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THE IMPACT OF NEW TECHNOLOGY ON THE LABORATORY'S CONTRIBUTION TO THE DIAGNOSIS AND MANAGEMENT OF MYCOBACTERIAL DISEASE

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From the time of the discovery of the tubercle bacillus in the late nineteenth century until the introduction of chemotherapy in the mid-twentieth, the role of the laboratory in the management of tuberculosis was limited because the treatment of the disease was nonspecific. The advent of specific chemotherapy and the recognition of human diseases caused by a number of mycobacterial species other than *M. tuberculosis* increased the scope and importance of the clinical laboratory in guiding the diagnosis and management of mycobacterial disease. This included the isolation of mycobacteria, the identification of the isolates, the determination of their susceptibilities to chemotherapeutic agents and their subtyping for epidemiologic purposes. In spite of the enhanced role it has played in the past forty years, the laboratory's contribution has been impeded by the slow growth of mycobacteria, which causes delays of weeks or months between submission of a specimen and the availability of a definitive report. In the meantime both the urgency and the complexity of diagnosis and management of mycobacterial disease have increased with the emergence of epidemics of these diseases associated with the acquired immunodeficiency syndrome (AIDS). This development has also increased the need for recognition of tubercle bacilli in such specimens as blood and stools[1)59)73)116)], which were only infrequently studied in past years.

Recent developments in microchemical and immunologic technology, and especially molecular biology, are greatly reducing the time needed to get information that contributes to diagnosis and management of mycobacterial disease. These include solid phase immunologic assays, sequencing of selected nucleic acid regions and development of specific probes, and exquisitely sensitive isotopic or enzymatic amplification techniques for the detection of traces of products.

DIAGNOSIS

Specific diagnosis may be based on detection of the host response to the mycobacteria by immunologic methods, detection of mycobacteria by culture, or detection of mycobacterial products by chemical or serologic means. But before we get into the details of methodologies, I want to point out the simple but critical fact that there are two ways of asking the diagnostic question. There is the narrow, specific question, "is *M. tuberculosis* the cause of this patient's disease?". The answer is yes or no. Or we can ask a much broader question, "what organism is the cause of this patient's disease?", with a choice of many different answers. The way you

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ask the question will be important in deciding which of the different diagnostic methods to use. As shall be discussed, there are some tests that are limited only to the detection of *M. tuberculosis* ; often the most important thing to know is if this organisms is indeed the etiologic agent of a given infection. But in other circumstances, where the patient's disease may be caused by a number of different mycobacteria and where good laboratory resources are available, the broader question is preferable.

Immunologic diagnosis. Immunologic diagnosis includes both skin and serologic tests. I am not going to say much about skin tests, because they are still not specific or sensitive enough to confirm the presence of active disease. Traditionally a negative test has helped to rule out tuberculosis, but many HIV positive individuals, as well as AIDS patients with proven tuberculosis, do not exhibit a positive tuberculin test[12)35)59)72)]. Modern genetic techniques permit the cloning of genes for specific mycobacterial proteins into the fast growing bacterium, *E. coli*. Studies of at least 10 cloned *M. tuberculosis* proteins in the 14 to 88 kD size ranges are providing insights into the epitopes that are most active at the T-cell level. This may eventually lead to a superior vaccine or a highly specific and sensitive skin test reagent[39)67)97)], but products with the necessary sensitivity and specificity are not yet available for practical application.

Serology for tuberculosis. The dominant serologic response of patients with tuberculosis is to antigens that are highly conserved and rich in polysaccharides[99)]. Unfortunately the dominant polysaccharide antigens are common to all species of mycobacteria[25)], including many that we routinely encounter in our environments, so the responses are not specific to *M. tuberculosis* infection. Serologic tests may prove useful in diagnosing tuberculosis through application of ELISA, RIA and dot blot tests with selected peptide antigens that are rich in those epitopes that are associated with antibody production by humans. These potentially useful peptide antigens can be recognized by cloning and western blot assay[39)45)97)]. T-cell reactivity of cloned *M. tuberculosis* antigens appears to be inversely correlated with the antibody responses to them[39)]. However, no blood tests are now available with sufficient predictive power to provide a highly reliable diagnosis of tuberculosis in low prevalence areas[6)13)15)43)44)78)103)105)].

