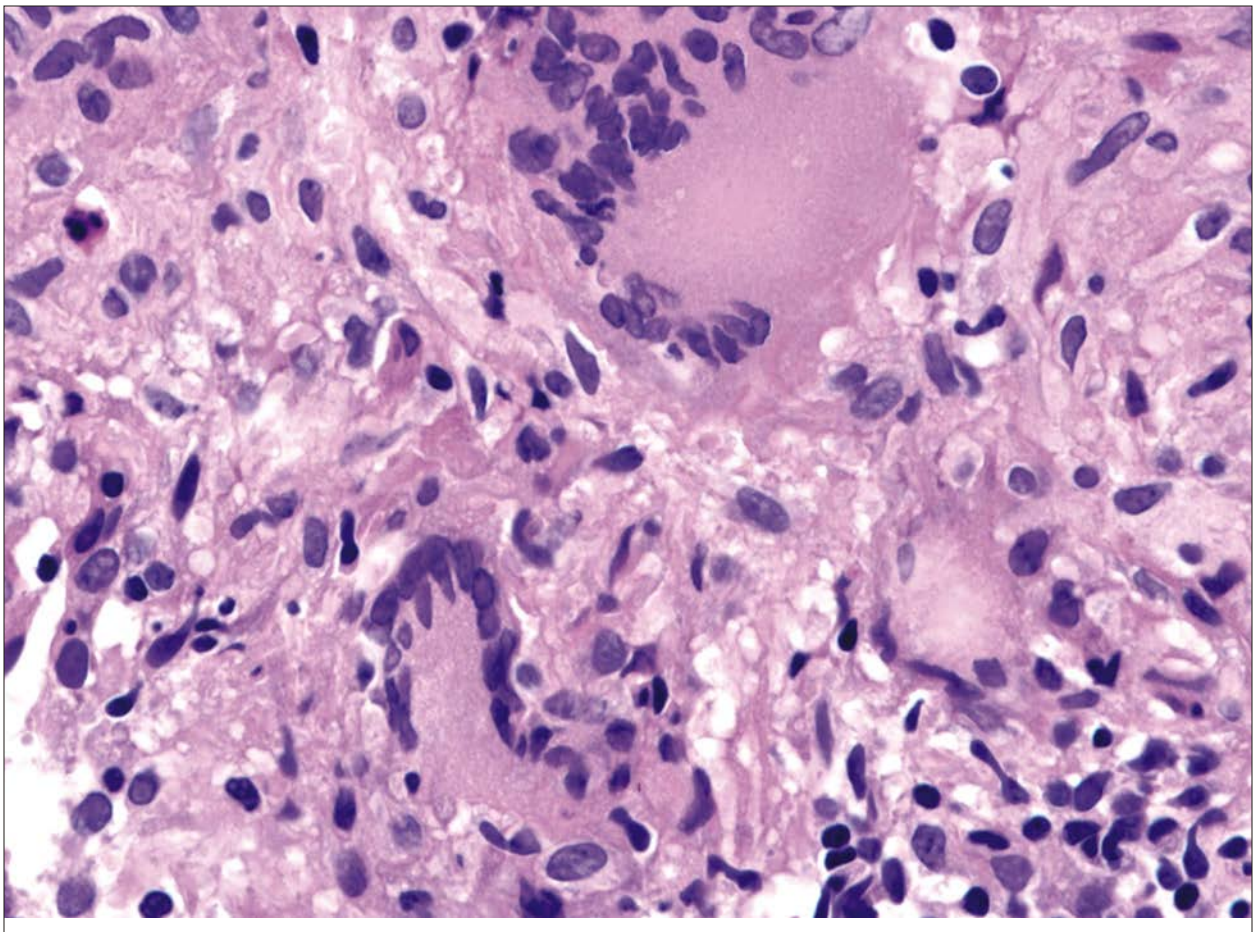

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Drug Resistant Tuberculosis and its Survielance

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ABSTRACT

TB is the most common cause of death due to a single infectious agent worldwide in adults. In 1993, the World Health Organization took an unprecedented step and declared TB to be a global emergency. 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.

