Drug Resistance in *Mycobacterium tuberculosis*

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Abstract

Anti-tuberculosis drugs are a two-edged sword. While they destroy pathogenic *M. tuberculosis* they also select for drug resistant bacteria against which those drugs are then ineffective. Global surveillance has shown that drug resistant Tuberculosis is widespread and is now a threat to tuberculosis control programs in many countries. Application of molecular methods during the last decade has greatly changed our understanding of drug resistance in tuberculosis. Application of molecular epidemiological methods was also central to the description of outbreaks of drug resistance in Tuberculosis. This review describes recommendations for Tuberculosis treatment according to the WHO guidelines, the drug resistance problem in the world, mechanisms of resistance to first line and second line drugs and applications of molecular methods to detect resistance causing gene mutations. It is envisaged that molecular techniques may be important adjuncts to traditional culture based procedures to rapidly screen for drug resistance. Prospective analysis and intervention to prevent transmission may be particularly helpful in areas with ongoing transmission of drug resistant strains as recent mathematical modeling indicate that the burden of MDR-TB cannot be contained in the absence of specific efforts to limit transmission.

Introduction

Drug resistance and global surveillance: history

Shortly after the first anti-tuberculosis (TB) drugs were introduced, streptomycin (STR), para-aminosalicylic acid (PAS), isoniazid (INH) resistance to these drugs was observed in clinical isolates of *Mycobacterium tuberculosis* (Crofton and Mitchison, 1948). This led to the need to measure resistance accurately and easily. The Pasteur Institute introduced the critical proportion method in 1961 for drug susceptibility testing in TB and this method became the standard method of use (Espinal, 2003). Studies on drug resistance in various countries in the 1960s showed that developing countries had a much higher incidence of drug resistance than developed countries (Espinal, 2003). By the end of the 1960s rifampicin (RIF) was introduced and with the use of combination therapy, there was a decline in drug resistant and drug susceptible TB in developed countries. This led to a decline in funding and interest in TB control programs. As a result, no concrete monitoring of drug resistance was carried out for the following 20 years (Espinal, 2003). The arrival of HIV/AIDS in the 1980s resulted in an increase in transmission of TB associated with outbreaks of multi-drug-resistant TB (MDR-TB) (Edlin *et al*., 1992; Fischl *et al*., 1992) i.e. resistant to INH and RIF. In the early 1990s drug resistance surveillance was resumed in developed countries, but the true incidence remained unclear in the developing world (Cohn *et al*., 1997).

The WHO/IUATLD global project on drug-resistance surveillance

In 1994 the Global Project on Drug-Resistance Surveillance was initiated to monitor the trends of resistance. The first report was published in 1997 and contained data from 35 geographical settings for the period 1994–1996 (World Health Organization, 1997; Pablos-Mendez *et al*., 1998). The report showed that drug resistance was present globally, and that MDR-TB ranged from 0% to 14% in new cases (median:1.4%) and 0% to 54% in previously treated cases (median:13%). A second report for the period 1996–1999, followed in 2000 and included surveillance data from 58 geographical sites (Espinal, 2003; World Health Organization, 2000). This report confirmed that drug resistant TB was a sufficient problem since MDR-TB ranged from 0–16% (median: 1%) among new cases and from 0% to 48% (median: 9%) in previously treated cases. The recently published third report has data on 77 geographical sites, collected between 1999 and 2002, representing 20% of the global total of new smear-positive TB cases (World Health Organization, 2003). Eight countries did not report any MDR-TB amongst new cases, while the highest incidence of MDR-TB amongst new cases occurred in Kazakhstan and Israel (14%). Significant increases in MDR-TB prevalence were seen in Estonia, Lithuania, Tomsk Oblast (Russian Federation) and Poland and significant decreasing trends in Hong Kong, Thailand and the USA. The highest prevalence of MDR-TB among previously treated cases was reported in Oman (58.3%, 7/12) and Kazakhstan (56.4%, 180/319). The annual incidence of MDR-TB in most Western and Central European Countries was estimated to be fewer than 10 cases each. Alarmingly, it is estimated that the annual incidence of MDR-TB for 2 provinces in China (Henan and Hubei) is 1000 and for Kazakhstan and South Africa it is more than 3000. According to the report, the most effective means to prevent the emergence of drug resistance is by implementing the direct observed therapy strategy (DOTS) (World Health Organization, 2003).

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Mycobacterium: Genomics and Molecular Biology | Book

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Expert scientists critically review the current and most recent advances in the genomics and molecular biology of mycobacteria. The focus is on the topical and most relevant aspects and the authors aim to give readers an insight into the current understanding of the subject and the future direction of research. Topics covered include strain variation and evolution, hypervirulent strains, electron transport and respiration, lipid biosynthesis, DNA repair, oxygen signaling, sulphur metabolism, protein secretion, the protein kinase family, and much more.

A valuable reference text for all microbiology laboratories and essential reading for all scientists and researchers involved with mycobacteria.

Reviews:

"This very timely book reviews the current knowledge about the genetics and cell biology of the mycobacterium species. It is clear that this information will lead to new treatment options and preventive strategies that may cure or prevent the serious disease of tuberculosis." *from* Doodys (2009)

"a select collection of reviews of mycobacterial 'hot topics' written by leaders in the respective fields. Each chapter is a thorough treatment of the topic, summarizing current understanding and highlighting gaps in knowledge. ... an excellent introduction to the topics covered and will be valuable for all mycobacteriologists." *from* Microbiology Today

Current recommendations for TB treatment by WHO

TB persists as a global public health problem and the main focus for the twentieth century is firstly to cure the individual patient and secondly to minimize the transmission of *M. tuberculosis* to other persons (World Health Organization, 2003; Blumberg *et al*., 2003). The ongoing TB problem has been due to the neglect of TB control by governments, inadequate access and infrastructure, poor patient adherence to medication, poor management of TB control programs, poverty, population growth and migration, and a significant rise in the number of TB cases in HIV infected individuals. Treatment of patients with TB is most successful within a comprehensive framework based upon the following five key components:

- government commitment
- case detection by sputum smear microscopy
- standardized treatment regimen of six to eight months
- a regular, uninterrupted supply of all essential anti-TB drugs
- a standard recording and reporting system.

These five key elements are the recommended approach by the World Health Organization (WHO) to TB control and are called the DOTS strategy (Walley, 1997). DOTS is an inexpensive strategy for the detection and treatment of TB. DOTS was implemented as part of an adherence strategy in which patients are observed to swallow each dose of anti-TB medication, until completion of the therapy. Monthly sputum specimens are taken until 2 consecutive specimens are negative. Currently there are four recommended regimens for treating patients with TB infection by drug-susceptible organisms. Each regiment has an initial phase of 2 months intensive phase followed by a choice of several options for the continuation phase of either 4 or 7 months. The recommended regimens together with the number of doses specified by the regimen are described in Table 1.

