Genetics and pulmonary medicine • 5

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Genetics of drug resistant tuberculosis

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The emergence of multidrug-resistant tuberculosis (MDR-TB), generally defined as resistance to at least isoniazid and rifampicin, has generated concern for the future of tuberculosis control.[1] The global magnitude of the problem is not well known. Most of the available studies are non-representative surveys of a population or a country, frequently failing to discriminate between primary and acquired resistance. However, emerging data (fig 1) suggest that, while multidrug resistance may not be a widespread problem, it remains a public health threat in areas with a high prevalence of tuberculosis and suboptimal tuberculosis control programmes.[2] Progress in understanding of the basis of drug action and resistance is the key to development of diagnostic strategies, novel drugs and treatment programmes, and to gaining insight into the pathogenicity of drug resistant strains.

Mechanisms of resistance and drug targets in tuberculosis

Bacteria use a number of strategies to achieve drug resistance. These can be roughly summarised into three categories: (1) barrier mechanisms (decreased permeability and efflux pumps); (2) degrading or inactivating enzymes—for example, β-lactamases; and (3) drug target modifications—for example, single mutation in a key gene. The genetic information for such properties may be acquired via exogenous mobile genetic elements such as plasmids or transposons, or it may reside in the chromosome.

Mycobacteria are not basically different from many other bacteria in that they use several of these strategies. Firstly, mycobacteria are characterised by a specialised cell wall which displays significantly reduced permeability to many compounds.[3] Secondly, mycobacteria produce degrading enzymes such as β-lactamases[4] and other drug-modifying enzymes. These are among the factors cited to explain the natural resistance of many mycobacterial species to frequently used antibacterial agents.

Resistance to agents used for the treatment of tuberculosis generally depends on the third general mechanism of resistance described—that is, modification by mutation of key target genes. Thus, acquisition of resistance in *Mycobacterium tuberculosis* derives from chromosomal mutational events. MDR-TB reflects the stepwise accumulation of individual mutations in several independent genes[5] and not the "block" acquisition of multidrug resistance.

A considerable amount of work has been devoted in the last few years to understanding mechanisms of resistance and to identifying the genes involved. The use of molecular data is already helping the development of novel ways of detecting MDR-TB earlier.[6 7] A summary of our current knowledge is presented in table 1.

RESISTANCE TO ISONIAZID

There is now a large body of information, both genetic and biochemical, on the multistep process involved in the activation of isoniazid prodrug into a potent derivative, and its final action on the mycolic acid biosynthesis.[8] Isoniazid is actively taken up by *M tuberculosis* and is oxidised by the mycobacterial catalase-peroxidase. Absence of catalase activity has long been recognised as a marker for isoniazid resistance and it has now been shown to result from mutation of this enzyme.[9 10] This phenomenon is observed in approximately 50% of clinical strains.

In the presence of an intact catalase-peroxidase an active intermediate is generated which will inhibit the activity of an enzyme involved in the synthesis of mycolic acids: the enoyl-ACP reductase, encoded by *inhA*.[8 11] Mutations in the *inhA* region appear to be responsible for resistance in approximately 25% of clinical isolates and are generally associated with low level isoniazid resistance (MIC ≤1 mg/ml) (table 1).[5 11 12] Most mutations result in upregulation of the *inhA* gene expression and thus in increased amounts of the corresponding enzyme which overwhelms the inhibitory action of the drug. Rarely, mutations have occurred at the site of interaction with the activated form of isoniazid.[8 11 13] Availability of the three-dimensional structure of the enoyl-ACP reductase has allowed a detailed analysis of the interaction of the enzyme with isoniazid, thus setting the basis for future rational drug design strategies.[13 14]

After the identification of the *katG* and *inhA* genes it was apparent that 10–20% of isoniazid resistant isolates lacked mutations in either gene. Search for additional genes led to the identification of the *ahpC* gene which encodes the alkyl hydroperoxide reductase.[12 15 16] Mutations in *ahpC*, identified in approximately 10–15% of clinical isolates,[12 17] may not have a causal role in resistance, and rather serve to identify major lesions in *katG*.[12 15] Unknown

