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Microm 496

Antibiotic Resistance and *Mycobacterium tuberculosis*

Introduction:

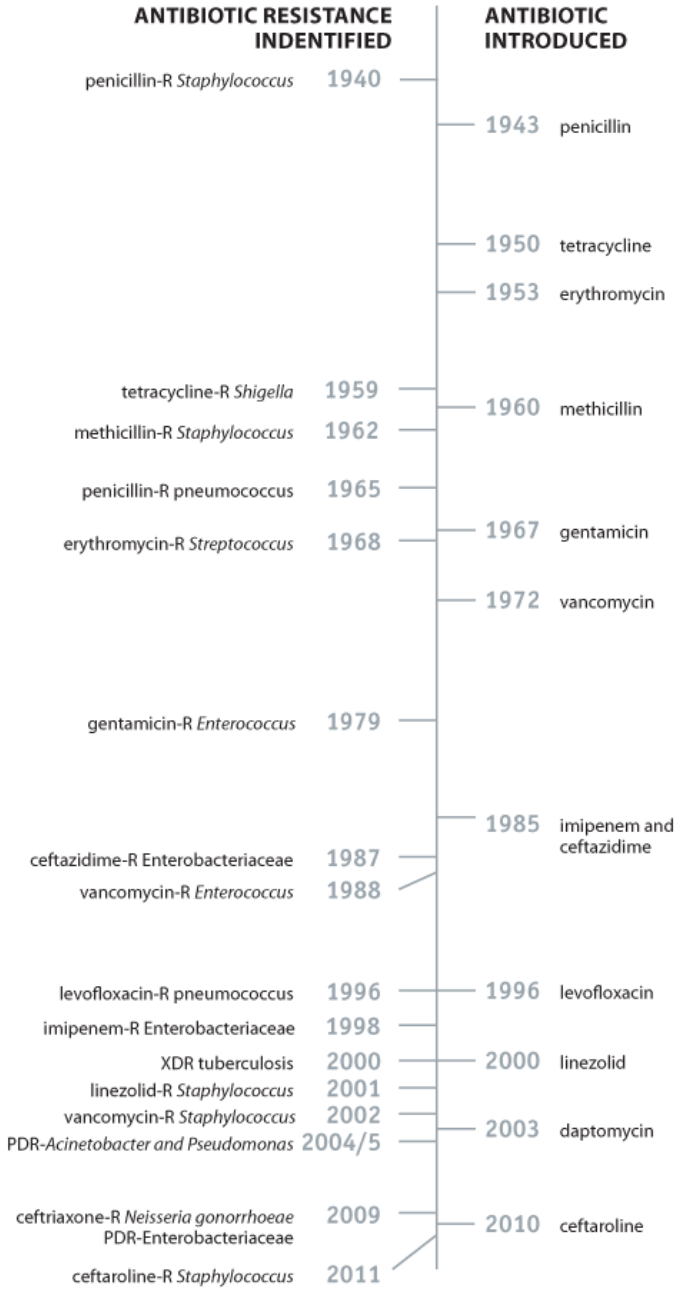

Figure 1. Timeline of Antibiotic resistance identification and introduction of antibiotic [1].

Antibiotic resistance has been a growing public heath concern in the world. This phenomenon caused by genetic mutations and gene transfer in bacteria has allowed these microorganisms to increase and in some cases acquire complete resistance to certain antibiotics that previously were enough to eradicate a bacterial infection. Resistance to the available drugs makes these bacterial infections very difficult, and sometimes impossible to cure, claiming the lives of at least 23,000 US residents every year according to the CDC [1].

Penicillin was the first antibiotic, introduced in 1943, during WWII and used effectively for many years afterwards to treat serious infections. By the 1960s, three more significant antibiotics were introduced; tetracycline, erythromycin, and methicillin[1].

Unfortunately, in the same decade, antibiotic resistance to all four previously mentioned drugs began to arise, limiting their usefulness to treat those infections. As seen in Fig. 1, this trend has repeated itself into present years, increasing the pressure to find new solutions to the ever growing problem.

One of the main driving forces in bacterial development of antibiotic resistance is incorrect prescription of antibiotics, and poor adherence to the treatment by the patient. The presence of antibiotics in low or incomplete dosages enriches an environment in which many bacterial strains are able to accumulate genetic changes that confer antibiotic resistance resistance in a Darwinian fashion [2]. This is particularly a serious problem with Mycobacterium tuberculosis (MTB), which has been found to not only develop resistance to first line drugs used in standard treatment- referred to as multiple drug resistant (MDR), but it also has been found to develop resistance to the second line drugs used in treatment, -referred to as extensively drug resistant (XDR). According to the WHO in 2016 there were 240,000 deaths reported globally due to MDR MTB, and 123 countries have reported at least one case of XDR MTB [3]. It is important to understand the mechanism by which these bacterial pathogens develop resistance to drugs in order to develop plans for a swift intervention.

Basics of Bacterial development of antibiotic resistance:

One of the ways in which an organism may develop antibiotic resistance is through mutations in the chromosome that can be passed on to daughter cells. Alternatively, it can be spread to other microorganisms by horizontal gene transfer (HGT). HGT includes three types of mechanisms. The first is bacterial conjugation. This is done through a complicated mechanism of proteins which ensure plasmid transfer from one bacteria to another by means of forming a mating pair. Only organisms with this machinery are able to perform conjugation[4]. The other

two are transformation and transduction. When it comes to being a pathway of antibiotic resistance acquisition they are less studied than the previously mentioned pathways, but have been found to support the theory that antibiotic resistance genes developed in the environment before finding their way into human pathogens[5].