The structural divergence of isofunctional proteins from different species of bacteria is reflected by the immunologic distances between them[75)] ; different enzymes from the species within a family show parallel evolutionary divergence of structure[18)] which means that some antigenic regions on analogous molecules are common to all of the species, and a small proportion of the epitopes are species specific. Our own studies on structural divergence of mycobacterial catalases[106)] and those of Tasaka and colleagues on alpha proteins[96)] suggest that rarely, if ever, will mycobacterial protein antigens be found that are completely specific for one species. Even if one is found, it may not be one to which all tuberculosis patients produce antibody. Detailed study of many proteins and selective production of peptides that are rich in those epitopes that are specific to members of the *M. tuberculosis* complex and to which patients' sera react may permit the development of an antigen mixture that will meet the criteria for a successful ELISA or dot blot test for diagnosis of pulmonary tuberculosis[97)].

Among the most urgent problems in diagnosis of mycobacterial disease is that of tuberculous meningitis, because the prognosis is so grave if treatment is delayed. Tests have been developed for detection of both antigen and antibody in CSF. Most of the studies on *antibody* detection used crude antigen mixtures, with a median sensitivity of about 70 % and specificity of about 90 %. Single antigens were less sensitive than crude products[3)14)21)42)66)74)103)]. The median sensitivity of *antigen* detection in a series of 7 reports was about 65 %, with a probable 98 % specificity[3)50)51)58)77)83)103)]. There was a steep decline in detection of antigen after about 4 weeks of therapy. It is yet to be determined whether that apparent decline was due to actual disappearance of antigen or to *in situ* development of immune complexes that masked the

presence of antigen. In pulmonary tuberculosis, there may be antimycobacterial IgG in cerebrospinal fluid (CSF) due to passive diffusion from the serum. However, when meningitis occurs there is also significant *in situ* production of antibody in the CSF[92]. Thus, when adjusted to constant IgG concentration, the *ratio* of specific activity of the antibody in CSF to that in the serum of tuberculous meningitis patients is elevated, and this may be diagnostically useful. In the face of urgency of diagnosis of tuberculous meningitis, the existing test antigens and capture antibodies may be useful, but high priority research to refine these tests could lead to rapid improvement in their diagnostic value. CSF assays for both antigen and antibody, as well as for specific immune complexes should be standardized, and all three applied to a given specimen. Furthermore, the test results should be converted to specific activities per mg of total IgG in both the serum and the CSF in order to distinguish between passive diffusion of circulating antibody and its *in situ* production[92].

A specific glycolipid antigen is available for serodiagnosis of leprosy[16] and tests with this antigen are positive in 91–96% of patients with active multibacillary/lepromatous disease ; the sensitivity is only half that in patients at the paucibacillary tuberculoid end of the rating scale[32].

In contrast to fairly good prospects for development of a satisfactory test for diagnosis of tuberculosis, there is less reason to hope for a serologic test that will confirm active disease caused by environmental mycobacteria such as *M. avium, M. intracellulare, M. scrofulaceum, M. kansasii,* etc. This is because many people produce antibody that appears to be due to subclinical colonization with these organisms and this is especially common in individuals with evidence of lung pathology, i. e. the ones most in need of good diagnostic procedures[105]. Pulmonary diseases due to environmental mycobacteria such as members of the *M. avium* complex occur most frequently in individuals with some other lung impairment[112].

Microbiologic diagnosis. Examination of a simple smear permits recognition of patients who are excreting the greatest numbers of tubercle bacilli, and are thus most likely to be spreading tuberculosis. It will continue to be a very important diagnostic tool in areas with a high prevalence of tuberculosis, but insensitivity and lack of specificity limit its value for routine and definitive diagnosis in low or variable prevalence situations[34)55)56]. The great advantage of culture methods is that they are not limited to the detection of a single selected pathogen, but permit detection of a variety of mycobacterial species. Conventional culture for detection of *M. tuberculosis* and most of the opportunistic mycobacteria on solid medium takes an average of 2 to 3 weeks under the best conditions and longer when the number of bacilli in a specimen is small[60]. The advantage of solid medium is that the number of colonies can be counted, which is important in judging the probable significance mycobacteria other than *M. tuberculosis.* Other advantages include he availability of colonies for identification procedures and drug susceptibility tests, as well as the recognition of mixed cultures and detection of mycobacteria in the presence of non–acid –fast contaminants.

An incremental breakthrough in rapid diagnosis was made with the introdution of radiometric methods for detection of viable mycobacteria[80]. Cultivation of mycobacteria in the BacTec liquid medium system and daily sampling of head gases for the evolution of radioactive CO_2 from the ^{14}C labelled palmitate substrate permits radiometric detection of growth well before there is any visual evidence of that growth. The *efficiency* of recovery of *M. tuberculosis* from pulmonary secretions by radiometric technique is comparable to that of conventional methods. However, the mean *time* required for radiometric detection of *M. tuberculosis* averages only 7 to 10 days with smear positive specimens and 14 to 21 days for smear negative specimens, i. e. about 10 days earlier than with conventional culture[2)76)80)85)86]. In studies of blood specimens from AIDS patients, *M. avium* complex was detected 3 to 5 days sooner in radiometric broth than on solid medium[54)88] ; continuous agitation of the broth

may produce even earlier growth[48].

