HISTORICAL REVIEW

Chemotherapy and diagnosis of tuberculosis

Cesare Saltini*

Department of Internal Medicine, University of Roma "Tor Vergata", Viale Oxford 81, 00133 Roma, Italy

Received 1 September 2006; accepted 14 September 2006

KEYWORDS
TB chemotherapy; Clinical trials; MDR-TB; TB molecular diagnosis; TB in vitro T-cell assays

Summary Since after the first streptomycin 1944 trials, anti-tuberculous chemotherapy research has been focused upon establishing drug combination regimens capable of overcoming drug resistance and amenable to ambulatory treatment in resource strapped countries. The first milestone being the 1959 Madras trial comparing home and sanatorium treatment in South India. Subsequently, the MRC trials led Fox and Mitchison to indicate rifampicin, isoniazid and pyrazinamide as the first line drugs for short course, 6 month, regimens and the 1982 Hong Kong Chest Service trials established intermittent therapy as the ambulatory treatment standard for directly observed therapy (DOT). The rising of the HIV epidemic at the beginning of the 1980s has refuelled tuberculosis spread in Africa and Asia and contributed to the expansion of drug-resistant tuberculosis worldwide making the development of new drugs and drug regimens for ambulatory treatment a top priority.

Led by biotechnological advances, molecular biology has been brought into TB laboratory diagnosis for the highly sensitive and specific rapid identification of Mycobacterium tuberculosis in biological samples. The field of immunological diagnosis of TB infection, dominated since the early 1900s by the intradermal tuberculin reaction has been put back in motion by the discovery of M. tuberculosis-specific proteins and peptides, now employed in blood tests of high sensitivity and specificity for the diagnosis of latent TB which may help with the identification of contacts at higher risk of active disease and the eradication of pandemic cases.

&2006 Elsevier Ltd. All rights reserved.

Chemotherapy

Soon after streptomycin discovery and its clinical application to tuberculosis treatment it became apparent that the bacillus was capable of rapidly developing drug resistance. It was then found that para-aminosalicylic acid (PAS) could be used in combination with streptomycin to prevent, or delay, streptomycin resistance.1-3 With the discovery of the anti-tuberculous bactericidal activity of isoniazid, it was found that it was much more powerful in combinations with streptomycin and PAS than when used alone and the first combination chemotherapy regimens were standardized. These included daily isoniazid, streptomycin and high
dose PAS: these regimens were shown to prevent streptomycin, isoniazid and PAS resistance and their entire course was extended to 18–24 months, using pneumothorax in the more advanced cases.4–6

The 1950s and the 1960s: from the sanatorium to home treatment

The introduction of highly effective chemotherapy regimens allowed the design of ambulatory interventions for active, AFB sputum positive, cavitary tuberculosis in order to reduce the need for sanatorium isolation, to make tuberculosis therapy less costly for health programs and more socially acceptable to patients and their families. A 12 month randomized controlled trial of isoniazid plus PAS in the same cachet (200 mg and 10 g, respectively for the average above 45 kg person) was launched at the Tuberculosis Chemotherapy Centre of Madras (India), where 193 active tuberculosis patients were enrolled of whom 96 were treated at home and 97 in sanatorium. Several aspects of this study established the role of chemotherapy, with combinations of rifampicin, isoniazid and thiacetazone administered in “trial mode” gave patients a 10% better bacteriological outcome at 12 months.11 Importantly, trial experience also showed that direct observation and follow up played a pivotal role in making domiciliary treatments effective. The comparison the results obtained by routine treatment services with those achieved in clinical trials by the East Africa Tuberculosis Investigation Centre and the British Medical Research Council in Kenya indicated that, although the three drug routine combination regimen with streptomycin, isoniazid and thiacetazone was more powerful, the two drug combination of isoniazid and thiacetazone administered in “trial mode” gave patients a 10% better bacteriological outcome at 12 months.11

