THE ORGANIZATION OF LABORATORY SERVICES FOR A "TUBERCULOSIS CONTROL PROGRAMME"

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INTRODUCFION

In order to support a "Tuberculosis Control Programme", an adequate laboratory service must be guaranteed. This should include peripheral laboratories capable of preparing and examining smears for acid fast bacilli (AFB) and one "Referral Laboratory" providing facilities for culture, identification and drug sensitivity tests for my. cobacteria.
The direct examination of sputum.smears represents the most important tool for detecting patients with tuberculosis, in order to control the spread of disease in the community.[1] However, to improve case-finding, especially when no AFB are found in direct smears, when a failure or relapse occurs during treatment and/or drug-resistance is suspected, culture of the sputum and drug-susceptibility tests should be performed[2,3].
Although laboratory procedures for the study of Mycobacterium tuberculosis as well as non-tuberculous mycobacteria (NTM) have been already established and detailed in several publications[3-7] in organizing a laboratory service for a "Tuberculosis Control Programme" several rules must be emphasized.

MICROSCOPIC EXAMINATION
The high cell-wall lipid content (up to 30%) of mycobacteria make them able to bind the dye carbolfuchsin and to resist de-staining with acid-alcohol.
All clinical specimens submitted for the diagnosis of a mycobacterial infection should be examined for AFB. Direct microscopy is much less sensitive than culture and a positive AFB smear provides only a presumptive diagnosis since the actual species of mycobacteria cannot be determined by morphological observation. However, as NTM diseases are rare in high incidence areas of tuberculosis, the acid-fastness of mycobacterium, together with their characteristic size and shape, is useful for the early detectioh of tubercular infection and can help to follow the patient's response to chemotherapy.
Examination of stained smears of sputum is useful also when making appropriate dilutions of decontaminated— concentrated samples for direct drug sensitivity testing[3,8]
Acid fast bacilli in specimens are usually rod-shaped and pleiomorphic (2.0-4.4 x 0.3 — 0.5 jz), which may appear bonded with dark-stained parts. In slides obtained from pure culture, M. tuberculosis may show strands of bacilli in "cord"' :— used[3,7]
Two types of acid-fast stain are commonly Carbolfuchsin stains by Ziehi-Neelsen (hot stain) or Kinyoun (cold-stain),
2) Fluorochrome stain auramine 0 with or without rodhamine.
Smears stained with carbolfuchsin procedures must be scanned with an oil-immersion objective for 100-300 fields (1x2 centimeters area). Smears stained with the fluorochrometechnique can be scanned

with 25 x objective which will increase the field of view, allowing inspection of a larger area in a shorter time.
As the smear examination, using a Ziehi Neelsen procedure is an essential part of a "Tuberculosis Control Programme", microscopic examination of sputa must be carried out meticulously and, in order to be meaningful, smear results must be quantified. In

Table 1. Number of Acid Fast Bacilli (AFB) found in Smears stained with Ziehl Neelsen Method.

Number of AFB observed In sputum None	Report No AFB observed
1–2/300 fields	must be repeated
1–9/100 fields	1+
1–9/10 fields	2+
1–9/1 field	3+
>9/1 field	4 +

Table 1 are the suggested interpretation for the reporting of smear results.
A clump of bacilli is considered a single colony forming unit, and must be taken in account as a single unit. In order to classify the slide as positive at least three AFB should be observed on the same slide; this shall avoid possible misinterpretations between bacillary forms and cellular wastes.
Since M. tuberculosis is released from lungs irregularly, at least three• separate specimens must be examined:
- one spot specimen when the patients first attend the clinic
- an early morning specimen.
- a second spot specimen collected when the early morning specimen is submitted for examination.

RECOVERY OF M. TUBERCULOSIS

Specimen collection

M. tuberculosis as well as non tuberculous mycobacteria can be found in a variety of clinical specimens including sputum, urine, pus and tissue biopsies from organs suspected of being the site of mycobacterial infection[2,3].
Recovery and identification of M. tuberculosis from clinical specimens provides the only definitive proof of tuberculous infection.
In areas of high incidence of tuberculosis nearly all the specimens to be examined come from the respiratory tract. Early morning expectorated sputum, as well as sputum samples induced by nebulized saline (10%) inhalation, are more useful specimens than the 24 hours sputum pools. In fact the former provides:
- faster detection during smear observation.
- better growth when culture media are inoculated.

- reduced contamination rates when culture is performed.

If patient's sputum cannot be obtained, gastric washing samples may be utilized[3,6].

Since sputa, urine and pus from fistulized localization are usually soiled by commensal bacteria, specimens may be collected in clean but not necessarily sterile screw-capped containers. Specimen digestion and decontamination

The high cell-wall ffpid content of mycobacterial cells make them more resistant to chemical agents such as alkali, acids and quaternary ammonium compounds than other types of bacteria which can contaminate sputum, urine and other clinical specimens[2,3,9].