Since the introduction of the DOTS strategy in the early '90s by the WHO, considerable progress has been made in global TB control (Sterling *et al*., 2003). In 1997, the estimated average treatment success rate world wide was almost 80%. However, less than 25% of people who are sick with TB are treated through the DOTS strategy (Bastian *et al*., 2000). A total of 180 countries (including both developed and undeveloped countries) had adopted and implemented the DOTS strategy by the end of 2002 and 69% of the global population was living in areas covered by the DOTS strategy (Blumberg *et al*., 2003). However, even though DOTS programs are in place, treatment success rates are very low in developed countries due to poor management of TB control programs and patient non-compliance (Lienhardt and Ogden, 2004; Bastian *et al*., 2003). Furthermore, the effectiveness of DOTS is facing new challenges with respect to the spread and increase of MDR-TB and the co-epidemic of TB/HIV (World Health Organization, 2003). WHO and partners have addressed these new challenges and have developed a new strategy called DOTS-Plus for the treatment of MDR-TB and its co-epidemic TB/HIV. The goal of DOTS-plus is to prevent further development and spread of MDR-TB and is a comprehensive management initiative built upon the DOTS strategy (Table 2). It is important to note that DOTS-Plus should only be implemented in areas were the DOTS strategy is in place as there can be no DOTS-plus without an effective DOTS program.

Drug susceptibility testing

Drug susceptibility testing is carried out on sub-cultured bacteria after the initial positive culture is obtained for diagnosis. It usually takes 3–6 weeks to obtain the initial positive culture with an additional 3 weeks for

Table 1. Drug Regimen for Culture-Positive Pulmonary TB Caused by Drug-Susceptible Organisms

Intensive Phase			Continuation Phase		
Regimen	Drugs	Doses	Regimen	Drugs	Doses
1	INH, RIF PZA, EMB	7 d/wk for 56 doses (8wk) or 5 d/wk for 40 doses (8wk)	1	INH/RIF	7 d/wk for 126 doses (18 wk) or 5d/wk for 90 doses (18 wk)
			1	INH/RIF	2d/wk for 36 doses (18wk)
			1	INH/ RPT	1 wk for 18 doses (18wk)
2	INH, RIF PZA, EMB	7 d/wk for 14 doses (2wks), then 2 d/wk for 12 doses (6wks) or 5 d/wk for 10 doses (2wk), then 2 d/wk for 12 doses (6 wk)	2	INH/RIF	2d/wk for 36 doses
			2	INH/ RPT	1 wk for 18 doses (18wk)
3	INH, RIF PZA, EMB	3d/wk for 24 doses (8wk)	3	INH/RIF	3 wk for 54 doses (18wk)
4	INH, RIF, EMB	7 d/wk for 56 doses (8wk) or 5 d/wk for 40 doses (8 wk)	4	INH/RIF	7 d/wk for 217 doses (31 wk) or 2d/wk for 62 doses (31 wk)

INH-isoniazid; RIF-rifampicin; RPT-rifapentine; PZA-pyrazinamide.
Note: Streptomycin (STR) efficiency is equal to that of EMB and was use as an interchangeable drug with EMB in the initial phase of treatment. Due to the increase of resistance the drug is rendered less useful. Thus, STR is not recommended to be interchangeable with EMB unless the organism is known to be susceptible to the drug or the patient is from a community in which STR resistance is unlikely. Extracted from Blumberg *et al*.(Blumberg *et al*., 2003).

Table 2. DOTS Compared to DOTS-Plus Strategy	
DOTS	DOTS-plus
DOTS prevent emergence of drug resistant TB and MDR-TB	DOTS-plus design to cure MDR-TB using second line drugs.
Make primarily use of 1st line drugs that are less expensive	Make use of 2nd line drugs that are more toxic and expensive,difficult to treat less effective to administrate and often poorly tolerated
	DOTS-plus needed in areas where MDR-TB has emerged due to previous inadequate TB control
	DOTS-plus only recommended in settings where DOTS strategy is fully in place to prevent against the development of further drug resistance

susceptibility testing (reduced to about 15 days when using the BACTEC system) (Rastogi *et al*., 1989; Siddiqi *et al*., 1985; Snider, Jr. *et al*., 1981; Tarrand and Groschel, 1985). Thus, susceptibility testing is time consuming and costly, and there are numerous problems associated with the standardization of tests and the stability of the drugs in different culture media (Martin-Casabona *et al*., 1997; Victor *et al*., 1997). The slow diagnosis of drug resistance may be a major contributor to the transmission of MDR-TB (Victor *et al*., 2002). The WHO recommended that drug susceptibility testing is done by the proportion method on Löwenstein-Jensen medium, but other media, such as Middlebrook 7H10, 7H11, 7H12 (BACTEC460TB) and other methods, including the absolute concentration and resistance ratio methods, may also be used (World Health Organization, 2001). For the ratio method, serial dilutions are cultured on 2 control media (without the drug) and 2 test media (with two different drug concentrations). The colonies on the different slants are counted after 21 and 40 days of growth. The proportion of resistant bacilli is calculated by comparing colony counts on drug free and drug containing media. For a resistant isolate the calculated proportion is higher and for a susceptible strain the calculated proportion is lower than the critical proportion (World Health Organization, 2001).

Molecular mechanisms of drug resistance

In order to control the drug resistance epidemic it is necessary to gain insight into how *M. tuberculosis* develops drug resistance. This knowledge will help us to understand how to prevent the occurrence of drug resistance as well as identifying genes associated with drug resistance of new drugs. The development of clinical drug resistance in TB is summarized in Fig. 1 and is classified as acquired resistance when drug resistant mutants are selected as a result of ineffective treatment or as primary resistance when a patient is infected with a resistant strain. Mutations in the genome of *M. tuberculosis* that can confer resistance to anti-TB drugs occur spontaneously with an estimated frequency of 3.5×10^{-6} for INH and 3.1×10^{-8} for RIF. Because the chromosomal loci responsible for resistance to various drugs are not linked, the risk of a double spontaneous mutation is extremely low: 9×10^{-14} for both INH and RIF (Dooley and Simone, 1994). MDR-TB defined as resistance to at least INH and RIF will thus occur mainly in circumstances where sequential drug resistance follows sustained treatment failure. Treatment can be divided into first line and second line drugs according to the WHO TB treatment regimen and the mechanisms of these will be discussed separately.