Members of the *M. tuberculosis* complex can be identified directly from the original radiometric culture with a nucleic acid probe[5)70)] or on subculture to NAP medium[95]. Centrifuged sediments from radiometric broth cultures have also been identified rapidly by use of nucleic acid probes that are specific for *M. intracellulare* and *M. avium*[5)19)70)]. Since nucleic acid probes are commercially available for only a few of the species likely to be encountered in clinical specimens, identification of the others requires subculture. This situation may change if other probes come on the market that permit identification of many species with a single small inoculum, as will be discussed below.

The disadvantages of the radiometric system are the ones inherent in any system that relies on growth in liquid medium instead of the appearance of individual colonies on solid. There is no reliable way to estimate radiometrically the numbers of viable bacilli that were present in the original inoculum ; different species, and even different strains of a species, have different growth rates. The condition of the specimen and its treatment will influence the growth lag phase, so the rates of evolution of CO_2 cannot be used to extrapolate back to an inoculum size. Contamination is two to three fold greater by radiometric than by conventional methods. Recognition of mixed cultures requires, with few exceptions[20], parallel inoculation of both solid and liquid medium[54] or subculture of serial dilutions of the broth culture to solid medium. Thus the radiometric method represents a great improvement in the speed of diagnosis, but a backup culture on solid medium should always be made in case problems arise in identification or interpretation of the significance of an isolate.

Because of the genetic determinants of rates of replication and metabolism of mycobacteria, we are probably close to the limit of speed with which we can detect these organisms by methods that depend on their growth. As a consequence, most efforts at improving diagnostic methodology are now being directed toward the detection of bacillary products that may be present in the original clinical specimen. These technologies may provide diagnostic information in a few hours, and include nucleic acid amplification and identification, antigen detection, and chromatography of bacillary lipids.

Nucleic acids. Polymerase chain reaction, or PCR, technology represents perhaps the most revolutionary of all modern techniques in terms of its potential contribution to rapid and precise diagnosis of a wide range of diseases. It is potentially the most sensitive of all procedures that have been applied to the detection of bacteria, because a single segment of DNA from a single organism can serve as a template upon which an unlimited number of copies of the molecule can be synthesized at a logarithmic rate, yielding detectable product in a very few hour. The principles and practical prospects of PCR in clinical diagnosis have been well reviewed by Persing[69]. With individual cycles taking only a few minutes, 30 cycles of replication can be accomplished in 2 or 3 hours, potentially yielding 10^9 copies from an original molecule of DNA in the original sample. By the selection of appropriate primers, characterization of the synthesized amplicon by its size, and/or use of very specific detection probes one might detect and identify a single cell of *M. tuberculosis* in a few hours under ideal conditions. Since conditions are never ideal and polymerase action is susceptible to inhibition by substances in clinical samples, technical limitations prevent the method from yielding precise quantitative results at present ; it would be most useful for diagnosis of disease due to such consistently pathogenic mycobacteria as *M. tuberculosis, M. leprae,* and probably *M. paratuberculosis,* but not common environmental mycobacteria, where the number of organisms detected helps determine their significance.

Various primers have been used to initiate PCR detection of *M. tuberculosis* in clinical specimens. They were directed toward amplification of sequences that code for known proteins[22)68)71)89)], and/or for DNA regions that were selected for specificity[22)28)41)89)]. Nested primers have been used for secondary amplifica-

tion where the primary PCR has not appeared to be sensitive enough[33)71)]. In addition to detecting *M. tuberculosis* in pulmonary specimens and in cerebrospinal fluid[52)], the method has found application in very specific diagnosis of leprosy[23)27)38)] and Johne's disease[101)], for which unique repeating sequences of *M. leprae* and *M. paratuberculosis* DNA were used as probes for identification of the amplified DNA.

