Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* (EUCAST document E.DEF 8.1) – Report of the Subcommittee on Antimicrobial Susceptibility Testing of *Mycobacterium tuberculosis* of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)

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ABSTRACT

This review describes the methods available for drug susceptibility testing of *Mycobacterium tuberculosis*. The methods have been developed over several decades and are restricted to specialised centres in most European countries, as they are technically demanding, require appropriate isolation facilities and can be difficult to interpret. The absolute concentration, resistance ratio and proportion methods can all give accurate results, provided that they are carefully quality-controlled and standardised. Automated rapid culture and molecular methods have been evaluated at large reference centres and in multicentre collaborations, and perform well for testing susceptibility to most first- and second-line anti-tuberculosis drugs. Accuracy is more important than rapid testing, and this is most reliably achieved if drug susceptibility tests are done in a small number of well-equipped, experienced laboratories that participate and perform well in an international drug susceptibility testing quality assessment scheme. The WHO Supranational Laboratory Quality Control Network offers a global scheme that assesses the ability of participating laboratories to identify isoniazid, rifampicin, ethambutol and streptomycin resistance. Second-line drug resistance testing is currently being standardised, and such testing should only be performed at the national reference laboratories in western and central European countries because of the relatively small number of cases and the concomitant difficulty of maintaining testing proficiency in multiple centres performing small numbers of tests. There is a need to expand international external quality assessment to include second-line drug susceptibility testing.

Keywords Drug resistance, EUCAST, *Mycobacterium tuberculosis*, review, susceptibility testing, tuberculosis

Accepted: 25 June 2007

Clin Microbiol Infect 2007; **13**: 1144–1156

INTRODUCTION

Tuberculosis (TB) is one of the commonest infectious diseases in the world today, with over 8 million new cases and 2 million deaths occurring annually [1–5]. More than 95% of cases occur in the developing world, and TB can cause *c.* 25% of all avoidable adult deaths from infection [6]. The steady decline in the number of cases of clinical TB in the developed world, as well as in parts of the developing world, ceased or reversed during the mid-1980s. Case rates rose in the USA, particularly in New York, and throughout much of western Europe [3,7–10]. TB notifications have continued to increase globally, particularly in Africa and Asia, but also in many eastern European countries of the former Soviet Union. In western Europe, these trends mainly reflect the

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migration of individuals from countries with a high incidence of TB and, in some settings, infection with human immunodeficiency virus (HIV). However, the increasing rates of TB in eastern Europe reflect problems in national control programmes as a consequence of rapid political transitions, non-standard treatment strategies, and socio-economic crises [2,3,5,11]. The highest estimated incidence and mortality rates occur in sub-Saharan Africa and southeast Asia. In contrast, mortality rates are generally <2/100 000 population in the industrialised parts of Europe, America and Australasia [1,2].

DRUG-RESISTANT TB

The real level of drug resistance in the world today is unknown, although national and regional studies and anecdotal evidence indicate that it has been increasing in recent years [12–19]. In particular, multiple drug-resistant TB (MDRTB) continues to be a serious problem, especially in developing countries in Asia [12], the Baltic region [20–22] and other parts of the former Soviet Union [23–28].

Analysis of drug susceptibility of *Mycobacterium tuberculosis* strains is essential: (i) for surveillance; (ii) to influence the development of treatment guidelines; (iii) to guide the clinical management of cases with proven resistance to first-line drugs and individuals with epidemiological risk-factors for drug resistance; (iv) to determine the type of hospital or the need for institutional isolation of patients; and (v) to determine the scope of institutional and community outbreak investigations required.

Landmark trials conducted by the UK Medical Research Council and others have established the therapeutic efficacy of current multidrug regimens, taken for periods of 6–12 months, depending on the location of disease. Cure rates in excess of 95% are possible, but the effectiveness of these standardised regimens is compromised when drugs are taken inappropriately because of poor patient compliance [29–34]. There have been few truly novel antimicrobial agents identified in recent years [35]. Morbidity and mortality rates are higher for drug-resistant cases, particularly if the organisms involved are multiple drug-resistant (i.e., resistant to isoniazid or rifampicin) [36] and there is co-infection with HIV [37–41]. Recent studies have demonstrated that survival can be improved by the rapid initiation of treatment with drugs to which the organism is susceptible [41–48].