First line drugs

Any drug used in the anti-TB regiment is supposed to have an effective sterilizing activity that is capable of shortening the duration of treatment. Currently, a four-drug regiment is used consisting of INH, RIF, pyrazinamide (PZA) and ethambutol (EMB). Resistance to first line anti-TB drugs has been linked to mutations in at least 10 genes; *katG, inhA, ahpC, kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance.

Isoniazid

KatG. INH or isonicotinic acid hydrazide, was synthesized in the early 1900s but its anti-TB action was first detected in 1951 (Heym *et al*., 1999; Slayden and Barry, III, 2000; Rattan *et al*., 1998). INH enters the cell as a prodrug that is activated by a catalase peroxidase encoded by *katG*. The peroxidase activity of the enzyme is necessary to activate INH to a toxic substance in the bacterial cell (Zhang *et al*., 1992). This toxic substance subsequently affects intracellular targets such as mycolic acid biosynthesis which are an important component of the cell wall. A lack of mycolic acid synthesis eventually results in loss of cellular integrity and the bacteria die (Barry, III *et al*., 1998). Middlebrook *et al*. initially demonstrated that a loss of catalase activity can result in INH resistance (Middlebrook, 1954). Subsequently genetic studies demonstrated that transformation of INH-resistant *Mycobacterium smegmatis* and *M. tuberculosis* strains with a functional *katG* gene restored INH susceptibility and that *katG* deletions give rise to INH resistance (Zhang *et al*., 1992; Zhang *et al*., 1993). However, mutations in this gene are more frequent than deletions in clinical isolates and these can lower the activity of the enzyme. Most mutations are found between codons 138 and 328 with the most commonly observed gene alteration being at codon 315 of the *katG* gene (Slayden and Barry, III, 2000). The Ser315Thr substitution is estimated to occur in 30–60% of INH resistant isolates (Ramaswamy and Musser, 1998; Musser *et al*., 1996; Slayden and Barry, III, 2000). The *katG* 463 (CGG-CTG) (Arg-Leu) amino acid substitution is the most common polymorphism found in the *katG* gene and is not associated with INH resistance.

ahpC. It has been observed that a loss of *katG* activity due to the S315T amino acid substitution is often accompanied by an increase in expression of an alkyl hydroperoxide reductase (*ahpC*) protein that is capable of detoxifying damaging organic peroxides (Sherman *et al*., 1996). Five different nucleotide alterations have been identified in the

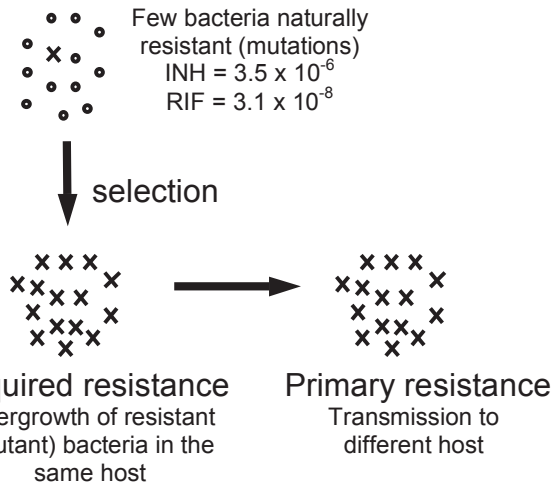

Fig. 1 Acquired resistance develops due to natural selection which is a function of ineffective treatment and non-compliance

promoter region of the *ahpC* gene, which lead to over expression of *ahpC* and INH resistance (Ramaswamy and Musser, 1998). *AhpC* overexpression exerts a detoxifying effect on organic peroxides within the cell and protects the bacteria against oxidative damage but does not provide protection against INH. *KatG* expression can also be up regulated under conditions of oxidative stress. The correlation between polymorphic sites in the *ahpC* regulatory region with INH resistance in *M. tuberculosis* requires further examination.

inhA. One of the targets for activated INH is the protein encoded by the *inhA* locus. InhA is an enoyl–acyl carrier protein (ACP) reductase which is proposed to be the primary target for resistance to INH and ethionamide (ETH) (Banerjee *et al*., 1994). ETH, a second line drug, is a structural analog of INH that is also thought to inhibit mycolic acid biosynthesis and several studies have suggested that low-level INH resistance is correlated with resistance to ETH. Activated INH binds to the InhA-NADH complex to form a ternary complex that results in inhibition of mycolic acid biosynthesis. Six point mutations associated with INH resistance within the structural *inhA* gene have been identified (Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro) (Ramaswamy and Musser, 1998; Basso and Blanchard, 1998). A Ser94Ala substitution results in a decreased binding affinity of *inhA* for NADH, resulting in mycolic acid synthesis inhibition. Although these mutations in the structural *InhA* gene are associated with INH resistance, it is not frequently reported in clinical isolates. *InhA* promoter mutations are more frequently seen and are present at positions -24(G-T), -16(A-G), or -8(T-G/A) and -15(C-T). These promoter mutations result in over expression of *inhA* leading to low level INH resistance. To date approximately 70–80% of INH resistance in clinical isolates of *M. tuberculosis* can be attributed to mutations in the *katG* and *inhA* genes (Ramaswamy and Musser, 1998).

kasA. There seems to be considerable dispute within the litreature as to the role of *kasA* as a possible target for INH resistance (Sherman *et al*., 1996). This gene encodes a β-ketoacyl-ACP synthase involved in the synthesis of mycolic acids. Mutations have been described in this gene that confer low levels of INH resistance. Genotypic analysis of the *kas*A gene reveals 4 different amino acid substitutions involving codon 66 (GAT-AAT), codon 269 (GGT-AGT), codon 312 (GGC-AGC) and codon 413 (TTC-TTA) (Ramaswamy and Musser, 1998; Mdluli *et al*., 1998). However, similar mutations were also found in INH susceptible isolates (Lee *et al*., 1999; Piatek *et al*., 2000). Nevertheless, the possibility of *kasA* constituting an additional resistance mechanism should not be completely excluded.

ndh. In 1998 another mechanism for INH resistance in *M. smegmatis* was described by Miesel *et al*. (Miesel *et al*., 1998). The *ndh* gene encodes NADH dehydrogenase that is bound to the active site of *inhA* to form the ternary complex with activated INH. Structural studies have shown that a reactive form of INH attacks the NAD(H) co-factor and generates a covalent INH-NAD adduct. Mutations in the *ndh* gene, encoding NADH dehydrogenase, cause defects in the enzymatic activity. Thus, defects in the oxidation of NADH to NAD result in NADH accumulation and NAD depletion (Lee *et al*., 2001). These high levels of NADH can then inhibit the binding of the INH-NAD adduct to the active site of the InhA enzyme (Rozwarski *et al*., 1998; Miesel *et al*., 1998). Prominent point mutations in the *ndh* gene at codons 110 and 268 (T110A and R268H) were detected in 9.5% of INH resistant samples. These similar mutations were not detected in the INH susceptible group (Lee *et al*., 2001).

