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New Tools for the Diagnosis of Tuberculosis

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The routine clinical approach to the diagnosis of tuberculosis has remained largely unchanged for the last fifty years. The majority of patients with tuberculosis have pulmonary disease. These patients are first evaluated clinically, when the classical symptoms of fever, weight loss and haemoptysis are sought. A chest X-ray is performed which again may show the typical features of upper lobe shadowing, with or without cavitation. A sputum sample or, when this is unavailable, bronchoscopic washings are obtained and examined by staining for acid fast organisms, although this yields positive results in less than half the cases. This material is also cultured to obtain confirmation that the acid fast organisms are indeed *Mycobacterium tuberculosis*, and to obtain information on the sensitivity of the organism to different anti-tuberculous drugs. This process takes two to three months to provide results.

A number of factors, in particular HIV infection, multi-drug resistance (MDR) and rapid air transport of people around the world, cause difficulties with this diagnostic approach rendering it too slow and unreliable. For example, patients with HIV-infection alone may have symptoms similar to those of tuberculosis. HIV-positive patients may also develop pulmonary tuberculosis presenting with atypical features and a non-classical chest X-ray. HIV-positive patients may develop respiratory infection with mycobacteria other than *M. tuberculosis* which require treatment by different drug regimes. In addition, there are now a growing number of outbreaks of multiple drug resistant tuberculosis and an appreciable incidence in many parts of the world of resistance to at least one drug. These factors combine with an increase in the mobility of the tuberculosis infected population to pose a growing problem for the clinician.

These concerns have lead to an urgent search for new techniques to assist in making the diagnosis of tuberculosis. This article will review progress in using molecular biology techniques to identify, type and establish the drug sensitivity of *M. tuberculosis* in sputum and other biological samples.

M. tuberculosis DNA Identification

The *M. tuberculosis* genome contains distinctive sequences which can be utilized to identify the organism. Many studies have taken advantage of this opportunity. Thus, following culture, it is possible to extract the DNA, digest it with enzymes and use specific probes for different mycobacteria to confirm which cultures are indeed *M. tuberculosis*, as opposed to other mycobacteria such as *M. avium intracellulare*. There are now commercial kits such as the Accuprobe system which can identify a culture as belonging to the *M. tuberculosis* complex in under two hours. This approach is widely used combined with the BACTEC system of rapid identification by liquid culture to provide results within two weeks (Woods & Witebsky 1995).

Polymerase Chain Reaction Amplification of DNA in Clinical Samples

The polymerase chain reaction (PCR) is a technique which allows sequences of DNA present in only 10–100 copies to be amplified, such that the amount of amplified DNA can be visualized and identified. Thus, if appropriate sequences, specific to *M. tuberculosis* are selected, 10–1000 *M. tuberculosis* organisms can be identified (Brisson-Noel et al 1989). The PCR methodology is rapid, offering the potential for results being available within a few hours of sample collection. There has been a large body of experimental work evaluating many different target genes. The most commonly used sequence is termed IS6110. This sequence is specific for the *M. tuberculosis* complex and is present up to twenty times in the genome, thereby offering multiple targets for amplification (Thierry et al 1990). Many studies suggest that PCR detection of IS6110 in sputum provides useful diagnostic information. For example, in one study the sensitivity, specificity and positive predictability for PCR when compared to culture was 83·5%, 99% and 94·2% respectively (Clarridge et al 1993). Some studies have extended the work on sputum to suggest that tuberculosis can be diagnosed by PCR analysis of peripheral blood (Schluger et al 1994). On the other hand, enthusiasm has been tempered by large inter-laboratory comparisons which suggest that the IS6110 PCR may not provide consistent, reliable results when used in different laboratories (Noordhoek et al 1994). This may relate to the problem of "carry over" in which amplified products from previous assays contaminate subsequent samples giving rise to false positive results, as well the difficulty of standardizing "in house" assays between laboratories. Commercial kits have been developed which aim to provide standardization and reduce contamination. The Amplicor *Mycobacterium tuberculosis* test system (Roche Molecular systems), uses biotinylated genus-specific primers to amplify a DNA sequence which encodes a portion of the 16S ribosomal RNA, which is then bound to a *M. tuberculosis*-specific probe. A second method, the Mycobacterium Tuberculosis Detection Test (Genprobe), combines amplification of rRNA with a detection method involving an acridinium ester-labelled DNA probe specific for the *M. tuberculosis* complex (Jonas et al 1993) can also be used on sputum. Both the Amplicor and the Genprobe methods have very high specificity and are very effective at confirming the presence of *M. tuberculosis* in smear positive sputum (Bodmer et al 1994; Carpentier et al 1995; D'Amato et al 1995; Dilworth et al 1996). They have yet to demonstrate high sensitivity in iden-

