



Tuberculosis: 7. Laboratory aspects of diagnosis

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The case

A 68-year-old woman has been complaining of a productive cough and weight loss of 3 kg over the past 3 months. She underwent mastectomy for breast cancer 3 years ago and is otherwise well. A sputum sample sent to the provincial laboratory tested positive for acid-fast bacilli. The physician explains the test result to the patient, who wants to know if she has tuberculosis. Should treatment be started?

According to the Laboratory Centre for Disease Control (LCDC), Health Canada, 1930 cases of new active and reactivated tuberculosis (TB) were reported in Canada in 1995.¹ This represents an annual incidence rate of fewer than 7 cases per 100 000 population, one of the lowest in the world; only Sweden, Australia, Iceland, Norway and a few small island jurisdictions have lower reported rates.² Because of the relative scarcity of TB, it is therefore not surprising that the typical Canadian physician seldom sees a patient with this disease. However, the low incidence of TB masks a heterogeneous distribution: foreign-born people account for 60% of all new and reactivated cases, and the TB rate of about 55 per 100 000 among Canadian aboriginal people is similar to rates in some countries of South and Central America.

The aim of this article is to inform general practitioners of the availability, timeliness, reliability and location of diagnostic laboratory services for TB in Canada and to review the suitability of various diagnostic techniques.

Diagnostic mycobacteriology is a complex and technically demanding branch of clinical microbiology. Unlike in some other infectious diseases, the TB clinical laboratory plays a critical role, not only in the diagnosis and management of the disease, but also in control and elimination strategies.

Laboratory diagnostic services for TB in Canada

In Canada there is a network of local, regional and national laboratories for the diagnosis of TB. The National Reference Centre for Tuberculosis of the LCDC has a central role in providing the diagnostic services that are required provincially and territorially, such as reference identification and susceptibility testing, as well as in training laboratory staff, in quality control and proficiency testing (as required by the provinces and territories), and in providing leadership in defining research issues and priorities.³

In its capacity as the World Health Organization Collaborating Centre for Tuberculosis Bacteriology Research and as the TB reference centre for the LCDC, the National Reference Centre for Tuberculosis coordinates these networks and provides proficiency testing both internationally⁴ and nationally.

An inventory of resources and services available within the network has been developed (see section entitled "Location and availability of TB diagnostic services"), and the coordination of an inventory of mycobacterial culture collections has been partly achieved. The creation and management of a centralized laboratory database for *Mycobacterium tuberculosis*, which would include information on drug resistance and DNA fingerprinting patterns, is in the planning phase.

Education

Éducation

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Conventional laboratory diagnostic tools

The examination of clinical specimens suspected of containing mycobacteria by conventional methods involves several diagnostic tools: microscopic examination, culture of samples, drug susceptibility testing and identification of isolates.

Microscopic examination

The use of microscopy to reach a rapid preliminary diagnosis of TB is of great value, especially in the detection of active, infectious cases. The turnaround time for smear microscopy is one working day from receipt of specimen to reporting of results. Because examination of direct smears can detect only concentrations of at least 10^5 acid-fast bacilli per millilitre of specimen,⁵ this method has limited sensitivity. In addition, its specificity varies with different patient populations, that is, specificity is very high in developing countries, but much lower in developed countries because of the frequent presence of mycobacteria other than those causing TB. Concentration of the specimen by centrifugation increases the capacity to detect mycobacteria by this method.^{6,7}

Usually 3 sputum specimens are collected: 2 "spot" specimens and 1 "morning" specimen. The morning specimen is more likely to yield positive results. Approximately 80% of patients who are ultimately smear positive will have a positive result on the first specimen, 15% have a positive result on the second specimen and 5% on the third.

To obtain a sputum sample, the physician gives the patient a labelled sputum container and takes him or her to a nearby open space, far away from other people, and gives the following instructions:

- Inhale deeply 2 or 3 times.
- Cough out deeply from the chest.
- Open the container, bring it close to the mouth and bring the sputum out into it.

Uses of laboratory results in tuberculosis (TB)

- detect and confirm disease
- follow up on treatment
- guide treatment in cases of relapse and treatment failure by providing information about drug resistance for such patients
- develop empirical chemotherapy
- define cases of TB, relapse and treatment failure, so as to monitor the progress of TB control and elimination strategies
- measure the success of treatment strategies by monitoring the development of multidrug resistance in the community
- define outbreaks by establishing relations between isolates, which allows identification of breaches in TB control measures.

- Do not put saliva or nasal excretions into the container.
- Close the container.