The 1970s: the introduction of rifampicin and the 6-month short course

The discovery of rifamycins and of rifampicin,12 with their introduction in the armamentarium of antituberculous drugs in 196613–15 prompted renewed investigation on the reduction of treatment duration: eventually, the 6 months short-course chemotherapy, with combinations of rifampicin, isoniazid and pyrazinamide became standard.
The first relevant clinical study to this end was carried out in East Africa and Zambia in association with the British Medical Research Council. In this study, four 6-month daily regimens with streptomycin, isoniazid and rifampicin (SHR); streptomycin, isoniazid and pyrazinamide (SHZ); streptomycin, isoniazid and thiacetazone (SHT) and streptomycin plus isoniazid (SH) were compared with a standard 18-month streptomycin, isoniazide and thiacetazone (STH for 2 months and then HT for 16 months) regimen. The study demonstrated the efficacy of the 6 months regimens and the superiority of the rifampin or pyrazinamide containing regimens, as the 6 month SHT and SH regimen had the highest relapse rates at 30 month follow up (22–32%), with the SHR regimen producing the lowest one (2%), that was not significantly different from that obtained with the 18 month standard treatment (3%).

Fox and Mitchison, with the Medical Research Council, developed a pharmacokinetic rationale from clinical and laboratory studies, whereby isoniazid and rifampin were considered as complete bactericidal drugs, being capable of killing bacteria in all environments, while streptomycin and pyrazinamide were “half” bactericidal drugs, being the former active in the more alkaline extracellular milieu and the latter active on the more acidic intracellular environment. Interestingly, streptomycin and pyrazinamide were perfectly complementary drug which, together, made a very powerful bactericidal combination. Following this reasoning, the East African SHR short-course regimen contained two and a half bactericidal drugs and the almost equally powerful SHZ regimen contained two full killers. In contrast, the SHT and SH less powerful 6-month regimens contained only one and a half killer. The East African trial so suggested that at least two full bactericidal drugs were required to successfully cut treatment duration from 12–18 months down to a 6-month short course.

A number of randomized trials followed suit, in East Africa, Madras, Hong Kong, Singapore and Algeria. In a second East African study, the 6-month SHR daily regimen, the best arm of the previous study, was compared with a similar regimen without streptomycin (HR daily for 6 months) and with two regimens where rifampicin was given only during the first 2 of the 6-month course, with the addition of pyrazinamide (SHRZ daily for 2 months plus TH daily for 4 months and SHRZ daily for 2 months plus SHZ twice per week for 4 months) in order to explore the efficacy of limiting rifampicin administration to the first 2 months of treatment. Interestingly, the study showed that 2SHRZ, the regimen with three full bactericidal drugs, produced better bacteriological results at 2 months, thereby suggesting that the use of such a combination in the initiation phase of treatment could lead to complete elimination of all susceptible organisms.

After a subsequent East African study of 4-month, short-course therapy using the same initiation scheme (2SHRZ) followed by three different, 2-month continuation schemes using either isoniazid, isoniazid and rifampin, isoniazid and pyrazinamide or the three drugs, was interrupted for an unacceptably high number of relapses, a fifth study was undertaken with which the role of pyrazinamide in short-course therapy was to be established. This study tested the same 2-month initiation therapy (2SHRZ) followed by four different continuation regimens of 4 months i.e., 4HR, 4HZ, 4H or 6H. The study showed that, after the common initiation phase with 2SHRZ, the 4HR continuation regimen was significantly better than all others, with only 2% relapses at 24 months after the completion of treatment. The 4HZ continuation regimen, with 8% relapses, was no better than the 4H with 10%. This, together with the finding that the 6H regimen resulted in only 3% relapses, suggested that the contribution of pyrazinamide was essential during the 2-month initiation phase of the short-course combination regimens, but not thereafter.

Similar results were obtained in studies of the Research Committee of the British Thoracic Association that found that streptomycin or ethambutol, with pyrazinamide added to isoniazid and rifampicin during the 2-month initiation phase (2SHRZ or 2EHRZ) followed by 4 HR equalled the standard 9HR with negligible relapse rates and by the Algerian working group’s study which found that a similar 6-month regimen had better results than a 12-month standard treatment.

The Singapore Tuberculosis Service/British Medical Research Council study also confirmed the efficacy of short-course regimens using streptomycin, isoniazid, rifampicin and pyrazinamide in the initiation phase with isoniazid plus rifampicin in the continuation phase: In addition, it confirmed that a 4 months course was ineffective. In this study the 2SHRZ, 4HRZ short-course regimen produced no relapses at 24 months after treatment cessation and the 2SHRZ, 4HR regimen 2.5%, while with the shorter regimens (2SHRZ, 2HRZ or 2HR) 8% and 11.8% relapses were observed, respectively.