tifying tuberculosis in the difficult group of patients who are smear negative but are subsequently found to be culture positive. The introduction of commercial kits to aid diagnosis should however improve inter-laboratory reproducibility.

Evaluation of Drug Sensitivity and Resistance

The long delay between collection of the sample and confirmation of the drug sensitivity of the organism may result in inappropriate treatment of patients and uncertainty over the requirement for isolation. A major advance in the understanding of drug resistance may offer a solution to this problem. A number of studies have identified genes in *M. tuberculosis* which include sequences associated with drug sensitivity or resistance. In the case of rifampicin, drug resistance is associated with mutation within a well defined 100-base pair region of the *rpoB* gene (Telenti et al 1993). Similarly, streptomycin resistance is associated with mutation in the *rpsL* gene (Finken et al 1993; Nair et al 1993; Honore & Cole 1994; Honore et al 1995) and ethionamide with the INH gene (Banerjee et al 1994). Isoniazid resistance is more complex, since it may develop following mutation of any of a large number of base pairs in the *katG* gene (Zhang et al 1992), or in the *inhA* gene (Banerjee et al 1994). Quinolone sensitivity is linked to the genes *gyrA* and *gyrB* (Takiff et al 1994). A variety of PCR-based methods attempting to offer rapid drug sensitivity testing have been evaluated. The technique of Single Stranded Conformation Polymorphism has been used to identify a number of different drug resistance patterns in selected organisms (Heym et al 1994), although the complexity of the method suggests that it will not prove suitable for routine clinical use. On a practical basis the important drug sensitivity is to rifampicin. If the organism is rifampicin-sensitive it is likely not to be MDR-TB and thus can be treated successfully with the first-line triple or quadruple therapy. Conversely, if the organism is rifampicin-resistant, it is likely that the organism is MDR-TB and more complex regimes will be required. The INNO-Lipa RIF.TB kit specifically detects rifampicin resistance (de Beenhouwer et al 1995). We have used this kit, with confirmation by automated sequencing, in the investigation of suspected nosocomial transmission, to identify rifampicin resistance within 48 h of receiving a clinical sample (Goyal, unpublished results).

Typing *M. tuberculosis* for Epidemiological Studies

The ability to examine *M. tuberculosis* DNA offers the opportunity to apply typing techniques. It is thus possible to identify whether two individuals are infected with an identical strain. Such an approach serves as a powerful epidemiological tool, and at least in theory may allow us to identify the places or patterns of human behaviour which promote transmission of the organism. The most frequently used molecular biology approach for typing *M. tuberculosis*, is restriction fragment length polymorphism (RFLP), using the IS6110 sequence as a target for probing (RFLP-IS6110). In this technique the organism is cultured and the DNA extracted. The DNA is digested enzymatically and the fragments are separated on a gel and incubated with a probe for the IS6110 sequence. Since this sequence is generally present in multiple copies, a ladder is seen on the gel and the relative position of the DNA bands on the ladder is specific to a given strain. For some strains there are five or fewer bands and a second typing method is required.