Transformation is the ability of a bacteria to take up and express DNA from the environment. One of the hypotheses about how antibiotic resistance made its way into human pathogens is that they came from commensal or environmental bacteria. These bacteria are thought to have produced antibiotics as a defense mechanism, and therefore had immunity to the chemical they produced. Since a lot of antibiotics used in medicine are derived from environmental microorganisms, DNA taken up by a human pathogen would confer resistance to those drugs. [6]

Finally, transduction is the process of a bacterial virus, or bacteriophage inserting its DNA into a bacteria and integrating itself within the chromosome. When the bacteria are in a stressful environment, the virus may enter the lytic cycle in which the viral DNA is excised and replicated, and capsules are produced in which DNA is packaged to make hundreds of phages until the cell bursts. During this DNA packaging, bacterial DNA can be mistakenly packed into a phage capsule, leading to transfer of a piece of bacterial DNA into another organism during infection [7].

In order for an antibiotic to be effective in an organism, there must be a target; such as a transporter or protein, and a certain concentration level, is able to inhibit its activity. Therefore, the two most efficient mechanisms of antibiotic resistance discovered have involved modifying the target through mutations in genes coding for such proteins and transporters, or acquiring the ability to reduce the concentration of antibiotic either by expulsion through efflux pumps or

inactivation or modification with bacterial enzymes[6]. One such case is the mechanism of resistance to Beta-lactam antibiotics. Beta-lactam antibiotics are in the family of penicillin and contain a beta-lactam ring. Microorganisms which carry a resistance gene that produces beta-lactamases are able to break the ring and inactivate the antibiotic. A gene coding for an extended spectrum of activity Beta-lactamase which was originally found in *E. coli*, for example, has found its way into other members of *Enterobacteriaceae*. Since it was found to be located in a highly mobile INCFII plasmid, it could be passed on to other cells through conjugation. It was also found to be in association with an insertion sequence, which is a type of mobile genetic element employed in bacterial transformation that can move between different positions on a chromosome or between two different other chromosomes, or plasmids [2]. Another set of genes conferring beta lactamases was also found by real time PCR in bacteriophage DNA in urban sewage and river water samples. These phage were able to transduce of these genes into susceptible *E.coli* strains, making them resistant to ampicillin [7].

Antibiotic resistance in tuberculosis (TB):

MTB has only been found to transfer resistance to daughter cells, as it develops resistance through mutations in the chromosome. Isoniazid, rifampicin, ethambutol and Pyrazinamide are used as first line drugs used in standard treatment for TB. Unfortunately, cases of resistance to all of them have been discovered thus far. Furthermore, through whole genome sequencing, it has been discovered to develop acquisition in a sequential manner. Most often initial acquisition of resistance confers resistance to isoniazid, then rifampicin or ethambutol, followed by pyrazinamide, and finally to the second and third line drugs used in the treatment [8].

Drug	Associated MIC (mg/L)	Mutation frequency among resistant isolates (%)	Compensatory mechanisms
Isoniazid: inhibition of cell wall mycolic acid synthesis			
katG	0.02–0.2	70	*oxyR′* and *ahpC*
inhA		~10	
kasA		~10	
Rifampicin: inhibition of RNA synthesis			
rpoB	0.05–1	95	*rpoA* and *rpoC*
Ethambutol: inhibition of cell wall arabinogalactan biosynthesis			
embB	1–5	~70	unknown
ubiA		~45, occurs with *embB* mutations	
Pyrazinamide: reduction of membrane energy; inhibition of trans-translation; inhibition of pantothenate and coenzyme A synthesis			
pncA	16–100	~99	unknown
rpsA		no clinical evidence	
panD		no clinical evidence	

Figure 2. List of gene mutations and their frequency of mutation associated with resistance to isoniazid, rifampicin, ethambutol, and pyrazinamide [8].

Isoniazid is a prodrug, meaning it requires activation inside the host before it can be an active inhibitor. In the case of isoniazid, it is activated by the catalase/peroxidase enzyme, therefore it requires that the organism is active. As seen in figure 2, two of the main genes involved in isoniazid resistance are *katG*, which encodes the catalase/peroxidase enzyme that activates isoniazid, and *inhA*, which encodes the NADH dependent enoyl-acyl carrier protein reductase which is inhibited by the active form of isoniazid. This inhibits mycolic acid synthesis, a major component of the cell-wall in this genus of bacteria. Therefore, the most common types

of resistance found in those strains that carry resistance to isoniazid have mutations in those two genes. Specifically, mutations in katG that during activation of isoniazid, result in an inefficient product, and mutations in inhA which either cause over expression of the reductase, or decreased affinity to isoniazid [8].

Rifampicin on the other hand, is able to act upon active and slow growing bacilli because it involves binding to the beta-subunit of the RNA polymerase, which in turn inhibits RNA synthesis. Hot spots of mutations have been found in several isolates between codons 507 and 533, most of which confer resistance by causing conformational changes in the B-subunit of RNA polymerase- encoded by *rpoB*, which decreases the affinity to rifampicin. Nevertheless, there have been mutations found outside of these hotspots, just as there have been studies that showed strains with no alteration in the rpoB coding region that still carried a resistance to rifampicin [8]. This suggests that there is more than one pathway that leads to resistance to this drug.