The 1980s: intermittent short-course regimens and DOTS

With the background that supervised, treatment administration in trial mode had proven superior to
the routine service mode, the high cost of rifampicin and pyrazinamide, pressed tuberculosis services throughout the developing world to find a short-course intermittent regimen that could be given to outpatients under supervision. Two important studies were carried out at the Hong Kong Chest Service with the collaboration of the British Medical Research Council to determine the best regimen for fully supervised outpatient treatment. In the first study, a fully intermittent 6-month regimen of streptomycin, isoniazid, rifampicin and pyrazinamide three times weekly for 4 months (4SHRZ3), followed by streptomycin, isoniazid and pyrazinamide (2SHZ3), resulted in a bacteriological relapse rate of 6% at 18 months in patients with fully drug-sensitive bacteria, with an even higher relapse rate with isoniazid-resistant strains.26 Another important study was carried out by the Singapore Tuberculosis Service, also in collaboration with the British Medical Research Council, with which it was established the role of pyrazinamide and streptomycin in short course, intermittent regimens. This study examined three treatment protocols with streptomycin, isoniazid, rifampicin, and pyrazinamide for two months (2SHRZ), or for 1-month (1SHRZ), or for 2 months without streptomycin (2HRZ), followed by three-times-weekly isoniazid and rifampicin (H3R3) up to 6 months for all patients. The data showed that, in patients with drug sensitive strains, relapse rates were less than 1% and that, importantly, they were only 3% in the isoniazid and streptomycin resistant.

A similar trial was carried out at the Tuberculosis Research Centre in Madras, India. In this study, three short-course chemotherapy regimens were evaluated in patients with newly diagnosed, bacteriologically positive pulmonary tuberculosis: patients were allocated to either rifampicin, streptomycin, isoniazid and pyrazinamide daily for 2 months, followed by streptomycin, isoniazid and pyrazinamide twice weekly for a total of 5 (2SHRZ/3SHZ2) or 7 months (2SHRZ/5SHZ2) or to the same 7 month regimen but without rifampicin (2SHZ/5SHZ2). The result at 5 years indicated a slightly higher percentage of bacteriological relapses, compared to the Hong Kong and the Singapore studies, with 7% for the 5-month rifampicin regimen, 4% for the 7-month rifampicin regimen and 7% for the 7 month no-rifampicin regimen. In addition, a 10% relapse rate was observed among isoniazid or streptomycin resistant patients.27

Eventually, the 1980s studies brought to the recommendations of the International Union Against Tuberculosis and Lung Disease (IUATLD), indicating isoniazid, rifampicin, pyrazinamide for the 2-month induction phase followed by isoniazid and rifampicin for the 4-month continuation phase of the 6-month daily regimen. The same recommendations also indicated adding ethambutol or streptomycin in the initial phase of the three times weekly regimen.29 Thus, with these trial-validated, intermittent short-course chemotherapy regimens, directly observed therapy (DOT) became feasible in the tuberculosis services of high prevalence countries. With the DOT strategy was developed by the IUATLD together with national tuberculosis programmes, the WHO recommended directly supervised treatment consisting of a 2-month daily regimen of isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin (2HRZE7 or 2SHRZE7) followed by 4-month continuation with either daily or intermittent isoniazid plus rifampicin (4HR7 or 4HR3), for patients with newly diagnosed smear
positive disease. As a second option, an entirely intermittent 6-month treatment was recommended (2HRZE	extsubscript{3}/4HR	extsubscript{3})	extsuperscript{30}

An 8-month regimen with the same induction phase (2HRZE	extsubscript{7} or 2HRZE	extsubscript{3}) and isoniazid plus thioacetazone in the continuation phase (6HT) was recommended for use in countries with limited financial resources. However, with the advent of the Human Immunodeficiency Virus (HIV) epidemic, being HIV-infected patients at increased risk of severe, in some cases fatal, dermatological toxic effects from thioacetazone, the WHO recommendations were modified and in the 8-month regimen above, ethambutol (6HE) was substituted for thioacetazone in the 6-month daily isoniazid plus thiacetazone continuation phase.