The emergence of MDRTB has re-focused attention on TB as a disease of continuing significance in the developed and developing world. Unfortunately, several methodological problems have prevented the development of a clear global picture, including the absence of adequate culture facilities, the use of non-standardised methodologies, the absence of quality control measures, the absence of longitudinal studies to detect trends, the failure of some studies to differentiate between primary and acquired drug resistance, and the inherent selection bias of many surveys, particularly those centred on large studies and specialised hospitals.

GLOBAL ANALYSIS OF DRUG RESISTANCE

In 1994, the WHO and the International Union Against Tuberculosis and Lung Disease initiated the Global Project on Anti-tuberculosis Drug Resistance Surveillance [15,16]. This project was designed to measure the prevalence of resistance according to standardised methods, guided by three overriding principles: (i) that the sample of TB patients must be representative of cases within an entire country; (ii) that laboratory performance is validated; and (iii) that primary and acquired drug resistance can be distinguished. In the recent third report of this project, data collected between 1999 and 2002 from 77 settings or countries, representing 20% of the global total of new smear-positive TB cases, were presented [16]. Data concerning new cases were available for 75 settings and 55 779 patients. The prevalence of resistance to at least one anti-TB drug (any resistance) ranged from 0% in some western European countries to 57.1% in Kazakhstan (median 10.2%). Median frequencies of resistance to specific drugs were streptomycin 6.3%, isoniazid 5.9%, rifampicin 1.4% and ethambutol 0.8%. The prevalence of multiple drug resistance (MDR) ranged from 0% in eight countries to 14.2% in Kazakhstan (51/359) and Israel (36/253) (median 1.1%). The highest frequencies of MDR were observed in Tomsk Oblast (Russian Federation) (13.7%), Karakalpakstan (Uzbekistan) (13.2%), Estonia (12.2%), Liaoning Province (China) (10.4%), Lithuania (9.4%), Latvia (9.3%), Henan

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Province (China) (7.8%) and Ecuador (6.6%). Trends in drug resistance among new cases were determined in 46 settings (20 with two data points and 26 with at least three). Significant increases in the prevalence of any resistance were found in Botswana, New Zealand, Poland and Tomsk Oblast (Russian Federation), while Cuba, the Hong Kong Special Administrative Region and Thailand reported significant decreases over time. Tomsk Oblast (Russian Federation) and Poland reported a significantly increased prevalence of MDR, while decreasing trends were observed in the Hong Kong Special Administrative Region, Thailand and the USA.

Data concerning previously treated cases were available for 66 settings. In total, 8405 patients were surveyed. The median prevalence of resistance to at least one drug (any resistance) was 18.4%, with the highest prevalence being 82.1% in Kazakhstan (262/319). Median frequencies of resistance to specific drugs were isoniazid 14.4%, streptomycin 11.4%, rifampicin 8.7% and ethambutol 3.5%. The median prevalence of MDR was 7.0%. The highest frequencies of MDR were reported in Oman (58.3%; 7/12) and Kazakhstan (56.4%; 180/319). Among countries of the former Soviet Union, the median prevalence of resistance to these four drugs was 30%, compared with a median of 1.3% in all other settings [16]. Given the small number of subjects tested in some settings, the prevalence of resistance among cases treated previously should be interpreted with caution. The use of standardised short-course chemotherapy was associated with a lower level of drug resistance. The recent emergence of extensively drug-resistant tuberculosis (resistance to isoniazid, rifampicin, any fluoroquinolone and amikacin or kanamycin or capreomycin) has caused a high mortality, particularly in individuals co-infected with HIV [17,18]. The properties and recommended doses of the principal anti-TB drugs that are currently available are summarised in Table 1.

