25 ml of Stock 2 is taken and 25 ml of 100% DMSO is added to it.	25
	0.5	Stock 4: Solution A: Take 25 ml of Stock 1 and add 25 ml of 100% DMSO and mix. 25 ml of solution A is taken and 25 ml of 100% DMSO is added.	50
	0.3	Stock 5: Take 25 ml of Stock 3 and add 25 ml of 100% DMSO.	25
	0.2	Stock 6: Take 25 ml from Stock 4 and add 25 ml of 100% DMSO.	50
	0.125	Stock 7: Take 5.078 ml of solution A and add 19.922 ml of 100% DMSO.	50
	0.06	Stock 8: Take 25 ml of Stock 6 and add 25 ml of 100% DMSO.	25

BDQ and CLZ drug concentrations, which were previously prepared in DMSO and frozen at -80ºC, were thawed at room temperature before the study. Remaining amounts of dissolved drugs were not used in subsequent studies. BDQ from tube 2 to tube 10 and tube 19 from tube 11 to tube 19 in sequence. 0.2 ml of CLZ drug concentrations were taken up to the tube and added to the tubes, respectively.

Passage was made from 0.5 ml main culture tube to AC tube. The purpose here is to ensure the viability of the strain, as no drug is added to the tube and no dilution is made. 0.5 ml of the B dilution was taken and added to each tube containing the drug concentration. 0.5 ml of C dilution was taken and added to the GC tube. In order to ensure that the total liquid volume of the GC tube has the same liquid volume with the tubes containing the drug, 0.2 ml of sterile 0.9% NaCl solution was also added. Thus, the total volume in the tubes was: 7.0 ml of MGIT broth + 0.8 ml of OADC + 0.2 ml of drug (or NaCl) + 0.5 ml of inoculum = 8.5 ml.

Table 3.2. Preparation of clofazimine stock solutions

	Concentrations (mg/L)	Preparation of Pharmaceutical Stock Solutions	Volume (ml)
Clofazimine	1.6	Stock 1: 13.6 mg of bedaquiline is dissolved in 50 ml of 100% DMSO.	25
	1	Stock 2: 8.5 mg of bedaquiline is dissolved in 50ml of 100% DMSO.	25
	0.6	Stock 3: 25 ml of Stock 2 is taken and 25ml of 100% DMSO is added to it.	25
	0.5	Stock 4: Solution A: Take 25 ml of Stock 1 and add 25 ml of 100% DMSO and mix. 25 ml of solution A is taken and 25 ml of 100% DMSO is added.	50
	0.3	Stock 5: Take 25 ml of Stock 3 and add 25 ml of 100% DMSO.	25
	0.2	Stock 6: Take 25 ml from Stock 4 and add 25 ml of 100% DMSO.	50
	0.125	Stock 7: Take 5.078 ml of solution A andadd 19.922 ml of 100% DMSO.	50
	0.06	Stock 8: Take 25 ml of Stock 6 and add 25ml of 100% DMSO.	25

Evaluation of Test Results:

Drug susceptibility assessment was performed between 4-13 days. The test in tubes that gave positive before four days was terminated due to the presence of contamination. These tubes were decontaminated and the test was repeated. In accordance with the drug sensitivity procedure, the test was continued until the growth amount in the GC tube reached 400 Growth Units (GU) so that the test could give a meaningful result. The test was terminated when the growths in the tubes containing the drug reached 100 GU. Bacteria were considered resistant to drug concentrations reaching 100 GU before the GC tube reached 400. After the GC tube reached 400 GU, it was waited for up to 7 days. Medication reaching 100 GU within this period, bacteria were considered intermediate to the concentration in the tubes. Bacteria were considered susceptible to drug concentrations in medicated tubes that did not reach 100 GU within 7 days after GC reached 400 GU. Tubes with a positive signal reaching 100 GU after the 13th day in medicated tubes were not considered positive. The reason for this is that the effectiveness of the drugs decreases after the 13th day.

3.2. Molecular diagnosis:

Mutations in M. tuberculosis pepQ, Rv0678 and Rv1979c genes, which are known to be responsible for bedaquiline and clofazimine resistance, were investigated. For this purpose, DNA isolation, PCR, visualization of the obtained amplicons on agarose gel and then Sanger sequencing were performed and the results were analyzed.

3.2.1. Extraction of DNA

1. Growth detected from Middlebrook 7H9 medium was taken into a 0.5ml microcentrifuge tube.
2. Bacteria were inactivated by incubating at 80°C for 30 minutes.
3. Spin at 15000xG for 15 minutes, supernatant discarded.
4. 0.5ml of 1xTE buffer was added.
5. Spin at 15000xG for 15 minutes, supernatant discarded.
6. 0.25ml of 1xTE buffer was added.
7. 50-100 l glass beads (SIGMA acid washed glass beads) were added to the tube.
8. Subject to cell lysis in Mickle (Mickle tissue disintegrator) device for 2 minutes held.
9. Spin for 10 minutes at 15000xG, supernatant was taken into a new microcentrifuge tube.
10. Stored at -20 C.

3.2.2. PCR amplification:

The sequences of genes including were analyzed by PCR: Rv0678 (Be), pepQ (Be), Rv1979c (Clz). Primers were used to Amplify in this study are listed in Table1.

Table 3.3. Primers

Primers	**Sequence (5′→3′)**
PepQ / Rv2535c	ATC AAT GCC CCC TGG AAC
PepQ / Rv2535c	GCA CGT TCT TCA ACT TGG TG
Rv0678_F	GTTCGATCCGAGCAGCATCGCT
Rv0678_R	GTTCGATCCGAGCAGCATCGCT
Rv1979c F	CGGTATCTTCCTAACTCCAGGT
Rv1979c R	GTTCGATCCGAGCAGCATCGCT

Table 3.4. PCR mix of genes.

PCR Mix solution	**Stock Concentration (mM)**	**Volume (µl)**
H2O	1	32,75
Reaction buffer incl. MgCl2	10	5,0
dNTP-Mix (3x d UTP)	2	5,0
Primer Forward	10	1,0
Primer Reverse	10	1,0
FastStart Roche Taq Polymerase	5	0,25
DNA-Insta Gene Extract (Kap. 32,43204)	5,0	5,0

Table 3.5. Rv0678 gene PCR conditions.

Temperature	**Duration**	**Number of Cycles**
96°C	10 s	25
94°C	5 s	
56°C	4 m	
4 °C	∞	

Table 3.6. PepQ gene PCR conditions.

Temperature	**Duration**	**Number of Cycles**
96°C	10 s	25
94°C	5 s	
56°C	4 m	
4 °C	∞	

Table 3.7. Rv1979c gene PCR conditions.

Temperature	**Duration**	**Number of Cycles**
96°C	10s	25
94°C	5 s	
56°C	4m	
4 °C	∞	

PCR product amplicons were loaded on agarose gel and checked. Amplicons were immediately sequenced or stored at -20ºC for studies.

3.2.3. Sanger Sequencing:

The presence of mutations in gene regions known to be responsible for bedaquiline and clofazimine resistance in *M. tuberculosis* were investigated by Sanger DNA sequencing method in Swiss strains and by WGS method in Turkish strains. Automated sequence analysis methods have allowed DNA to be analyzed in a shorter time. In these methods, the reactions were carried out in a single tube containing all four dd NTPs, each labeled with dyes that radiate at different wavelengths (dye-terminator sequencing) and laser reading was achieved (Rodriguez et al., 2002).

Purification of PCR Products

We purified the obtained PCR products by using the "Exo SAP-IT" kit, by adding 2µl of enzyme to 5µl PCR product, in accordance with the company's instructions, after incubation at 37°C for 15 minutes, and then incubating at 85°C for 15 minutes.

Cycle Sequence PCR Amplification

We performed the single-chain amplification of the purified PCR products using the forward primer with the Big Dye ® Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems) containing the dNTP and dd NTP reaction mix, using the volumes indicated in Table 3.9.

