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WORLD HEALTH ORGANIZATION

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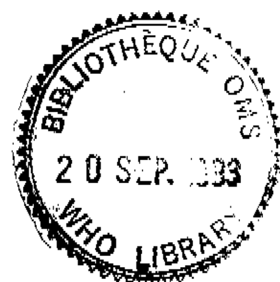
LABORATORY EVALUATION OF DRUG RESISTANT TUBERCULOSIS

Basil P. Vareldzis, MD, MPH[1]
Jacques Grosset, MD[2]
Isabel de Kantor, ScD[3]
John Crofton, MD[4]
Adalbert Laszlo, PhD[5]
Michael Felten, MD[1]
Mario C. Raviglione, MD[1]
Arata Kochi, MD, MPH[1]



Tuberculosis Programme, World Health Organization, Geneva, Switzerland[1]
Faculté de Médecine Pitié-Salpetriere[2]
Instituto Panamericano de Proteccion de Alimentos y Zoonosis, Buenos Aires, Argentina[3]
John Crofton, Professor Emeritus, University of Edinburgh, Scotland[4]
National Reference Centre for Tuberculosis, Ottawa, Ontario, Canada[5]

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INTRODUCTION

Reports of multi-drug resistant (MDR) tuberculosis (TB) arising from the United States have caused great concern owing to the very high case-fatality rate[1-6]. It is possible that poor patient compliance combined with inadequately funded TB control programmes in the age of AIDS may have lead to the emergence of increased numbers of resistant strains. If these spread increasingly in a community, TB may become progressively uncontrollable using currently available chemotherapy.

Drug resistance rates have decreased in developing countries over the period 1962 - 1985, while resistance is increasing now. The initial decrease in resistance was seen in well functioning National Tuberculosis Control Programmes (NTP), while the recently observed increase may be due either to understaffed, resource poor programmes or to the effect of the HIV epidemic or to both. The HIV epidemic may overwhelm the NTP resulting in decreased programme efficiency and increased drug resistance. Resistance surveillance appears to be a good measure of programme efficiency.

For research purposes, primary drug resistance surveys should be done on a sample of relevant patients which includes and distinguishes between HIV-positive and HIV-negative patients. At this time, there is not enough information to warrant a recommendation regarding HIV testing of TB patients for surveillance purposes.

Currently, therapy for tuberculosis involves multi-drug chemotherapy for a period of several months. The most effective treatment regimens include isoniazid (H) and rifampicin (R), the two most effective drugs available in the anti-tuberculosis armamentarium, at least for part if not all of the treatment regime [7, 8]. Other potent drugs in common use include pyrazinamide (Z), streptomycin (S), and ethambutol (E).

In order to better evaluate the extent of the problem and its implications from the laboratory perspective a review of the current literature on drug resistant tuberculosis was undertaken. This review, intended for the research community, will focus on laboratory measurement of resistance to H and R, as both these drugs are of greatest clinical consequence. Resistance measurements for other first line drugs will be mentioned when they relate to H and R. Standard definitions of terms and techniques will be proposed in order to encourage the scientific community to publish results that are comparable internationally.

DEFINITIONS

Consensus on definitions related to drug resistant TB does not exist, and, as a result, some confusion has been generated in the literature. For the purposes of this report, the following definitions will be used [8 - 10]:

Anti-tuberculosis chemotherapy: refers to treatment based on the most used drugs like H, R, S, Z, E and thiacetazone (Tb1). These six drugs are the most widely used anti-tuberculosis chemotherapeutic agents available at this time. The treatment regimen recommended by the World Health Organization (WHO) is referred to as short course chemotherapy (SCC) and consists of 2 months of H, R and Z plus a fourth drug (usually S or E) followed by four months of dual drug therapy with H and R, or alternatively followed by 6 months of H and E or Tb1 [7]. Newer drugs which are not yet in widespread use, such as the quinolones or the long acting rifamycins will not be discussed as there are not enough published data on their use in TB treatment at this time.

Wild strain is defined as a strain of M. tuberculosis complex which has never been exposed to any antibacterial drug.

Naturally resistant strains, that should be clearly distinguished from **wild type resistant mutants**, are wild strains resistant to a particular drug without ever having been in contact with it. Thus, neither the patient with the naturally resistant bacilli nor his source of infection has had chemotherapy in the past. Examples of this type of resistance include M. bovis resistance to Z [11] and M. africanum resistance to Tbl. Natural resistance is a species specific resistance which can be used as a taxonomic marker, useful in species identification.