Commensal bacteria, which usually replicate every 20 minutes can overgrow the slowly growing mycobacteria (average replication time: 20 hQurs). To eliminate undesirable bacteria and liquify mucus, before preparation of the culture, contaminated specimens must be treated with a decontaminating agent capable of killing microorganisms other than mycobacteria.

The agents utilized for digestion and decontamination of clinical specimens are usually alkali and acids such as NaOH (4%), sulphuric acid (4%) and oxalic acid (5%). Because of the high toxicity of strong acid and alkaline solutions, many mycobacteria are also killed. Therefore, carefully controlled period of decontamination and proper neutralizing procedures are mandatory.

N-acetyl-L-cysteine or dithiotreitol (Sputolysin; Calbiochem, La Jolla, Calif.) employed simultaneously with 2% NaOH permits a milder alkali treatment, while liquifying tenacious sputum.

Sulphuric acid (4%) and oxalic acid (5%) are useful in the processing respectively of urine samples and of specimens containing Pseudomonas aeruginosa as a contaminant[3].

Alternatively trisodium phosphate (13%) combined with benzalkonium chloride (Zephiran) or the chloride or bromide salt of cetyipridiniurn (1%) plus Naci (2%) appear to be milder decontaminating agents[10]. With the latter methods careful timing is not required.

Cetyipridinium chloride (CPC), a quaternary ammonium compound, may be added to the clinical specimen before shipment from outpatient clinics to the "Referral Laboratory". Decontamination, due to the CPC and liquefation of the sputum due to Naci, occur in transit. M. tuberculosis can survive 8 days transit without any significant loss of viability. Concentration and inoculation can begin upon arrival in the laboratory, without further processing the clinical specimens.

Since CPC is bacteriostatic for mycobacteria inoculated onto agar-base media, specimens processed with this chemical compound should be inoculated only onto egg-base[10].

After treatment with decontaminating solution, mixtures are then centrifuged to concentrate mycobacteria. Before this, to dilute toxic substances and decrease the specific gravity of the specimen, the digested-decontaminated sample must be diluted with sterile distilled water or sterile buffered normal saline.

However, for optimal recovery of mycobacteria, because many of these remain in suspension following centrifugation and can be poured off with the discarded supernatant fluid a relatively high centrifugal force such as 3000 x g has been recommended[5].

Sterile body fluids and tissue biopsies can. be homogenized and inoculation on to culture media can be done without any decontamination. Recovery and identification of M. tuberculosis

The growth requirements of M. tuberculosis are such that it will not grow on primary isolation on simply chemically defined media. The only media which allow abundant cultures are those supplemented with blood, bovine serum, bovine albumin or homogenized hen's egg. Of these, egg-enriched media containing glycerine and asparagine, or agar media supplemented with serum or bovine albumin seem to be the most suitable[2,9].

The most commonly used media for isolation of M. tuberculosis and NTM are:

1) egg-base media (Lowenstein-Jensen, Petragnani, American Thoracic Society),

2) agar-base media (Middlebrook and Cohen, 7H-10 and 7H-11),

3) liquid media (7h-9 broth, Dubos broth),

4) selective media (the Guft modification of
Lowenstein—Jensen, Mycob actosel, selective 7H-11), It has been shown that an increased amount of CO2 (5—10%) in the incubation atmosphere improves the growth of mycobacteria on egg-media, and is essential for the growth on 711-10-7H-11 agar.
According to Sommers and Good (1985) ideal culture media for the recovery of M. tuberculosis and NTM from clinical specimens should include one egg-base medium such as LowensteinJensen, a non selective agar medium and one selective medium. However, since the cost of media supplemented with serum or bovine albumin are very high, an egg-enriched medium, such as the standard Lowenstein-Jensen, as prescribed by the International Union against Tuberculosis seem to be the most suitable in a "Tuberculosis Control Programme"[9].
Lowenstein-Jensen medium is very sensitive, inexpensive and can be stored at 4° C for several months[5].
Media inoculated with the largest possible quantity of the sample should be incubated at 35—37°C and examined after 3 and 7 days and thereafter weekly for 6—8 weeks. Cultures that show grossly visible characteristic colonies, are removed for acid fast staining and further identification[2,3,5,6].
M. tuberculosis and NTM, may be identified by their acid fastness, growth rate, optimal growth, temperature, colonial photoreactivity, biochemical properties and growth inhibition tests[7].
All mycobacteria have cataIasic activity which is thermolabile for M tuberculosis and strong and thermostable 'for NTM. M. tuberculosis reduces nitrates to nitrites, is naturally resistant to 2 mg/L of thiophen-2-carboxylic acid hydrazide (TCH), to 500mg/L of 4(p)-nitrobenzoic acid (PNB), and produce a large amount of niacine[1].