Table 3.8. PCR Amplification components

PCR Amplification components	**volume**
Ready Reaction Mix (RR-100) 5x	2µl
Sequencing Buffer 5x	2µl
Primer*-F	2µl
dH2O	2µl
PCR products	2µl
Total volume	10µl

Components and volumes used for Cycle Sequence PCR Amplification: * Forward primer of the Gene Region to be Sequenced.

We performed the amplification of the samples prepared for the cycle sequence in the thermal cycler device in the following heat cycles.

Table 3.9. Cycle Sequence Amplification Stages

Temperature	**Duration**	**Number of Cycles**
96°C	10 s	25
94°C	5 s	
56°C	4 m	
4 °C	∞	

Purification of Cycle Sequence PCR Products and Loading into Sequence Analysis Device: We purified the Cycle Sequence products again using the sephadex spin column method before loading them into the sequencing device. We carried out the purification process according to the following protocol by loading the amplicons into the columns formed with the mixture obtained by adding 14 ml of water to 1gr of sephadex chemical.

- ➢ We placed the empty spin columns in 1.5ml collection tubes.
- ➢ We added 750 µl of sephadex mix to each column.
- ➢ 2 min at 4500 rpm. We performed centrifugation.
- ➢ We loaded the amplicons without damaging the sephadex column formed by draining the liquid in the collection tube.

- 3 min at 4500 rpm. After centrifugation, the pure cycle sequencing product obtained in the collection vessel was loaded into the ABI Prism 3730 sequencing device and we worked in accordance with the device usage procedures.

We analyzed the obtained results in the Sequencing Analysis 5.1 (Applied Biosystems) program on the ABI Prism 310 DNA sequencer (Applied Biosystems).

3.2.4. Epidemiology diagnosisSpoligotyping:

In vitro amplification of spacer DNA by PCR

After DNA extraction, the spoligotyping method was applied for the 100 selected isolates of *M. tuberculosis*. we started with the amplification of DR regions of 36 bp.

DRa: 5'-GGT TTT GGG TCT GAC GAC-3' (labeled with biotin at the 5' end)

DRb: 5'-CCG AGA GGG GAC GGA AAC-3'

The primer targeting the DR region was synthesized. A positive control (M. tuberculosis H37Rv, M. bovis BCG or clinical isolate with a known genotype) and a negative control (dH2O) were used in each cycle of the study. The following amount of reaction mixture was prepared for each strain.

9.5ml dH2O

12.5ml 2x PCR Master Mix (Fermantas)0.25ml DRa (25pmol/ml)

0.25ml DRb (25pmol/ml)2.50ml DNA

The tubes containing the PCR reaction mix were placed in a thermal cycler (Applied Biosystem AB) and run in the following program:

95°C 5 min

. 94°C 1 min

55°C 1 min /40 cycle72°C 45 sc.

72 °C 10 min

4 °C µ

The PCR reaction mixture was prepared in a sterilized cabinet free of mycobacterial DNA. The DRa primer being labeled with biotin, it was stored at +4°C. The DRb primer was aliquoted and stored at -20°C.

Solutions

0.5M EDTA (pH 8.0)

EDTA 46.53g

d H2O 250ml

The pH was adjusted to 8.0 and autoclaved.

20mM EDTA (pH 8,0)

dH2O 960ml 0.5M EDTA 40ml

20x SSPE (pH 7,4)

0,2M Na2HPO4 28,39gr/L

3,6M NaCl 210,24gr/L

dH2O 960ml

0,5M EDTA 40ml (autoclave)

2xSSPE

20 × SSPE 100ml

dH2O 900ml

%10 SDS

dH2O 250ml Preheated (60°C)SDS 25g

2xSSPE / %0.1SDS

dH2O 890ml

20 × SSPE 100ml

% 10 SDS 10ml

SSPE solution and SDS solution were measured in separate measuring tapes.

2xSSPE / %0.5SDS

dH2O 850ml

20 × SSPE 100ml

%10 SDS 50ml

%1 SDS

% 10 SDS 100ml

dH2O 900ml

10 × TE

Tris HC l 15,764g (100mM)0,5M EDTA 20ml (10mM)

dH2O 980ml

Hybridization with PCR product and detection

After the amplification of the DR region by the use of biotin-labeled primers, the hybridization of the amplicons was carried out.To make the products visible after hybridization, we added the enzyme alkaline phosphatase streptavidin and the chemifluorescent substrate phosphate as follows.

1) All buffer solutions were brought to the desired temperature beforehand.Quantities required for a membrane:

   80ml 2xSSPE / 0.1% SDS, 60°C
   160ml 2xSSPE / 0.5% SDS, 60°C
   160ml 2xSSPE / 0.5% SDS, 42°C
   160ml 2xSSPE, room temperature

2) 20ml of PCR product was added onto 150ml of 2xSSPE / 0.1% SDS.
3) The PCR products were denatured at 99°C for 10 minutes.
4) When denaturing PCR products, membrane (ISOGEN) with 80ml 2xSSPE / 0.1% SDSIt was washed at 60°C for 5 minutes.
5) While placing the sponge pad (Immunetiks Plastic Cushion PC200) and the membrane into the miniblotter (Miniblotter-3024), care was taken to ensure that the slots of the miniblotter wereperpendicular to the oligonucleotides connected to the membrane as a line.
6) Residual fluid in the slots was removed by aspiration.
7) The slots were filled with the diluted hot PCR product. The miniblotter was hybridized (FINEPCR-combi-SV120) for 60 minutes at 60°C in a horizontal position.
8) Samples were aspirated from the slots and removed from the upstream miniblotter using plastic forceps.
9) The membrane was washed twice with 80ml 2xSSPE / 0.5% SDS at 60°C for 10 minutes.
10) The membrane was put in a bag file.
11) On 5ml 2xSSPE / 0.5% SDS, 1.25ml streptavidin-alkaline phosphatase conjugate (500U/ml) (Promega) was added, the mixture was poured onto the membrane inside the three-sided sealed bag file, the air bubbles were removed and the open edge of the bag file was closed by heating.

. It was incubated at 42°C for 45-60 minutes.

12) The membrane was incubated twice with 80ml 2xSSPE / 0.5% SDS for 10 minutes at 42°C, rinsed with 80ml 2xSSPE for 5 minutes at room temperature.
14) 5ml of ECF substrate (Attophos AP Flourescent Substrate, Promega) was added onto the membrane, incubated in the bag file for 60 minutes.

15) The membrane was wrapped with stretch film and exposed for 20 seconds (QUANTUM-ST4 3020-WL/BLUE/20M) in the appropriate illumination cassette (450nm).

Imaging and Interpretation of the Membrane

The spoligotyping method is spacer during the interpretation of the results. The presence of spacer regions was evaluated by taking into account the blotting that occursas a result of hybridization of the PCR product with the membrane to which the oligonucleotides arecovalently attached.

The results were converted to the "Octal code" format consisting of 15 numbers between 0-7 using the Octal coding key specified in Table 3. Clusters and families (clades) were determined using the obtained data SpolDB4 database.

Regeneration of Membrane

In order for the membrane to be reused after hybridization and imaging, the following washing and regeneration steps were applied.

1) The membrane was incubated twice with 1% SDS at 80°C for 30 minutes.
2) The membrane was incubated with 20mM EDTA pH 8 for 15 minutes at room temperature.

The membrane was stored in 20mM EDTA pH 8 in a bag file, with the number of usesand the date written, and stored at +4 C.

4. RESULT AND DISCUSSION

Tuberculosis is an infectious disease transmitted by Koch's bacillus, which affects 10.4 million people worldwide. Each year, WHO estimates that the number of reported cases is less than the number of actual cases. The disease is transmitted by air, which facilitates the risk of contamination (Shetye et al., 2020). The standard treatment for susceptible tuberculosis isbased on the administration of a therapy which, when it is well conducted, offers an optimal chance of recovery. Poor treatment on the other hand, whether in terms of drugs, non-compliance or poor duration of treatment, generates resistance.