Meanwhile, wild type resistant mutants occur as a result of genetic mutations that precede contact with the drug. These spontaneous mutants are found in wild M.tuberculosis strains with frequencies which vary for different drugs: they are in the order of 10^{-6} for H, and 10^{-8} for R.

Initial resistance is defined as the presence of drug resistance to one or more anti-tuberculosis drugs in a new tuberculosis patient who presents to a treatment centre. This category includes those patients with **primary resistance** as well as those patients with undisclosed **acquired resistance** who either do not remember prior treatment, refuse to divulge the information on past treatment, or were not appropriately asked about the treatment history. Initial resistance does not include chronic patients. It can be stated, from a programmatic point of view, that initial resistance is the result of national tuberculosis control programme (NTP) that were inefficient in the past [8]. The frequency of primary drug resistance to one or more drugs is estimated to be about 5% in technically advanced countries while it is substantially higher (15% or even higher) in some developing countries [8, 12].

Acquired resistance is defined as resistance to one or more anti-tuberculosis drugs which arises during the course of treatment, usually as a result of non-adherence to the recommended regimen or faulty prescribing. Emergence of acquired resistance in a patient receiving chemotherapy is serious and one of the frequent reasons for treatment failure [9]. From the programmatic point of view, a high level of this type of resistance is a mark of a poorly functioning NTP at the current time [8].

Chronic patient is defined as a patient who has failed a long, often irregular, course or courses of therapy, and who is now likely to present with acquired resistance to one or more anti-tuberculosis drugs. Alternatively, this is defined as a patient who remains smear-positive after completing a re-treatment regimen under supervision.

Multi-drug resistance refers to resistance to at least both H and R. This type of resistance, which is man-made, can occur both as primary and secondary resistance and is often associated with HIV infection as well as with chronic patients. Multi-drug resistance in the setting of HIV infection can have very serious consequences often resulting in death within a very brief period of time.

MECHANISMS OF RESISTANCE

Resistant mutants to any single antibiotic occur readily at random in bacilli undergoing replication and may be selected for by not using the adequate combination of drugs. In the tubercle bacilli, the sites of resistance to TB drugs are chromosomally located, and are not plasmid borne. Thus, the likelihood of occurrence of a mutant resistant simultaneously to two drugs is the product of individual probabilities [13].

Drug resistant mutants exist at different frequencies for given drugs and have a selective advantage in the setting of monotherapy as they are able to survive therapy while the drug susceptible bacilli are killed. When two drugs are included in the treatment, mutants resistant to one drug are killed by the other drug.

In a population of 1 million bacilli, one may expect to find 10 to 50 mutants capable of surviving in the presence of 0.05 mg/l of H [9]. If the population is only 100, on the other hand, then the probability of finding one resistant organism to the same concentration of H is near zero. Thus, from a laboratory methodology point of view, one must specify not only the drug concentration, but also the inoculum size, if the outcome (*i.e.*, >20 resistant colonies) is to be quantified.

The probability of resistance to multiple drug is multiplicative. If the mutation rate is 10^{-6} per reproductive event per bacillus, then the probability of developing resistance simultaneously to three drugs assuming that they act independently is the product of the three independent probabilities, namely 10^{-18} per reproductive event per bacillus. A tuberculous cavity will normally harbour 10 million to 1 billion (10^7 to 10^9) bacilli. Thus, it is theoretically improbable that double or triple spontaneously occurring resistant mutants can be found in most patients [9].

The resistant mutants do not have a selective advantage unless they are exposed to a drug to which they are already resistant while the mass of bacilli is susceptible. In such an instance, the sensitive bacilli are killed while the resistant organisms continue to grow and eventually become the dominant forms of the infecting organism found in that particular patient. Future therapy with the drug in question will be futile as the bacilli will remain resistant. If the patient is then exposed to a second course of drug therapy with yet another drug, mutants resistant to the novel drug may be selected for again, and the patient may end up with bacilli resistant to two and eventually more drugs as future courses of chemotherapy are used. This scenario, referred to as serial selection of drug resistance, is the predominant mechanism accounting for multidrug resistance to 2 or more drugs. The patients harbouring multi-resistant organisms constitute a pool of chronic drug resistant infectious cases, propagating multidrug primary resistance.

