RAPID DIAGNOSIS OF DRUG-RESISTANT TUBERCULOSIS*

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NATURE AND SCALE OF THE PROBLEM OF MULTI-DRUG RESISTANT TUBERCULOSIS

It is estimated that one third of the world's population is infected by tuberculosis and that three million people die annually of this disease,[1] with this resurgence being due in part to the increase in human immunodeficiency viral (HIV) infection.[2] Partial treatment, curtailed duration and inadequate compliance with prolonged courses of medication have contributed to an increasing problem of drug resistant disease. Multi-drug resistant tuberculosis (MDR-TB), which is characterised by infection by mycobacterial isolates resistant to a minimum of rifampicin and isoniazid, has a high mortality. Thus in a study of 173 patients with MDR-TB, treated between 1983 and 1994, HIV-positive patients had a 72% mortality and HIV-negative patients a 20% mortality, with a median duration of survival of 20 months.[3] In less developed countries, with inadequate diagnostic facilities and without access to a full range of therapeutic options, the mortality may be considerably higher.

The epidemic of drug-resistant disease of the 1980s and 1990s in New York has been ascribed to the breakdown of the tuberculosis care programmes and appears to be resolving with the widespread introduction of directly-observed therapy (DOT).[4] Drug resistance is present in all countries surveyed by the WHO.[5] In some less developed countries there is evidence of high rates of multi-drug resistant disease. For example, in Baku prison in Azerbeijan, all patients with tuberculosis unresponsive to therapy are infected by organisms resistant to at least one drug, and in 17 of 38 unselected patients, organisms were also resistant to at least one drug.[6] In one hospital in Mumbai, (formerly Bombay), approximately half the mycobacterial isolates tested were resistant to isoniazid and rifampicin.[7] The ease of air travel and complex international links of people living in major metropolitan centres suggest that MDR-TB strains will present in cities such as London, despite long-established tuberculosis control programs.

Between 1987 and 1997, tuberculosis notification rates in many parts of inner London have more that doubled.[8] The rate of MDR-TB is higher in London than in the rest of the UK, and is rising. In 1993-6 the overall rate of MDR-TB rose from 0.6% to 1.7%, with the average rate in London for the period being 1.9% compared with 0.7% for the UK outside the capital.[9] The importance of appreciating that the nature of the tuberculosis caseload varies in different areas of the UK is further illustrated by the uneven distribution of the overlap between tuberculosis and HIV infection: on a national level this overlap occurs in less that 5% of cases, whereas our data suggest that in certain clinics in London the overlap may exceed 20%.

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THE CLINICAL PROBLEM

The challenge for the clinician is to select patients who have MDR-TB and identify those who require long-term treatment in negative pressure facilities and the use of complex, as well as potentially toxic, drug regimens. These will have to be distinguished from those who will respond to conventional therapy and who can be regarded as non-infectious following the first two weeks of conventional treatment. The key to solving this problem is to develop a rapid test which identifies *M. tuberculosis* and predicts rifampicin resistance/sensitivity. At least in the UK, rifampicin resistance is a good marker for MDR-TB, because isolated rifampicin resistance is very rare.

EXISTING APPROACHES TO THE DIAGNOSIS OF MDR-TB

The conventional approach to the diagnosis of tuberculosis involves a stained sputum smear followed by culture. Using this approach, definitive organism identification and drug sensitivity prediction may take over two months. Drug sensitivity testing may be accelerated by measuring mycobacterial growth using the Bactec system which identifies radiolabelled carbon atoms released from palmitic acid in liquid culture,[10] or by the mycobacteria growth indicator tube (MGIT) system, in which fluorescent oxygen consumption is measured.[11] As yet, neither of these methods can offer drug sensitivity testing results within two weeks of sputum collection.

A novel and sensitive method to detect viable organisms has been developed in which a bacteriophage, which contains the enzyme luciferase, is inoculated into cultures of mycobacteria: where the organism is viable, emission of light occurs and can be appreciated by a photosensitive cell. This approach can be combined with exposure to different antibiotics to provide *in vitro* drug sensitivity testing.[12] This method has yet to be developed to the point where rapid results can be obtained within the context of a routine laboratory service.