Key words: MDR, XDR, tuberculosis, isoniazid, second line drugs

INTRODUCTION

Global efforts to control TB were reinvigorated in 1991, when a World Health Assembly (WHA) resolution recognized TB as a major global public health problem (WHO, 1993). Two targets for TB control were established as part of this resolution-detection of 70% of new smearpositive cases and cure of 85% of such cases, by the year 2000. In 1994, the internationally recommended control strategy, later named DOTS, was launched (WHO, 1994). MULTIDRUG-resistant tuberculosis (MDR-TB) is defined as a form of tuberculosis (TB) due to *Mycobacterium tuberculosis* that is resistant to at least isoniazid and rifampicin, the two most powerful anti-TB drugs. This form of TB was documented in nearly every country surveyed by the World Health Organization (WHO)/International Union Against Tuberculosis and Lung Disease (IUATLD) Global Drug Resistance Surveillance Project during the period 1994-2000. Some settings, such as those in the former Soviet Union, show a high proportion of MDR-TB cases among new TB cases (Espinal *et al*., 2001). Other settings show a lower proportion, but have a considerable MDR-TB burden in terms

of total number of patients due to the size of the population and the magnitude of TB as a whole (Dye *et al.*, 2002). The diversity in the epidemiology of MDR-TB poses a challenge for its management in various settings. Drug resistance in bacteria is a natural phenomenon, but selective pressure induced by man-made mechanisms is the primary cause of MDR-TB. Drug resistance in TB (to isoniazid, para aminosalicylic acid [PAS], streptomycin and capreomycin) was documented shortly after the advent of these chemotherapeutic agents and principles to manage such patients were proposed accordingly (Styblo *et al.*, 1968). 4-6 With the implementation of the internationally accepted DOTS† strategy for TB control and its essential component of standardized Short-Course Chemotherapy (SCC), a comprehensive control strategy is available that, when followed properly, prevents the emergence of drug resistance. All currently recommended regimens are based upon the first-line drugs isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin.

After the widespread use of rifampicin for more than two decades, multidrug-resistant tuberculosis (MDR-TB; resistance to rifampicin and ionized) was recognized as a clinical problem in the early 1990s. Two decades later, MDR-TB is now prevalent over 80 countries worldwide. Thus, the WHO estimates that of about 10 million total episodes of tuberculosis in 2007, approximately 5% had MDR-TB3 and of these, 40 000 (6.6%) are estimated to have had extensively drug-resistant (XDR-TB, defined as *Mycobacterium tuberculosis* resistant to isoniazid, rifampicin, any fluoroquinolone and at least one of three injectable second-line drugs) (WHO, 2006).

Drug resistance-history: In 2008, an estimated 390 000-510 000 cases of MDRTB emerged globally (best estimate, 440 000 cases). Among all incident TB cases globally, 3.6% (95% confidence interval (CI): 3.0-4.4) are estimated to have MDR-TB. These estimates, which lie in the same range as the previous ones, are based on more data and a revised methodology. Almost 50% of MDR-TB cases worldwide are estimated to occur in China and India. In 2008, MDR-TB caused an estimated 150 000 deaths. 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 . 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. 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 multidrug- resistant TB (MDR-TB) (Edlin *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). Raja *et al.* (2009) reported that the new antibiotics active against drug resistant bacteria are required. Bacteria have lived on earth for several billion years and encountered by wide range of naturally available drugs. To survive in these environment microbes develops drug resistant mechanism (Hoskeri *et al.*, 2010).