SUPRANATIONAL REFERENCE LABORATORY NETWORK

A key part of the Global Programme (see above) was the creation of a Global Network of Supranational Reference Laboratories (SRLs) to serve as reference centres for external quality assessment/control (EQA) of drug susceptibility testing in national surveys. Several annual EQA distributions, focusing on resistance to isoniazid, rifampicin, ethambutol and streptomycin, have been completed by the network. There is no EQA for pyrazinamide at present, reflecting the difficulty in reliably and consistently measuring resistance to this agent.

In each EQA distribution, identical sets of ten isolates of *M. tuberculosis* (20 cultures in early distributions, 30 cultures in recent distributions) were distributed to all SRLs. This sample size was calculated to yield a significance level of 95% in order to detect true differences among laboratory methods with a power of 90% [15,16,49]. In turn, the SRLs supported national surveys or ongoing surveillance in their own countries, and also supported national reference laboratories worldwide in conducting surveys with the national TB programme. Regional networks of laboratories were formed in the Western Pacific Region and in Europe, coordinated by the SRLs in London (UK), Stockholm (Sweden), Paris (France) and Borstel (Germany).

European countries have various degrees of centralisation of facilities for measuring drug resistance. For example, most hospital laboratories in England submit isolates of *M. tuberculosis* and other mycobacteria to the Health Protection Agency *Mycobacterium* Reference Unit, or to one of the other Regional Centres for Mycobacteriology, for speciation and drug susceptibility testing. A system for the surveillance of drug resistance in isolates of *M. tuberculosis*, i.e., the UK Mycobacterial Resistance Network (Mycobnet), was established in 1993–1994 to link the principal reference units in the UK. In initial studies, isoniazid and rifampicin resistance rose from 4.6% (157 cases) to 6.1% (221 cases), and from 0.6% (22 cases) to 1.8% (65 cases), respectively, between 1993 and 1996 [19]. Initial MDRTB rates in the same period increased from 0.6% to 1.6%, and combined clinical resistance or period prevalence (the total level of resistance occurring in 1 year) rose from 0.6% to 1.7% [17,50]. In 2003, susceptibility test results with isoniazid and with rifampicin for 4928 isolates (99.7% of the actual total), obtained from cases at the start of treatment, revealed that 388 (7.9%) were resistant to one or more of the first-line drugs; 346 isolates (7.0%) were isoniazid-resistant and 68 (1.4%) were MDR (UK Mycobnet, http://www.hpa.org.uk/infections/topics_az/tb).

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Table 1. Front-line anti-tuberculosis drugs for adults

Drug	Route[a]	Daily dose[b]	Intermittent twice-weekly	Three times weekly	Major side-effects[c]	Monitoring[d]
Isoniazid	PO IM IV	300 mg 5 mg/kg	15 mg/kg max. = 900 mg	15 mg/kg max. = 900 mg	Peripheral neuropathy, hepatitis, CNS effects, increased phenytoin levels, interaction with disulphiram, hepatic enzyme elevation	LFT; levels of interacting drugs[e]
Rifampicin	PO IV	600 mg 10 mg/kg	10 mg/kg max. = 600 mg	10 mg/kg max. = 600 mg	GI upset, hepatitis, rash, bleeding, contact lens and body fluids coloured orange/pink; decreases serum levels of warfarin, methadone, contraceptive hormones, dapsone, ketoconazole and theophylline; influenza-like syndrome	LFT; levels of interacting drugs
Pyrazinamide	PO	1.5–2.5 g 15–30 mg/kg	2.5–3.5 g 50–70 mg/kg	2–3 g 50–70 mg/kg	GI upset, increase in hepatic enzyme levels, rash, joint pain, hyperuricaemia (gout rarely); may complicate control of diabetes mellitus	LFT; uric acid (if needed)
Ethambutol	PO	2.5 g (max.) 15–25 mg/kg	50 mg/kg	30 mg/kg	Red/green colour blindness, optic neuritis, decreased visual activity, rash	Colour vision, visual acuity
Streptomycin[f]	IM IV	15 mg/kg	25–30 mg/kg	25 mg/kg	Nephrotoxicity, ototoxicity, hypokalaemia, hypomagnesaemia	Blood chemistry, renal function, audiometry