We speak of multi-resistance to designate the inability to treat tuberculosis with the two main antibiotics of the standard treatment for susceptible tuberculosis. Currently, treatment against multi-resistant tuberculosis is based on the combination of 4 to 5 anti-tuberculosis drugs, which are administered over a long period, longer than that of a susceptible tuberculosis due to the lesser effectiveness of these treatments. After 4 decades without major therapeutic innovation in this field, and faced with the emergence of increasingly resistant strains, the intensification of research has led to the discovery of bedaquiline, an antibiotic whose mechanism of action is currently revolutionizing treatment of multi-resistant tuberculosis (Özer et al., 2020).

After a few reminders on tuberculosis, its physiopathology and an overview of current therapeutic regimens; we will discover how bedaquiline, with the help of clinical studies, has demonstrated its effectiveness and made its use an absolute necessity, even if phase III clinical studies are still currently underway. MDR-TB is an emergency alarm in the global strategy for the fight against tuberculosis. In the case of a patient with confirmation of the presence of MDR-TB strains through standard diagnostics, treatment periods are long, with antibiotics that are toxic and more expensive than first-line antibiotics. According to the U.S. Food and Drug Administration, when the standard antibiotics are not available, bedaquiline can be an alternative, short treatment against MDR and sensitive TB-strains (Prasad et al., 2021).

Bedaquiline and clofazimine are new drugs, used against tuberculosis, so it is very important to discover the mechanisms of bacterial resistance to apply molecular tests in the diagnostic routine and also to find solutions in cases of failure. The changes in antibiotic sensitivities of the population must always be under control.

Our first objective is to improve the genetic bibliography on the mechanisms of resistance to antibiotics. This is why our work was based on the search for the mutations responsible for the cross-resistance between bedaquiline and clofazimine, and more particularly the gene variants of pump efflux. one cannot exclude the importance of phenotypic diagnosis through mics, and also the importance of diagnostic epidemiology. through the three parts we could build a table which develops the bibliography.

4.1. Drug Resistance Profiles:

we can start with the results of the sensitivity tests with the four" SIRE" antibiotics, and that to be sure that our strains are MDR -TB strains. Our table shows the resistance profiles for each strain in relation to the year of isolation. we can confirm that our strains are at least resistant to two antibiotics isoniazid and rifampicin based on the definition of MDR. We included as control 20 sensitive TB-isolates to use it as references in our analysis steps. The clinical sample types and numbers from which *M. tuberculosis* strains were isolated are given in Table 4.3.

Table 4.1. Clinical samples from which *M. tuberculosis* strains are isolated

Isolates	Drug Resistance Profile	Year of Isolation
58	I,R	2021(39)/2022(19)
10	S,I,R	2021(5)/2022(5)
8	S,I,R,E	2021(4)/2022(4)
4	I,R,E	2021(2)/2022(2)
20	S	2021

Table 4.2. Sample Types

Sample Type	Number
Sputum	75
Induced sputum	18
BAL	7

4.2. Bedaquiline and Clofazimine susceptibility distributions of Turkish TB-strains: MIC values

The susceptibility results for bedaquiline are shown in Figures 4.2 and table. In the graph created by the software, the bacterial growth amount in the growth control tube reached the threshold value of 400 on the 8th day. Growth in the tube containing 0.25 µg/ml bedaquiline occurred on the 4th day and the strain exceeded the 100 GU threshold. Therefore, this strain is resistant to 0.25 µg/ml of bedaquiline. The occurrence of adverse effects was also similar between the addition of BDQ to a treatment for multidrug-resistant tuberculosis and the administration of a 2nd line treatment for MDR-TB.

The increase in hepatic transaminases that had already been observed during preclinical studies was more frequently observed in the BDQ group than in the placebo group. The occurrence of these adverse events suggests that the use of BDQ in patients known to have underlying liver disease should be considered with caution. The current data suggest, close monitoring of patients during concomitant administration of BDQ with drugs with liver toxicity such as pyrazinamide for example. Furthermore, it has been established that the presence of BDQ in the treatment regimen of

patients was associated with a moderate prolongation of the mean QT interval, which raises an important cardiac toxicity problem, since a prolongation of the QT interval is considered to be a potentially fatal risk factor, called torsade de pointe. There is a risk of prolonged prolongation of the QT interval when combining BDQ with other medicinal products which also prolong the QT, such as fluoroquinolones or 4-aminoquinoline antimalarials.

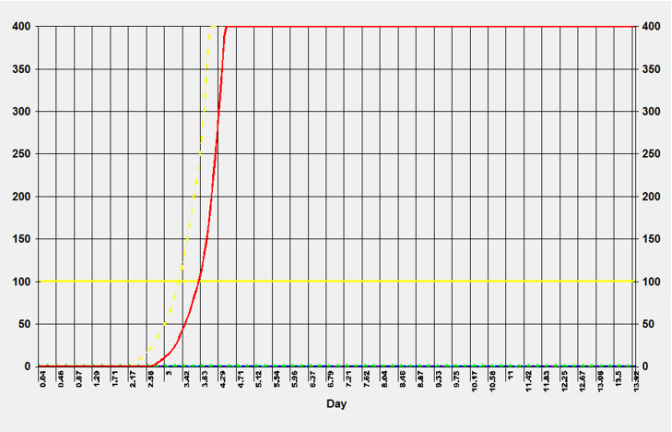


Figure 4.1. Bedaquiline-Resistance in MDR-TB strain with software detection.

A medium resistant strain to different bedaquiline concentrations is shown in Figure 4.3. M. tuberculosis strain is seen. After the 4th day when the growth control tube reached GC 400 GU, the growth in the tubes containing bedaquiline 0.25, 0.4, 0.65 and 0.8 µg/ml exceeded the 100 GU threshold in the first 7 days. Accordingly, this strain is moderately resistant to 4 different bedaquiline concentrations with growth detected. Similarly, Clofazimine susceptibility of the strains was determined with the same software (Figure 4.2).

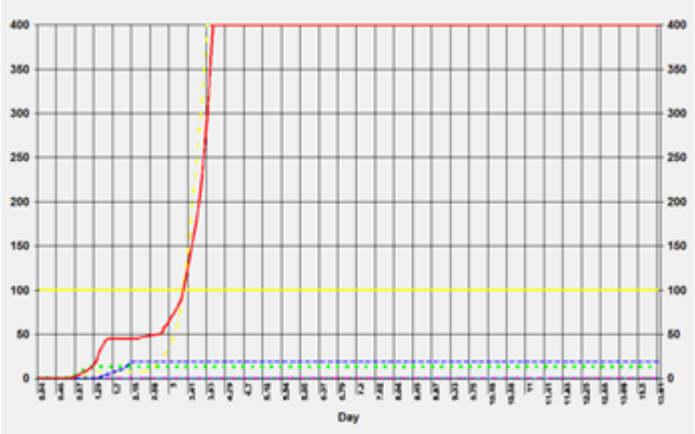


Figure 4.2. Clofazimine-Resistance in MDR-TB strain with software detection.

Species of mycobacteria are naturally resistant to BDQ, these are: *M. xenopi*, *M. novocastrense*, *M. Shimoidei* (Pang et al, 2012). The natural resistance of these species would be explained by the genetic diversity observed on the atpE gene coding for the c subunit of ATP-synthase which modifies the binding possibilities of BDQ on these sites, thus hindering its action. BDQ appears to be specific to mycobacteria. Much higher MICs have been observed in other bacterial species (Table 4.3). It will therefore have no activity on other non-mycobacterial bacteria. A few examples show this well, indeed its MIC for *Corynebacterium* and *Helicobacter pylori* is around 4.0µg/ml and that of *Escherichia coli* is greater than 32µg/ml (Lewis et al., 2008).

Bedaquiline is the first antibiotic against tuberculosis which acts by inhibiting the energy production (ATP) of a bacterium. This new drug inhibits ATP synthase mycobacterial inducing the death of tubercle bacilli, both those in replication and those which are dormant. It has the advantage of three weekly administrations which improves compliance rather than daily administration (Vija et al., 2013).