APPLICATION OF DNA TECHNOLOGY TO DIAGNOSIS OF TUBERCULOSIS

The use of molecular biology techniques to identify and track individual strains of MDR-TB has been made possible by a number of key developments. These include the development of rapid amplification methodologies such as the polymerase chain reaction (PCR),[13] the sequencing of the entire genome of *M. tuberculosis*,[14,15] and the linking of specific genetic polymorphisms to drug resistance.[16]

The development of a rapid test which can identify the presence of *M. tuberculosis* in sputum and other clinical

samples has been approached in a variety of ways.[17,18] Many of these tests are still under evaluation and are being developed further in an attempt to achieve the 'optimal trade-off' between sensitivity and specificity.

Two direct amplification tests have been approved by the US Food and Drugs Administration for use on smear-positive samples. Both the Gen-Probe Amplified Mycobacterium tuberculosis Direct test (Gen-Probe, CA USA),[19] and the Amplicor test from Roche,[20] have high specificity, sensitivity and positive predictive value for sputum-smear positive samples (>95%), but sensitivity is only 40-70% in smear-negative patients.[21]

NOVEL APPROACHES TO THE DIAGNOSIS OF DRUG-RESISTANT DISEASE

Resistance to all the major antituberculous agents has been linked to genetic polymorphisms on at least one mycobacterial gene.[22] For the majority of drugs, the polymorphisms conferring resistance to the drug are multiple and extend across a large region of DNA, often with mutations within different genes. In the case of rifampicin resistance, the majority of organisms with resistance have mutations within a small 69 base pair region of the gene encoding the beta unit of RNA polymerase (rpoB).[23] PCR amplification of this region in combination with a rapid screening system for mutations offers the possibility of rapid detection of rifampicin resistance. A commercial detection kit, INNO-LiPA-Rif.TB (Innogenetics, Belgium) has been designed to detect the common mutations in rpoB which are associated with rifampicin resistance.

In our laboratory, the combination of this technique with automated sequencing to confirm the presence of the mutation has been able to provide a result within one week. This has proved useful in the investigation of possible outbreaks of MDR-TB. In one example, samples from two possible contacts of a known case of MDR-TB were received. It was possible to confirm the presence of rifampicin-resistant infection in one, and rifampicin-susceptible disease in the other, and to inform the clinicians within 96 hours of sample receipt.[24] A second commercial method, MisMatch Detect II (Ambion, Austin, Tex), has a detection rate for rifampicin-resistant organisms similar to INNO-LiPA-Rif.TB.[25] Rapid testing for mutations associated with resistance to rifampicin is now available in the UK for selected patients from the Public Health Laboratory Service (PHLS), which claims to be able to provide results within three to four days.[26]

MOLECULAR BIOLOGY IN THE STUDY OF THE EPIDEMIOLOGY OF DRUG RESISTANT TUBERCULOSIS

An appreciation of subtle variations in the DNA sequence of organisms offers the opportunity to track the spread of drug-resistant organisms through the community. The majority of the studies have used the approach of restriction-length polymorphism in which DNA from cultured mycobacteria is enzymatically digested and probed to reveal similar, or differing, patterns on a gel. This approach has stimulated a number of important epidemiological studies. For example, a study of 1,953 *M. tuberculosis* isolates from New York revealed 273 (of which 259 were resistant to four or more drugs) to have similar RFLP typing patterns, indicating that they were closely related.[27] It is thus possible to envisage the development of international collaboration to track the spread of individual isolates of MDR-TB as they move through communities and infect individuals in different countries.

Other approaches have used combinations of PCR-based techniques which have the advantage that they can be developed for use directly on sputum. One technique termed 'spoligotyping' involves amplifying spacer regions between a number of directly-repeated sequences. The presence or absence of the spacers provides a pattern, which is specific to a subset of isolates of *M. tuberculosis*. This technique has been used in epidemiological studies in Africa[28] and London.[29] More recently, the combination of spoligotyping with the second technique of heminested inverse PCR[30] has been used in an epidemiological study in London, which documented a recent transmission rate of 19%.[31] Another study has combined spoligotyping with double repetitive element PCR and found this technique to be as descriminatory as RFLP.[32]

The power of rapid PCR-based techniques such as spoligotyping in the management of suspected outbreaks of MDR-TB, is that they can refute or support suggestions of an epidemiological link within a few days of sample collection.[29] This allows scarce resources to be focused only on those where there is a high probability of infection from an index case of MDR-TB.