PO, oral; IM, intramuscular; IV, intravenous; CNS, central nervous system; GI, gastrointestinal.
[a]Possible routes of administration; in practice, all drugs are given orally wherever possible.
[b]The daily dose is quoted for an adult male of average weight; all doses are adjusted in accordance with a patient's weight.
[c]Isoniazid causes increased elimination of pyridoxine, leading to peripheral neuropathy, particularly in alcoholics, in the malnourished, and during pregnancy. Daily doses of 10 mg/day are sufficient to compensate for this loss.
[d]LFT, liver function test; specific monitoring points are given. At appropriate intervals, the patient should be monitored clinically, radiologically and bacteriologically. A full blood count including platelets should be performed if there is any bleeding tendency.
[e]Aluminium-based antacids reduce absorption.
[f]Streptomycin in patients aged >60 years is more likely to lead to side-effects, and daily doses should be limited to 10 mg/kg, with a maximum dose of 750 mg. Closer observation of hearing loss and renal function may be necessary in this age group.

In general, drug-resistant cases of TB were more likely to be seen among males, individuals who were previously TB-positive or who were HIV-positive (biased by outbreaks), those born abroad and those in the black African ethnic group (rates were lowest among the white ethnic group). Drug-resistant cases, by trend and absolute numbers, were identified predominantly in London. Similar risk-factors apply in respect of increases in the incidence of TB seen in other west European countries.

MEASURING DRUG RESISTANCE IN *M. TUBERCULOSIS*

Drug resistance occurs spontaneously in *M. tuberculosis* at a different rate for each drug. For example, mutations resulting in resistance to rifampicin occur at a rate of 10^{-10}/cell division, compared with 10^{-7}–10^{-9} for isoniazid. Overall, this creates an estimated frequency of resistant organisms in drug-free environments of 1 in 10^8 and 1 in 10^6, respectively, for the two drugs [51]. As lung cavities frequently contain 10^7 bacilli, resistant bacilli emerge naturally without antimicrobial pressure, and the use of anti-mycobacterial drugs then selects the resistant population [30,33,51]. By the 1950s, it was established that combination chemotherapy could prevent the emergence of clinical resistance that was observed when patients were treated with a single drug. Today, mono-drug therapy effectively occurs only when inappropriate chemoprophylaxis or treatment is given, or in cases where there is poor adherence to therapy, an irregular drug supply or drug malabsorption [52].

The lack of uniformity and reproducibility in the methods used for testing susceptibility to anti-TB drugs was first noted in the late 1950s and 1960s. Thus, for isoniazid, at least eight criteria were used to define resistance. In studies that examined the major susceptibility testing

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methods available at the time, significant differences were noted in the media used, the inoculum size, the minimum concentrations of drug tested, and the criteria used to establish resistance. Symposia organised by the WHO and the International Union Against Tuberculosis produced agreed definitions for drug resistance, and three categories of acceptable methods were defined:

(1) the absolute concentration method (effectively the MIC);
(2) the resistance ratio method;
(3) the proportion method.

Detailed descriptions of these methods have been published for first- and second-line drugs [53–61]. These documents form the bedrock of drug susceptibility testing for *M. tuberculosis* internationally. The performance of drug susceptibility tests in a limited number of expert centres is necessary, as getting the right result is difficult (requiring good standard operating procedures, internal and external quality control systems and proficiency testing). The WHO Global Network of SRLs was established to act as a quality control network and to maintain a high level of proficiency in the diagnosis of drug-resistant TB. In practice, all three principal methods can perform adequately, provided that the technical protocols published are followed exactly [15,16,57].

The absolute concentration method

Drug is incorporated into solid agar or Lowenstein–Jensen medium as two-fold dilutions, or is used in a broth dilution method. Solid media methods are standardised more easily. Resistance is defined as the lowest concentration of the drug that inhibits growth (<20 colonies). Drug concentrations, and particularly inoculum size, must be carefully standardised with reference to wild-type cultures. Variation in inoculum size is the major source of error in this method [57,60].