It is difficult to evaluate the relative efficiency of PCR for detection of tubercle bacilli in clinical specimens. The only definitive standard with which it can be compared is the culture. However, the procedures that are used for decontaminating the usual clinical specimens can kill up to 90% of the bacilli, which means that specimens that contain 10 or fewer bacilli may yield negative results. The PCR procedure does not require viable bacilli, so it would be expected to be more sensitive than culture. In almost every diagnostic trial reported, the PCR procedure has been as sensitive or more sensitive than the culture. However, it is hard to interpret positive PCR results when cultures are negative. The method is so sensitive that contamination of a lab with traces of the very stable, repeatedly generated amplicon segments of DNA could be a serious problem by causing "false positive" reactions[69)]. Such contamination is almost inevitable given the numbers of amplicon copies produced in a single test and the likelihood that pipetting procedures will produce micro-aerosols that can contaminate the pipettors and reagents. Novel techniques have been developed to "sterilize" the amplicons that are produced in this test, preventing them from influencing subsequent procedures; this problem and some methods for protecting against amplicon contamination were reviewed by Persing[69)]. All attempts to evaluate PCR procedures for detection of *M. tuberculosis* in real clinical specimens must include very stringent control procedures.

The major value of PCR lies in the unprecedented speed with which a diagnosis can be made. The procedure, from processing of the clinical specimen through the *amplification* of the DNA, takes less than 8 hours. At present, the detection of the amplicons, and the proof that they are indeed derived from *M. tuberculosis* and not some other mycobacteria involve agarose gel electrophoresis and detection either by direct stain or by blotting and visualization with labelled probes, and add a day or more to the time required for a definitive diagnosis[26)28)]. The specific identification may be based on the size of the amplicon, as estimated from its electrophoretic mobility and/or its response to very specific probes, or even on sequence analysis of the product[4)]. However, eventually a procedure based on the principles of the Gen-Probe for specific identification of nucleic acid in the original liquid preparation will probably be developed and permit the entire PCR procedure, from processing of specimen to identification of the *M. tuberculosis* gene product to be completed in one working day.

PCR is presently very expensive for application on a large scale and requires sophisticated laboratory personnel. Although a number of investigators have evaluated the technique with different primer and detection systems and shown it to be very fast and sensitive, I am not aware that the technique has ever been licensed for diagnosis of tuberculosis. In the meantime, in January of 1992, an isothermal method for amplification of *M. tuberculosis* DNA was described as an alternative to PCR. By using two primers with a restriction endonuclease and an exonuclease-deficient form of the large fragment of *E. coli* DNA polymerase (ex-Klenow polymerase), Walker and colleagues[102)] achieved logarithmic amplification of target DNA from *M. tuberculosis* at a constant temperature by nicking amplification products at defined sites and both extending and displacing DNA as it is generated with the deficient Klenow polymerase. The existence of two alternate procedures for DNA amplification may accelerate the development and commercial availability of a test that has such potential importance in helping control and even eradicate tuberculosis.

Even when nucleic acid amplification becomes readily available for routine clinical detection

of *M. tuberculosis,* and assuming that the problem of amplicon contamination can be avoided, there will still be a need for cultures for drug susceptibility testing. Cultures will probably be needed for recognition of mixed infections as well. The applicability of PCR to diagnosis of infections caused by other mycobacteria is problematic. Primers that bracket regions of the mycobacterial DNA that code for the hypervariable regions of 16S rRNA can be used to induce amplification of DNA from any species of mycobacteria[8], and the amplicons will have sequences that are specific to each of the known species[4]. The actual sequence determination or the use of probes for these specific sequences of that amplified DNA can be used to recognize the amplicons derived from any mycobacteria in a specimen, presumably even when two or more are present. However, just demonstrating that a *Mycobacterium* other than a member of the *M. tuberculosis* complex, *M. paratuberculosis* or *M. leprae* is present does not prove that it is causing disease. Knowledge of the actual numbers of bacilli present is important to the interpretation. Thus the need for quantitative estimates of the numbers of bacilli of each species detected will still represent a problem in ascribing significance to the respective isolates.

Antigens. Attempts have been made to detect mycobacterial antigens in clinical specimens. Poor sensitivity was achieved in the detection of *M. tuberculosis* antigens in sputum[113], and very little has been done to improve upon and exploit this approach. Because of the urgency of diagnosis of tuberculosis meningitis, greater efforts have been expended to detect mycobacterial antigens in cerebrospinal fluid, using polyclonal antisera, with some success[3)50)51)58)77)83)103], as was mentioned earlier in this report.

The very specific and well characterized phenolic glycolipid surface antigen of *M. leprae* has been detected in the serum of leprosy patients by both ELISA and chromatographic methods. The results were positive in 71% to 100% of multibacillary patients, depending on the type of pathology seen ; significant declines in serum glycolipid antigen levels were seen after chemotherapy[17)81]. *M. leprae* antigens have also been detected in urine and nasal washings of leprosy patients toward the LL end of the Ridley–Jopling scale[93].