For the realization of these clinical studies, it was first essential to establish a safety profile of bedaquiline through a preclinical study, conducted on healthy volunteers. Then it took confirm its efficacy and tolerance profile. Phases IIa and IIb of the C208 study on the bedaquiline demonstrated its superiority over placebo on the criterion of main judgment (conversion of bacteriological culture) at 83 days against 125 days for the placebo, as well as on the secondary endpoints (percentage of of bacteria cultures) at 79% for bedaquiline and 58% for placebo (Pozniak et al., 2003).

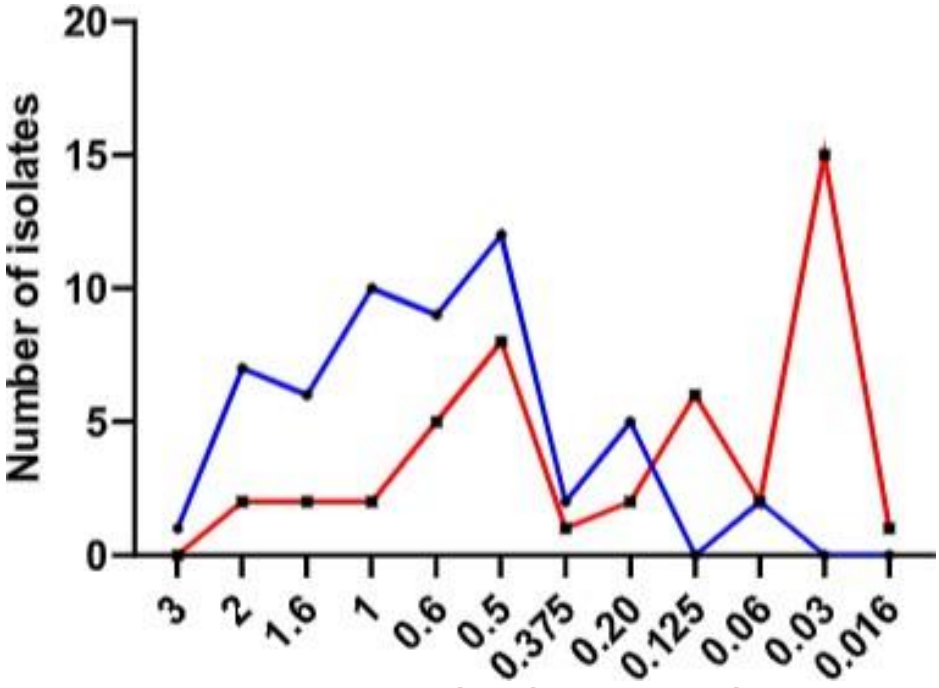


Figure 4.3. Correlation between CLZ and BED

Table 4.3. MIC values of bedaquiline and clofazimine

NO	DST	MIC CLZ µg/ml	MIC BED µg/ml	NO	DST	MIC CLZ µg/ml	MIC BED µg/ml
1	S,I,R	2	0.25	26	I,R	0.3	0.8
2	I,R	0.5	0.8	27	I,R	0.6	0.6
3	S,I,R,E	0.5	2	28	I,R	0.5	1
4	I,R	0.5	4	29	I,R	0.125	0.25
5	S,I,R	0,60	0.25	30	I,R	1	1
6	S,I,R	0.5	1	31	I,R	0.125	0.4
7	I,R	0.5	2	32	S,I,R,E	2	4
8	I,R	0.3	1	33	S,I,R	0.5	1
9	I,R	0.6	0.8	34	I,R	0.5	0.8
10	S,I,R	2	0.25	35	S,I,R,E	2	0.25
11	I,R	0.5	0.4	36	S,I,R,E	1.6	0.8
12	I,R	1	2	37	I,R	0.5	1
13	I,R	2	4	38	I,R,	1,.6	0.6
14	I,R	0.06	0.25	39	I,R,E	0.6	1
15	I,R	2	6.4	40	I,R,	1.6	0.6
16	I,R	0.3	0.6	41	sensitive	0.03	1
17	I,R	0.5	1	42	sensitive	0.03	0.25
18	I,R	0.5	0.8	43	sensitive	0.03	0.25
19	I,R	1	0.4	44	sensitive	0.03	0.25
20	I,R	1	0.25	45	sensitive	0.03	0.25
21	I,R	0.125	6.4	46	sensitive	0.03	0.25
22	I,R	0.6	0.25	47	sensitive	0.03	1
23	I,R	1	2	48	sensitive	0.03	0.6
24	I,R,E	1	2	49	sensitive	0.125	0.6
25	I,R	1	0.25	50	sensitive	0.6	1
NO	**DST**	**MIC CLZ** µg/ml	**MIC BED** µg/ml	**NO**	**DST**	**MIC CLZ** µg/ml	**MIC BED** µg/ml
51	S,I,R	0.5	0.25	76	I,R	0.5	0.8
52	I,R	0.2	0.4	77	I,R	0.5	0.25
53	S,I,R,E	2	0.8	78	I,R	0.2	0.6
54	I,R	0.6	0.25	79	I,R	0.2	4
55	S,I,R	0.6	0.25	80	I,R	0.2	1
56	S,I,R	0.6	0.25	81	I,R	0.125	0.25
57	I,R	0.6	0.6	82	S,I,R,E	2	0.8
58	I,R	0.2	6.4	83	S,I,R	1	2
59	I,R	0.6	0.4	84	I,R	0.5	0.25
60	S,I,R	1	0.25	85	S,I,R,E	0.5	2
61	I,R	1.6	0.6	86	S,I,R,E	0.2	0.6
62	I,R	1.6	2	87	I,R	0.5	0.25
63	I,R	0.5	6.4	88	I,R,	1,.6	0.25
64	I,R	0.06	0.25	89	I,R,E	0.6	0.8
65	I,R	3	0.4	90	I,R	1.6	0.25
66	I,R	0.3	0.6	91	sensitive	0.03	0.4
67	I,R	0.2	0.8	92	sensitive	0.03	1
68	I,R	0.5	0.25	93	sensitive	1	0.25
69	I,R	1	0.6	94	sensitive	0.03	0.6
70	I,R	0.6	0.4	95	sensitive	0.016	1
71	I,R	0.125	4	96	sensitive	0.06	0.25
72	I,R	0.6	2	97	sensitive	0.03	0.6
73	I,R	1	0.25	98	sensitive	0.03	1
74	I,R,E	2	2	99	sensitive	0.03	0.25
75	I,R	1	0.8	100	sensitive	0.06	0.4

MIC values of bedaquiline and clofazimine of our TB strains were determined as < 0.25 µg/m:>6.4 µg/ml and <0.016 µg/ml :>3 µg/ml, respectively shown in the table. We tested 80 MDR TB strains and 20 drug-susceptible strains, which were isolated between 2021 and 2022. To avoid the waste of antibiotics, laboratory materials, and especially our project budget, we chose 7 to 12 concentrations (the table 4.3). For bedaquiline, the mean MIC for the susceptible strain was 0.25 mg/litter, and the median was 0.4 mg/litter. for MDR TB strains have a MIC value of 0.6 mg/liter. For clofazimine, the MIC value for the susceptible phenotype was 1 mg/litter; the overall median

was 0.25 mg/litter. for MDR was between 1.6 mg/litter and 3 mg/litter. A bedaquiline-associated resistance mutation was found in the Rv0678 (fMet1Ala) gene, which also confers cross-resistance to clofazimine and which had a drug MIC of 6.4 mg/liter.