DRUG SUSCEPTIBILITY TESTS

Drug resistance in mycobacteria is independent of exposure to antibiotics and arises by spontaneous mutations that occur at random in the wild bacteria population[8,9,11,12]. The proportion of these mutants can be relatively high also in culture of drug-susceptible bacilli isolated from previously untreated patients (Primary Drug Resistance). Their frequencies have been estimated, for example at about from 10-5 to 10-6 for isoniazid (INH), 10-5 for streptomycin (SM) and 10-7 for rifampicin (RM)[13]. The resistant mutants do not survive well and remain in minority in the normal environment[11]. However, when a patient is treated with active concentrations of a single antibiotic, susceptible bacteria are inhibited and resistant microorganisms are selected (Acquired Drug Resistance).
Because strains resistant to a given antibiotic are sensitive to other drugs, the frequency of resistant mutants to more than one drug decreases and could be estimated in about 1 every 10 bacteria when, for example, INH and SM are simultaneously used. Advantage is taken of these properties in combined therapy of tuberculosis[9,12].
Therefore, the only difference between M. tuberculosis strains which are sensitive or resistant to a single drug is the presence of a higher number of resistant mutants in resistant isolates than in sensitive strains. Clinical drug trials have statistically demonstrated that if more than 1% of a patient's tubercle bacilli are "in vitro" resistant to an antibiotic, the drug is not clinically useful. For such reasons the assessment of susceptibility to antibiotics with conventional diffusion techniques (total end point) are not suitable for mycobacteria: with these it is necessary to utilize a method allowing calculation of the proportion of tubercie bacilli, derived from the patient's specimen, which are resistant "in vitro" to each drug to a lesser or greater extent than 1% (pro'portion method: 1% end point).
The proportion method is the only method that enables an exact assessment of the rate of M. tuberculosis sensitive or resistant to a given drug. This technique, which is utilized in highly specialized laboratories (especially. in Italy, France and United States) gives results which, not only are reproducible, but also correlate well with the clinical data. However, in order to calculate precisely the proportion of resistant mycobacteria, procedures must be followed correctly; several dilution of the

inoculum have to be inoculated both in drug containing and in control media[5]. With a correctly performed proportion method it is possible to perform susceptibility test either from sputa rich in AFB (direct test) or from cultures of M. tuberculosis (indirect test).
With the direct test the inoculum size must be adjusted on the basis of the number of AFB seen in the smear[9,14].
Recommended dilutions for preparing inoculum from decontaminated concentrated sputum for the direct mycobacterial sensitivity test are included in table II.

Table II. Selected dilutions of Sputum concentrate for direct Mycobacterial Susceptibility Test.

Carbolfuchsin stained smear (Ziehl–Neelsen) No. of organisms per field (oil immersion objective 100 X)	Dilution
0 or <1	10^{-0} to 10^{-1}
1 to 10	10^{-1} to 10^{-2}
> 10	10^{-2} to 10^{-3}

The direct susceptibility test reflects the true situation of tubercle bacilli distribution in patient's lesions and makes it possible to know the results of a susceptibility test in three to four weeks, whereas almost 8 weeks are required when using the indirect procedure.
The indirect sensitivity method, that uses organisms previously grown on culture medium, is performed when smears are negative but cultures positive and when growth on control medium in direct sensitivity test is inadequate. In order to maintain the true situation of tubercie bacilli distribution as in patient's lesions, the inoculum for indirect susceptibility test should be prepared by selecting a proportion of all the colonies growing on the culture tube.
The inoculum is adjusted by turbidimetiy to yield countable colonies on one of the used dilutions.
Drugs under test are usually incorporated into the culture medium. The less expensive egg-based Lowenstein-Jensen medium is the most widely utilized. However, to avoid drug inactivation during the inspissation procedure, as well as the loss of potency of the antibiotic cornplexed to egg proteins, several laboratories recommend the use of 7H-10 agar medium[5]. This easily prepared medium must be icubated in 5 to 10% CO2; drugs ate added after autoclaving and just before pouring the plates, thus fully preserving antibiotic activity.
Drugs have to be added to the culture medium at critical concentration which inhibits the growth of sensitive tubercle bacilli without affecting multiplication of all resistant mutants[8,9].
Pyrazinamide, which is only active in acid environment (pH 5.5) needs to be incorporated in acid

culture media. However, as the acid pH of the medium itself could affect the growth of M. tuberculosis one acid medium without pyrazinamide should be included as a control[9].