APPROACH TO INVESTIGATING SPUTUM FROM PATIENTS WITH POSSIBLE MDR-TB

Cost prohibits the use of novel molecular biology investigations on all patients with a possible diagnosis of tuberculosis. However, the costs of providing the resources needed by patients with MDR-TB are high and need to be targeted at those with the correct diagnosis. The algorithm in Figure 1 extends that of Drobniewski (1998)[26] and places the onus on the clinician to identify whether, in the community from which the patient comes, there is a high, intermediate or low risk of MDR-TB, and to prioritise the use of expensive molecular diagnostic techniques accordingly. An identical algorithm might be applied to bronchoscopy samples.

FUTURE DEVELOPMENTS

The main advance, which is likely to improve our ability to detect drug-resistant organisms, is the application of micro-array technology. In this technique, microscopic amounts of many tens of thousands of different small sequences of DNA (oligonucleotides) are adhered to a small chip. DNA in the sample binds only to those sequences with exactly the correct matching sequence. Binding is detected using a thin beam of laser light, and the results analysed by a computer.[33,34] By this means, precise identification of mutations in the organism's DNA can be defined on the basis of binding to the oligonucleotides containing the specific mutations, or to the oligonucleotides with the 'wild type' sequence.

This micro-array technology has the potential ability to identify mutations on a range of genes, and thus in the future may offer drug resistance prediction for a number of drugs in addition to rifampicin.

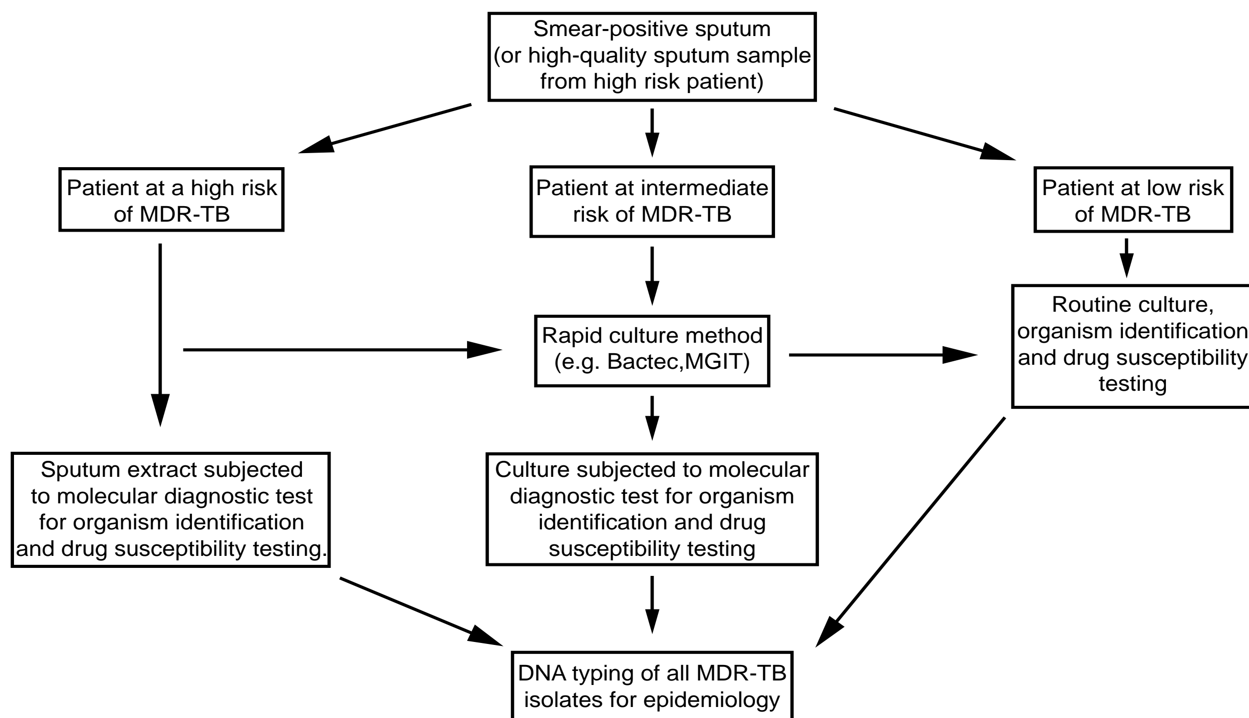

FIGURE 1
Approach to investigating sputum from patients with possible MDR-TB.

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