Lipid chromatography. A third approach to the accelerated detection of mycobacterial products is analysis by gas chromatography with very sensitive selective ion mass spectrometric monitoring of fatty acids. This technique requires concentration of the bacilli in a specimen, extraction of the cells in the sediment, and identification of one or more components by chromatography. The lower limit of detection is about 1000 bacilli[66], which is about ten times more sensitive than the smear, but much less sensitive than culture or DNA amplification. The main value of the method is its speed, with results available within 5 hours after receipt of the specimen[29]. The most frequently studied product is tuberculostearic acid (TSA), which is produced by many different species of mycobacteria as well as some members of other genera[29)61], i. e. it is not specific to *M. tuberculosis.* The most promising application of the TSA detection system is in the examination of cerebrospinal fluid. Mycobacterial meningitis is almost always associated with *M. tuberculosis* so the lack of specificity is not a serious problem. Reports have ranged from 83 to 95% sensitivity, with 90% or better specificity and some improvement is reported when whole carboxylic acid profiles, rather than the single TSA peak are used for diagnosis[9)29]. The method is probably too labor intensive for use with specimens other than CSF, and it requires very careful calibration of peaks to avoid error[9]. The test tends to revert to negative within as little as a week after specific chemotherapy has been started[9].

The various methods just described for ultrasensitive and rapid detection of bacterial products may not be practical for the recognition of mixed infections and do not eliminate the need for a culture for drug susceptibility testing. They are also not very quantitative, so while they may be useful for diagnosing disease due to nonenvironmental mycobacterial pathogens such

as *M. tuberculosis* or *M. leprae,* they offer less definitive answers with other species of mycobacteria, where the number of organisms seen helps determine their significance. Any one or all of these methods may be valuable for rapid diagnosis, but a culture should still be made for assessment of significance and follow-up studies.

Identification of isolates. Identification of mycobacterial isolates has traditionally required phenotypic characterization of living cultures with a panel of tests that may require weeks to complete[104]. The main contribution of new technology to phenotypic identification has been the computerized processing of data to permit identification of a variety of species on the basis of a large number of properties, and with a defined order of reliability through the use of probability matrix programs[104)108)~110)]. This approach is especially suited to the broad diagnostic question since, as new species are discovered, a simple adjustment of the matrix can permit their inclusion without requiring the preparation of new specific reagents. To use the Bayesian probability matrix for identification of mycobacteria[104)108~110)], the results of 15 or 20 tests are entered into the computer, and a printout is provided which shows which species it most resembles. It gives a normalized list of ID probability product scores with all of the species that it resembles. If the ID score of the strain to the most likely species is 100 fold or more greater than that of the second most likely species, the ID score is considered satisfactory. However, the program also compares the probability product of the unknown strain to that expected of a "perfect" strain of the most likely species, and the ratio of perfect to test score must be less than 100 for the identification to be definitive. The program also lists the test properties of the strain, shows which results deviate from those of the closest species, and, if needed, it suggests additional tests that might increase the ID score to make it definitive.

Some new tests that do not require growth or active metabolism can reduce the time required for identification of cultures to a few hours. Examples include nucleic acid probes, specific cross absorbed antibodies to detect epitopes on bacterial products that are unique to each species, and computer analysis of chromatographic patterns of bacillary extracts.

Nucleic acid probes. Nucleic acid probes are presently available commercially for only a few species. To make them, the manufacturer determines the nucleotide sequences of the variable region of the ribosomal RNA (rRNA) of the *Mycobacterium* and identifies a region of the molecule that is unique to the desired species or complex. A single strand DNA fragment that is complementary to that region is then synthesized. A sample of the culture to be identified is disrupted sonically to release its RNA, and an excess of the probe is allowed to react with this extract. If the probe encounters a complementary region of RNA, i.e. one that corresponds to the species for which it is specific, hybridization occurs and a duplex is formed. Two methods can then be used to detect the duplex. The probe may be synthesized with a radioisotopic label and is mixed with the specimen and a solid adsorbent, hydroxyapatite. The hybridization conditions are such that only a nucleic acid duplex, but not a single stranded molecule will stick to the hydroxyapatite. The non-hybridized free probe is washed away, and the amount of radioactivity sticking to the hydroxyapatite is measured to determine if hybridization has occurred. Alternatively, an unlabelled probe is mixed with a fluorescent dye and the specimen. Whenever a hybrid is formed, some of the dye is trapped in the duplex. The remaining free dye is destroyed by mild chemical treatment, but the dye that is intercalated into the hybrid is protected from degradation. Only the protected dye will fluoresce, so the presence of duplexes can be detected in a fluorometer.