The new drugs and regimens of the 1990s

Are the intermittent regimens developed during the past 30 years up to the task of curing 85% of pulmonary tuberculosis patients, as per the goals of the WHO tuberculosis programme?

In the late 1990s studies have been carried out on intermittent regimens with newer rifamycin derivatives. A Centres for Disease Control and the Tuberculosis Trials Consortium trial compared once-weekly rifapentine, in combination with high dose isoniazid (15 mg/kg) in the continuation phase of a standard intermittent regimen, with twice-weekly isoniazid and rifampicin. The study reported 2-year crude rates of failure or relapse of 9.2% in the rifapentine arm and of 5.6% in the control treatment.

A Hong Kong study compared 600 mg rifapentine plus 15 mg/kg isoniazid, once-weekly or every two of 3 weeks, with trice-weekly isoniazid plus rifampicin in the continuation phase of a standard trice weekly streptomycin, isoniazid, rifampicin and pyrazinamide regimen (2SHRZ	extsubscript{3}). This study found 5-year relapse/failure rates of 10.8% and 11.7%, respectively, for the rifapentine arms compared to 4.2% for the standard HR	extsubscript{3} regimen.

A trial of rifabutin 300 mg plus isoniazid twice-weekly as the continuation phase of a standard daily/intermittent regimen of isoniazid plus pyrazinamide and ethambutol, with rifabutin substituted for rifampicin. The 2-year projection of the failure/relapse rate was of 6.6%. Strikingly though, notwithstanding the directly observed administration of all drugs doses, eight of the nine relapses observed were due to acquired rifampicin resistance.

Concerns about the ultimate efficacy of intermittent therapy regimens have been raised by a Cochrane review and by a recent systematic review that analyzed 20 rifampicin-containing short-course chemotherapy regimens including 32 study groups of non-multi-drug resistant, non HIV tuberculosis. These studies point to the increased relapse rates observed with standard 6-month intermittent regimens, where the total dose of anti-tuberculous drugs appears to be related with the bacteriological outcome. These observations suggest that streamlined intermittent treatments may pose the threat of increased rifampicin resistance, hence of selecting multiple drug resistant strains, unless careful pharmacokinetic evaluations of the new treatments are used in the study design.

The changing epidemic: multi-drug resistant tuberculosis (MDR-TB)

With the introduction of streptomycin, it rapidly became apparent that the mycobacterium would inevitably acquire drug resistance during the course of chemotherapy and it was with the introduction of PAS that it became understood that combination drug treatment was mandatory to prevent, or delay, the arising of drug resistant Mycobacterium tuberculosis strains. In the late 1960s trials, 6% of the patients enrolled had primary resistance to at least one drug and only 0.4% had strains resistant to all three drugs used i.e., streptomycin, isoniazid and PAS.

In the 1980s, streptomycin or isoniazid resistance was seen in about 10% of clinical trial subjects and in 5% resistance was to both drugs. However, rifampicin-based, standard short-course regimens obtained 90% or greater cure rates in streptomycin and isoniazide resistant subjects. With widespread use of rifampicin-containing regimens, resistance to multiple drugs, notably against the two major bactericidal drugs isoniazid and rifampicin, appeared. In the US, 0.5% of new cases and 3.0% of recurrent cases were resistant to both isoniazid and rifampicin in 1982 but this proportion increased to 3.1% and 6.9% by 1991 and similar figures have been reported worldwide. Hence, the growth of MDR-TB prevalence has become the major threat that the tuberculosis epidemic poses today.