Such an event is illustrated by the MDR-TB outbreaks among HIV seropositive individuals and contacts that have been seen in some large cities in the USA recently and in a percentage of chronic patients in several developing countries (G. Cauthen, pers. comm., 1992) [14].

MEASUREMENT OF RESISTANCE IN THE LABORATORY

From a purely laboratory and bacteriological perspective, resistance can be defined as a "decrease in sensitivity of sufficient degree to be reasonably certain that the strain concerned is different from a sample of wild strains of human type that have never come into contact with the drug" [18, 19]. There is evidence that a diminished clinical response or even a clinical deterioration under therapy may occur when resistance in the above-mentioned bacteriological sense is demonstrated in the laboratory [19].

Drug sensitivity testing has four main uses: 1) scientific; 2) epidemiologic; 3) planning wide-scale treatment; and 4) providing clinically useful information for the management of the individual patient [19].

This paper is primarily concerned with items two and three in the above mentioned list. The reader is referred to excellent reviews by Canetti *et al.* for further discussion of the these issues [19, 20].

Technical problems exist in determining drug resistance because of a lack of standardization of methods for drug susceptibility testing. Culture media and conditions should be standardized so that data collected by different laboratories can be compared. Finally, details about the drug susceptibility procedure used by different laboratories are often omitted from reports and there is a tendency to stray from standardized, time tested methodology.

The sensitivity and specificity of the drug sensitivity testing must be set at such a level as to be able to detect clinically significant resistance without falsely labelling susceptible strains as resistant, or the reverse [19, 21].

Another major issue revolves around the reliability of measurement techniques which are currently used to measure drug resistance. H and R resistance can be measured reliably and consistently within the same laboratory. On the other hand, resistance to Z, E and S is more difficult to measure in a consistently reliable manner at this time due to limitations of the techniques (G. Cauthen, pers. comm., 1992) [19].

There are three widely used methods for drug sensitivity testing on Lowenstein-Jensen medium: 1) the absolute-concentration method [also referred to as minimal inhibitory concentration (MIC) method]; 2) the resistance-ratio method; and 3) the proportion method as described by Canetti *et al.* in 1963 [19]. There are other methods as well: agar-based semi-synthetic media and liquid media, and vertical diffusion methods. A variant of the proportion method, the BACTEC radioactive liquid medium, is the fastest way to measure resistance, yielding results within less than 10 days after isolation of the organism; but it is also the most resource intensive and is not yet routinely available in the developing world.

<u>The absolute concentration method (MIC):</u> For this sensitivity test, a 2 milligram inoculum is taken from a Lowenstein - Jensen culture within 2 weeks of the culture slopes initially becoming positive. The inoculum is then discharged into 0.4 ml of sterile distilled water in a 7 ml screw capped bottle. This is then suspended and a loopful of the suspension is spread on the surface of each slope of the sensitivity test. Slopes are incubated at 37 degrees Celsius and a preliminary reading is made at 2 weeks. A final reading is made at 4 weeks.

Growth is defined as the presence of 20 or more colonies at the end of 4 weeks. This test uses absolute concentrations of a given drug, for example 0.2, 1.0, 2.0, 5.0 and 50 mg / l of H. Growth of 20 or more colonies on the drug containing medium is the only definition of resistance using this technique, making it especially sensitive to variations in inoculum size.

<u>The resistance - ratio method</u>: This technique was developed as a way to control for the variability in growing conditions by adding a reference control strain. In this technique, similar to the absolute-concentration method, a 2 milligram inoculum is taken from a Lowenstein - Jensen culture within 2 weeks of the culture slopes initially becoming positive. The inoculum is then discharged into 0.4 ml of sterile distilled water in a 7 ml screw capped bottle. It is then suspended and a loopful of the suspension is spread on the surface of each slope of the sensitivity test. A drug free slope is then set up for each strain to be tested as a control. The standard strain of <u>M. tuberculosis</u>, H37Rv, is tested in each set of tests. Slopes are

incubated at 37 degrees Celsius and a preliminary reading is made at 2 weeks. A final reading is made at 4 weeks.