The nucleic acid probes are very specific and easy to use. The presently available 16S rRNA directed products require a separate probe tube and a separate sample of specimen for each species to be identified, and probes for only 4 species are now available. They have proven especially useful in identifying strains that were isolated from clinical samples in the Bactec

radiometric broth system, thus taking advantage of the great speed offered by both of the techniques, achieving isolation and identification in three weeks or less[5)19)70)]. Saito and his colleagues have also used the Gen-Probes for *M. avium* and *M. intracellulare* to confirm the redistribution of serovars among these two species and he showed that some of the higher numbered serovars in the *M. avium* complex do not react with either of these two species probes[84)]. A probe has recently been introduced that reacts with these strains[62)], and Ferguson and colleagues have presented evidence from 23S rRNA studies that these may represent subspecies of *M. intracellulare*, rather than a new species[30)], results that are consistent with our own, as yet unpublished observations based on serology of catalase.

Instead of identifying a mycobacterial nucleic acid segment by probing it is possible to determine the actual nucleotide sequence of the variable region of 16S rRNA either directly or by inference from the structure of the region of the DNA that codes for the RNA[4)7)8)82)]. The advantage of this approach is that no actual reference samples are needed against which to test the sample ; the relevant sequences of all of the known mycobacteria can be entered in the computer, and the data base searched for the sequence that corresponds to the one determined on the test specimen. The obvious disadvantage is the sophisticated equipment and technology that are needed to perform this task, and the consequent high cost of performing these analyses on a routine basis.

Antibody probes. An alternative to analysis of nucleic acids is the use of antibody probes to specific regions of protein molecules. The intrinsic enzyme dot system developed in our laboratory uses a detection system that is based on the reaction of an organism's own enzyme, which has bound to a dot on a membrane that corresponds to specific antibody to that enzyme[107)]. We have isolated the T-catalase[106)] from 11 different species of mycobacteria to date and immunized rabbits with each of them. Dots of IgG from these sera were placed on a membrane strips, which were then soaked in crude extracts of mycobacteria, washed and incubated in a developing solution of diaminobenzidine and H_2O_2 ; catalase in the crude extract that bound to the IgG dots appeared as a dark spot. Crude antibody to catalase from any *Mycobacterium* tends to cross react with the enzyme from most other species. However, we had previously shown[107)] that all of these catalases had some epitopes that were unique to each species. Therefore we cross absorbed the crude IgG preparations with catalases isolated from heterologous species to remove the antibody against epitopes that were common to other species. The titers dropped dramatically after cross absorption, demonstrating that most of the catalase protein structure is identical for all of the species, but some antibody remained that was specific. To identify an unknown organism, we placed drops of a selected concentration of each of the cross absorbed antibodies, as well as unabsorbed polyvalent antibody to serve as an internal standard, onto a membrane strip and immersed the strip in sonic extract of the unknown organism. After incubation of the strip in developing reagent color developed at two sites, the polyvalent internal standard dot and the dot of antibody that corresponded to the species to which the test organism belonged. Four species are illustrated in Fig.; *M. asiaticum, M. avium, M. intracellulare* and *M. kansasii*. This intrinsic enzyme dot assay incorporating many dots, each corresponding to a single species on a single strip, requires the use of a smaller initial sample of the clinical isolate than if a number of separate and individual test had to be run. The technique also permits use of very crude specimen extracts, since other proteins do not interfere with the specificity or sensitivity of the test, and it requires fewer steps than is required for a second antibody type of detection system. Thus the test provides much information for a low cost in materials and labor.

The use of cross absorbed antibodies to a sharply defined antigen, has also been the basis of the recent alpha protein studies. These antigens are produced by most mycobacteria, but immunodiffusion analysis provided evidence

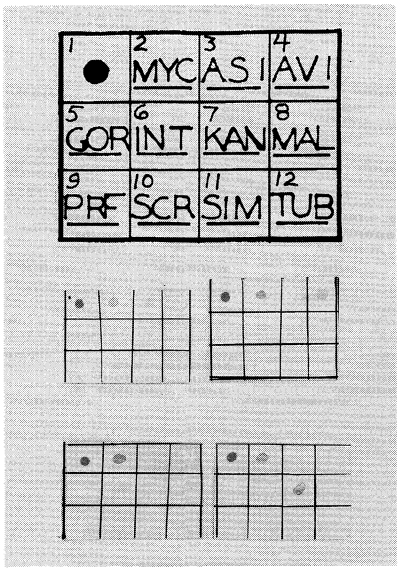

Fig. Example of intrinsic enzyme (T-catalase) immunoassays for identification of four mycobacterial strains. Dot 1 is a dye dot for orientation of strip. Dot 2 is a polyvalent antibody dot to confirm presence of T-catalase. Dots 3 to 12 : ASI, *M. asiaticum* ; AVI, *M. avium* ; GOR, *M. gordonae* ; INT, *M. intracellulare* ; KAN, *M. kansasii* ; MAL, *M. malmoense* ; PRF, "M. paraffinicum" ; SCR, *M. scrofulaceum* ; SIM, *M. simiae* ; TUB, *M. tuberculosis*. (Note, the MAL dot was not satisfactory with a known *M. malmoense* sample and should be disregarded)