The genes coding for resistance to isoniazid are katG, a gene involved the scavenging toxic peroxides produced by phagocytes that’s thought to be required for protection against oxygen free radicals within the macrophage and that’s required for the generation of isoniazid toxic products, the inhA, the oxyR, the kasA and ahpC genes. The rpoB gene has been identified as the gene responsible for
resistance to rifampicin, rpsL and rrs for streptomycin, embB for ethambutol and pncA for pyrazinamide, inhA for ethionamide, rrs for kanamycin and, finally, the gyrA and gyrB genes for fluoroquinolones.43

Multi-drug resistance can be conferred by a single mutational event the genes coding for multi-drug efflux transporters, membrane proteins that recognize dissimilar toxic compounds and pump them out of eukaryotic and bacterial cells. Although efflux pump genes have been identified in mycobacteria, and *M. tuberculosis*, in particular, they do not seem to play a major role in the emergence of MDR strains.44–46 To the contrary, multi-drug resistance is thought to be the consequence of stepwise accumulation of random mutations in the chromosome selected under the environmental pressure of chemotherapy. These finding have important implications for treatment program strategy.47 In this regard, it was observed that short-course chemotherapy regimens including four or five drugs (rifampicin, isoniazid, pyrazinamide and ethambutol or streptomycin) were still effective in the presence of resistance to isoniazid alone and longer course protocols could still treat patients with rifampicin mono-resistance,48,49 in contrast, resistance to isoniazid and rifampicin combined i.e., multi-drug resistance or MDR-TB dramatically impacts upon treatment. With MDR-TB the course of treatment needs prolongation from the standard 6 months to 18–24 months and the cure rate decreases from nearly 100% to less than, or equal to, 60%.40

From the 1990s to the present day, a number of reports have described the treatment of cases of MDR-TB. In the retrospective study of Goble and colleagues on the treatment of 171 MDR-TB patients admitted to a specialized centre from 1973 to 1983, bacteriological conversion was observed in 65% of cases and cure, defined as bacteriological negativity for at least 3 months, in 56%. However, if the default rate of 21% is taken into account, the ‘intention to treat’ cure rate would have been a mere 43%.45 Higher ‘intention to treat’ cure rates were observed, almost 20 years later, in Yew’s et al. Hong Kong study of 106 patients carried out between 1990 and 2000 [78% cure rate41], in the 31 cases of the inpatient arm of the 1994–1997 USA study of Narita and coworkers [79% cure rate52], in the 75 cases of the 1996–1999 Peruvian domiciliary study of Mitnick and coworkers [73% cure rate53] and in the 204 cases of the 2000–2001 Latvian study of Lemaine and colleagues, who reported an ‘intention to treat’ cure rate of 66%.54 The Turkish study of Tahaoglu and colleagues of 158 cases treated between 1992 and 1999 also reported a higher ‘intention to treat’ cure rate 77%.55 However, the interpretation of the study results is hampered by having MDR-TB been defined clinically i.e., by the lack of sputum conversion after 5 months of standard treatment instead of bacteriologically. The above recent studies included, albeit in a non randomized fashion, (i) more than five drugs, (ii) the fluoroquinolones cyprofloxacin, ofloxacin or levofloxacin in most treatments and (iii) second line drugs such as aminoglycosides, cycloserine and ethionamide.

Some other studies reported instead dimmer outcomes and the, such as the French nationwide observational 1994 study of Flament-Saillour and colleagues, who reported an ‘intention to treat’ cure rate of 33% among their 84 cases,56 the 1997–1999 Indian study of Subhash and coworkers who reported an ‘intention to treat’ cure rate of 32% in their 100 cases57 and the 1998–2000 South Korean study of 142 cases reported by Park and colleagues whose ‘intention to treat’ cure rate was of 44%, due the very high default rate.58

The Stop TB Department of WHO estimates that there were 458,000 (95% confidence limits, 321,000–689,000) incident MDR-TB cases (including new and re-treatment cases) in 2003 worldwide.41 According to the WHO surveys, drug resistance was strongly associated with previous treatment. In previously treated patients, the probability of any resistance was over 4-fold higher, and of MDR-TB over 10-fold higher, than for untreated patients. The overall prevalence of drug resistance was often related to the number of previously treated cases in the country. Among countries with a high burden of TB, previously treated cases ranged from 4.4% to 26.9% of all patients registered in DOTS programmes. In the two largest high-TB burden countries (China and India), re-treatment cases accounted for more than 20% of sputum smear-positive cases.59 In detail, in patients never previously treated, the median prevalence of resistance to any of the first-line drugs, most commonly streptomycin and/or isoniazid, was 10.7% (range 0–57.1%); although 20 survey sites exceeded 20%. The median prevalence of MDR-TB was 1.2% (range 0–14.2%); with 11 sites exceeding the 6.5% threshold for extreme values. In patients previously treated, the median prevalence of any resistance was 23.3% (range 0–82.1%) and of MDR-TB, 7.7% (range 0–58.3%).50