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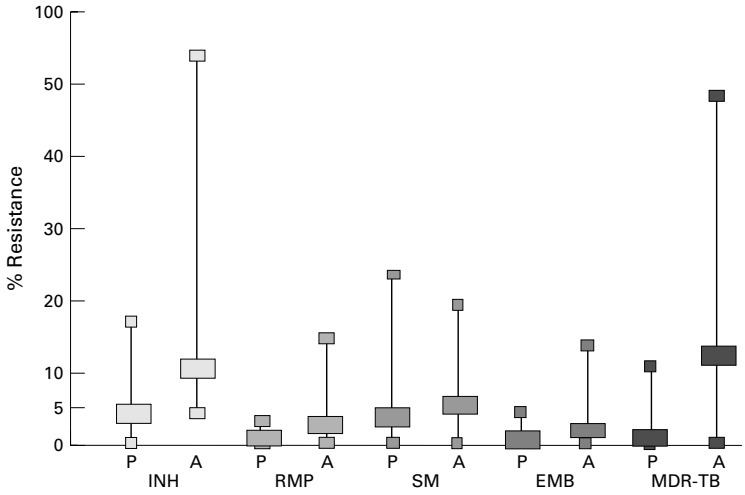

Figure 1 Global rates of drug-resistant tuberculosis. Median, upper and lower range values reported in various studies are shown. For each drug primary (P) and acquired (A) resistance values are presented separately. WHO-IUATLD Global Surveillance Program 1985–1994.[1]

mechanisms may account for ⩽10% of clinical resistance, and several genes are being investigated as potentially relevant to the action and resistance to isoniazid: *kasA* (ketoacid synthase), *ceoA* (UDP galactopyranose reductase), and the mycobacterial NADH and malate dehydrogenases.[18–20]

RESISTANCE TO RIFAMPICIN

Rifampicin is a broad spectrum antimicrobial agent which acts by interfering with the synthesis of mRNA by binding to the RNA polymerase. Different bacteria—for example, *Escherichia coli*, *Staphylococcus aureus*, *Neisseria meningitidis*—achieve resistance to rifampicin using a shared strategy: mutation in a defined region of the RNA polymerase subunit β. Mycobacteria are no exception and mutations have been found in the *rpoB* of >97% of resistant clinical isolates of *M tuberculosis* and *M ieprae*.[21 22] Although *rpoB* mutations have been described in rifampicin resistant *M avium*,[23] many isolates from the *M avium* and *M intracellulare* group present a significant level of natural resistance to rifampicin as a result of an efficient permeability and exclusion barrier.[24] Ribosylation, a degradative mechanism of resistance to rifampicin, has been described in rapidly growing mycobacteria.[25 26]

RESISTANCE TO STREPTOMYCIN

The most frequent mechanism of resistance to aminoglycosides in clinically relevant bacteria is the acquisition of aminoglycoside-modifying enzymes via plasmids or transposons. However, as discussed earlier, exogenous acquisition of resistance determinants has not been described in the tubercle bacillus. Rather, *M tuberculosis* becomes resistant by mutating the target of streptomycin in the ribosomes. The principal site of mutation is the *rpsL* gene, encoding the ribosomal protein S12.[27 28] The loops of 16S rRNA that interact with the S12 protein constitute a secondary mutation site. Mutations in those structures are identified in 50% and 20% of clinically resistant isolates, respectively. A third mechanism accounting for low level resistance remains unidentified.[29]

RESISTANCE TO ETHAMBUTOL

Ethambutol specifically inhibits biosynthesis of the mycobacterial cell wall. Resistance to ethambutol is associated with changes in a defined genomic region, the *embCAB*,[30] which encodes arabinosyltransferases involved in the synthesis of unique mycobacterial cell wall components arabinogalactan and lipoarabinomannan.[31] Resistance results from an accumulation of genetic events determining overexpression of the Emb proteins and structural mutation in EmbB.[30] Mutations, identified in up to 65% of clinical isolates of *M tuberculosis*,[30 32] are associated with high level resistance. Lower levels of resistance (<10 mg/ml) are the most frequent finding for the 35% resistant isolates not presenting with EmbB mutations.[33] Natural susceptibility or resistance to ethambutol among non-tuberculous mycobacteria is also determined by the Emb region.[33]