Rifampicin

RIF was fist introduced in 1972 as an anti-TB drug and has excellent sterilizing activity (Rattan *et al*., 1998; Ramaswamy and Musser, 1998). The action of RIF in combination with PZA has allowed a shortening of routine TB treatment from 1 year to 6 months. RIF in combination with INH forms the backbone of short-course chemotherapy. It is interesting to note that mono resistance to INH is common but mono resistance to RIF is quite rare. It has thus been proposed that resistance to RIF can be used as a surrogate marker for MDR-TB as nearly 90% of RIF resistant strains are also INH resistant (Somoskovi *et al*., 2001). RIF interferes with transcription by the DNA-dependent RNA polymerase. RNA polymerase is composed of four different subunits (α, β, β' and σ) encoded by *rpoA, rpoB, rpoC* and *rpoD* genes respectively. RIF binds to the β-subunit hindering transcription and thereby killing the organism. Extensive studies on the *rpoB* gene in RIF resistant isolates of *M. tuberculosis* identified a variety of mutations and short deletions in the gene. A total of 69 single nucleotide changes; 3 insertions, 16 deletion and 38 multiple nucleotide changes have been reported (Herrera *et al*., 2003). More than 95% of all missense mutations are located in a 51bp core region (Rifampicin resistance determining region) of the *rpoB* gene between codons 507–533 with the most common changes in codons Ser531Leu, His526Tyr and Asp516Val. These changes occur in more than 70% of RIF resistant isolates (Rattan *et al*., 1998; Ramaswamy and Musser, 1998; Herrera *et al*., 2003). Furthermore, the minimal

inhibitory concentration (MIC) showed that high level of RIF resistance is associated with mutations in codon 526 and 531, whereas alterations in codon 511,516, 518 and 522 result in low level RIF resistance.

Pyrazinamide

PZA, a nicotinamide analog, was first discovered to have anti-TB activity in 1952. PZA targets an enzyme involved in fatty-acid synthesis and is responsible for killing persistent tubercle bacilli in the initial intensive phase of chemotherapy (Somoskovi *et al*., 2001). However, during the first two days of treatment, PZA has no bactericidal activity against rapidly growing bacilli (Zhang and Mitchison, 2003). PZA on the other hand has effective sterilizing activity and shortens the chemotherapeutic regiment from 12 to 6 months. PZA is a prodrug which is converted to its active form, pyrazinoic acid (POA) by the pyrazinamidase (PZase) encoded by *pncA*. The activity of PZA is highly specific for *M. tuberculosis,* as it has no effect on other mycobacteria. *Mycobacterium bovis* is naturally resistant to PZA due to a unique C-G point mutation in codon 169 of the *pncA* gene. PZA is only active against *M. tuberculosis* at acidic pH where POA accumulates in the cytoplasm due to an ineffective efflux pump. Accumulation of POA results in the lowering of intracellular pH to a level that inactivates a vital fatty acid synthase (Zimhony *et al*., 2004). Cloning and characterization of the *M. tuberculosis pncA* gene by Scorpio *et al.* (Scorpio and Zhang, 1996) showed that *pncA* mutations conferred PZA resistance. Various *pncA* mutations have been identified in more than 70% of PZA resistant clinical isolates scattered throughout the *pncA* gene but thus far no mutational hot spot has been identified (Scorpio and Zhang, 1996; Sreevatsan *et al*., 1997b; Scorpio *et al*., 1997). In a study from Peru it was found that 59% of MDR patients also had *M. tuberculosis* resistant to PZA(Saravia *et al*., 2005). PZA susceptibility testing is not done routinely in many countries due to technical difficulties. Thus the extent of PZA resistance globally is largely unknown. A study done by Louw *et al.* (Louw *et al*., 2006). showed that PZA resistance is common amongst drug-resistant clinical *M. tuberculosis* isolates from South Africa**.** PZA resistance was shown to be strongly associated with MDR-TB and therefore it was concluded that PZA should not be relied upon in managing patients with MDR-TB in this setting. PZA resistant isolates had diverse nucleotide changes scattered throughout the *pncA* gene. Mutations in the *pncA* gene correlate well with phenotypic resistance to PZA. However, PZA resistant isolates without *pncA* mutations were also observed suggesting that another mechanism may be involved in conferring PZA resistance in these isolates. In addition, not all mutations (e.g. $Thr_{114}Met$) were associated with PZA resistance. In summary, the complexity of PZA resistance makes the development of molecular methods for rapid diagnosis difficult.

Ethambutol

EMB, a first line drug, is used in combination with other drugs and is specific to the mycobacteria. EMB inhibits an arabinosyl transferase (*embB*) involved in cell wall biosynthesis (Takayama and Kilburn, 1989). Telenti *et al.* (Telenti *et al*., 1997) identified 3 genes, designated *emb*CAB, that encode homologous arabinosyl transferase enzymes involved in EMB resistance. Various studies have identified five mutations in codon 306 [(ATG-GTG), (ATG-CTG), (ATG-ATA), (ATG-ATC) and (ATG-ATT)] which result in three different amino acid substitutions (Val, Leu and Ile) in EMB-resistant isolates (Lee *et al*., 2002; Sreevatsan *et al*., 1997c; Mokrousov *et al*., 2002b; Ramaswamy *et al*., 2000). These five mutations are associated with 70–90% of all EMB resistant isolates (Ramaswamy and Musser, 1998). Missense mutations were identified in three additional codons: Phe285leu, Phe330Val and Thr630Ile in EMB resistant isolates. MIC's were generally higher for strains with Met306Leu, Met306Val, Phe330Val and Thr630Ile substitutions than those organisms with Met306Ile substitutions. Mutations outside of codon 306 are present but quite rare. In a study recently done by Johnson *et al*. (Johnson *et al*., 2006) it was shown that genotypic analysis identified mutations at codon 306 of the *embB* gene rendering resistance to EMB. However, routine phenotypic analysis failed to identify EMB resistance in 91.4% of resistant isolates in this setting and confirm the difficulty of EMB phenotypic testing. The inability to accurately detect true EMB resistance by the culture based method have a negative impact on the TB control program. Molecular-based methods offers a rapid diagnosis of EMB resistance and could thereby benefit the management of TB patents within days. However a number of EMB phenotypic resistant isolates (about 30%) still lack an identified mutation in *embB*. There is therefore a need to fully understand the mechanism of EMB resistance in clinical isolates.