A drug regimen to treat TB cases resistant to isoniazid and rifampicin is currently >50 times more costly than the cheapest short-course regimen for drug-susceptible cases, although negotiated through the World Health Organization’s Green Light Committee. Thus, it could cost more to treat the new TB cases that are MDR than all other
new TB cases together. Adding in the cost of managing MDR TB cases that have previously failed treatment, would demand in many countries a significant fraction of the overall budget for TB control. The same may happen in industrialized countries, where drug resistant and MDR-TB are often associated with social problems such as homelessness, illegal immigration, drug abuse or mental illness all rendering domiciliary care unfeasible. Drug resistance dramatically increases the cost of TB care as it requires, in addition to using more expensive drugs, lengthy in-hospital isolation, the major component of hospital costs.

The new epidemic of the 1990s: HIV infection and tuberculosis

Since the first cases of HIV infection were recognized 25 years ago, the AIDS epidemic has become one of the major world’s health problems: an estimated 39 million people live with HIV at the end of 2005, world-wide, with four million newly infected with HIV and three million loosing their lives to AIDS. The HIV epidemic and tuberculosis have fuelled each other in Africa, almost doubling tuberculosis incidence from the 100–200 cases per 100,000 population incidence of the 1980s to the 200–400 per 100,000 of 2005.

Since the beginnings of the epidemic, laboratory and clinical studies characterized the HIV infection for being dominated by progressive impairment of antigen-specific T-cell mediated immune responses, and by progressive susceptibility to infections as well. Subsequent immunological studies, showing that mycobacteria have the ability to activate HIV replication within the mononuclear phagocytes and to accelerate the spread of viral infection, established a link between the increased susceptibility to tuberculosis, brought about by HIV infection, and accelerated HIV progression, caused by M. tuberculosis.

In this regard, clinical and epidemiological studies have elucidated the role played by HIV infection in tuberculosis morbidity and mortality. The first series of tuberculosis cases in HIV-infected persons were described in the early 1980s in the US and Europe among population groups at higher risk of tuberculosis reactivation, such as the immigrant from high prevalence areas or intravenous drug users, and it was found that HIV co-infection confers upon the carriers of latent M. tuberculosis infection a greater than 100-fold risk of developing active tuberculosis.

The HIV epidemic had and will have a major impact upon TB, and may be also a major factor for the spread of MDR-TB. In the New York MDR-TB epidemic of the 1990s it was found that, contrary to observations in the HIV non-infected, HIV infection was associated with a higher proportion of primary drug resistance. It is thought that HIV-infected persons may be more exposed to drug resistant isolates for their very risk factors, such as homelessness and drug abuse. In addition, HIV-seropositive patients infected with resistant organisms may rapidly progress to active disease, even though resistant organisms might be less virulent than susceptible organisms. Third, as HIV-infected tuberculosis patients are less likely to be cured, they may be at higher risk of developing drug resistance, as the recent rifapentine trial has indicated. As a result, a number of MDR-TB outbreaks involving a majority of HIV-infected patients have occurred in recent years in hospitals and clinics, shelters and the community, a warning that MDR-TB might become epidemic.

Diagnostics

Microbiology, from acid fast stains to DNA amplification

The diagnosis of M. tuberculosis infection is more difficult to establish than with almost every other common bacterial infection. The specific microbiological stains and culture procedures still widely used for M. tuberculosis date back to 1882 Robert Koch’s announcement to the Berlin Physiological Society that he observed and isolated the causative organism of tuberculosis.

Examination of sputum smears for acid-fast bacilli (AFB) still is the most rapid way to obtain evidence of tuberculous infection. However, not all infected individuals have detectable M. tuberculosis in AFB-stained smears and acid fastness does not distinguish M. tuberculosis from non-tuberculous mycobacteria. Cultures in selective media are more sensitive, but their outcome is delayed by the extremely low growth rate of mycobacteria. Contrary to a number of environmental mycobacteria that are rapid growers, yielding colonies in 7 days or less, M. tuberculosis exhibits a slow growth rate, requiring 14–21 days to generate visible colonies and does not produce any pigment.