Again, growth is defined as the presence of 20 or more colonies at the end of 4 weeks. The resistance ratio is defined as the minimal concentration inhibiting growth of the test strain divided by the minimal concentration inhibiting growth of the standard sensitive (control) strain in the same set of tests. A resistance ratio of 2 or less is defined as sensitive, while 8 or more is resistant. For H, concentrations of 0.2, 1.0, 5.0, and 50 mg/l are used. A resistance ratio of 2 or less is equivalent to the presence of less than 20 colonies growing on 0.2 mg / l of H in the absolute concentration method [19]. Resistance is defined as a resistance ratio of 8 or more or a resistance ratio of 4 or more followed by a resistance ratio of 4 or more upon repeat testing. Variations in inoculum size are difficult to prevent.

The proportion method: For this method, the bacilli are cultured on two media, one with and one without drug: the ratio of the number of colonies obtained on the drug-containing medium to the number of colonies obtained on the drug-free medium indicates the proportion of resistant bacilli. This allows for control of inoculum size. Below a certain proportion, the strain is sensitive, above it is said to be resistant. This technique uses the same growth medium, namely Lowenstein-Jensen. The results of the test are first read 4 weeks after inoculation; if the result at 4 weeks shows suceptibility, a second reading is made 6 weeks after inoculation.

For both H and R, 1% is the criterion for resistance using this technique. That is to say that if 1 out of every 100 bacilli being tested are able to grow above a certain concentration of drug in the growing medium the strain is said to be resistant. For H, this concentration is defined as 0.2 mg / l, and for R as 40 mg / l. For S, the resistance criterium adopted is usually 10%, because of the inherent instability of dihydrostreptomycin in the culture medium and the variation in growth characteristics observed in wild strains on S-containing Lowenstein Jensen medium which would cause a large numbers of falsely labeled resistant organisms [19].

Importance of standardization of inoculum size

Standardization of the size of the inoculum is of the greatest importance. There is a large difference from culture to culture in the number of colonies that grow from a similar inoculum specified by weight or opacity [20]. This can be attributed to differences in the number of organisms in the inoculum and, to some degree, to clumping of the bacilli. The basic requirement for reliability of testing techniques is information on the number of viable cells growing on drug-free versus the drug-containing medium. Wild strains of *M. tuberculosis* contain approximately 1 mutant per 10^6 viable cells growing on Lowenstein-Jensen medium with 0.2 mg of H / l [20]. If the criterion chosen for calling a strain H resistant is >20 colonies on 0.2 mg per l, and the inoculum consists of 100 million cells, a wild strain of normal susceptibility yielding 100 colonies onto 0.2 mg / l H-containing medium will be misinterpreted as resistant. Thus, it is very important to provide information on the actual number of cells growing on the drug-free and on the drug-containing slopes. Variability in the inoculum is the major cause of variability in results in the absolute concentration and the resistance ratio methods.

Methods to use to test each drug

For H and R, all the above mentioned methods are good at detecting resistance because there is a large *in vitro* difference between susceptible and resistant strains although the proportion method is recommended as the best (G. Cauthen, pers. comm., 1992) [12, 19, 20]. For Z, because of the poor growth of mycobacteria at low pH and the inactivation of Z above pH 5.5, the results of sensitivity testing are often unreliable even with the proportion method. Sensitivity testing to Z is therefore not recommended in routine practice. For E, the *in vitro* difference between sensitive and resistant strains is very small and thus the resistance ratio method is not good [19]. For a particular drug, levels of resistance attained are usually higher in acquired resistance cases than in primary resistance [19].

The therapeutic index for a given drug is the difference between the *in vitro* MIC and the drug levels obtained in blood. This index is high for H and R, giving a good safety margin in results, but is very low for certain second line drugs such as ethionamide, cycloserine, viomycin and PAS. This makes it more difficult to distinguish between sensitive and resistant strains and often leads to misinterpretation of results [20]. For this reason, resistance testing for the second line drugs is not routinely performed [20].

Choice of culture medium

Another important factor involves the culture medium [20]. Studies must use the same medium for susceptibility testing. For example, Lowenstein-Jensen medium may allow higher proportions of strains to grow on it than on Dubos-agar medium or Middlebrook-Cohn 7H10 agar, and the inhibitory concentrations of drugs vary in the different media.

Despite the occasional discrepancies, results obtained with the three described drug susceptibility procedures are roughly comparable. The same can be said for the proportion method performed on Middlebrook 7H10 or 7H11 agar and for the Bactec Method which is also based on the proportion method.