Streptomycin

STR, an aminocyclitol glycoside, is an alternative first line anti-TB drug recommended by the WHO (Cooksey *et al*., 1996). STR is therefore used in the retreatment of TB cases together with the four drug regimen that includes INH, RIF, PZA and EMB (Brzostek *et al*., 2004). The effect of STR has been demonstrated to take place at the ribosomal level (Telenti *et al*., 1993). STR interacts with the 16S rRNA and S12 ribosomal protein (*rrs* and *rpsL*) (Escalante *et al*., 1998; Finken *et al*., 1993; Sreevatsan *et al*., 1996; Abbadi *et al*., 2001), inducing ribosomal changes, which cause misreading of the mRNA and inhibition of protein synthesis. Although STR is a recommended anti-TB drug, is it less effective against *M. tuberculosis* than INH and RIF. Point mutations in STR resistant isolates have been reported in *rrs* and *rpsL* genes in 65–67% of STR resistant isolates (Ramaswamy and Musser, 1998). In the *rrs* gene a C-T transition at positions 491, 512 and 516, and a A-C/T transversion at position 513 were observed in the highly conserved 530 loop. The 530 loop region is part of the aminoacyl–tRNA binding site and is involved in the decoding process (Carter *et al*., 2000). The C-T transition at codon 491 is not responsible for resistance to STR as it occurs in both STR resistant and susceptible isolates but is strongly associated with the global spread of *M. tuberculosis* with a Western Cape F11 genotype (van Rie *et al*., 2001; Victor *et al*., 2001). Other mutations in the 915 loop [903 (C-A/G) and 904 (A-G)] have also been reported to have an association with STR resistance (Carter *et al*., 2000). Mutations in the *rpsL* gene at codon

43 (AAG-AGG/ACG) (Lys-Arg/Thr) and codon 88 (AAG-AGG/CAG) (Lys-Arg/Gln) are associated with STR resistance. MIC analysis of STR resistant isolates indicate that amino acid replacements in the *rpsL* genes correlate with a high level of resistance, whereas mutations in the *rrs* gene correlate with an intermediate level of resistance (Cooksey *et al*., 1996; Meier *et al*., 1996). In addition, it has been suggested that low levels of STR resistance are also associated with altered cell permeability or rare mutations which lie outside of the *rrs* and *rpsL* genes.

Second line drugs used in TB treatment

According to the WHO the following drugs can be classified as second line drugs: aminoglycosides (kanamycin and amikacin) polypeptides (capreomycin, viomycin and enviomycin), fluoroquinolones (ofloxacin, ciprofloxacin, and gatifloxacin), D-cycloserine and thionamides (ethionamide and prothionamide) (World Health Organization, 2001). Unfortunately, second-line drugs are inherently more toxic and less effective than first-line drugs (World Health Organization, 2001). Second line drugs are mostly used in the treatment of MDR-TB and as a result prolong the total treatment time from 6 to 9 months (Cheng *et al*., 2004). The current understanding of molecular mechanisms associated with resistance to second line drugs are summarized in Table 3. The phenotypic methods to detect resistance to second line drugs are less well established and the molecular mechanisms of resistance are also less defined.

Fluoroquinolones

Ciproflaxin (CIP) and ofloxacin (OFL) are the two fluoroquinolones (FQs) used as second-line drugs in MDR-TB treatment (World Health Organization, 2001). The quinolones target and inactivate DNA gyrase, a type II DNA topoisomerase (Cynamon and Sklaney, 2003; Ginsburg *et al*., 2003; Rattan *et al*., 1998). DNA gyrase is encoded by *gyrA* and *gyrB* (Rattan *et al*., 1998; Takiff *et al*., 1994) and introduces negative supercoils in closed circular DNA molecules (Rattan *et al*., 1998; Ramaswamy and Musser, 1998). The quinolone resistance-determining region (QRDR) is a conserved region in the *gyrA* (320bp) and *gyrB* (375bp) genes (Ginsburg *et al*., 2003) which is the point of interaction of FQ and gyrase (Ginsburg *et al*., 2003). Missense mutations in codon 90, 91, and 94 of *gyrA* are associated with resistance to FQs (Takiff *et al*., 1994; Xu *et al*., 1996). A 16-fold increase in resistance was observed for isolates with a Ala90Val substitution, a 30-fold increase for Asp94Asn or His94Tyr and a 60-fold increase for Asp94Gly (Xu *et al*., 1996). A polymorphism at *gyrA* codon 95 is not associated with FQ resistance, and is used, with the *katG*463 polymorphism, to classify *M. tuberculosis* into 3 phylogenetic groups (Sreevatsan *et al*., 1997a).

Aminoglycosides

Kanamycin (KAN) and Aminokacin (AMI) are aminoglycosides which inhibit protein synthesis and thus cannot be used against dormant *M. tuberculosis*. Aminoglycosides bind to bacterial ribosomes and disturb the elongation of the peptide chain in the bacteria. Mutations in the *rrs* gene encoding for 16s rRNA are associated with resistance to KAN and AMI. Nucleotide changes at positions 1400, 1401 and 1483 of the *rrs* gene have been found to be specifically associated with KAN resistance (Suzuki *et al*., 1998). An A→G change at codon 1400 in the *rrs* gene showed resistance to KAN of MICs more that 200 µg/ml (Taniguchi *et al*., 1997; Suzuki *et al*., 1998).

Ethionamide

Ethionamide (ETH) is an important drug in the treatment of MDR-TB, and is mechanistically and structurally analogous to INH. Like INH, ETH is also thought to be a prodrug that is activated by bacterial metabolism. The activated drug then disrupts cell wall biosynthesis by inhibiting mycolic acid synthesis. Mutations in the promoter of the *inhA* gene are associated with resistance to INH and ETH (Morlock *et al*., 2003). *EthA* catalyses a two step activation of ETH and gene alterations leading to reduced EthA activity lead to ETH resistance (Engohang-Ndong *et al*., 2004; Morlock *et al*., 2003; Vannelli *et al*., 2002). The expression of *ethA* is under the control of the neighbouring *ethR* gene encoding a repressor. *EthR* negatively regulates the expression of *ethA*, by binding upstream of *ethA* to suppress *ethA* expression (Engohang-Ndong *et al*., 2004).