Mycobacteria are neither Gram-negative nor positive but they are characterized by acid fastness: that depends upon the composition and the integrity of the mycobacterial cell wall i.e., killed or fragmented mycobacteria may not be acid fast. The most popular acid fast staining methods have
used either carbol fuchsin, as the Ziehl-Neelsen and the Kinyoun techniques, or auramine O, a fluorescent dye. Since fluorochrome-stained slides may be viewed at lower magnification (250–450 x ) thus enabling the microscopist to rapidly screen a high number of fields, fluorescent staining is more sensitive than basic fuchsin staining. The lower limit of detection of AFB sputum smears has been estimated at 0.5–1 x 104 microorganisms per ml of sample, although higher speed centrifugation has been shown to improve recovery up to 90%,79,80 and this has been the lower diagnostic detection limit for over a century.

The diagnostic gold standard for microbiological confirmation of the diagnosis of tuberculosis is the growth in selective culture media is.81 Culture is significantly more sensitive and specific than acid fast smears and systems have been developed, in the 1980s such as the BACTEC and the biphasic culture systems, that provided faster recovery than the traditional cultures in solid media.82,83

However, the most important progress in tuberculosis diagnosis was brought about by the introduction of nucleic acid amplification assays by 1985 by Saiki et al. using the polymerase chain reaction (PCR). The PCR was the first nucleic acid amplification procedure capable of generating in vitro of millions of copies of a specific DNA segment84 allowing the rapid detection and quantification of few DNA copies with very high sensitivity and specificity, hence becoming the most important research advance of the last decades for the identification of infectious agents in biological and environmental samples.

In 1989, the first attempt at the identification of M. tuberculosis in clinical samples was reported by Brisson-Noël and coworkers, using a 65 kDa mycobacterial gene that allowed both the detection of mycobacterial DNA and the differential diagnosis of M. tuberculosis vs. non tuberculous mycobacteria.85 A number of other genes, among them the highly popular IS6110 repeated sequence,86 has been used in clinical research laboratories, however susceptibility of amplification procedures to both false positives and negatives hampered clinical use of these tests87,88 until automated, robust commercial tests became available.89-94 With these tests in the clinical setting using standardized clinical indicators of TB pre-test probability, nucleic acid amplification has finally been shown to be amenable to provide clinically relevant positive and negative predictive powers.95,96 Ten years later, the clinical utility of nucleic acid amplification tests has been certified by the USA Centers for Diseases Control, that recommended the use of nucleic acid amplification tests on sputum samples for the diagnosis of pulmonary tuberculosis. In particular, the use of nucleic acid amplification using the GenProbe MTD test and the Roche Amplicor were recommended for the rapid identification of M. tuberculosis in AFB-positive sputum smears, where the diagnosis of non-tuberculosis mycobacterial infection may be suspected. Furthermore, the Amplicor test was recommended for the determination of the presence of amplification inhibitors in the sputum sample in the case of a negative amplification of an AFB-positive sputum. In addition, the use of the MTD test was recommended for the diagnosis of tuberculosis in AFB- sputum samples, in the presence of clinically suspected of tuberculosis.97

Delayed type hypersensitivity testing: skin and blood tests

The tuberculin skin test is one of the oldest diagnostic tests, as it was developed by R. Koch and subsequently described by von Pirquet in 1907.98 The test identifies subjects infected by M. tuberculosis. The intensity of the skin reaction may grossly discriminate between M. tuberculosis and M. avium or other non tuberculous infections,99 but the test is susceptible to inter-operator variability, to variability due to administration modalities such as the multi-puncture or the intradermal injection and to false negativity due to a number of clinical conditions causing immune compromise.100,101 Most importantly, cross reactivity with M. bovis BCG represents the major limitation in surveying BCG vaccinated populations, as vaccination induced immunity may last for over 15 years.102,103