In addition, monoresistance to rifampicin is quite rare. As discussed above, MTB tends to amass resistance to standard treatment drugs in a sequential manner starting with isoniazid before moving to rifampicin [9]. Therefore, rifampicin is used as a surrogate marker for MDR TB because the hotspots of mutations conferring resistance have been well documented and fairly consistent through whole genome sequencing [8].

Figure 3 shows a global representation of the consistently high levels of resistance to both isoniazid and rifampicin in strains of MTB as compared to monoresistance to rifampicin, monoresistance to isoniazid, and susceptibility to both in MTB strains.

Table 1 Geographic origins and drug susceptibility patterns of *M. tuberculosis* strains by the proportion method and the INNO-LiPA Rif.TB tests

Origin (*n*)	Proportion method				Isolates resistant by LiPA
	INH^r/RMP^r	INH^r/RMP^s	INH^s/RMP^r	INH^s/RMP^s	
Asia (462)*	165	125	3	19	37/38
North Africa (78)†	35	10	1	32	22/22
Sub-Saharan Africa (176)‡	60	22	2	92	54/56
Western Europe (251)§	37	12	7	195	26/26
Eastern Europe (258)¶	97	65	3	93	35/35
Central Europe (267)**	115	60	6	86	56/57
South America (21)††	4	4	3	10	7/7
Control strains (49)‡‡	24	12	1	12	25/25
Total (1562)	537	310	26	689	262/266

* Bangladesh, Kazakhstan, Pakistan.
† Algeria, Egypt, Libya, Tunisia.
‡ Benin, Burkina-Faso, Burundi, Congo, Democratic Republic of Congo, Guinea, Senegal, Rwanda.
§ Belgium, France, Luxembourg.
¶ Abkhasia, Azerbaidjan, Georgia, Ossetia (south).
** Romania, Russia (Siberia).
†† Colombia, Honduras, Peru.
‡‡ Strains from WHO quality control study for drug resistance.[11]
LiPA = line probe assay; INH^r = strains resistant to INH; RMP^r = resistant to RMP; RMP^s = susceptible to RMP; INH^s = susceptible to INH.

Figure 3. Proportion of isolates with co-resistance to isoniazid vs rifampicin (INHr/RMPr), resistance to isoniazid and suceptibility to rifampicin (INHr/RMPs), resistance to rifampicin and susceptibility to isoniazid (INHs/RMPr), and suceptibility to both drugs (INHs/RMPs).

Ethambutol, like isoniazid also only acts on multiplying bacilli because it disrupts the biosynthesis of arabinogalactan, another important component in the cell wall of MTB. As figure 3 points out, one of the main genes involved in the mechanism is *embB*, a part of the *embCAB* operon that encodes the arabinosyl transferase enzyme necessary for the production of the cell wall component. Certain aminoacid substitutions in codon 306 have been found to confer differing degrees of moderate resistance to ethambutol. Higher levels of reisistance have been found when mutations in *embB* and *ubiA* are present. *ubiA* is a gene which encodes a protein involved in cell wall synthesis. Mutations in *ubiA* are reported to be most predominant in African isolates, as they are lineage specific [12].

Pyrazinamide is the fourth first line drug used in standard TB treatment and the newest one, having been introduced in the early 1950's. It was an important novel drug when introduced, due to its ability to act upon semi dormant bacilli allowing the treatment to be shortened to the current standard of six months. The mechanism by which pyrazinamide works is still a debated topic, however mutations in *pncA* have been found to be the most common type of mutation in resistant strains [12].

There are many more alternative antibiotics used in the treatment of TB, they are also known as second line drugs, and are used in cases of MDR MTB. Some examples include fluoroquinolones -which act by inhibiting the topoisomerase II, Kanamycin- which acts by inhibiting protein synthesis, and Cycloserine- which inhibits synthesis of peptidoglycan [12]. What is very troubling is that even though there are so many alternatives to the first line drugs, resistance has been identified in most of them and patients continue to die from XDR TB.

Diagnosis of drug resistance and its impact:

When it comes to identifying drug resistance in microorganisms, one of the most widely used methods involves growth of culture on a plate with disks infused with different doses of the antibiotic to be tested. For most bacteria, this takes no longer than a few days including the steps from the process of isolation of the pathogen to the growth on media infused with antibiotic through the diffusion disk [12]. However, MTB grows very slowly and it may take several weeks or months to get accurate assessments. This results in the treatment plan being inferred through empirical data based on a combination of past medical or social history of the patient and the local prevalence of resistance, which may turn out to be an inappropriate approach. This causes the potential for further development of resistance through exposure [8]. In addition, the standard treatment for tuberculosis consists of a six month course of several drugs, some of which are

highly toxic, with potential adverse effects such as irreversible hearing loss. This highly discourages patient adherence to treatment, which is essential to avoiding an environment that is encourages development of antibiotic resistance during the treatment of any bacterial infection. The reason for the length of tuberculosis treatment in particular is that Mycobacterium tuberculosis has the ability to go into a dormant state in which it is not replicating, leaving most of the antibiotics used in he treatment useless because they require cell growth. If treatment is halted early, and sometimes even after the standard treatment period is finished, cells that were dormant during the treatment may re-infect the host as they become active again [13].