D-Cycloserine

D-cycloserine (DCS) is a cyclic analog of D-alanine which is one of the central molecules of the cross linking step of peptidoglycan assembly (Ramaswamy and Musser, 1998; Feng and Barletta, 2003; David, 2001; Caceres *et al*., 1997). DCS inhibits cell wall synthesis by competing with D-Alanine for the enzymes D-alanyl-D-alanine synthetase (Ddl) and D-alanine racemase (Alr) and also inhibiting the synthesis of these proteins. Over expression of *alr* cause DCS resistance. A G→T transversion in the *alr* promoter may lead to the overexpression of *alr* (Feng and Barletta, 2003; Ramaswamy and Musser, 1998).

Peptides

Viomycin (VIO) and capreomycin (CAP) are basic peptide antibiotics that inhibit prokaryotic protein synthesis and are used as second-line anti-TB drugs. Earlier studies have shown that resistance to VIO in *M. smegmatis* is caused by alterations in the 30S or 50S ribosomal subunits (Taniguchi *et al*., 1997). Mutations in the *rrs* gene that encodes the 16S rRNA is associated with resistance to VIO and CAP, specifically a G→A or G→T nucleotide change at codon 1473 (Taniguchi *et al*., 1997).

Molecular methods to predict drug resistance

M. tuberculosis is a very slow growing organism and the use of molecular methods for the identification of mutations in resistance-causing genes may offer a means to rapidly screen *M. tuberculosis* isolates for antibiotic resistance. Mutation screening methods are fast and include methods such as DNA sequencing, probe based hybridization methods, PCR-RFLP, single–strand conformation polymorphism (SSCP), heteroduplex analysis (HA), molecular beacons and ARMS-PCR (Victor *et al*., 2002). The end results for each of these methods are given as a combined photo in Fig. 2.

Table 3. Properties of Resistance to Various Second-line Anti-TB Drugs							
Second-line drug	Gene locus	Gene product	Known polymorphism	Most frequently mutated codons associated with resistance	MIC [a] (µg/ml)	Methods for genotypic detection of resistance	Reference
Fluoroquinolones Ofloxacin Cipromycin	*gyr*A	DNA gyrase	*gyrA* 95	*gyr* 90, 91, 94	OFL: 1.0–2.0 CIP: 0.5–4.0	Cloning & expression PCR-SSCP[b] DNA sequencing	(Takiff *et al*., 1994; Pletz *et al*., 2004; Rattan *et al*., 1998; Ginsburg *et al*., 2003; Cheng *et al*., 2004)
Aminoglycosides Kanamycin Amikacin	*rrs*	16 S rRNA		*rrs* 1400	KAN: > 200 AMI: > 256	IS*6110*-RFLP PCR-RFLP[c]	(Ramaswamy and Musser, 1998; Takiff *et al*., 1994; Taniguchi *et al*., 1997; Suzuki *et al*., 1998; Ramaswamy *et al*., 2004; Vannelli *et al*., 2002)
Ethionamide	*inhA*	Enoyl-ACP reductase		*inhA*21, 94, 44	≥ 25	DNA Sequencing	(Baulard *et al*., 2000; Morlock *et al*., 2003; Cynamon and Sklaney, 2003)
	ethA	Flavin monooxygenase			≥200		
	ethR	Transcriptional repressor					
D- cycloserine	*alr*	D-alanine racemase			≥ 300	Cloning & expression DNA Sequencing	(Caceres *et al*., 1997; Feng and Barletta, 2003)
	ddl	D-alanine: D-alanine ligase					
Viomycin	*rrs*	16S rRNA			20	DNA Sequencing	(Ramaswamy and Musser, 1998; Taniguchi *et al*., 1997; Suzuki *et al*., 1995)
a) Minimum inhibitory concentration. b) Polymerase chain reaction- single strand conformation polymorphism. c) Polymerase chain reaction- Restriction fragment length polymorphism.							