Bedaquiline exhibits activity against Mycobacterium tuberculosis with a minimum inhibitory concentration (MIC) between ≤ 0.008 and 0.12 mg/l for both susceptible and resistant strains (multi-resistant strains including pre-ultra-resistant strains and ultra-resistant strains). The N-mono dimethyl metabolite (M2) is not considered to contribute significantly to clinical efficacy, given its lower average exposure in humans (23% to 31%) and anti-mycobacterial activity (3 to 6 times weaker) compared to those of the parent compound. The intracellular bactericidal activity of bedaquiline in primary peritoneal macrophages and in a macrophage cell line was greater than its extracellular activity (Luthra et al., 2018). Bedaquiline also has bactericidal activity against dormant tubercle bacilli (not in replication). In the mouse model of TB infection, bedaquiline demonstrated bactericidal and sterilizing activities. Bedaquiline is bacteriostatic for many nontuberculous mycobacterial species. *Mycobacterium xenopi, Mycobacterium novocastrense, Mycobacterium shimmied* and non-mycobacterial species are considered inherently resistant to bedaquiline (Hameed et al., 2018).

4.3. Low-level BED-CLZ cross-resistance in MDR-TB strains:

4.3.1. PCR Products: PepQ, Rv0678, Rv1979c

In the other side of our work, genetic studies are based on the amplification of 3 genes pepQ, Rv0678 and Rv1979c responsible for cross resistance by PCR. we obtained the bands of each gene in the electrophoresis gel, using markers to clearly specify the size of base pairs.

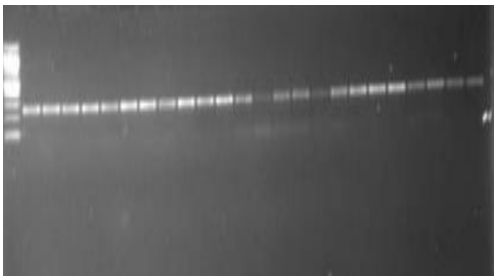


Figure 4.4. PCR products of amplified Rv1979c gene

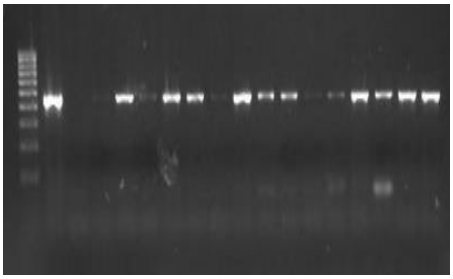


Figure 4.5. PCR products of amplified Rv0678 gene

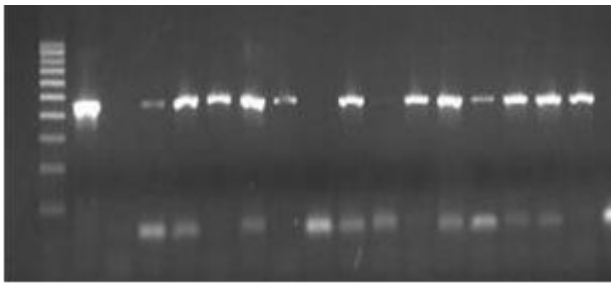


Figure 4.6. PCR products of amplified pepQ gene

4.3.2. Sanger sequencing of genes thought to be responsible for cross resistance bedaquiline-clofazimine

As a result of Sanger sequencing performed on TB-strains that are thought to be responsible for bedaquiline and clofazimine resistance, mutations in their genes were detected in strains with high MIC values.

PepQ is a 372 amino acid (aa) protein that consists of two domains: a 100-aa N-terminal alpha/beta domain and a 250-aa C-terminal peptidase domain (Fig. 4). the first area is creatines which hydrolyses creatine into sarcosine and urea, and which does not require metal ions to be active.

But peptidase activity depends on metal ions and that they bind to cations in the active site, selected mutations occurred in both areas. Three mutations are very important, the first is the frameshift mutation in Ala14, the second is the non-synonymous L44P mutation occur in the N-terminal domain, and the last one was the frameshift mutation in Arg271 occur in the catalytic domain The figure shows where these mutations are located in the structure of pepQ.

Table 4.4. PepQ mutations

Pep Q	
Nucleotides	**Amino Acid**
INS27A/INS30A/INS42A/INS47C/INS75C/INS84A/INS58C/INS63C/G69A/C87A/G104T/C71A/G74A/INS81A/T96C/INS133C/INS141C/INS166A/INS172T/INS177C/INS180G/INS182G/INS191GT/INS221G/INS230G/INS243G/INS247C/INS282G/INS288CGC/T296G/INS305G/G1010T	V82M/R337L
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T	V85M/R437L/T438N
TA53AC/INS58A/INS75C/G890A	V82M
TA53AC/INS58A/INS75C	V82M
INS27A/INS30A/INS42A/INS47C/INS75C/INS84A/INS58C/INS63C/G69A/C87A/G104T/C71A/G74A/INS81A/T96C/INS133C/INS141C/INS166A/INS172T/INS177C/INS180G	No mutation
no mutation	No mutation
no mutation	No mutation
no mutation	No mutation
no mutation	No mutation
no mutation	No mutation
no mutation	No mutation
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T /G32016T/DEL216T/A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G/ GG174CT/AA178CG/DEL180GC/G194C/DEL198A/GC204CA /DEL207ATC/DEL213G/DEL220CC/DEL228G/DEL232A/C233T/DEL244GCA/DEL251C	V82M/R337L
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A/C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T/INS178C /INS180G/INS182G/INS191GT/INS212G/INS221G/INS230G/INS239G/INS243G/INS247C/INS288CGC/INS282G/T296G/INS305G/G1010T	R337L
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A/C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T	V85M/R437L/T438N
INS15T/G20A/INS27A/INS31A/INS36C/INS47A/TA53AC/INS58A/INS75C/G890A/G1010T	V76M/R337L
INS183GT/INS170A/INS238G/INS189GT/T298G/INS305G/	V83M/G605A
INS132GT/INS288CGC/INS224G/T296G/INS246C/INS305G	V83M
INS191GT/INS212G/INS221G/INS229G/INS288CGC/T296G/INS305G	V83M/G605A/G619M/A610Q
G415T	V83M/G605A
G415T	V83M/G605A
DEL207ATC/DEL213G/DEL220CC/DEL228G	No mutation
DEL207ATC/DEL213G/DEL220CC/DEL228G	No mutation
DEL207ATC/DEL213G/DEL220CC/DEL228G	No mutation
G1010T	R337L
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T /G32016T/DEL216T/A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G	V85M/R437L/T438N
T203C/C206G/GC208TT/G211T	No mutation
C206G/GC208TT	no

	mutation
T203C	no mutation
INS27A/INS30A/INS42A/INS47C/INS75C/INS84A/INS58C/INS63C/G69A/C87A/G104T/C71A/G74A/INS81A/T96C/INS133C/INS141C/INS166A/INS172T/INS177C/INS180G	no mutation
	no mutation
no mutation	no mutation
A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G	V85M/R437L/T438N
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A	R437L
G1010T	V85M/R437L/T438N
G415T	V83M/G605A
TA53AC/INS58A/INS75C/G890A/G1010T	V85M/R437L/T438N
INS140C/INS167A/INS173T	V83M
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A/C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T	V83M
A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G	V83M/G605A
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T /G32016T/DEL216T/A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G	V83M
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
INS27A/INS30A/INS42A/INS47C/INS75C/INS84A/INS58C/INS63C/G69A/C87A/G104T/C71A/G74A/INS81A/T96C/INS133C/INS141C/INS166A/INS172T/INS177C/INS180G/INS182G/INS191GT/INS221G/INS230G/INS243G/INS247C/INS282G/INS288CGC/T296G/INS305G/G1010T	V82M/R337L
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T	V5M/R437L/T438N
INS191GT/INS221G/INS230G/INS243G/INS247C/INS282G/INS288CGC/T296G/INS305G/G1010T	V82M
INS282G/INS288CGC/T296G/INS305G/G1010T	V82M
G69A/C87A/G104T/C71A/G74A/INS81A/T96C/INS133C/INS141C/INS166A/INS172T/INS177C	V82M
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A	V82M
G415T	V83M/G605A