The need of an atmosphere with 5% CO_2 for incubating cultures in Middlebrook 7H10 agar, as well as the variation in quality of Lowenstein Jensen medium according to eggs used and temperature or time for its coagulation should all be taken into account.

While inhibitory concentrations may vary according to the medium used, the diagnosis of resistance or susceptibility testing should remain unchanged if the tests are properly calibrated and controlled. If modifications in technique are introduced or if critical drug concentrations and resistance proportions are arbitrarily changed, then the tests become non-comparable and highly misleading.

Choice of drugs to evaluate

The specific drug to which resistance has developed is extremely important. Resistance to H or R is a far more serious situation than resistance to only S or even H or one of the second line drugs because H and R are the two most potent anti-tuberculosis drugs available at this time. They are currently used in the vast majority of treatment regimens for tuberculosis accounting for over 99% of the bacillary killing within two

months of initiation of treatment [8, 15]. Loss of one or both of these two agents leaves one with drugs that are far less effective, with higher side effects, and which result in very high mortality rates especially in the HIV setting [8, 13, 15].

The commonly used tuberculosis chemotherapeutic drugs can be ranked into three groups based on the probability of development of drug resistance following exposure of M. tuberculosis to a given drug (Table 1). Resistance to H and R is a far more serious situation than resistance to only S or even to H plus one of the second line drugs. The drug with the lowest probability of resistance is R (10^{-8}). H along with S, E, kanamycin and sodium para aminosalicylate (PAS) all have similar probabilities of resistance (10^{-6}), while Tbl and the remaining agents have very high resistance rates of 1 per 1000 (especially in M. africanum) [16, 17]. Resistance appears to develop most readily to the agents that are least effective (Table 2).

Bactericidal activity measures the speed with which the bacilli are killed during the initial phase and is defined as the proportion of sputum cultures that are negative at 2 months after the start of treatment. The sterilizing activity measures the capacity to eliminate the last few bacilli and is defined as the proportion of relapses that occur after chemotherapy has been stopped. Rifampicin, H and E are the best bactericidal agents, while R is far the best sterilizing agent [15]. However, in clinical practice, resistance to R will occur just as readily unless it is combined with effective companion drugs to which the patient's bacilli are sensitive.

CONCLUSION

Although the magnitude of the resistance problem and the appearance of multidrug resistance [to both isoniazid (H) and rifampicin (R)] in association with HIV is cause for alarm in both resource rich or resource poor countries, drug resistance to anti-tuberculosis chemotherapy is not a new problem per se.

There exists a great need for a systematic survey of drug resistance data from adequate samples of representative populations reflecting the distribution of tuberculosis in the world. This could be obtained from a series of national surveys in countries having the highest TB burdens. Specific attention should be focused on resistance to H and R, using standardized laboratory techniques which specify inoculum size and are calibrated between laboratories.

It is imperative that standardized laboratory methods be used and reported so as to enable public health agencies and NTP to better evaluate the situation of drug resistance. Drug resistance surveys, consisting of collection and analysis of purely bacteriological data are of limited public health interest. They must be linked with clinical information in order to distinguish between initial and acquired resistance.

Given the impact that HIV is having on MDR-TB, research on primary or initial drug resistance should be done on a sample of newly diagnosed patients presenting for treatment which includes and distinguishes between HIV-positive and HIV-negative patients. Increasing reports of initial resistance from countries such as the United States, as seen in Figure 1, must be able to evaluate the effect of HIV, as it is becoming a major confounder due to the high number of dually infected people.

In order for resistance surveys to be relevant from the public health perspective, the proportion of patients presenting for treatment having previously received treatment must be known. The meaningful assessment of initial (or primary) drug resistance surveys should be the *number of people never having received prior TB treatment with resistant bacilli* divided by the *number of new patients presenting for treatment*.

From a programme evaluation perspective, one ultimately needs to know how many patients who start treatment will develop acquired resistance. To do this, we propose that acquired resistance be calculated as the *number of patients who started treatment with drug susceptible bacilli and have become resistant six months later*. This includes both patients who have completed therapy as well as those who have absconded or been lost to follow up, highlighting the responsibility of control programmes to ensure that all patients who begin treatment must be followed to completion, wherever feasible. The numerator should consist of *number of patients with drug resistant bacilli 6 months following the start of therapy* while the denominator would consist of the *number of patients who started treatment with drug sensitive bacilli*.