Novel studies on new technology:

In 2010, the WHO endorsed a new disposable cartridge based nucleic acid amplification test that detects resistance to rifampicin. This test, known as Xpert MTB/RIF, is suitable for point of care testing as it has made most of the steps involved in susceptibility testing automated and can diagnose resistance in about two hours. This has allowed for a reported 23 million Xpert tests procured in 130 countries, which has greatly increased accurate testing for MDR TB and resulted in more appropriate treatment of patients with it [14]. It works by amplifying the rifampicin resistance-determining region of the rpoB gene. Five different specific molecular beacons are used to detect the presence of MTB and the most common mutations that confer resistance to rifampicin. Molecular beacons work by emitting fluorescence during a polymerase chain reaction (PCR) when they bind to a complementary section of the DNA being amplified.

While this technique has been very helpful in increasing appropriate treatment of TB, it has some shortcomings. For example, a study conducted in 2014 using pooled data to analyze the specificity and sensitivity of Xpert MTB/RIF. Specificity reflects the ability of the assay to correctly identify a sample with no MTB, and sensitivity is its ability to show a positive result for

presence of MTB in a person who has TB. This study showed only 67% sensitivity when used on patients with a smear negative sputum sample and 79% in HIV positive patients [15].

Covariate (Number of studies)	Pooled median sensitivity (95% credible interval)	Pooled median specificity (95% credible interval)
Smear status		
Smear + (21)	98% (97, 99)	***
Smear - (21)	67% (60, 74)	99% (98, 99)
Difference (Smear+ minus Smear-)	31% (24, 38)	***
P (Smear+ = Smear-)	1.00	***
HIV status		
HIV- (7)	86% (76, 92)	99% (98, 100)
HIV+ (7)	79% (70, 86)	98% (96, 99)
Difference (HIV- minus HIV+)	7% (-5, 18)	1% (-1, 3)
P (HIV- = HIV+)	0.90	0.85

Figure 4. Sensitivity and Specificity of Xpert assay in Smear positive, and negative samples, and samples from HIV positive and negative patients [15]..

Specificity, however remained high across the board, as seen in figure 4, meaning that the assay can correctly identify that a patient does not have an MTB infection.

Cepheid Inc. has been working on improving the sensitivity and specificity of their product with a new cartridge called Xpert MTB/RIF Ultra assay. In a study conducted in 2017 they were able to compare the limit of detection (LOD) of the Xpert and Ultra cartridges by spiking serial

dilutions of quantified colony forming units (CFU) of known MTB strains into patients' sputum samples.

