Novel approaches to the diagnosis of mycobacterial infections

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The methods available for diagnosis of tuberculosis (TB) and other mycobacterial infections are under rapid development. We briefly review the present and the possible future laboratory tools for the diagnosis of mycobacterial infections.

Conventional laboratory diagnostic methods

A preliminary diagnosis of TB can be obtained by the detection of acid-fast bacilli by direct microscopy (DM); in developing countries this is often the only available method. Microscopy is a rapid method but lacks sensitivity and does not distinguish between different species of mycobacteria, i.e. M. tuberculosis complex and atypical mycobacteria. The sensitivity of DM is often not more than 25–40% compared with culture [1]. Under optimal conditions, it is possible to achieve a sensitivity of 60–70% compared with culture [2, 3].

Culture is still considered as the reference method for the detection of tubercle bacilli and other mycobacteria. However, mycobacterial culture is laborious, expensive and slow. The sensitivity of culture is difficult to estimate. It may be not more than 50% in clinically diagnosed TB patients [4]. The long time required for bacteriological diagnosis by culture is due to the extremely long generation time of mycobacteria. Traditional culture on Löwenstein Jensen egg media, used by most laboratories in Europe, takes up to 8 weeks. The introduction of the (Bactec) radiometric respirometry technique has drastically decreased the time needed both for isolation and susceptibility testing [5]. In our laboratory, the median time for demonstration and susceptibility testing of M. tuberculosis was reduced from 8–9 weeks to 4 weeks. For the M. avium complex the time for demonstration of mycobacterial growth was cut from 4 weeks to less than 2 weeks by this method [6].

To further improve the speed of the laboratory diagnosis, culture-independent methods have been sought.

Identification of mycobacterial cell wall components

Gas-chromatography (GC) - either alone or in combination with mass spectrometry (MS) - has been used for the detection of specific mycobacterial lipids, such as tuberculostearic acid (TSA), in clinical samples [7, 8]. TSA is present not only in mycobacteria but also in other micro-organisms which constitute the normal microbial flora in various body sites. Hence, this method is unsuitable for the diagnosis of TB in sputum and other nonsterile specimens, because of low specificity [9], but the application of GC/MS may hold some promise for the diagnosis of extrapulmonary TB [10–12]. However, the expensive equipment and the specialized staff required for GC/MS limit the value of this technique.

Immunological assays for detection of mycobacterial antigens

Mycobacterial antigens can be detected in clinical samples, using specific polyclonal or monoclonal antibodies. Recently, this approach has been applied for quantification of a M. leprae specific phenolic glycolipid in urine [13]. Similar approaches applicable to TB infections have been reported by several laboratories [14–16]. The lack of sensitivity and/or specificity of these assays limits their practical usefulness. However, in our laboratories as well as in others, efforts are made to improve these tests to achieve rapid diagnosis (within 1 day).

Serological diagnosis of TB

Mycobacteria are rich in antigens that stimulate antibody production, and several assays have been used to detect specific antibody responses in patients with mycobacterial diseases [17]. Enzyme-linked immunosorbent assay (ELISA) measuring the antibody response to semi-purified mycobacterial antigens has been most commonly used. Recently, crude antigens have been superseded by more purified antigens that are specific to certain mycobacterial species [18, 19]. The specificity of serological assays can also be improved by measuring antibodies directed towards individual antigenic epitopes by competitive inhibition of the binding of monoclonal antibodies.

A serological test for the diagnosis of TB must be significantly better than DM (i.e. specificity >99% and sensitivity >70%) to be diagnostically useful. There is at the present time no serological test that meets these requirements. However, further development of serological tests is justified considering that such a technique could...
Nucleic acid probes

The genetic information of all cells is carried in their deoxyribonucleic acid (DNA). The recent development in the knowledge of the molecular genetics of mycobacteria has made it possible to identify particular sequences of DNA that are specific for individual mycobacterial species. These unique DNA sequences can be detected using labelled oligonucleotides that are exactly complementary to the nucleotide sequence in the mycobacterial genomic DNA. Normally, the DNA double helix is very stable, but when it is heated the two chains separate. Upon subsequent cooling, rehybridization of the two complementary strands occurs. It is, therefore, possible to use short synthetic and highly specific, single-stranded, labelled DNA oligonucleotides as probes which will specifically hybridize with the target DNA in the sample.

Such DNA probes can, with high specificity, identify genus or species specific bacterial DNA sequences. Commercial probes are currently available for identification of several species of mycobacteria [20, 21]. Although such probes have been shown to be highly specific, they lack sensitivity and cannot be used directly on clinical samples. However, the combination of culturing mycobacteria in liquid medium - using radiometric respirometry for monitoring mycobacterial growth - followed by species identification with DNA probes has significantly shortened the time needed for the laboratory diagnosis to the species level of mycobacterial infections (table 1).

Polymerase chain reaction (PCR)

At the present time, much attention is focused on the use of the polymerase chain reaction (PCR), and this method has been applied for rapid detection of mycobacterial DNA by several groups [22–28, 29].