To assess the value of treatment in the community, on the other hand, overall resistance can be calculated with *the number of patients with drug resistant bacilli after 6 months of treatment* in the numerator while the denominator should consist of *those starting treatment with drug susceptible bacilli*.

The need to implement effective short course chemotherapy (SCC) throughout the world is becoming more urgent. WHO's Global targets of 85% cure rates and 70% case detection rates need to be adhered to the maximum extent possible.

ACKNOWLEDGEMENT

The authors would like to thank the following individuals who contributed to the development of this paper: Dr George Cauthen of the U.S. Centers for Disease Control and Prevention, and Mr Fumio Omori, Drs Fabio Luelmo, Paul Nunn, and Sergio Spinaci of the WHO Tuberculosis Programme.

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Low	Medium	High
rifampicin	*isoniazid* *streptomycin* *ethambutol* *kanamycin* *P.A.S.*	*thiacetazone* *ethionamide* *capreomycin* *viomycin* *cycloserine*

Source: Shimao (16)

Table 1: Probability of developing Mycobacterial resistance to commonly used drugs.

Grade	Prevention of Resistance	Early Bactericidal Activity	Sterilizing Activity
High	*isoniazid* *rifampicin*	*isoniazid*	*rifampicin* *pyrazinamide*
Medium	*ethambutol* *streptomycin*	*ethambutol* *rifampicin*	*isoniazid*
Low	*pyrazinamide* *thiacetazone*	*streptomycin* *pyrazinamide* *thiacetazone*	*streptomycin* *thiacetazone* *ethambutol*

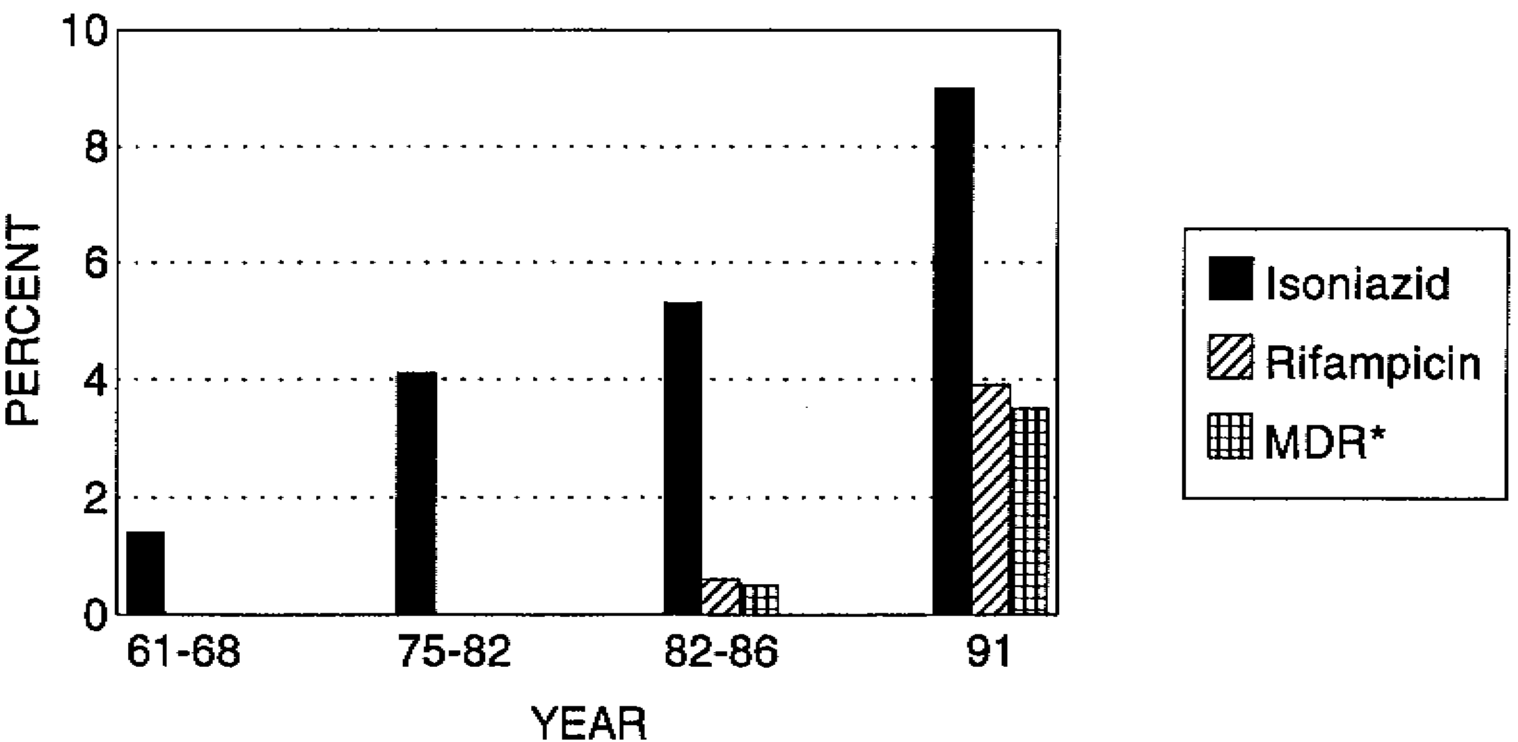
Source: Mitchison (15)