of epitopes that were unique to individual species[31)114)115)]. Tasaka etal have since applied the modern methods of Western blotting[96)] and ELISA to extend this work to identification of isolates.

Chromatography. Analysis by chromatography of extracts has also been used to identify cultures. Early attempts used gas-liquid chromatography of methyl derivatives of mixed organic acids in saponified extracts ; interpretation was based on a dichotomous key of ratios of selected peak height or complex patterns. These methods yielded only partial identification of most of the clinically significant mycobacteria ; supplemental biochemical testing was needed for satisfactory identification[63)98)]. Butler and Kilburn have applied a similar concept to the analysis p-bromophenacyl esters of mycolic acids by HPLC. Each mycolate peak is assigned a number and a computer program calculates the peak height ratios for each peak pair in the elution pattern[10)11)]. These peak pair ratios are used to track a strain through a dichotomous identification scheme with 98% accuracy. The main concern with dichotomous schemes is that a strain with a single discrepancy will be thrown into the wrong limb of the tree, and no diagnosis, or a wrong diagnosis may be made.

DRUG SUSCEPTIBILITY

The conventional methods for drug susceptibility testing are based on growth from standardized inocula on solid culture medium containing the desired concentrations of the different drugs. These tests take an average of 3 weeks to yield results. Although different criteria for resistance are used in different institutions, consistent application of a method in a given institution will yield results that can correlate well with clinical experience with infections due to *M. tuberculosis*. The *in vitro* susceptibility tests are generally less reliable in predicting therapeutic efficacy in diseases due to other mycobacteria.

The only major practical innovation in susceptibility testing in the past decade has been the application of the radiometric culture method[91)] to this task. By incorporation of various concentrations of drugs into the Bactec medium and monitoring the rate of evolution of radioactive CO_2 in parallel with that from drug free controls, the susceptibility of subcultures can be estimated an average of 2 weeks earlier than by conventional methods[76)80)]. Mycobacteria appear to be slightly more sensitive to drugs in liquid than on solid media, so adjustments must be made in the interpretation of results to account for this[46)]. Of special interest is a report of the estimation of susceptibility of *M. tuberculosis* to the important drug pyrazinamide in a pH 6 radiometric broth[87)] ; susceptibility to this drug has been very difficult to estimate on solid medium.

There has been a report of the use of the Gen-

Probe DNA/RNA probe to assess the susceptibility of *M. tuberculosis* to isoniazid[53]. The liquid medium containing the drug was seeded with a standard inoculum of *M. tuberculosis*. After 3 to 5 days of incubation, the amount of growth, as reflected in the amount of rRNA detected with the probe, was compared for control and drug containing media.

Attempts may be made to define regions in the chromosome or other nucleic acid structures of mycobacteria that are associated with resistance to different drugs, but it remains to be seen whether they will lead to PCR and probe methods for rapid recognition of resistance. The problem would be complicated by the fact that resistance to a drug can be the result of either the acquisition of a resistance factor, or the loss of a susceptibility factor, such as catalase for isoniazid[65)90)] or pyrazinamidase for PZA[57]. Such tests would also be difficult to interpret, since all populations of predominantly susceptible bacteria contain some resistant mutants, and the relative proportions of susceptible and resistant clones play a major role in determining whether a given drug or combination will be clinically effective.

EPIDEMIOLOGY AND STRAIN MARKERS

In attempting to establish point sources of tuberculosis infections one must identify individual strains beyond the level of species. Among strain markers that have been used in the past for epidemiologic purposes are drug susceptibility patterns and phage patterns. The drug patterns are too changeable and not specific enough, although they have been used in conjunction with phage patterns to track outbreaks of tuberculosis[36]. While useful information for tracking miniepidemics has been derived from phage typing of *M. tuberculosis*[94], the phage patterns are limited in number, and standard methods must be rigidly adhered to if results are to be comparable between different laboratories[79]. Detection of plasmids by electrophoresis has found some utility in epidemiologic studies of the *M. avium* complex, especially comparing strains from AIDS patients with strains from other sources[49]. The method obviously is useful only for strains that carry plasmids, and the patterns are relatively limited. Plasmids are found more frequently in disease associated strains of the *M. avium* complex than in environmental organisms[49], and perhaps even more frequently when associated with AIDS[40].