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 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 resistance: Of 114 countries that provided information between 1994 and 2009 on resistance to first-line anti-TB drugs, 109 countries reported data on resistance occurring among new TB cases. Of these 109 countries, 102 also provided data among previously treated cases. Five countries (Australia, the Democratic Republic of the Congo, Fiji, Qatar and the Solomon Islands) did not report drug resistance data disaggregated by treatment history (i.e., for new and previously treated cases) but provided data for all TB cases combined. Countries reporting data on first-line drug resistance are distributed in the 6 WHO regions (Table 1). 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: INH or isonicotinic acid hydrazide, was synthesized in the early 1900s but its anti-TB action was first detected in 1951 (Slayden and Barry, 2000). 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. 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 *et al*., 1998). 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

Table 1: No. of countries reporting data on resistance to first-line anti-TB drugs, by WHO region

WHO region (No. of countries)	No. of countries reporting first-line anti-TB drug resistance (%)
African (46)	22 (48)
Americas (35)	20 (57)
Eastern Mediterranean (21)	8 (38)
European (53)	44 (83)
South-East Asia (11)	6 (55)
Western Pacific (27)	14 (52)
Total (193)	114 (59)

(Zhang *et al.*, 1993). 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). 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) (Basso and Blanchard, 1998).

Rifampicin: RIF was fist introduced in 1972 as an anti-TB drug and has excellent sterilizing activity. 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. 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 (Herrera *et al.*, 2003).

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. 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 and Zhang (1996) showed that *pncA* mutations conferred PZA resistance.

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.* (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). These five mutations are associated with 70-90% of all EMB resistant isolates. 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. STR interacts with the 16S rRNA and S12 ribosomal protein (*rrs* and *rpsL*) (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).

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) (WHO, 2001). Unfortunately, second-line drugs are inherently more toxic and less effective than first-line drugs (WHO, 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 phenotypic methods to detect resistance to second line drugs are less well established and the molecular mechanisms of resistance are also less defined.

Role of multidrug transporters: Multidrug transporters comprise four families of transmembrane efflux proteins that actively pump out a broad range of structurally unrelated compounds from the interior of the cell, using either proton motive force or ATP supplied energy. These proteins are expressed by all organisms ranging from prokaryotes to higher eukaryotes, including human cells. They mediate both intrinsic and acquired resistance to various drugs of a multitude of organisms such as *Pseudomonas* sp., *Candida* sp., *Plasmodium* sp. and cancer cells74. P-glycoprotein is a human analogue of these multidrug transporters and is expressed on immune effector cells75. It has been observed that infection of experimental cell lines by *M. tuberculosis* results in increased expression of P-glycoprotein and decreased accumulation of isoniazid inside the cells76. Apart from the up regulation of host cell P-glycoprotein, *M. tuberculosis per se* expresses at least three multidrug transporter proteins Tap, Lfr *A* and Mmr 77-79. The potential contribution

of these multidrug transporter proteins in the causation of MDR-TB merits further evaluation. These transmembrane efflux proteins also appear to be novel targets for drug therapy in future.

Guidelines for the management of patients with MDR-TB: When MDR-TB is suspected on the basis of history or epidemiological information, the patient's sputum must be subjected to culture and anti tuberculosis drug sensitivity testing and the WHO re-treatment regimen15 or the empirical regimens employing second-line reserve drugs suggested by the American Thoracic Society, Centers for Disease Control and Prevention and the Infectious Diseases Society of America (ATS/CDC/ IDSA). When susceptibility testing reports are available and there is resistance to isoniazid and rifampicin (with or without resistance to streptomycin) during the initial phase, a combination of ethionamide, fluoroquinolone, another bacteriostatic drug such as ethambutol, pyrazinamide and aminoglycoside (kanamycin, amikacin, or capreomycin) are used for three months or until sputum conversion. During the continuation phase, ethionamide, fluoroquinolone, another bacteriostatic drug (ethambutol) should be used for at least 18 months after smear conversion. If there is resistance to isoniazid, rifampicin and ethambutol (with or without resistance to streptomycin) during the initial phase, a combination of ethionamide, fluoroquinolone and another bacteriostatic drug such as cycloserine or PAS, pyrazinamide and aminoglycoside (kanamycin, amikacin, or capreomycin) are used for three months or until sputum conversion. During the continuation phase, ethionamide, ofloxacin, another ba teriostatic drug (cycloserine or PAS) should be used for at least 18 months after smear conversion.

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