INS47C/INS75C/INS84A/INS58C	no mutation
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T /G32016T/DEL216T/A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G/ GG174CT/AA178CG/DEL180GC/G194C/DEL198A/GC204CA /DEL207ATC/DEL213G/DEL220CC/DEL228G/DEL232A/C233T/DEL244GCA/DEL251C/G1010T	V82M
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A/C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T/INS178C/INS180G/INS182G/INS191GT/INS212G/INS221G/INS230G/INS239G/INS243G/INS247C/INS288CGC/INS282G/T296G/INS305G	no mutation
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A/C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T	V82M
INS15T/G20A/INS27A/INS31A/INS36C/INS47A/TA53AC/INS58A/INS75C/G89A	V76M
INS183GT/INS170A/INS238G/INS189GT/T298G/INS305G/	V83M/G605A
INS132GT/INS288CGC/INS224G/T296G/INS246C/INS305G	V83M
INS191GT/INS212G/INS221G/INS229G/INS288CGC/T296G/INS305G	V83M/G605A/G619M/A610Q
G121T/INS132C/INS140C/INS167A/INS173T/INS178C/INS180G/INS182G/INS191G	
no mutation	no mutation
no mutation	no mutation
no mutation	no mutation
G32016T/DEL216T/A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G	
DEL207ATC/DEL213G/DEL220CC/DEL228G/DEL232A/C233T/DEL244GCA/DEL251C/G1010T	G605A/G619M/A610Q
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T /G32016T/DEL216T/A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G	V83M/G605A
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A/C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T	no mutation
GC208TT/G211T	V83M/G605A
INS221C/T203C/C206G/GC208TT/G211T	V83M/G605A
no mutation	no mutation
no mutation	no mutation
INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A/C104A/INS101A/T113C/G121T	no mutation
DEL244GCA/DEL251C/G1010T	G605A/G619M/A610Q
INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94A	V83M
/INS221C/T203C/C206G/GC208TT/G211T	no mutation

G415T	V 83M
no mutation	n o mutation
C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T	
INS26A/INS29A/INS31A/INS36C/INS48C/INS54C/INS59A/INS75C/G89A/C91A/G94 A/C104A/INS101A/T113C/G121T/INS132C/INS140C/INS167A/INS173T	V 83M
G69A/C87A/G104T/C71A/G74A/INS81A/T96C/INS133C/INS141C/INS166A/INS172 T/INS177C	
C104A/T219G/G201C/INS221C/T203C/C206G/GC208TT/G211T /G32016T/DEL216T/A228T/A231G/C233G /DEL235T/DEL156T/ATC161TAT/C167T/C172G	V 83M
no mutation	n o mutation
no mutation	
G415T	R 437L
no mutation	
no mutation	
no mutation	
no mutation	
no mutation	
no mutation	
no mutation	

Until now, the mechanism that causes reduced sensitivity to bedaquiline and clofazimine through mutations in pepQ is unclear. Mutations Rv0678 up conferring cross-resistance to bedaquiline and clofazimine is the cause of mmpL5-mmpS5(Rv0676c-Rv0677c) depression. mmpL5-mmpS5 encode a transporter capable of exporting bedaquiline and clofazimine outside the bacterium. it cannot be concluded that the mutations in Rv0678 and the mutation in pepQ are associated with the overexpression of mmpL5 or mmpS5. Our results indicate the importance of including pepQ in the genetic analysis of strains resistant to bedaquiline and clofazimine and especially in routine clinical diagnostics. We need in-depth studies of the biological function of pepQ in *M. tuberculosis* and the mechanism by which pepQ mutations confer reduced susceptibility is substantiated.

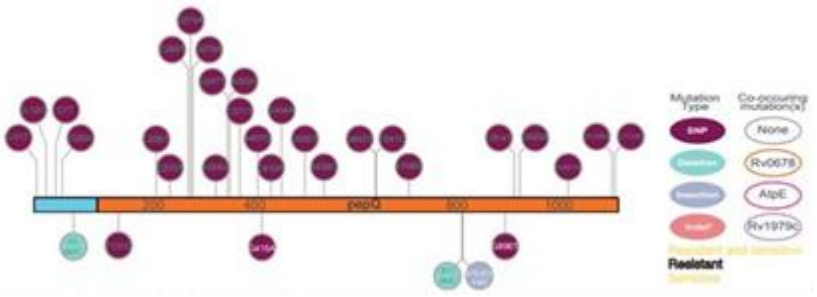


Figure 4.7. Lollipop figure with all observed variants across the pepQ gene

According to the study of Ismail, the results have shown that after in vitro culture of *M. tuberculosis* with clofazimine they found a mutant in the pepQ gene. But in parallel there are no phenotypic studies to confirm the correlation between the mutation and the value of MIC. The mutations of Rv0678 and pepQ are the cause of the increase in the values of MIC for clofazimine and bedaquiline, it is almost 4 times. Dueto this increase of MICs, the efficacy of BED and CLZ are reduced in vivo. on the other side, it cannot be said that the mutations of pep and RV0678 are responsible for the complete resistance to these drugs because increasing doses introduce greater treatment results. On based -of the data in the table, although the mechanism of resistance against the two antibiotics is common. The bactericidal effect is very important and clear, against TB-strains, but the combination of BED and CLZ in the treatment remains more efficient (Ismail et al. ,2019).

Table 4.5. Efflux Pump Gene mutations

Genetic variants of Rv0678	Number of Clinicalisolates
107C→T, 158C→T, 176C→T, 185C→T, 296C→T, 193G→A,	15
337G→A and 350T→G	5
136T→C and 248T→C	2
11A→C, 14A→G, 165A→C, 331A→G, 67A→G, 11C→A, 53C→A, 220C→G, 251C→T, 268C→T, 279C→A, 406C→G, 109G→A, 149G→C, 194G→C, 196G→T, 245G→A, 259G→A, 352G→A, 358G→A, 393G→C, 417G→A, 421G→C, 61G→A, 20T→A, 240T→G, 254T→G, 274T→G, 416T→C, 437T→G, 469T→G, 59T→C	45
4A→T, 172A→C, 247C→G, 253G→T, 259G→C, 326G→T, 362G→A, 7G→A, 236T→C, and 278T→C	6
78T→G (non-sense)	1
67A→C and T25→4C	10
119T→C	8
798T→G (silent)	2
425T→G	5
Genetic variants of Rv1979c	Number of Clinicalisolates
733A→C	18
1226G→A	23
114G→C	11
155A→C, 562C→T, 1216G→A, 724G→A, 1057T→G,	8
311T→C, and 824T→C	3
403A→G, 20G→A, 187A→G, 1432C→G	16
520C→T	2
798G (silent)	9
114G→T or C	6
151T→A	1
857A→G	3

Acquired resistance mechanisms that affect bedaquiline MICs include mutations in the atpE gene that encodes the ATP synthase target and in the Rv0678 gene that regulates expression of the MmpS5-MmpL5 efflux pump. Mutations in the gene encoding ATP synthase generated in preclinical studies lead to an 8 to 133-fold increase in the MIC of bedaquiline, causing MICs to vary between 0.25 and 4 mg/l. Mutations in the gene encoding the efflux pump have been observed in

pre-clinical and clinical isolates. These mutations lead to a 2 to 8-fold increase in the MICs of bedaquiline, causing the MICs of bedaquiline to vary between 0.25 and 0.5 mg/l. The majority of isolates with a bedaquiline-resistant phenotype show cross-resistance to clofazimine (Haver et al., 2015). Isolates resistant to clofazimine may, however, be sensitive to bedaquiline. The impactof high baseline MICs of bedaquiline, the presence of mutations in the Rv0678 gene prior to treatment, and/or increased bedaquiline MICs after initiation of therapy treatment on microbiological outcomes is unclear due tothe low incidence of such cases in phase II trials (Luthra et al., 2018).