Recent studies on the distribution of restriction fragment length polymorphism (RFLP) patterns of DNA extracted from tubercle bacilli and other mycobacteria suggest that this technique may be the long sought method for typing of these organisms for epidemiologic purposes. It is relatively easy to standardize and perform. Cultures that have been isolated from the patients or environmental sources under study are extracted to release their DNA. If they are all derived from the same strain, the DNA from all should be identical. If they do not represent a single strain, there may be minor differences in some of the sequences present on the genome. All bacteria have some nucleotide sequences that are recognition sites for different bacterial restriction endonucleases. That is, each enzyme has a specific sequence of about 4 to 6 nucleotides that it recognizes and it cuts DNA at the site of that sequence. A very small change in DNA structure, i. e. deletion of only one site can produce a major difference in pattern ; i. e., it can yield a *difference* of three bands corresponding to the disappearance of one large fragment as it is nicked into two new small fragments. The RFLP method, whether one uses a single restriction endonuclease or several in combination, allows one to determine whether two cultures represent the *same* or *different* strains, but one must be cautious in trying to infer much about the quantitative differences or evolutionary distances between them solely on the basis of RFLP patterns.

A number of significant epidemiologic observations have resulted from RFLP typing of mycobacteria. A single RFLP type of *M. avium* is dominant among the strains of members of the *M. avium* complex that are responsible for infections in patients with AIDS in Europe and North America[37]. It is not clear whether this reflects transmission between AIDS patients, or

a particular level of invasiveness of a common environmental strain for individuals with this type of immunologic impairment. It is of interest, though, that a variety of serovars were represented within the single dominant RFLP type. A single RFLP pattern is seen among strains of *M. leprae* from leprosy patients over wide range a wide geographic range, suggesting the existence of a single highly conserved strain of this species throughout the world[111]. This observation is compatible with the extremely fastidious nature of the organism, and the extremely narrow ecological niche that it occupies. The method has proven exceptionally valuable in tracking miniepidemics of tuberculosis i. e. disease due to *M. tuberculosis* and identifying source cases in selected populations[24)64)100)]. This is especially important in view of the contagiousness of this organism and the emergence of multiple-drug resistant cases, especially among HIV positive individuals.

SUMMARY

Diagnosis of tuberculosis with a serological blood test is not yet a routine procedure, but there is hope for the emergence of a polyvalent yet specific antigen, through one or another of the methods of modern biotechnology. There is probably already enough information available to permit standardization of a four pronged test for tuberculous meningitis based on detection of antigen, antibody and immune complex, and relating it to total IgG present in CSF. Because of common colonization by environmental mycobacteria, the prospects for a diagnostic serologic test for disease due to mycobacteria other than *M. tuberculosis* are less encouraging.

A number of different primers and probes have been reported for use in PCR based diagnosis of tuberculosis, but it remains to be seen which, if any of these will be licensed for manufacture. At least one other DNA amplification technique has also been applied to *M. tuberculosis*. DNA amplification technology offers the greatest promise for extremely rapid and specific diagnosis of tuberculosis. Here also, the ubiquity of most other potentially pathogenic mycobacteria in the environment raises the question whether application of these exquisitely sensitive amplification techniques to the environmental species would provide definitive diagnostic information.

The diagnosis of disease due to environmental mycobacteria will probably continue to depend on cultural isolation of these organisms, in combinations of radiometric broth for rapid detection, and on solid media to permit estimation numbers and recognition of mixed cultures. The identification of these cultures can be accelerated through the use of either nucleic acid probes or intrinsic enzyme serologic probes, or a combination of both.

Drug susceptibility tests continue to depend on bacterial growth, but the results can be hastened by the use of radiometric broth.

The greatest promise for epidemiologic characterization of strains of all species of pathogenic mycobacteria appears to lie in the analysis of RFLP patterns, since every organism will yield such a pattern, and the range of different patterns is essentially unlimited.

Modern technology has yielded a number of innovative sensitive and specific methods for clinical mycobacteriology. Some may have to be considered as supplements to, rather than substitutes for conventional culture, offering speed of diagnosis in many cases, but needing backup cultures for some purposes. The final selection of which of these methods will reach common acceptance will be determined by their respective predictive values, cost, ease of performance, availability of materials.

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