The principle of the PCR technique is based on the amplification of a given DNA sequence to such numbers of copies that it can be identified by separation on gel electrophoresis, and subsequently either with or without probing with a labelled oligonucleotide specific for the amplified DNA fragment. By PCR, a target fragment from one single DNA molecule can be multiplied $10^{12}$ fold [30].

To amplify the selected DNA sequence, the PCR technique utilizes DNA polymerases to synthesize many copies of the DNA replicate. DNA polymerases are able to elongate and make copies of single-stranded DNA starting from short stretches of double-stranded DNA. The strands in the double-stranded DNA are first separated by heating. Next, two synthesized short oligonucleotides, so called primers (each complementary to a given sequence in each of the strands of the target DNA), are hybridized with the separated DNA strands by cooling. Thereafter, one copy of each of the two DNA strands is produced by addition of DNA polymerase and nucleotide building blocks (deoxyribonucleotides). The "chain reaction" is achieved by repeating this sequence of events over and over again, for e.g. 40 cycles, until a sufficient number of copies of the desired DNA sequence is reached.

The principle for this method has been known for quite a long time, but its application has been both laborious and expensive, since the DNA polymerase enzymes used have been heat sensitive, and have had to be added de novo after each temperature cycle. A large step forward was the introduction of the Taq polymerase [31]. This polymerase is produced by the thermophilic bacterium Thermus aquaticus, which was originally found in hot water springs. Its DNA polymerase proved to be heat-stable to the temperatures that are needed to separate double-stranded DNA molecules. The discovery of the Taq DNA polymerase revolutionized the PCR technique, in that it allowed automatization using automatic thermocyclers.

The use of specific DNA probes, as well as the PCR, for diagnosis requires detailed examination of the molecular genetics of the organisms, especially the identification of those DNA sequences that are specific for the organism in question. During the last decade, several such unique sequences have been reported for the M. tuberculosis complex [22–27].

Provided that the DNA sequence is appropriately chosen, the PCR method may be highly specific and sensitive. Recent studies of sputum samples have reached a sensitivity of around 95% [9, 32]. A critical factor for the sensitivity is the method used for DNA extraction, since mycobacteria have an unusually compact lipid-rich cell wall.

The specificity of the test is dependent on the DNA sequence chosen for amplification. However, false positives are in most instances due to other factors than the DNA sequence. An important factor is the risk of contamination with (amplified) mycobacterial DNA. Much effort has been made to circumvent this risk, which, however, is still a reality [33].

A crucial factor, when a new method is evaluated, is the sensitivity of the "gold standard". Normally, the standard is conventional culture. The significance of a PCR positive result when the culture is negative is
evaluated in several ongoing prospective studies of PCR positive, culture and microscopy negative, cases. It is important to bear in mind that the PCR technique detects both live and dead bacteria, and the test can be positive several months after initiation of an effective treatment.

Recently, another gene amplification test for direct identification of M. tuberculosis has become commercially available (AmpliProbe, GenProbe Inc., San Diego, Cal, USA). This test combines amplification of M. tuberculosis recombinant ribonucleic acid (rRNA) with detection through a specific chemiluminescent DNA probe. The first evaluations of this system indicate a sensitivity and a specificity similar to those obtained by conventional PCR [34–36].

The cost of a PCR test is at present still high, but varies with the methods used, and is likely to decrease with the advent of more automated systems.

Fingerprinting of mycobacterial strains

Molecular genetic methods are also of use in studies of the epidemiology of tuberculosis and other mycobacterial diseases. Recent outbreaks of multidrug-resistant tuberculosis have been traced by DNA “fingerprinting” of TB isolates, using restriction fragment length polymorphism (RFLP) [37]. The principle of the method is to extract the mycobacterial DNA from cultured organisms, digest it with chosen DNA cleaving restriction enzyme(s), separate the DNA fragments produced by gel electrophoresis, whereafter certain repetitively occurring DNA sequences (insertion sequences) are identified by specific probes [38]. This results in a “fingerprint” highly specific for each individual mycobacterial strain. This molecular genetic identification on the subspecies level now makes it possible to trace the spread of specific strains (clones) in the community.

In conclusion, identification of specific DNA sequences allows identification of mycobacterial isolates on genus, species and subspecies level. In fact, each individual (myco)bacterial strain has its unique pattern, which may be identified and used for epidemiological studies. Instead of amplification of live bacteria by culture, molecular biological methods (e.g. PCR) allow the amplification of specific DNA sequences for identification of mycobacteria in clinical samples. Such DNA amplification/identification is potentially more sensitive than culture. It is also rapid, which, considering the slow growth of mycobacteria, may be even more important. It can, however, not replace culture when susceptibility testing is required. This molecular method is particularly useful for epidemiological purposes, where the structure of the TB laboratories, especially in developing countries, will play an important role in the overall strategy for the control of tuberculosis.

In the community, such DNA amplification/identification may be of use in the control of tuberculosis, but it has to be remembered that a positive test is only of value if the diagnosis of the disease is confirmed by conventional diagnostic methods.

References