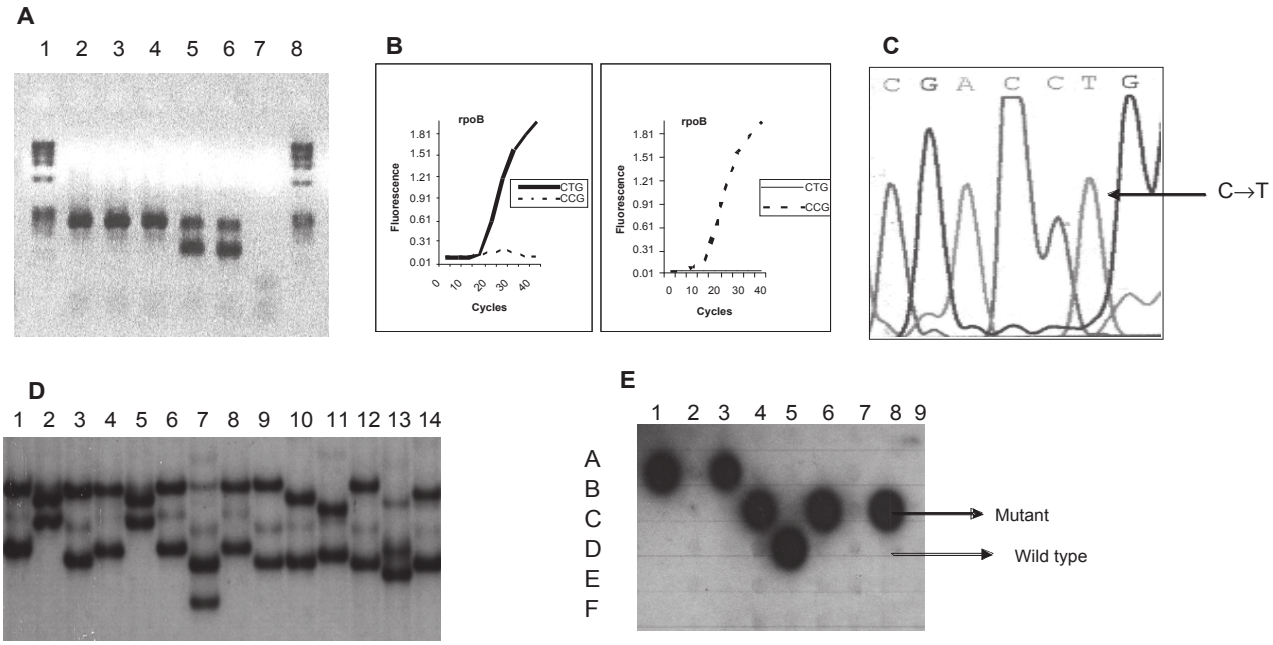

Fig. 2 Molecular methods for detecting gene mutations associated with resistance to anti-TB drugs**.** A–E are typical examples of final results obtained by PCR-based mutation screening methods. The DNA template can be pure DNA extracted from a culture, sputum or crude DNA templates prepared from culture or sputum. In all the examples PCR amplification of the DNA is followed by the different mutation detection methods (A-E). A: ARMS-PCR analysis of *embB* gene. Lane 1 and 8 = Molecular marker and Samples 5 and 6 are mutants. B: Molecular beacon genotyping of the *rpoB* gene showing a T→C mutation. C: Sequence analysis showed a C→T mutation at nt161 in the *pncA* gene D: SSCP analysis with samples having different mutations (visualized as different band mobility shifts) in the of *rpoB* gene. Heteroduplex analysis would give similar band mobility shifts on the gel. E: DOT-BLOT analysis with *rpsL 43* mutant probe showing wild type (lane D8) and mutant (lane C8) controls and mutant (STR resistant) clinical isolates in B1, B3, C4, D5, C6 and C8.

Sequencing

PCR amplification followed by DNA sequencing is the most widely used technique to identify mutations associated with drug resistance in TB (Victor *et al*., 2002). This technique is costly and require expertise, which make it unpractical for use in routine laboratories, especially in developing countries, where simple, cost effective drug susceptibility testing is needed (Victor *et al*., 2002).

Probe-based hybridization methods

In these assays, amplified PCR products of genes known to confer drug resistance are hybridized to an allele-specific labeled probe that is complementary to the wild type or mutant sequence of the gene. This can then be visualized by autoradiography, enhanced chemiluminescence, alkaline phosphatase or other detection systems. These methods include the Dot-blot and Line blot essays and the commercially available INNO-LIPA RIF-TB test (Innogenetics, Belgium) (Victor *et al*., 1999; Mokrousov *et al*., 2004).

PCR-restriction fragment length polymorphism (PCR-RFLP)

Mutations associated with resistance can be identified by digestion of amplified PCR products with a restriction enzyme that cuts at the specific polymorphic DNA sequence followed by gel electrophoresis. Since not all mutations result in the gain or loss of a restriction site, general use of RFLP to screen for mutations associated with drug resistance is limited (Victor *et al*., 2002).

Single stranded conformation polymorphism analysis (SSCP)

SSCP is a gel based method that can detect short stretches of DNA approximately 175–250bp in size. Small changes in a nucleotide sequence result in differences in secondary structures as well as measurable DNA mobility shifts that are detected on a non-denaturing polyacrylamide gel. To date various studies have applied PCR-SSCP to identify mutational changes associated with drug resistance in *M. tuberculosis* for frontline drugs like, RIF and INH (Kim *et al*., 2004; Cardoso *et al*., 2004; Fang *et al*., 1999; Heym *et al*., 1995; Pretorius *et al*., 1995). However, PCR-SSCP analysis has been found to be technically demanding and not sufficiently sensitive. Furthermore SSCP conditions must be carefully evaluated since not all mutations will be detected under the same conditions.

Heteroduplex analysis (HA)

HA depends on the conformation of duplex DNA when analysed in native gels. Heteroduplexes are formed when PCR amplification products from known wild type and unknown mutant sequences are heated and re-annealed. The DNA strand will form a mismatched heteroduplex if there is a sequence difference between the strands of the wild type and tested DNA. These heteroduplexes have an altered electrophoretic mobility when compared to homoduplexes, since the mismatches tend to retard the migration of DNA during electrophoresis. There are two types of heterodoplexes. The “bubble” type is formed between DNA fragments with single base differences and the bulge type is formed when there are deletions

or insertions present within the two fragments. Recently, temperature mediated HA has been applied to the detection of mutations associated with mutations in *rpoB, katG, rpsL, embB* and *pncA* genes (Mohamed *et al*., 2004; Cooksey *et al*., 2002). Neither HA nor the SSCP analysis are 100% sensitive although Rosetti *et al.* found that HA detected more mutants (Nataraj *et al*., 1999). However, HA has certain disadvantages in that it has been found to be insensitive to G-C rich regions and is very time consuming (Nataraj *et al*., 1999).

Molecular beacons

Molecular beacons are single-stranded oligonucleotide hybridization probes which can be used as amplicon detector probes in diagnostic assays. A beacon consists of a stem-loop structure in which the stem contains a fluorophore on one arm and a quencher on the other end of the arm. The loop contains the probe which is complementary to the target DNA. If the molecular beacon is free in a solution it will not fluoresce, because the stem places the fluorophore so close to the non-fluorescent quencher that they transiently share electrons, eliminating the ability of the fluorophore to fluoresce. However, in the presence of complementary target DNA the probe undergo a conformational change that enables them to fluoresce brightly. Different colored fluorophores (different primers) can be used simultaneously to detect multiple targets (each target will give a different color) in the same reaction. Molecular beacons are very specific and can discriminate between single nucleotide substitutions. Thus they are ideally suited for genotyping and have been used in the detection of drug resistance in *M. tuberculosis* (El Hajj *et al*., 2001; Piatek *et al*., 2000; Piatek *et al*., 1998).

Amplification refractory mutation system (ARMS)-PCR

ARMS also known as allelic specific PCR (ASPCR) or PCR amplification of specific alleles (PASA) is a well established technique used for the detection of any point mutation or small deletions (Newton *et al*., 1989). ARMS-PCR, is usually a multiplex reaction where three (or more) primers are used to amplify the same region simultaneously. One of the three primers is specific for the mutant allele and will work with a common primer during amplification. The mismatch is usually located at or near the 3′ end of the primer. The third primer will work with the same common primer to generate an amplified fragment which is larger than the fragment from the mutant allele primer – this serves as an internal control for amplification. Amplification is detected by gel electrophoresis and the genotypic classification is determined by assessing which amplification products are present. An amplification product should always be present in the larger internal control amplified fragment; if this is the case then the absence or presence of the smaller product will indicate the presence or absence of a mutant allele. This technique has successfully been used for the detection of mutations associated with RIF resistance in *M. tuberculosis* (Fan *et al*., 2003). Fig. 2A indicates how the amplified products in the multiplex reaction are distinguished on a gel.