Table 2: Grading of effectiveness of anti-Tuberculosis drugs.

Figure 1: Percent Initial Resistance by Drug

United States

1961 - 1991



*resistance to both Isoniazid and Rifampicin
Sources: CDC: Toman (8); Snider et al (22); Bloch et al (23).
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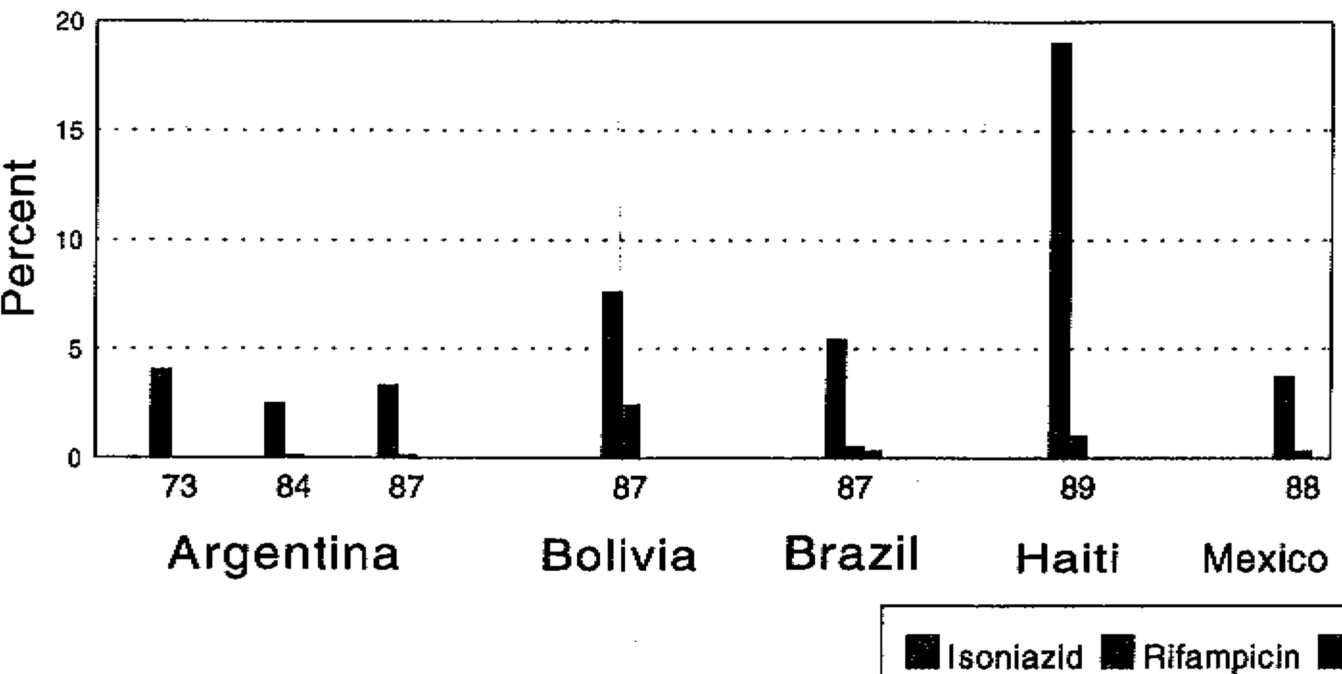
Percent Initial Drug Resistance

Isoniazid, Rifampicin and MDR
Americas



* MDR is resistance to both INH and RIF

Where ranges are provided midpoint of data is plotted.