The resistance ratio method

This is a refinement of the absolute concentration method, in which variations in the MIC for a given isolate are controlled when the isolate is tested on different batches of drug-containing medium. The resistance ratio is defined as the MIC for the test isolate divided by the MIC for a standard susceptible strain, e.g., H37Rv, or for recently isolated susceptible wild-type strains. If the ratio is 2 or less, or 8 or more, the isolate is fully susceptible or highly resistant, respectively. Intermediate or low-level resistance is difficult to measure accurately [53,54,57]. Inoculum size needs to be standardised, but the critical concentration does not need to be determined because of the direct comparison with susceptible isolates [57].

Proportion method

In this method, the strain is classified as susceptible if its constituent cell population contains below a critical proportion of resistant cells, and as resistant if above this proportion. The proportion varies with different drugs, e.g., 1% for isoniazid and rifampicin. This correlates with an effective clinical outcome. In practical terms, the proportion of drug-resistant mutants is obtained from the ratio of the number of colonies growing on drug-containing medium and on drug-free medium [53,54,60].

The introduction of the broth-based radiometric BACTEC 460 system led to the development of the proportion method for use with this system, using the ratio of growth indices obtained by inoculation of the test isolate in drug-containing medium and inoculation of a 100-fold dilution (1%) of the isolate in drug-free medium [61,62]. Standardisation of the inoculum is not as critical in this method, although individual colonies must be visible when using solid media.

Rapid, non-radiometric, automated culture methods

In addition to these three accepted methods, rapid, non-radiometric, automated methods have been used increasingly for diagnostic culture, and studies using these systems to determine resistance to isoniazid and rifampicin, as well as to other first-line drugs, have been published [63–77]. Their use is likely to increase, but mainly only in large reference centres because of the higher cost as compared with solid culture-based methods. All of these systems use a modification of the proportion method. Most studies have utilised indirect assays (i.e., on cultured bacteria), with greatest success for the determination of isoniazid and rifampicin resistance. However, smear-positive material can be analysed in direct assays on

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solid media, and this may be almost as fast as indirect assays and is less expensive. The capital costs of automated systems are high, and laboratory containment facilities must be of the highest order, which increases the cost even further.

The use of such systems for second-line drug susceptibility testing has only recently been explored, and it appears that the WHO 2001 guidelines concerning second-line drug susceptibility testing [59] may have been premature, as a subsequent multicentre analysis concluded that results obtained for second-line drug susceptibility testing were not reliable [74]. An earlier multicentre study, employing the BACTEC 460 system, demonstrated consistency for a range of second-line drugs [75], but until recently, there had been no comparative study of the MGIT 960 system and solid media-based methods, such as the resistance ratio or proportion methods. These studies have now been completed [72,76], with good correlation for most, but not all, second-line drugs between the solid medium-based resistance ratio and proportion methods and the MGIT 960 system.

Other methods have been explored for use in early bactericidal assays as surrogates for the above methods, with some success [78–80]. International quality assessment programmes for second-line drug testing are now being developed [81] and the WHO guidelines [59] are under revision and will be released in late 2007.

MOLECULAR METHODS FOR DETECTING DRUG RESISTANCE

Novel molecular assays for detecting drug resistance offer several potential advantages, including lower turnaround times and minimal (or possibly no) initial culture. Many of the key gene mutations conferring resistance have been identified, permitting the development of in-house and commercial molecular tests [82–99]. Considerable problems remain in the development of tests for clinical use. The majority of these tests are more costly than current methods, the exact ratio of resistant to susceptible organisms that produces clinical resistance is unclear, and the presence of common gene mutations is not always associated with drug resistance (i.e., silent mutations). Nevertheless, the mutations associated with resistance are now well-known for some drugs. Novel non-culture-based drug resistance tests can be divided into genotypic systems, in which the drug target and nature of the gene mutation is known, and phenotypic systems, in which an outcome (i.e., death of the bacillus) is measured and previous knowledge of the precise underlying resistance mechanism is not required.