Table 1 Mechanisms of drug resistance in Mycobacterium tuberculosis

Antimycobacterial agent	*Mechanism of action*	*Genes involved in resistance*	*Frequency of mutations associated with resistance*	*Mechanism of resistance*
Isoniazid	Inhibition of mycolic acid biosynthesis	(i) *katG* (catalase-peroxidase)	(i) 42–58%	(i) Mutations in *katG* result in failure to generate an active intermediate of isoniazid
		(ii) *inhA* (enoyl-acyl carrier protein reductase)	(ii) 21–34%	(ii) Over expression of *inhA* allows continuation of mycolic acid synthesis
		(iii) *ahpC* (alkyl hydroperoxide reductase)	(iii) 10–15%	(iii) *ahpC* mutations may just serve as a marker for lesions in *katG*
Rifampicin	Inhibition of transcription	*rpoB* (β subunit of RNA polymerase)	96–98%	Mutations in *rpoB* prevent interaction with rifampicin
Streptomycin	Inhibition of protein synthesis	(i) *rpsL* (ribosomal protein S12)	(i) 52–59%	Mutations prevent interaction with streptomycin. Resistance not associated with mutation in *rpsL* or *rrs* is usually low level
		(ii) *rrs* (16S rRNA)	(ii) 8–21%	
Ethambutol	Inhibition of arabinogalactan and lipoarabinomannan biosynthesis	*embcAB* (arabinosyl transferase)	47–65%	Over expression or mutation of EmbB allow continuation of arabinan biosynthesis. Resistance not associated with EmbB mutation is usually low level
Pyrazinamide	Unknown	*pncA* (pyrazinamidase-nicotinamidase)	72–97%	Loss of pyrazinamidase activity results in decreased conversion of pyrazinamide to pyrazinoic acid, the putative active moiety
Fluoroquinolones	Inhibition of the DNA gyrase	*gyrA* (DNA gyrase subunit A)	75–94%	Mutations in *gyrA* prevent interaction with fluoroquinolones Mutations in *gyrB* and efflux may contribute to resistance

Table 2 Alternative and potential compounds for multidrug-resistant tuberculosis

Drug	*Comments*
Established 2nd and 3rd line drugs	
Fluoroquinolones	The fluoroquinolones are the most active drugs of this category
Ethionamide	
PAS	
Clofazimine	
Kanamycin, amikacin	
D-Cycloserine	
Thiazetazone	
Capreomycin	
Anecdotal effectiveness	
Imipenem	There is initial in vitro, experimental and clinical data to support a role of β-lactam antibiotics in the management of MDR-TB.
Amoxicillin-clavulanate	Possible synergistic value in combination treatment
Clarithromycin	
Potential use under specific circumstances	
Isoniazid	Low level resistance (≤1 μg/ml), *inhA* mutation?
Ethambutol	Low level resistance (≤1 μg/ml), absence of EmbB mutations. May be useful irrespective of MIC
Rifabutin or KRM1648	Useful in the presence of particular *rpoB* mutations conferring rifampin resistance
Interferon γ	Some rationale to support its exceptional utilisation
Under development	
5-chloropyrazinoic esters of PZA	Active against pyrazinamide resistant strains
PA824	Nitroimidazolpyran analogue related to metronidazole
Oxazolidinones	New drug group with antituberculous activity
Thiolactomycins	New drug group with antituberculous activity

RESISTANCE TO PYRAZINAMIDE

There is a good understanding of the basis of resistance in *M tuberculosis* (acquired) and *M bovis* (constitutive) by disruption of the enzyme pyrazinamidase/nicotinamidase.[34–36] Susceptible strains of *M tuberculosis* produce the enzyme pyrazinamidase which converts pyrazinamide to pyrazinoic acid, the putatively active moiety. It is thought that the action of pyrazinoic acid is the combined effect of its specific activity and the ability to lower the pH below the limits of tolerance of the target organism. However, while the basis of resistance in most strains is clear, the exact mechanism of action of the drug has not been firmly established.

RESISTANCE TO FLUOROQUINOLONES

The recent outbreaks of MDR-TB brought the fluoroquinolones to prominence as second line antituberculosis agents.[37] Unavoidably, their use in the management of patients with MDR-TB, and perhaps the frequent utilisation the fluoroquinolones in the community as general antibacterial agents, is generating a pool of fluoroquinolone-resistant *M tuberculosis* strains.

The molecular basis of resistance to fluoroquinolones is a complex multistep process. Research in other bacteria[38] have conclusively shown the presence of resistance mutations in (1) the DNA gyrase (composed of subunits GyrA and GyrB), (2) the topoisomerase IV, and (3) cell membrane proteins that regulate the intracellular concentration of the drug by mediating drug permeability and efflux. Stepwise accumulation of mutations in several of these genes is necessary to achieve high levels of resistance. Experience with *M tuberculosis* indicates a similar pattern of resistance development: a multistep process where the presence of *gyrA* mutations predicts clinically significant levels of resistance to ciprofloxacin[39] and cross resistance to other fluoroquinolones such as ofloxacin.[40] The recent characterisation of a mycobacterial efflux pump, the *IfrA* gene (which confers low level quinolone resistance)[41] and of *gyrB* mutations[42] contribute to a more complete understanding of the mechanisms of resistance to fluoroquinolones in mycobacteria.