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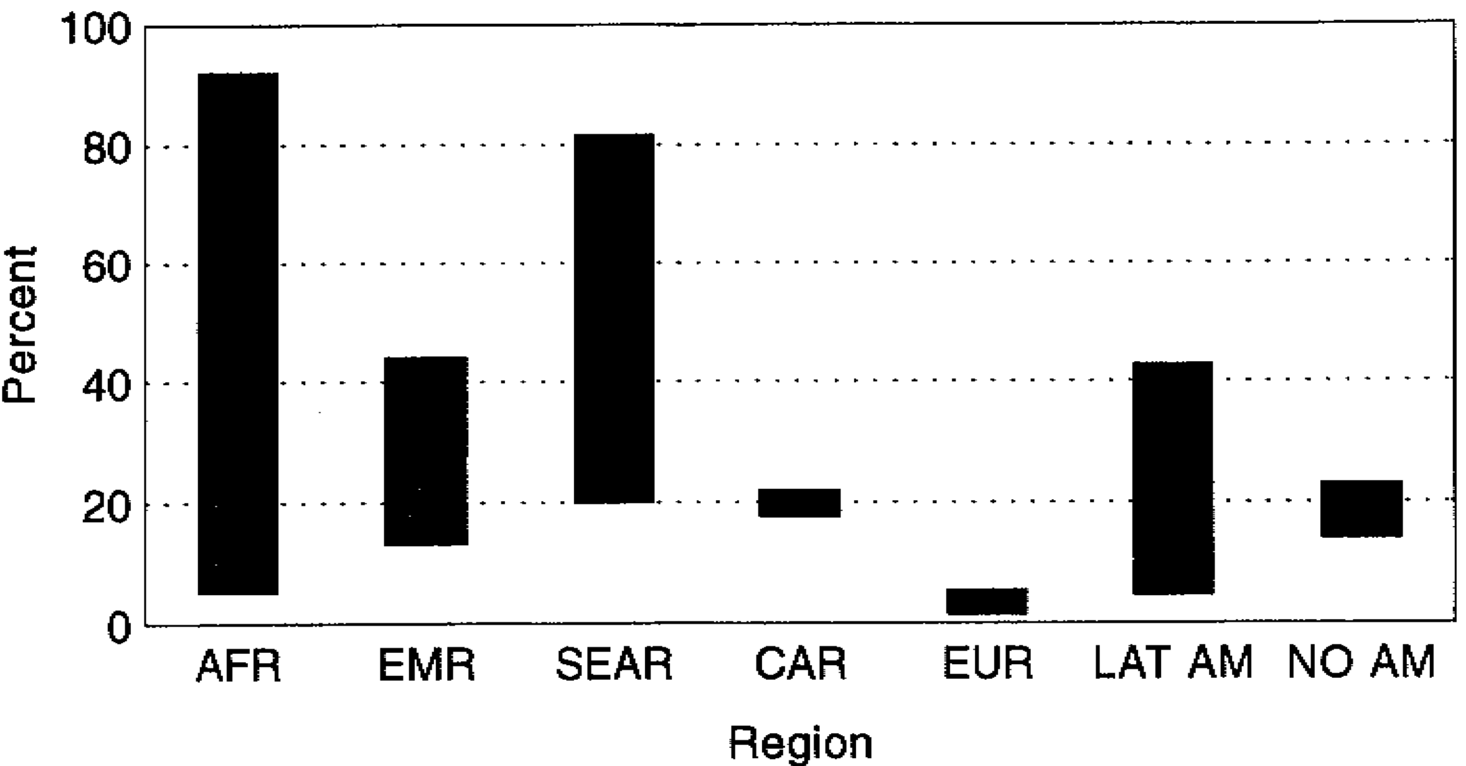
Range of Initial TB Drug Resistance Data

World Regions
1988 - 1992



Regions: AFR=Africa; EMR=Eastern Mediterranean; SEAR=South-East Asia; CAR=Caribean; EUR=Europe; LAT AM=Latin America; NO AM=North America

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