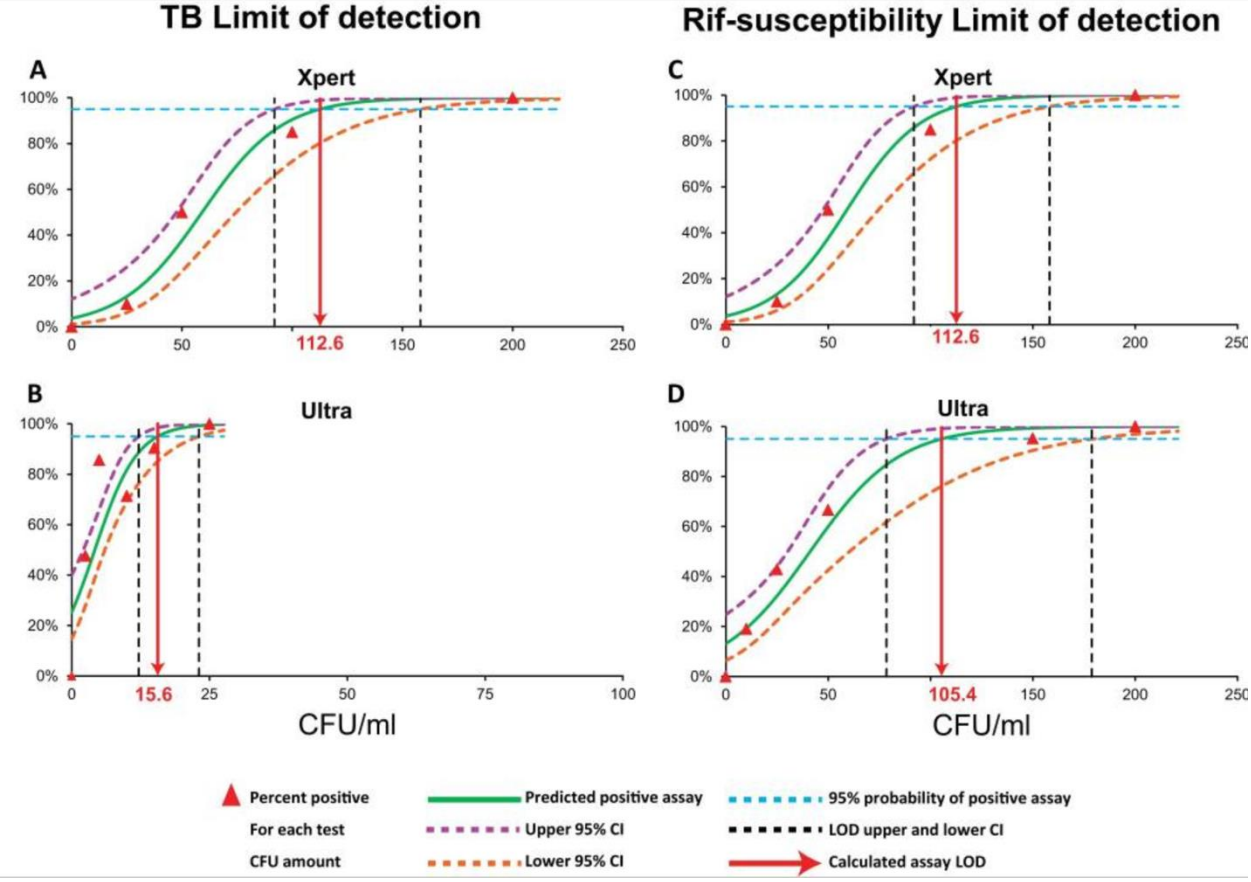

Figure 5. Limit of detection of MTB and RIF susceptibility at increasing concentrations of MTB CFU/ml with Xpert cartridge vs Xpert Ultra [14]

Above results on figure 5 showed a great improvement in the LOD of MTB and a significant improvement in the LOD of rifampicin susceptibility in MTB. Both cartridges were 100% successful at detecting the presence of MTB at concentrations above 200 CFU/ml. However, while Xpert had 10% correct detection of positive specimens at 25 CFU/mL, Ultra maintained 100% accuracy. In terms of detecting rifampicin susceptibility, the difference was less dramatic, with Xpert's LOD being 112.6 CFU/ml, compared to Ultra's 105.4 CFU/ml [14]. The biggest difference was that at concentrations below their respective LODs, Ultra produced more positive assay results.

In order to increase specificity of rifampicin resistance detection, they refined the three pre-existing, rpoB molecular beacons (rpo1-3) and added a fourth probe (rpo4). In order to distinguish silent mutations in the rifampicin resistance-determining region (RRDR) from mutations conferring rifampicin resistance, rpo1 was redesigned so that its melting temperature would increase when hybridizing to the silent mutations as compared to hybridization to wild type DNA. Melting temperature can be identified by the peaks shown in figure 4. The new probe was designed to be more complementary to the mutation in S531L, resulting in a much higher melting temperature in the presence of that mutation as compared to wild type DNA as can be seen by comparing graph A and F in figure 6.

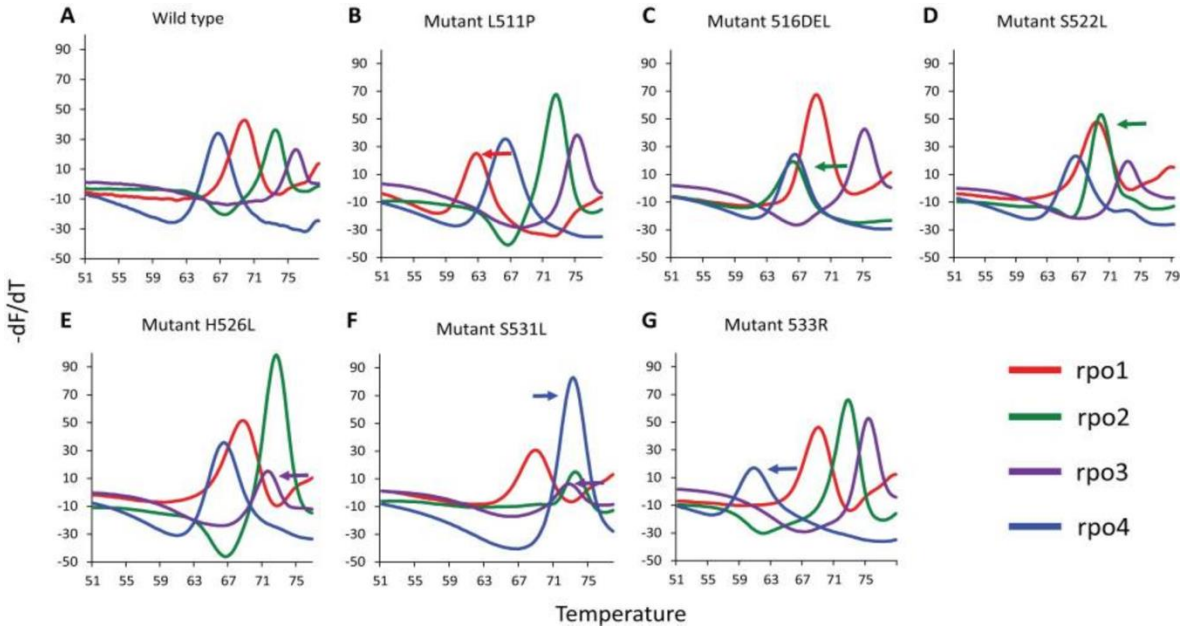

Figure 6. Melting temperatures of rpo probes in each common mutant gene are represented by peaks. Shifts in the peaks as compared to peaks in wild type MTB DNA (A) are a signal that there is a mutation in the zone targeted by the rpo probe. [14]

Specificity and exclusivity of Ultra was tested by running 30 isolates of nontuberculous mycobacteria (NTM) and 18 gram positive and negative bacteria through the assay. All tests came back negative, reporting: “MTB not detected”. In addition, they tested for the possibility

that NTM would be reported as positive for MTB detection by mixing 10^6 CFU/ml of several clinically relevant NTM with 50 CFU/ml of rifampicin resistant MTB. All tests showed presence of rifampicin resistant MTB. This was achieved by adding two probes for M. tuberculosis specific genes IS6110 and IS1081. The inclusion of these two targets also allowed for higher sensitivity. Samples that are positive for those targets but negative for the rpo targets fall into a new category, called "trace", indicating paucibacilliary tuberculosis.