To assess the presence of the drug in the medium, organisms of known susceptibility, such as a drug susceptible strain of M. tuberculosis H 37 Rv, should be included.

Sensitivity test cultures .are incubated at 35-37° C and results reported at 3 weeks. As resistant colonies often grow more slowly than susceptible ones, test results can be reported in less than 3 weeks only if drug resistance is shown[5].

Drug susceptibility tests are not generally performed for new cases of tuberculosis. If the incidence of primary resistance in a given population is less than 5%, a correct combined 3 drugs therapy can cover the risk of selective resistant mutant bacilli. For such a reason the American Thoracic Society suggests that susceptibility tests should usually be requested only in cases of tuberculosis which have been cured for a long period of time and which are likely to have acquired resistance.

Table III. Critical Drug concentrations for Proportion Method of Susceptibility Testing in Lowenstein-Jensen and Middlebrook 7H10.

Drug	Drugs final concentration (μg/ml)	
	Lowenstein Jensen	Middlebrook 7H 10
Streptomycin (SM)	4.0	2.0
Isoniazid(INH)	0.2	1.0
Ethambutol (EMB)	2.0	5.0
Rifampicin (RF)	30.0	1.0
Pyrazinamide (PZ) at pH 5.5	50.0	25.0

Table IV. When to perform Drug Susceptibility Tests.

–	when relapse or retreated case
–	when patient's specimens remain positive after 4 to 5 months of treatment
–	when patient's specimens become positive again after being negative
–	when primary drug resistance is suspected

Table III-VI shows such suggestions.
However, if epidemiological studies on the frequency and nature of Primary Drug Resistance have to be carried out, systematic antibiogramme must be performed in several new cases of tuberculosis selected according to an appropriate statistical procedure.

NON CONVENTIONAL PROCEDURES

There are some new exciting developments in the mycobacteriology laboratory. Procedures utilized to identify mycobacteria, such as thin-layer chromatography (TLC), mycolic and fatty acid analyses require sophisticated instruments and very large amounts of mycobacterial cells[4,16]. DNA probes are under evaluation, but up to now they lack the needed specificity and sensitivity. Monoclonal antibodies are being investigated to develop specific serologic tests and skin tests for hypersensitivity.
Fluorimetric assay of enzimatjc activity, TLC of mycobactins and serologic procedures measuring the immunologic relatedness of the mycobacterial catalase, are under evaluation[7]. Phage typing for epidemiological studies are so far utilized only by highly specialized laboratories[17,18].
Among the non conventional procedures one of the most widely used is the Bactec radio-metric System (Johnston Laboratories Inc., Cocheysville, M.O.). This method uses media containing ^{14}C-labelled substrata which, when metabolized by bacteria, yield detectable levels of CO_2. The radioactivity of the carbon dioxide is measured in the automatable ion chamber, and the relative value is printed out in Growth Index (G.I.) units, 100 of which are equivalent to 0.025 mCi of liberated $^{14}CO_2$ [19]. .

The same principle was employed to detect growth in pure culture[20,21], and for drug susceptibility testing[16,20,22] of M. tuberculosis by using a Middlebrook 7H-12 liquid medium, which contains palmitic-1-14 C acid as a labeled substratum.
This method was further developed to provide a rapid differentiation of tubercie bacffli from non-tuberculous mycobacteria by selective susceptibility to p-nitro-a-acetylamino-b-hydropropiophenone[23].
The radiometric procedure has been reported to reduce the time required to report culture results of M. tuberculosis from sputum[20,21] and from extrapulmonary specimens[22]. Results are available in 5 to 10 days compared with an average[23] days required by the conventional tests. Cultures can be also identified as M. tuberculosis in 5 to 7 days, and tubercie bacilli can be recovered and susceptibility test completed in an average time of 18 days, compared with the average 38 days of the conventional

procedures[4].
This new method and the many other techniques under evaluation can reduce the time required both for definitive diagnosis of tuberculosis and susceptibility testing. Their high costs, however, make them unsuitable for a “Tuberculosis Control Programme”.

CONCLUSIONS

In a “Tuberculosis Control Programme” the patient’s response to therapy should be always carefully monitored. If after 4 or 5 months of regular drug intake the patient’s smears or cultures are still positive the drug-regimens should pro. bably be changed. The role of the “Referral Laboratory” at this stage is essential. It is therefore important to emphasize that cultures, as well as susceptibility tests for M. tuberculosis hive to be performed only by reliable experienced laboratories. Those have to be proficient in mycobacterial studies, with internal quality control check, and at least 10 susceptibility tests done each week[11].
Only in such conditions the proficiency of the laboratory may be best maintained and major mistakes avoided. Wrong results, in fact, could be more dangerous for the patient than total absence of results.

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