Realistically, a combination of both types of test is required. Mutations within an 81-bp region (codons 507–533) of the *rpoB* gene, encoding the β chain of the DNA-dependent RNA polymerase, confer rifampicin resistance in *c.* 90–95% of all clinical isolates examined [86–89,91,93–105]. The presence of a mutation can be detected by a genotypic test and, in nearly all cases, is predictive of clinical drug resistance. However, isoniazid drug resistance is more complex, involving mutations in at least four genes or gene complexes, with not all mutations affecting the phenotype [82,84,85,87,91,92,96,97,103–105]. Insufficient information concerning the mechanism(s) of resistance means that although the best genotypic systems will predict rifampicin resistance in 90–95% of isolates, they would currently fail to predict streptomycin, isoniazid and ciprofloxacin resistance in *c.* 40%, 10–15% and 25% of isolates, respectively. Known or postulated genes involved in drug resistance are listed in Table 2.

Most genotypic assays involve three main steps: (i) sample preparation, which may be as simple as mechanical disruption by boiling a small volume of cells in water, or may involve full DNA purification; (ii) amplification of a specific region of a gene; and (iii) detection of the mutation. Once appropriate primers have been selected, the region of interest can be amplified and mutations detected as described below.

DNA sequencing and other rapid molecular genotyping assays

DNA sequencing is the reference standard, as all mutations will be detected and, unless they are silent, will be predictive of resistance. Automation has simplified the process, bringing sequencing within the capability of large academic and reference centres, although automated analysers are relatively expensive to purchase and operate. Automated analysers using fluorescent chemistry methods can provide accurate sequence data within 48 h. As indicated in Table 2, automated sequencing has been used in both research and

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Table 2. Molecular detection of drug resistance in *Mycobacterium tuberculosis*

Drug	Known or probable targets	Mutations in genes conferring resistance	Function of gene	Molecular assays
Rifampicin	RNA synthesis	*rpoB*	DNA-dependent RNA polymerase (β subunit)	PCR-SSCP, heteroduplex, microarray, macroarray, line probe, sequencing
Isoniazid	Mycolic acid biosynthesis	*katG* *inhA/mabA* *ahpC* *oxyR*	Catalase/peroxidase Fatty-acid biosynthesis Alkylhydroperoxide C reductase Oxidative stress regulator	PCR-SSCP, microarray, macroarray, sequencing
Streptomycin	Protein synthesis	*rrs* *rpsl*	16S rRNA ribosomal protein S12	PCR-SSCP, microarray sequencing
Ethambutol	Cell wall synthesis	*embA,B,C*	Lipoarabinomannan and arabinogalactan synthesis	Sequencing
Ethionamide	Cell wall synthesis	*inhA* + ?	Cross-resistance associated with *inhA* mutations	Sequencing
Pyrazinamide	Pyrazinamidase	*pncA* + ?	Pyrazinamidase	Sequencing
Ciprofloxacin	DNA synthesis	*gyrA* *gyrB*	DNA gyrase subunits A and B (prinicipally)	PCR-SSCP, microarray, macroarray, sequencing

PCR-SSSP, PCR single-strand conformation polymorphism.

clinical settings to identify mutations for resistance to most first-line TB drugs.

Microarrays and macroarrays using a solid-phase hybridisation approach offer an alternative approach to direct sequencing as part of initial screens for mutations (see below). Other alternative techniques for mutation identification include PCR single-strand conformation polymorphism analysis [89], heteroduplex analysis [90], pyrosequencing (a short-read sequencing assay developed and applied as a screen to detect common mutations in *katG* and *rpoB* in early cultures) [92], mutation-specific priming, and restriction enzyme analysis. These methods are generally PCR-based, with amplification of the key regions involved in drug resistance being followed by a method that detects wild-type sequences or mutations in the amplified fragments.

Solid-phase hybridisation: microarrays and macroarrays

Using knowledge of the sequence in susceptible and resistant strains, it is possible to design probes that can be immobilised on a membrane support. Resistance can then be detected by a reverse hybridisation principle [23,99–105]; i.e., a region of DNA that contains mutations associated with drug resistance can be amplified and allowed to hybridise with the probes. Failure of hybidisation is caused by the presence of a mutation, and thus is predictive of drug resistance.