Following this improvement, a study done by a group of researchers not affiliated with Cepheid conducted a multicenter, diagnostic accuracy study recruiting adults with pulmonary TB symptoms in South Africa, Uganda, Kenya, India, China, Georgia, Belarus and Brazil.
The study found that sensitivity in Xpert Ultra was much higher than Xpert when it came to detecting MTB presence in sputum smear negative samples of pulmonary tuberculosis patients. They also found that the Xpert Ultra cartridge increased detection of MTB in HIV positive patients by 13% [16]. However this increase in sensitivity came at a cost to specificity. Although

	Sensitivity		Specificity		
	All culture-positive (95% CI; n/N)	Smear-negative, culture-positive (95% CI; n/N)	All culture-negative (95% CI; n/N)	No history of tuberculosis (95% CI; n/N)	Any history of tuberculosis (95% CI; n/N)
Xpert	83%(79–86; 383/462)	46%(37–55; 63/137)	98% (97–99; 960/977)	98%(97–99; 715/727)	98%(95–99; 244/249)
Xpert Ultra	88%(85–91; 408/462)	63%(54–71; 86/137)	96% (94–97; 934/977)	96%(95–98; 701/727)	93%(89–96; 232/249)
Xpert Ultra, no trace	86%(82–89; 395/462)	54%(45–63; 74/137)	98% (96–98; 953/977)	98%(96–99; 709/727)	98%(95–99; 243/249)
Xpert Ultra, conditional trace†	88%(85–91; 406/462)	61%(53–70; 84/137)	97% (95–98; 945/977)	96%(95–98; 701/727)	98%(95–99; 243/249)

Figure 7.Sensitivity of Xpert and Xpert Ultra when assaying culture positive samples and smear-negative, culture positive samples. Specificity of Xpert an dXpert ultra when assaying culture negative samples, samples from patients with no history of TB, and samples from patients with a history of TB. This table also contrasts the change in sensitivity and specificity when "trace " is accepted as a positive result.

the two new targets IS6110 and IS1081, help differentiate NTM from MTB, they yield false positive results for patients with a medical history of tuberculosis treatment more than half the time. This specificity is expected to increase with time since completion of treatment and can take up to seven years to reach the level of sensitivity that Xpert has [16].

The increase in sensitivity is due to the new result category "trace". Different approaches to accepting "trace" as a positive result affect the specificity. As seen in figure 7, when trace is excluded from the result, sensitivity decreases for smear-negative samples from 63% to 54%. However, specificity is recovered, increasing from 93% in patients with a history of tuberculosis, back to 98% by excluding "trace". It can also be observed in figure 8 that Xpert Ultra has the lowest specificity, but when the "trace" result is excluded, the specificity is on par with Xpert.