Clofazimine, an antibiotic also used to treat lepromatous leprosy, reaches high tissue concentrations, which may contribute to long-lasting antimicrobial activity long after treatment has ended (Rodriguez et al., 2002). Furthermore, since previous preclinical studies have already suggested powerful synergies between other anti- tuberculosis treatments and clofazimine, the researchers here are testing rifapentine in combination - which has also just demonstrated higher efficacy over a short period of time, than rifampin (another antibiotic used in the treatment of bacterial infections including TB, leprosy and legionellosis). The CLO-FAST (CLO for clofazimine) clinical trial launched by the world's largest HIV research network, AIDS Clinical Trials Group (ACTG), will test a 3-month treatment protocol for patients with tuberculosis (TB) pharm-co sensitive. Composed of a dose of clofazimine (antibiotic already used to treat TB) and a high dose of rifapentine (antibiotic, idem), it is the first anti-TB treatment regimen based on preclinical evidence of long-lasting efficacy less than 4 months to be studied in a clinical trial (Mardassi et al., 2005). Tuberculosis caused by acid- fast microorganisms included in the M. tuberculosis complex has a high morbidity and it is among the most important causes of death, especially in young adults, despite the improvements in diagnosis and treatment methods in general protects. As well as the development of early and highly sensitive diagnostic methods in the prevention of tuberculosis and the prevention of the epidemic, which has become a global threat, typing methods that will monitor the movement of the microorganism in the community, allowing diagnosis at the clonal level, and epidemiological surveillance using these methods are needed. However, WHO reports that 86% of patients with active tuberculosis live in developing countries and that 95% of tuberculosis deaths occur in these countries, underlining that well-functioning control programs based on epidemiological surveillance are not implemented in these countries.

Only less than half of the patients living in these 22 developing countries, mostly in Africa and South East Asia, can be diagnosed, and less than half of those diagnosed can be treated. In addition, 80% of infected individuals in developed countries are 50 years and older. While 77% of the patients in developing countries are in the 15-50 age group, which is the most productive age group, this puts the insufficient production resources in these countries into trouble. Socioeconomic problems, migrations, neglect of tuberculosis control programs in the last 30 years, regional wars or civil wars.

There has been an increase in the incidence of tuberculosis in many developed countries with the mass migrations due to the conflicts and especially the emergence of the HIV/AIDS epidemic. As a result of poor control programs, drug resistance has become widespread and some genotypes such as the multi-drug resistant

M. tuberculosis Beijing family that has emerged in some regions have moved out of their regions and have become an important threat to public health all over the world. This global threat re-emphasizes Tuberculosis control programs all over the world.

led to the win. These programs increase the sensitivity in diagnosis and personnel, and cross-country and regional testing of MTK strains. It aims to monitor their movements. Phenotypic characters in monitoring MTBC strains such as genotypic-based spoligotyping and MIRU-VNTR took molecular-based methods.

Good results in studies using these methods emergence of XDR strains with frequent MDRs in countries with control programs very low rates of Beijing strains, which are considered the greatest potential threat to has been reported68. Horn of Africa with countries at risk for M. Tuberculosis from Kampala Uganda, 18 years and older. They typed 344 strains isolated from patients with the spoligotyping method.In their studies, they found that isolates concentrated in 33 spoligo clusters, 57 isolates (Aslan et al.,2022).

They found that they gave a special spoligotyping pattern. This group is the most common families, 70% (241 strains) T2, 3.5% CAS1-Clay, 2.6%. They reported LAM9 and 2.6% CAS1-Delhi families. 1.2% (4strains). In this study, in which the Beijing genotype was reported, 26.7% of the strains were HIV seropositive. isolated from patients, 8.1% INH and 4.4% RIF resistant reported. On the other hand (Campos. et al.,2003). In Tuscany, Italy.248 MTBC isolates collected from hospitalized patients for 1 year with the Spoligotyping method evaluated in their study, it was found that 116 isolates were single-membered special cluster and 12 isolates were unidentified isolates, and 166 isolates constituted 34 clusters (67%), have reported. This group included the T1 family with 11.6%, the H3 family with 7.2% and the LA9 family with a rate of 11.6%.

As the 3 most dominant spoligotyping families in Tuscany with 5.2 ratios. They defined, they identified 7 (2.8%) strains belonging to the Beijing family70. Molina-Torres and colleagues in Monterrey Mexico in 2010, Monterrey. By using the Spoligotyping method, 180 M. tuberculosis isolates collected from rural areas have been genotyped. As a result, this group resulted in 176 isolates with 44 spoligo international tip, that 4 isolates are orphan types that do not have a counterpart in SpolDB4. In addition, T1 is the second most common gene family with 43 (23.8%) strains. They reported that the family with 28 (15.5%) strains was X1 family.

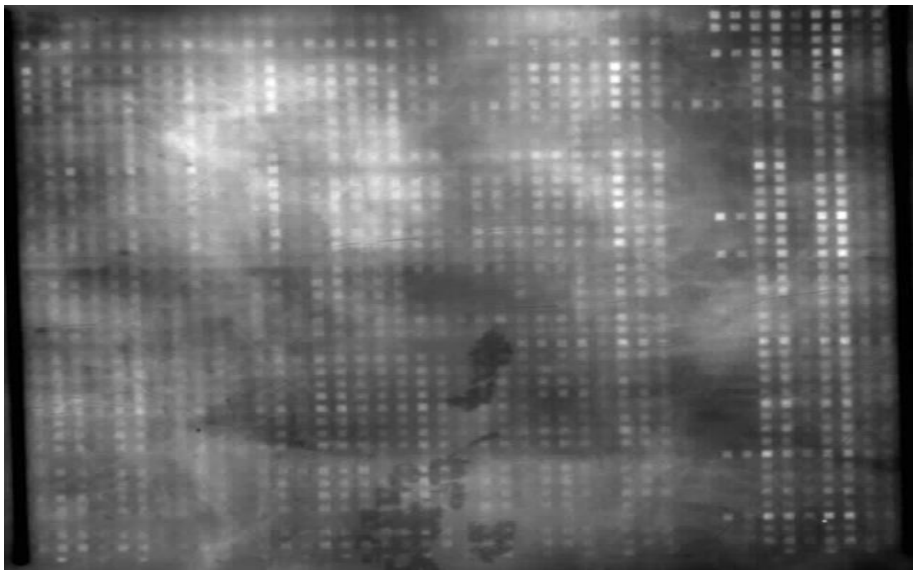


Figure 4.8. Spoligotyping profiles

At the end of the analysis, we conducted to determine the genotypic characteristics of our MTB complex isolates that we included in the study; We found that the strains were distributed into 9 different spoligotypes. We found that families with multiple members were Ghana families with 45.9% members and LAM families with 28.6% members. All the different spoligotypes of 100 isolates showed in table 4.8.

Table 4.6. Spoligotyping results

N°	Spoligotyping		N°	Spoligotyping	
	Species	**Lineage**		**Species**	**Lineage**
1	*M.tuberculosis*	H37Rv	26	*M.tuberculosis*	Ghana
2	*M.tuberculosis*	Ghana	27	*M.tuberculosis*	LAM
3	*M.tuberculosis*	Ghana	28	*M.tuberculosis*	Ghana
4	*M.tuberculosis*	LAM	29	*M.tuberculosis*	Ghana
5	*M.tuberculosis*	LAM	30	*M.tuberculosis*	Ghana
6	*M.tuberculosis*	LAM	31	*M.tuberculosis*	Ghana
7	*M.tuberculosis*	LAM	32	*M.tuberculosis*	UgandaI
8	*M.bovis*	Bovis	33	*M.tuberculosis*	Ghana
9	*M.tuberculosis*	Ghana	34	*M.tuberculosis*	Ghana
10	*M.tuberculosis*	TUR	35	*M.tuberculosis*	Ghana
11	*M.tuberculosis*	TUR	36	*M.tuberculosis*	Beijing
12	*M.tuberculosis*	Ghana	37	*M.tuberculosis*	Ghana
13	*M.tuberculosis*	LAM	38	*M.africanum*	WestAfrican 2
14	*M.tuberculosis*	Ghana	39	*M.tuberculosis*	Ghana
15	*M.tuberculosis*	Ghana	40	*M.tuberculosis*	LAM
16	*M.tuberculosis*	Ghana	41	*M.tuberculosis*	UgandaI
17	*M.tuberculosis*	Ghana	42	*M.tuberculosis*	Ghana
18	*M.tuberculosis*	Ghana	43	*M.tuberculosis*	Ghana