Resistance and bacterial fitness

The likelihood of a normal host developing disease following exposure to MDR-TB has not been well defined. Indeed, none of the more than 100 outbreaks of tuberculosis reported by 1965 had been caused by a drug resistant strain.[43] The first community outbreak caused by MDR-TB was reported in 1981—prior to the AIDS epidemic—and involved a catalase positive isoniazid resistant strain.[44] Molecular analysis of the epidemiology of tuberculosis in Holland indicates an underrepresentation of drug resistant strains in transmission clusters,[45] suggesting limited pathogenicity for those organisms. Today, outbreaks of MDR-TB occur mainly among HIV infected individuals. This phenomenon probably indicates a summation of facts: (1) particular epidemiological niches favouring transmission, (2) compliance and drug absorption issues determining inadequate drug levels, (3) rapid progression of disease which facilitates observation of clustering, and (4) the exquisite susceptibility of the host to opportunistic or low virulence organisms.

A recent study using mice could not demonstrate a consistent loss of virulence of MDR-TB, but rather described a wide range of virulence for these strains. Unfortunately, the isolates studied were genetically uncharacterised; no information was available on the identity and location of the resistance mutations.[46] In contrast, in a study with well characterised isogenic (originating from the same parenteral strain) isolates of *M bovis*, loss of virulence for mice was associated with a loss of catalase activity but not with mutations in the *inhA*, which also confers resistance to isoniazid.[47]

Thus, available data would suggest that the virulence of MDR-TB is dependent on the resistance genotype of the strains and on the immune status of the host. This may explain the protracted evolution in a proportion of

non-immunosuppressed HIV negative patients infected with MDR-TB, and the acceptable response to second and third line treatment combinations in some cohorts of patients.[48 49] These considerations notwithstanding, specific MDR-TB strains such as strain "W" implicated in several nosocomial outbreaks in New York, do represent a real threat to health care workers and other HIV negative exposed individuals.[50]

Treatment of MDR-TB: lessons from observing the molecular basis of resistance

Analysis of the mechanisms of action and resistance of antituberculous drugs provides useful insights for managing patients with MDR-TB (table 2). Firstly, in agreement with the previous section, molecular characterisation may provide information on the potential for the virulence of a particular strain. Thus, an MDR-TB strain resistant to isoniazid by means of an *inhA* mutation (catalase positive) will probably represent a greater threat to the patient and to the exposed contacts and health personnel than an isoniazid resistant strain mutated in the catalase peroxidase. The strain with an *inhA* mutation may also present lower levels of resistance ($\leqslant 1$ μg/ml) which, in a situation of limited treatment options, may allow continuation of the use of isoniazid in the therapeutic regimen.

With regard to rifampicin, clinicians may take advantage of the association of particular *rpoB* mutations and retained susceptibility to rifabutin and the new rifamycin KRM1648.[51 52] This would be important in the management of epidemic strains carrying those specific mutations.

Ethambutol may prove to be of particular interest. It remains useful in the management of *M avium* infection despite suboptimal in vitro susceptibility results.[53] This is attributed to the fact that ethambutol disorganises the cell wall and thus increases susceptibility to other drugs. Indeed, resistant mutants that grow in the presence of ethambutol may display defects of the cell wall—that is, loss of the lipoarabinomannan.[54] A further factor which suggests the usefulness of ethambutol relates to the existence of low level resistant mutants resulting from overexpression of Emb proteins and displaying MIC values of <10 μg/ml. While this level of ethambutol may not be achievable in plasma, it may be reached intracellularly.[55 56] Thus, despite unfavourable susceptibility profiles, isoniazid and ethambutol induced cell wall damage may assist second and third line drugs exerting their effect. There is initial in vitro, experimental, and anecdotal clinical reports to support a role of β-lactam antibiotics such as imipenem or amoxicillin-clavulanate in the treatment of immunocompetent hosts with MDR-TB.[57] This may be a reflection of enhanced efficacy of such drugs when the dynamics of cell wall permeability and interaction with β-lactamases are modified. Other strategies for manipulation of permeability barriers using inhibitors of efflux pumps (reserpine, calcium channel blockers, cycloserine A, and other compounds) remain poorly investigated in bacteriology. A therapeutic role for compounds such as clarithromycin[58] and even immunomodulators such as interferon γ[59–61] has not been defined (table 2).

Finally, drug development is being streamlined by detailed analysis of the molecular targets. Novel lead compounds such as 5-chloropyrazinoic esters of pyrazinamide, active against pyrazinamide resistant strains, and PA824, a nitroimidazolpyran analogue related to metronidazole (both compounds from Pathogenesis Corporation, Seattle), oxazolidinones such as U-100480, U-100592 and U-100766 (from Upjohn Co, Kalamazoo),[62 63] and thiolactomycins[64] are being developed with deep understanding of their molecular mechanisms of action. The availability of the complete genome of *M tuberculosis* will also provide a formidable tool for future development of antituberculous agents.[65]

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