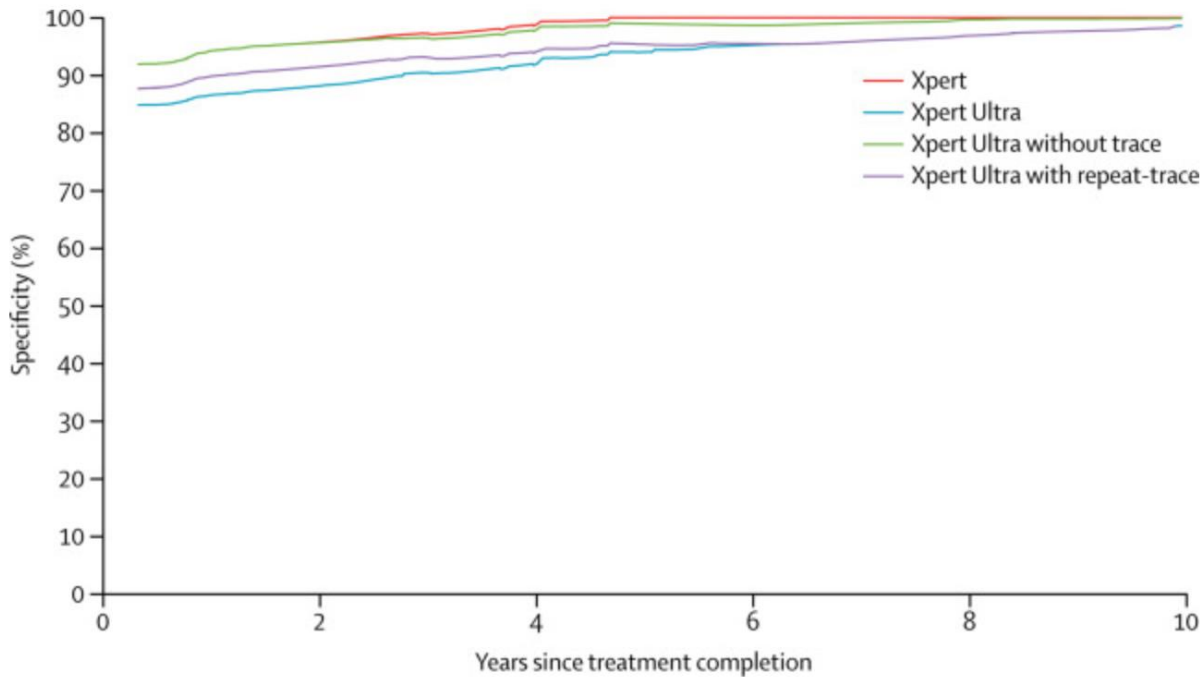

Figure 8. Percent Specificity of Xpert, Xpert Ultra, and Xpert Ultra without trace in years following treatment. Specificity of Xpert Ultra witout trace is highly comparable to that of Xpert, whereas Ultra with trace has the lowest specificity during the first years after treatment [16].

Specificity in this assay is important to avoid exposure of patients to unnecessary treatment which is highly toxic and inconvenient. However, in places where mortality due to TB is high, or in individuals with low sputum bacilliary burdens such as children and people with extra-pulmonary TB, the Ultra cartridge can be the best approach. This becomes more evident in the graph below from another study which found that the deaths averted by Ultra when compared to Xpert were almost proportional to the mortality rates of the countries. In the WHO's Global Tuberculosis Report from 2018, they released a list of 30 high TB burden countries. Included were India, South Africa, and China, which had a mortality rate of 31, 39, and 2.6 deaths per 100,000 population respectively [17]. Figure 9 shows data from a modeling study conducted in 2017 on the impact of Ultra as compared to Xpert [18]. On the leftmost graph in figure 9, it can be observed that deaths averted in China are much lower than in India and South Africa. Although they still showed a great increase in unnecessary treatments, it is much more justifiable when reducing the mortality rates in these burden countries.

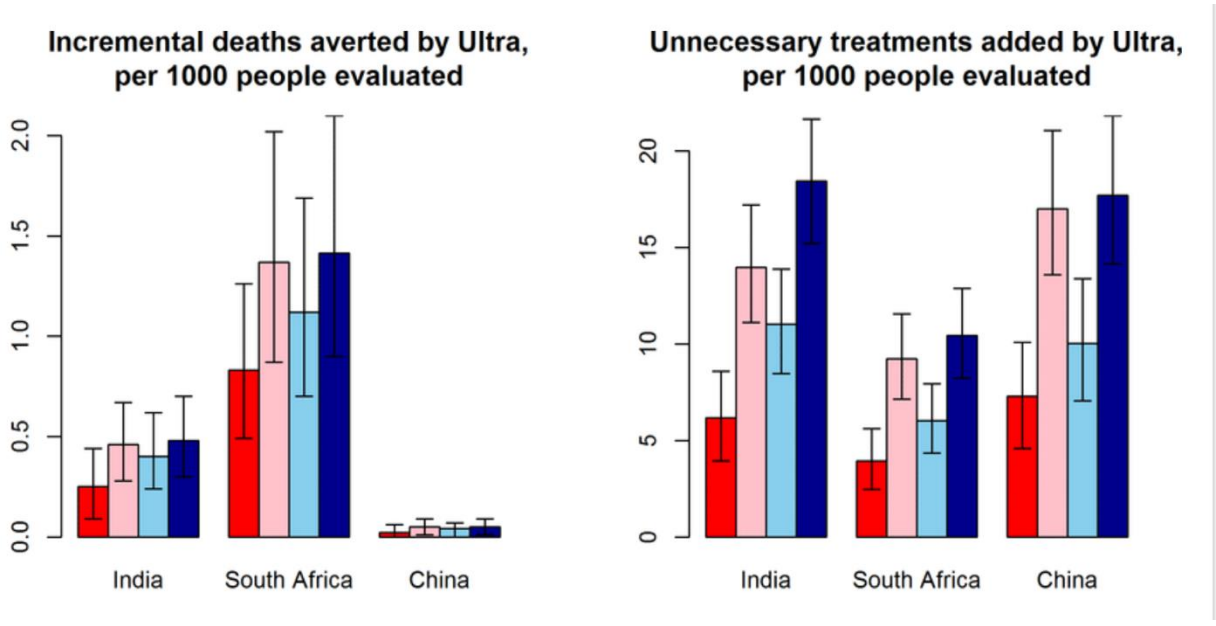

Figure 9. Deaths and unnecessary treatments averted per 1000 people evaluated by using Ultra cartridge as compared to Xpert. Unnecessary treatments are high in all three countries evaluated, but deaths averted are high in South Africa, making the use of Ultra more worth the risk.

Conclusion:

MDR TB is a man made phenomenon, developed through mutations that happen because of inappropriate or inconsistent treatment. It is a global burden costing thousands of lives every year. Immediate and appropriate treatment is not only important to avoid progression and mortality of the disease, but also to avoid the development of more antibiotic resistance in the microorganism. Advances in understanding the mechanisms by which resistance develops can have a major impact in understanding transmission patterns and the development of new anti-TB drugs. New technology like Xpert has been helpful in quickly identifying TB infection and providing a significantly accurate estimate of drug resistance in order to provide proper treatment. This is important to avoid unnecessary exposure of MTB to antibiotics, creating an ideal environment for MTB to develop resistance to the drugs. Xpert Ultra is not yet approved or available in the United States, but it has been shown to avert deaths in countries with high mortality and shows promise as a way to assay individuals with low MTB count if used in the proper setting. Xpert and Xpert Ultra both have different strong suits, and have to be tested in settings with different TB incidence and mortality rates in order to figure out how to more appropriately use this new technology. Used in conjunction, these two assays may significantly lift the burden of TB from the most affected countries and help to slow the rate of antibiotic resistance development through inappropriate patient treatment.