19	*M.tuberculosis*	Ghana	44	*M.microti*	Vole
20	*M.tuberculosis*	Ghana	45	*M.tuberculosis*	LAM
21	*M.tuberculosis*	Ghana	46	*M.tuberculosis*	LAM
22	*M.tuberculosis*	TUR	47	*M.tuberculosis*	LAM
23	*M.tuberculosis*	TUR	48	*M.tuberculosis*	LAM
24	*M.tuberculosis*	UgandaI	49	*M.bovis*	Bovis
25	*M.tuberculosis*	Ghana	50	*M.tuberculosis*	Ghana

N°	**Spoligotyping**		**N°**	**Spoligotyping**	
51	*M.tuberculosis*	Ghana	76	*M.microti*	Vole
52	*M.tuberculosis*	H37Rv	77	*M.tuberculosis*	LAM
53	*M.tuberculosis*	Ghana	78	*M.tuberculosis*	LAM
54	*M.tuberculosis*	Ghana	79	*M.tuberculosis*	TUR
55	*M.tuberculosis*	LAM	80	*M.tuberculosis*	UgandaI
56	*M.tuberculosis*	LAM	81	*M.tuberculosis*	Ghana
57	*M.tuberculosis*	Ghana	82	*M.tuberculosis*	Ghana
58	*M.tuberculosis*	Ghana	83	*M.tuberculosis*	LAM
59	*M.tuberculosis*	LAM	84	*M.tuberculosis*	Ghana
60	*M.tuberculosis*	LAM	85	*M.tuberculosis*	Ghana
61	*M.tuberculosis*	LAM	86	*M.tuberculosis*	Ghana

62	*M.bovis*	bovis	87	*M.tuberculosis*	Ghana
63	*M.tuberculosis*	Ghana	88	*M.tuberculosis*	Ghana
64	*M.tuberculosis*	TUR	89	*M.tuberculosis*	Ghana
65	*M.tuberculosis*	TUR	90	*M.tuberculosis*	Ghana
66	*M.tuberculosis*	Ghana	91	*M.tuberculosis*	Ghana
67	*M.tuberculosis*	LAM	92	*M.tuberculosis*	TUR
68	*M.tuberculosis*	Ghana	93	*M.tuberculosis*	TUR
69	*M.tuberculosis*	Ghana	94	*M.tuberculosis*	UgandaI
70	*M.tuberculosis*	Ghana	95	*M.tuberculosis*	Ghana
71	*M.tuberculosis*	Ghana	96	*M.tuberculosis*	Ghana
72	*M.tuberculosis*	LAM	97	*M.bovis*	Bovis
73	*M.tuberculosis*	UgandaI	98	*M.tuberculosis*	Ghana
74	*M.tuberculosis*	Ghana	99	*M.tuberculosis*	LAM
75	*M.tuberculosis*	Ghana	100	*M.tuberculosis*	LAM

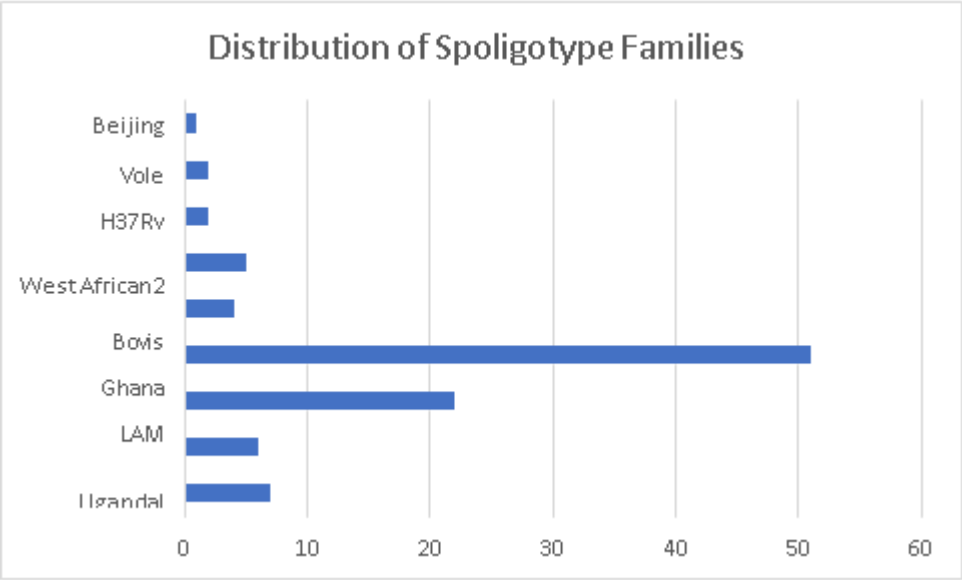


Figure 4.9. Distribution of Spoligotype Families

In order to be able to effectively treat TB, it is of great importance to determine the susceptibility of M. tuberculosis strain to the antituberculosis drugs used in the treatment before the treatment is started, in orderto prevent the formation of resistant strains.

As a matter of fact, WHO recommends performing ADT in every newly positive case. WHO recommends that phenotypic TB drug susceptibility be done according to the "critical drug concentration" they determined by compiling them from studies in the literature. Critical drug concentration is defined as the lowest drug concentration that inhibits the growth of the phenotypically wild M. tuberculosis strain by 99% in vitro. Due to sensitivity determination based on a single concentration, resistance to small amounts of TB drugs is common and drugs are excluded from the treatment protocol; In other words, drugs are lost. Today, the idea of evaluating the ADT result according to the "clinical threshold value" rather than this critical drug concentration is becoming widespread by TB researchers . WHO defines the clinical threshold as an MIC value higher than the critical drug concentration. Although the bacterium is resistant, the patient may or may not respond to treatment when treated with higher doses of the drug. The MIC value here; It is defined as the lowest drug concentration that inhibits the growth of a resistant or susceptible strain by 99% in vitro. The clinical threshold value of a drug can be obtained by clinical data in the literature and researches.

It is determined by evaluating the MIC distributions, genetic factors of the patient and the pharmacokinetic/pharmacodynamic values of the drug dose together. A drug that is found to be resistant at a low dose can reach the maximum tolerated dose. It can be given in higher doses by increasing solvable. Clinicians' individualized treatment protocol for TB patients that they can apply it while determining; however, in ADT surveillance studies. It is stated by WHO that the use of the threshold value is not appropriate. For allthese reasons, sensitivity according to critical concentration

with phenotypic ADT methods is detected. In order to be able to effectively treat TB, it is of great importance to determine the susceptibility of *M.tuberculosis* strain to the antituberculosis drugs used in the treatment before the treatment is started, in order to prevent the formation of resistant strains.

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5. CONCLUSION

According to the Critical Drug Concentrations Report of the World Health Organization, all strains in our study were found to be sensitive to bedaquiline according to their mean MIC values, while they were resistant to delamanid and clofazimine. The mutation responsible for cross-resistance in strains with high MIC values of bedaquiline and clofazimine could not be detected by Sanger sequencing, further studies are required to detect a new mutation causing resistance. A WGS protocol optimization study was conducted to determine the resistance profiles in the bacterial genome against all drugs. Resistance-associated variants in the genome of 25 strains could be detected. In conclusion, in this study, preliminary susceptibility data of Turkish M. tuberculosis strains were obtained for new drugs that are expected to be used in our country in the near future in the treatment of MDR-TB. It is thought that the research software integrated with the MGIT-960 system and the optimized WGS protocol can be used to determine the personalized treatment of MDR-TB patients.

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