Microarrays are high-density oligonucleotide arrays. An initial PCR produces fluorescently labelled DNA fragments which, when hybridised to the corresponding sequence on the microarray, emit a fluorescent signal that is detected by a scanner. This technique has been applied to the identification of mycobacteria [98,106,107] and to the detection of drug resistance. The latter results were in concordance with those obtained by sequencing of the *katG*, *inhA* (isoniazid resistance), *rpoB*, *rpsL* and *gyrA* genes. Eventually, these techniques should enable the simultaneous detection, identification, susceptibility profiling and assessment of the probable virulence of a strain, but costs are high, and the approach is limited by the current extent of knowledge relating to the mechanisms of resistance for many of these drugs.

There have been several reports concerning the use of commercial macroarrays with cultures [23,100–105]. For example, in an initial evaluation in the UK, 100% (16/16) of rifampicin-resistant cultures were identified correctly by the Innolipa assay [100]. This assay has also been used successfully to analyse rifampicin resistance directly in primary specimens [100–102]. In a retrospective follow-up study of 2287 consecutive specimens from 2110 patients, performed between 1 January 1999 and 31 December 2002, the overall concordance, sensitivity, specificity, positive predictive value and negative predictive value for TB diagnosis were 91.2%, 85.2%, 96.2%, 95.7% and 86.7%,

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respectively. For the detection of rifampicin resistance in specimens yielding *M. tuberculosis* complex on culture, the Innolipa assay had concordance, sensitivity, specificity, positive predictive value and negative predictive value of 99.1%, 95.0%, 99.6%, 92.7%, and 99.7%, respectively (Health Protection Agency National *Mycobacterium* Reference Unit, unpublished data).

Non-commercial macroarrays have been developed and used successfully for the detection of rifampicin and isoniazid resistance in cultures [23,104,105]. In a sample of 233 isolates from patients in Russia, 46.5% possessed mutations in both the *rpoB* and *katG* (or *inhA*) genes. Results from macroarrays demonstrated overall concordance with resistance defined phenotypically for 95.4% and 90.4% of isolates with rifampicin and isoniazid resistance, respectively [104].

Other molecular drug resistance detection methods

PCR single-strand conformation polymorphism analysis can be performed using either a manual or an automated system. A radioactive detection system is often used, but silver-staining now offers a sensitive alternative. Single-stranded DNA folds into a complex tertiary structure, the shape of which is dependent on the DNA sequence. If two single strands of DNA differ by one or more bases, i.e., a mutation, they will fold into structures with different mobilities on a polyacrylamide gel, and this can be used to detect the mutation [89]. Tests for drug resistance and viability determination have been developed, based on mRNA detection following RT-PCR [108,109] and the use of molecular beacons [110–112].

NOVEL PHENOTYPIC METHODS

Jacobs *et al.* [113] showed that drug susceptibility could be assessed by measuring the production of photons produced by viable mycobacteria infected with phages expressing the firefly luciferase gene. Untreated mycobacteria, or drug-resistant mycobacteria in the presence of the corresponding drugs, continue to express luciferase, which catalyses the reaction of luciferin with ATP to generate photons of light. Death of mycobacteria leads to cessation of light production. This approach has the advantage that knowledge of the underlying genetic basis of resistance is not required. Screening of novel antimycobacterial drugs would also be simplified using this technique. Recent research has reduced the cost of this technique by the development of the 'Bronx Box', involving a microtitre plate format with a photographic film detection system [114]. Research to increase the sensitivity of the assay and to reduce the capital costs is currently ongoing.