References:

1. "Antibiotic / Antimicrobial Resistance (AR / AMR)." *Centers for Disease Control and Prevention*, Centers for Disease Control and Prevention, 10 Sept. 2018, www.cdc.gov/drugresistance/about.html.
2. Shaikh, Sibhghatulla, et al. "Antibiotic Resistance and Extended Spectrum Beta-Lactamases: Types, Epidemiology and Treatment." *Saudi Journal of Biological Sciences*, vol. 22, no. 1, 2015, pp. 90–101., doi:10.1016/j.sjbs.2014.08.002.
3. "Drug-Resistant TB: Global Situation." *World Health Organization*, World Health Organization, 9 Feb. 2018, www.who.int/tb/areas-of-work/drug-resistant-tb/global-situation/en/.
4. Elena Cabezón, Jorge Ripoll-Rozada, Alejandro Peña, Fernando de la Cruz, Ignacio Arechaga; Towards an integrated model of bacterial conjugation, *FEMS Microbiology Reviews*, Volume 39, Issue 1, 1 January 2015, Pages 81–95.
5. Martinez, Jose L. "General Principles of Antibiotic Resistance in Bacteria." *Drug Discovery Today: Technologies*, vol. 11, 2014, pp. 33–39.
6. Martínez, José Luis, and Fernando Baquero. "Emergence and Spread of Antibiotic Resistance: Setting a Parameter Space." *Upsala Journal of Medical Sciences*, vol. 119, no. 2, 2014, pp. 68–77., doi:10.3109/03009734.2014.901444.
7. Balcazar, Jose Luis. "Bacteriophages as Vehicles for Antibiotic Resistance Genes in the Environment." *PLoS Pathogens*, vol. 10, no. 7, 2014, doi:10.1371/journal.ppat.1004219.
8. Navisha Dookie, Santhuri Rambaran, Nesri Padayatchi, Sharana Mahomed, Kogieleum Naidoo; Evolution of drug resistance in *Mycobacterium tuberculosis:* a review on the molecular determinants of resistance and implications for personalized care, *Journal of Antimicrobial Chemotherapy*, Volume 73, Issue 5, 1 May 2018, Pages 1138–1151.
9. Medicine, The Lancet Respiratory. "Health Inequality: a Major Driver of Respiratory Disease." *The Lancet Respiratory Medicine*, vol. 5, no. 4, 2017, pp. 235–360., doi:10.1016/s2213-2600(17)30092-9.
10. H. Traore, K.Fissette, I. Bastian, M. Devleeschouer, F. Portaels. "Detection of rifampicin resistance in *Mycobacterium tuberculosis* isolates from diverse countries by a commercial line probe assay as an initial indicator of multidrug resistance" *The International Journal of Tuberculosis and Lung Disease.* 12,1999.

11. Palomino, Juan, and Anandi Martin. “Drug Resistance Mechanisms in Mycobacterium Tuberculosis.” *Antibiotics*, vol. 3, no. 3, 2014, pp. 317–340., doi:10.3390/antibiotics3030317.
12. Syal k, Mo M, Yu H, Iriva R, Jing W, Guodong S, Wang S, Grys TE, Haydel SE, Tao N. Current and emerging techniques for antibiotic susceptibility tests. *Therapostics.* 2017; 7 (7): 1795-1805. Doi: 10.7150/thno.19217.
13. Connolly, Lynn E, et al. “Why Is Long-Term Therapy Required to Cure Tuberculosis?” *PLoS Medicine*, vol. 4, no. 3, 2007, doi:10.1371/journal.pmed.0040120.
14. Chakravorty, Soumitesh, et al. “The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium Tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing.” *MBio,* vol. 8, no. 4, 2017, doi:10.1128/mbio.00812-17.
15. Steingart, Karen R, et al. “Xpert® MTB/RIF Assay for Pulmonary Tuberculosis and Rifampicin Resistance in Adults.” *Cochrane Database of Systematic Reviews*, 2014, doi:10.1002/14651858.cd009593.pub3.
16. Dorman SE, Schumacher SG, Alland D, et al.; study team “Xpert MTB/RIF Ultra for detection of *Mycobacterium tuberculosis* and rifampicin resistance: a prospective multicenter diagnostic accuracy study”. *The Lancet Infectious Diseases*, vol 18, Issue 1, 1,2018, pp. 76-84.
17. Global tuberculosis report 2018. Geneva: World Health Organization; 2018. Licence: CC BY-NC-SA 3.0 IGO.
18. Kendall, Emily A., et al. “Estimated Clinical Impact of the Xpert MTB/RIF Ultra Cartridge for Diagnosis of Pulmonary Tuberculosis: A Modeling Study.” *PLOS Medicine*, vol. 14, no. 12, 2017, doi:10.1371/journal.pmed.1002472.