Applications

One of the major advantages of PCR based methods is the speed by which the result can be obtained (Siddiqi *et al*., 1985; Snider, Jr. *et al*., 1981; Tarrand and Groschel, 1985). It is envisaged that molecular techniques may be important adjuncts to traditional culture based procedures to rapidly screen for drug resistance. Prospective analysis and intervention to prevent transmission may be particularly helpful in areas with ongoing transmission of drug resistant strains as reported previously (van Rie *et al*., 1999). In addition, molecular prediction may also be useful in drug surveillance studies to further improve the confidence limit of the data in these studies if this test is performed on a subset of the samples. Enhanced efforts are necessary to better understand the molecular mechanisms of resistance to second line anti-TB drugs in clinical isolates. However, implementation for both rapid diagnosis and surveillance requires proper quality control guidelines and controls, which is currently not in place yet for molecular prediction of drug resistance in TB. Although molecular methods are more rapid, and can be done directly from a clinical sample there are important limitations when compared to conventional phenotypic methods. These include a lack of sensitivity since not all molecular mechanisms leading to drug resistance are known, therefore not all resistant isolates will be detected. Molecular methods may also predict resistance genotypes that are expressed at levels that may not clinically be relevant (Victor *et al*., 2002).

Transmission and epidemic drug resistant strains

There is much debate about the relative contribution of acquired and primary resistance to the burden of drug resistant TB in different communities. This controversy focuses on whether MDR strains are transmissible or whether the mutations that confer drug resistance also impair the reproductive function of the organism (fitness of the strain). Evidence that MDR strains do have the potential for transmission comes from a series of MDR-TB outbreaks that have been reported over the past decade. These have been identified in hospitals (Fischl *et al*., 1992; Edlin *et al*., 1992; Bifani *et al*., 1996; Cooksey *et al*., 1996), amongst health care workers (Beck-Sague *et al*., 1992; Pearson *et al*., 1992; Jereb *et al*., 1995) and in prisons (Valway *et al*., 1994) and have focused attention on MDR-TB as a major public health issue. Application of molecular epidemiological methods was central to the identification and description of all these outbreaks.

The most extensive MDR-TB outbreak reported to date occurred in 267 patients from New York, who were infected by Beijing/W genotype (Frieden *et al*., 1996). This cluster of cases included drug resistant isolates that were resistant to all first-line anti-TB drugs. The authors speculate that the delay in diagnosis and administering appropriate therapy resulted in prolonging infectiousness and placed healthcare workers and other hospital residents (or contacts) at risk of infection for nosocomial infection. This difficult-to-treat strain has subsequently disseminated to other US cities and Paris and the authors showed by using molecular methods, how this initially fully drug susceptible strain clonally expanded to result in a MDR phenotype by sequential acquisition of resistance conferring mutations in several genes (Bifani *et al*., 1996). Since then, the drug resistant Beijing/W genotype has been the focus of extensive investigations and Beijing drug resistant and susceptible genotypes have been found

to be widely spread throughout the world (Glynn *et al*., 2002), including in South Africa (van Rie *et al*., 1999) and Russia (Mokrousov *et al*., 2002a). Beijing/W genotypes can be identified by their characteristic multi-banded IS*6110* restriction fragment-length polymorphism (RFLP) patterns, a specific spoligotype pattern characterized by the presence of spoligotype spacers 35–43 (Bifani *et al*., 2002) and resistance conferring gene mutations. Although these data led many to propose that Beijing/W strains behaved differently from other strains, more recent work suggests that MDR outbreaks are not limited to the Beijing/W genotype. Smaller outbreaks involving other MDR-TB genotypes have been reported in other settings such as the Czech Republic, Portugal and Norway (Kubin *et al*., 1999; Portugal *et al*., 1999). However, since much of the MDR burden falls in developing countries in which routine surveillance does not usually include molecular fingerprinting, little is known about the characteristics of circulating drug resistant strains in much of the world. It is therefore possible that there are other MDR strains, as widespread as Beijing/W, which have not been recognized and reported as such.

Future

Enhanced efforts are necessary to better understand the molecular mechanisms of resistance in second line anti-TB drugs in clinical isolates. The next generation of molecular methods for the prediction of drug resistance in *M. tuberculosis* will possibly consists of matrix hybridization formats such as DNA oligonucleotide arrays on slides or silicon micron chips (Castellino, 1997; Vernet *et al*., 2004), particularly if these systems can be fully automated and re-used. This may be particularly useful for mutations in the *rpoB* gene, which can serve as a marker for MDR-TB (Watterson *et al*., 1998) and also for the multiple loci that are involved in INH resistance (Table 1). Selection of a limited number of target mutations which enable the detection of the majority of drug resistance (van Rie *et al*., 2001) would be useful in this strategy. It is essential that developments for new techniques must consider the fact that the majority of drug resistant cases occur in resource-poor countries (Raviglione *et al*., 1995) and therefore the methodologies must not only be cheap but also robust.

There are other rapid methods which do not depend on the detection of mutations to predict drug resistance. One promising method is phage amplification technology in which mycobacteriophages (bacteriophages specific for mycobacteria) are used as an indicator of the presence of viable *M. tuberculosis* in a clinical specimen (Albert *et al*., 2002; Eltringham *et al*., 1999; McNerney *et al*., 2000). The phage assay can also be adapted for the detection of drug resistance.

Application of rapid methods to break chains of ongoing transmission of drug resistant TB will increasingly become important as recent mathematical modeling indicate that the burden of MDR-TB cannot be contained in the absence of specific efforts to limit transmission (Cohen and Murray, 2004; Blower and Chou, 2004). This may include rapid detection of drug resistance by molecular methods.

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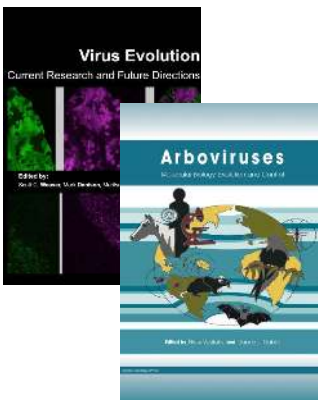

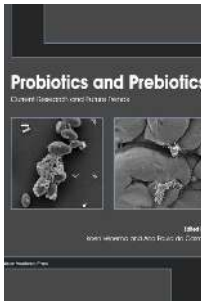

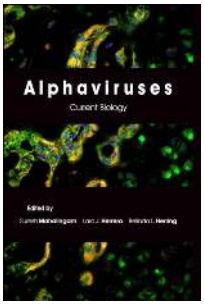

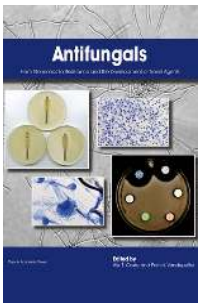

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