In the last decade, genomic studies have identified M. tuberculosis genome regions that are absent from the genome of M. bovis BCG and from that of most non-tuberculous mycobacteria. The region of differentiation (RD)-1, that’s shared only by M. tuberculosis, M. szulgai, M. marinum and M. kansasii, encompasses the ESAT-6 (early secretory antigen target-6) and the CFP-10 (culture filtrate protein-10) proteins that are target of the specific immune response against M. tuberculosis.104,105 Using these proteins, T-cell based, highly sensitive and specific diagnostic test were developed that measured interferon (IFN)-γ released by memory and effector T-cells in the blood of M. tuberculosis infected individuals. Of such tests, the QuantiFERON (QTF-TB) has been developed by Cellestis Ltd., (Carnegie, Victoria, Australia) and the T-SPOT.TB (TS.TB) by Oxford Immunotec (Abingdon, UK), in addition to a number of home-brewed T-cell-based tests.106
Overall, interferon-γ-based tests show sensitivities varying between 85% and 95% i.e., far greater than that of the tuberculin skin test, when subjects with active tuberculosis are tested as referents. Importantly, both the QFT-TB and the TS TB have been shown to be insensitive to BCG vaccination, with a resulting specificity of 98–100%. With regard to the identification of contacts of TB cases, in vitro blood tests show remarkable concordance with the tuberculin skin test. Even more importantly, they show a stronger correlation with exposure level and duration than the tuberculin skin test. The new tests do also perform better than the tuberculin skin test in the identification of latent TB infection in immuno-compromised patients. The more laborious Elispot test, by using determined numbers of blood mononuclear cells in the assay, is less susceptible to false negative results in the lymphopenic patients. Accordingly the test has shown greater sensitivity than the tuberculin test in active tuberculosis of HIV co-infected subjects, both adults and children.

The clinical utility of the new immunologic tests for latent TB infection has been recently acknowledged by the USA CDC which recommended the QFT-TB test for use in all of the instances where the tuberculin skin test was insofar recommended. Furthermore, the British National Institute for Health and Clinical Excellence (NICE) recently published a document that, based upon a cost benefit analysis, recommends the use of in vitro blood tests (i) to confirm an unreliable tuberculin skin test, (ii) where the prevalence of false positive skin reactions is expected to be high, e.g., in BCG vaccinated populations and laboratory logistics would allow the use of the new tests.

Serology: a 1940s promise not yet delivered

The antibody response to M. tuberculosis is generally thought not to reflect anti-tuberculous cell mediated immunity, although the rising of antibody titers against selected antigens derived from purified protein derivative (PPD) of tuberculin seems to follows the appearance of skin reactivity in BCG vaccinated subjects and in M. tuberculosis exposed individuals, a type of response not observed with highly purified antigens.

Back to the future

Er Griegg proposed some years ago a historically enticing epidemiologal hypotesis where M. tuberculosis epidemic wave spreads into naive populations limited by the death of the susceptible and the emergence of immune resistance in the population, until it subsidizes in the span of 2–3 centuries. The current epidemic, originated at the dawn of the industrial revolution in the 17th century’s England, reached its peak there at the end of the 18th century to make its way into continental Europe and Northern America during the first half of the 19th century, then into Eastern
Europe, the Americas, Africa and Asia, with an annual death rate of 1% of the population at the peak of the wave, to slowly decline thereafter.\(^{133}\) A century of medical discoveries, with Brechmer’s pioneering sanatoria in the mid 1800s, Koch’s isolation of \textit{M. tuberculosis} in 1882,\(^ {77}\) Forlanini’s invention of pneumothorax in 1884,\(^ {134}\) Schatz and Waksman’s discovery of streptomycin in 1944,\(^ {135}\) has been accompanied by a steady decline in TB morbidity and mortality in Western Europe and the USA. The past 50 years of tuberculosis research has brought rapid diagnosis to identify all new cases and powerful chemotherapy to cure it. Now \textit{M. tuberculosis} could be eradicated, economical, social and political conditions permitting.

References

29. International Union Against Tuberculosis and Lung Disease. Anti-tuberculosis regimens of chemotherapy: recommen-
Chemotherapy and diagnosis of tuberculosis

65. Lane HC, Depper JM, Greene WC, Whalen G, Waldmann TA, Fauci AS. Qualitative analysis of immune function in patients with the acquired immunodeficiency syndrome.
Chemotherapy and diagnosis of tuberculosis