RFLP-IS6110 is proving extremely powerful in monitoring infection throughout a whole community. Small et al (1994) examined isolates from 473 patients who presented with tuberculosis in 1991 and 1992. Of the 473 patients, 191 patients had *M. tuberculosis* isolates which, on DNA typing, were shown to be identical with isolates obtained from at least one other patient. The authors made the assumption that all patients who were infected by an organism which was identical to that isolated from samples from another patient constituted a cluster of cases. Since it was extremely unlikely that two patients who had been infected by the same strain in childhood should reactivate their tuberculosis in the same city at the same time, it was postulated that all clusters represented recent infection. The authors were then able to compare various epidemiological data between patients in clusters where recent infection was suspected, and those non-clustered cases which were thought to comprise both cases of reactivation of tuberculosis as well some case of recent transmission. An association was found in patients under 60, between recent infection by *M. tuberculosis* and black or hispanic race, birth in the USA, as well as a diagnosis of AIDS. A similar study in New York gave similar results (Alland et al 1994). In this study there was again a correlation between the presence of recent infection and seropositivity for HIV, infection with drug-resistant tuberculosis, younger age, and residence in areas with a median household income below $20 000. The power of this typing methodology is that it not only documents past events but serves as a method to monitor public health interventions to prevent spread of tuberculosis, as such measures should result in a reduction in the numbers of cases in clusters. It is, however, likely that the situation is more complex than can be explained by the underlying assumptions of the above studies. It is possible that in some isolated areas, a limited number of strains have been present for many decades and that a small number of epidemiologically unrelated cases may result from infection with these endemic strains.

Another powerful application of what may be termed molecular epidemiology has been the ability to track infection by specific strains. A study of 1953 *M. tuberculosis* isolates from New York revealed that 273 (of which 259 were resistant to four or more drugs) had similar RFLP typing patterns, indicating that they were closely related (Bifani et al 1996). It has been possible, using molecular typing, to follow this strain (W strain), and monitor the disease directly attributable to it (Plikaytis et al 1994). It is possible to speculate that in the future, we may be able to provide very detailed epidemiological data specific to infection with different specific strains of *M. tuberculosis*.

RFLP analysis has, however, a number of limitations. Unfortunately, a number of strains are not suitable for examination by RFLP-IS6110 due to the small number of copies of IS6110. In these circumstances other more complex laboratory methods have been employed (Chaves et al 1996). A further disadvantage of the RFLP-IS6110 methodology is the dependence on previous culture of *M. tuberculosis*. The results of the analysis are usually available no sooner than four to six months after the patient presented to the clinician. This time delay limits the ability of the technique to provide a rapid response in

the investigation of a suspected outbreak of epidemiologically related tuberculosis.

PCR-based Typing

Recently a PCR-based typing method has been developed (Kamerbeek, unpublished). This technique termed "spoligotyping" is based on the presence or absence of specific spacer regions of DNA, located between a number of directly repeated sequences. Thus, by amplifying the gaps between the direct repeats, a different number of spacers will be amplified for any given strain. This technique has the advantage that it can be applied directly to sputum samples. The technique is also rapid. It is possible to confirm or refute within a few hours, the possibility that two patients are infected by a common strain (Goyal, unpublished results). The method is not as effective as RFLP-IS6110 at differentiating *M. tuberculosis* strains. We have found that of samples from 167 patients, isolates sharing DNA patterns with other isolates, which raised the possibility of clusters of cases, were found using RFLP-IS6110 in 56 patients, using spoligotyping in 100 patients, and using both techniques in 32 patients (Goyal et al 1997). Conversely, unique DNA patterns were found in 111 patients by IS6110-RFLP, 67 by spoligotyping and 135 by combining both methods. However, the advantages in terms of speed of spoligotyping suggest that an optimal approach may involve spoligotyping with later confirmation by IS6110-RFLP.

Conclusions

Molecular biology tools have the potential to offer a number of major advances to the field of diagnosis of mycobacterial disease and in particular tuberculosis. It is possible to speculate that in future, samples from all smear positive patients will be screened by molecular biology techniques to identify drug-resistant disease and to determine whether the patient has been infected with a strain of known high pathogenicity. Similarly, large data bases of strain-type information would allow the points of transmission of tuberculosis to be precisely defined, permitting public health measures such as improving hostel, prison and hospital ventilation systems, to be targeted to areas of greatest need. There are many constraints to the implementation of such an approach. The major limitation is financial and it is likely that even in the developed countries only a limited number of centres will be able to afford to offer these services and then possibly only for patients in pre-determined high risk categories.

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