An alternative approach, the 'phage amplified biologically' (PhaB) assay, has been described for the diagnosis and detection of drug resistance [115,116]. This approach utilises skills and resources that are readily available within the diagnostic laboratory, without a need for expensive luminometers. The PhaB assay can be used for the diagnosis of *M. tuberculosis* in patient samples, as well as for direct drug susceptibility testing of strains isolated previously by conventional culture. The concept of the method is simple. In the first step, a test culture or sample is inoculated with mycobacteriophage. If viable mycobacteria are present in the sample, they become infected with mycobacteriophage. Subsequently, the mycobacteriophages remaining outside the mycobacteria are inactivated by a specific chemical treatment, whereas those inside the mycobacteria are protected. The protected mycobacteriophages then replicate within viable bacilli, and eventually cause the host cells to lyse and release the new generation of mycobacteriophages. The released mycobacteriophages are plated on a lawn of the rapidly growing related organism *Mycobacterium smegmatis.* The mycobacteriophages infect and replicate in this related organism and, after overnight incubation, are detected as clear areas of lysis or plaques in the turbid growth of the *M. smegmatis* lawn. The procedure is potentially rapid and simple, taking as little as 48 h, compared with the weeks required for conventional culture. The assay has been calculated to detect 10–100 resistant mycobacteria/mL of sample, which is at least as sensitive as conventional culture. The assay involves the use of simple equipment and, as the assay proceeds, the number of viable infectious *M. tuberculosis* cells declines, so that the assay actually becomes safer as it continues. In a recent evaluation, the assay correctly assigned and identified 8/8 multiple drug-resistant and 1/1 rifampicin-resistant, isoniazid-susceptible isolates. When used to test susceptibility to

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isoniazid, the PhaB assay correctly identified 15/17 (88.2%) isoniazid-resistant, rifampicin-susceptible isolates, and 17/21 (81%) isoniazid-susceptible isolates. The results can be obtained in as little as 2 days from receipt of a patient sample [115,116]. Correlation with the resistance ratio method for streptomycin, ethambutol and pyrazinamide has been reported to be 96%, 88% and 87%, respectively [116]. These assays have now been commercialised [117]. Other simpler and inexpensive assays involving the use of dyes, e.g., Alamar blue and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, have also shown promise [118,119].

CONCLUSIONS

Methods for drug susceptibility testing of *M. tuberculosis* have evolved over several decades. They are currently performed in specialised centres in most European countries, as they are technically demanding, require appropriate isolation facilities, and can be difficult to interpret. The absolute concentration, resistance ratio and proportion methods can all give accurate results, provided that they are carefully quality-controlled and standardised (the results from the WHO Global Programme on Drug Resistance in Tuberculosis indicated the problems of reproducibility that occur when inexperienced laboratories perform drug susceptibility testing or modify methods without a clear understanding of the needs for standardisation and quality control). Novel automated rapid culture and molecular methods have been evaluated at large reference centres and in multicentre collaborations, with great success being demonstrated for rifampicin and isoniazid testing, and some success for other first- and second-line drugs.

Drug susceptibility testing for first-line drugs is recommended for all new cases, with specimens taken: (i) before initiating treatment; (ii) if the patient continues to be culture-positive after 2–3 months; and (iii) if there is a history of previous TB treatment (a major risk-factor for drug resistance). Individual circumstances may dictate additional testing. Accuracy is more important than speed, and drug susceptibility test results should be done by a small number of well-equipped, experienced laboratories that participate and perform well in an international drug susceptibility testing quality assessment scheme. The WHO Supranational Laboratory Quality Control Network offers the greatest global coverage, and assesses participating laboratories for their ability to identify isoniazid, rifampicin, ethambutol and streptomycin resistance correctly.

Across Europe, early identification of mycobacterial growth as '*M. tuberculosis* complex' (principally *M. tuberculosis* and *Mycobacterium bovis*) and testing for rifampicin resistance should be the initial priorities, as rifampicin resistance invalidates standard 6-month short-course chemotherapy and is a useful marker in most countries for MDRTB. For patients with MDRTB, or for individuals who are genuinely unable to tolerate first-line therapy, second-line therapy should be instituted. There remains a need to standardise second-line drug resistance testing, and such testing should be performed only at the national reference laboratories in western and central European countries, because of the relatively small number of cases and the concomitant difficulty of maintaining testing proficiency in multiple centres performing small numbers of tests. This argument also holds for smaller eastern European countries, e.g., the Baltic States, where overall case numbers are small, but additional qualified centres will be needed for the largest countries in eastern Europe. There is a need to further develop international EQA for second-line drug susceptibility testing in order to complement existing schemes for first-line drugs.

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