Between 2 and 5 mL of sputum should be collected. The type of sputum container is important; it should have a wide mouth, it should be made of break-resistant plastic, and it should have a screw cap to prevent leakage, desiccation and aerosol formation. The presence of acid-fast bacilli in such a specimen does not automatically imply the presence of *M. tuberculosis*. In fact, more often than not, the acid-fast bacilli are mycobacteria other than tuberculosis, and confirmatory testing is therefore unavoidable.

Fluorescence acid-fast staining is more expensive than conventional Ziehl-Neelsen staining but is associated with a higher rate of detection because the slides can be examined faster at lower magnifications. Fluorescence-positive slides are confirmed by Ziehl-Neelsen staining (Fig. 1).

Isolation of mycobacteria by culture

A definitive diagnosis of TB can be obtained only by culturing clinical specimens and testing the isolates further after preliminary identification. The specimens should be sent to the laboratory as soon as possible, because mycobacteria in sputum die within a few days. Culture of clinical specimens is associated with higher case detection rates, because the sensitivity of culture is much higher than that of smear microscopy; with this technique, concentrations of 10 to 100 bacilli/mL can be detected. Culture methods based on a combination of liquid or biphasic (solid and liquid) media, together with solid media, are used to ensure maximum sensitivity of detection and are considered the current "gold standard" for culture. The mean time for detecting the *M. tuberculosis* complex is about 2 days for the solid media currently in use.

Rapid methods can be used to complement conventional methods for the recovery of mycobacteria. The only well-established rapid method for detecting mycobacteria in clinical specimens is the BACTEC 460TB system (Becton-Dickinson Diagnostic Instruments Systems, Maryland). This system is based on the detection of radioactive carbon



Fig. 1: Ziehl-Neelsen stain of *M. tuberculosis* in sputum.



dioxide produced by bacterial metabolism of palmitic acid labelled with carbon 14. Growth of the mycobacteria can be detected within as few as 3 days, and the mean time to detect the *M. tuberculosis* complex is about 14 days,^{8,9} so a reasonable turnaround time for the culture and presumptive identification of this complex is 18 days from receipt of the specimen. Table 1 compares detection time and isolation rates for the BACTEC 460TB system and conventional culture media. In practice, more than half of mycobacterial isolates detected in public health laboratories in Canada are mycobacteria other than tuberculosis.

Testing of mycobacterial isolates for drug susceptibility

Isolates of the *M. tuberculosis* complex are first tested against first-line antituberculosis agents, specifically isoniazid, streptomycin, rifampin, ethambutol and pyrazinamide. Drug-resistant *M. tuberculosis* isolates are then tested for susceptibility to second-line drugs, including amikacin, capreomycin, clofazimine, cycloserine, ethionamide, kanamycin, ofloxacin and rifabutin. Testing of isolates of the *M. avium* complex for susceptibility to amikacin, clarithromycin, clofazimine, ethambutol, rifampin, rifabutin and streptomycin is also performed. Testing for susceptibility to other agents is performed at the National Reference Centre for Tuberculosis upon request and on an experimental basis. As a rule, testing of susceptibility to isoniazid and rifampin is more reliable than testing of susceptibility to streptomycin, ethambutol and pyrazinamide. Furthermore, testing of susceptibility to second-line drugs is less reliable than testing of susceptibility to first-line drugs.

To generate drug susceptibility results within a week, the testing is performed with the BACTEC 460 rapid radiometric method. The average turnaround time is less than 30 days from the time of specimen receipt (2 to 3 weeks for primary isolation and 1 week for drug susceptibility testing).

Identification of mycobacterial isolates

Conventional cultural and biochemical tests, along with newer methods (including high-performance liquid chro-

matography, growth inhibition in the BACTEC 460 NAP test (NAP is *p*-nitro- α -acetylaminobeta-hydroxypropionophenone, a selective growth inhibitor of the members of the *M. tuberculosis* complex) and DNA probes) are used to identify the *M. tuberculosis* complex. Typical turnaround time for confirmation of *M. tuberculosis* is 21 days from receipt of the specimen in the laboratory. Conventional cultural and biochemical testing is also useful for identifying species of mycobacteria other than *M. tuberculosis*, although turnaround times may be longer, depending on the species.

New diagnostic tools

Several new methods based on molecular biology techniques are increasingly being used in diagnostic TB bacteriology.

The polymerase chain reaction, which is based on DNA amplification methods, has been proposed for rapid detection of mycobacterial DNA in clinical specimens as a replacement for culture and identification of the *M. tuberculosis* complex. This technique has been extensively tested because it has the potential to shorten the time required for diagnosis from 2–3 weeks to 24 hours. So far it has not lived up to expectations, because in its present format it is not as sensitive or as specific as was originally thought.^{10,11}

Nucleic acid probes with nonradioactive detection systems have gained increased acceptance in the clinical TB laboratory as a replacement for fastidious, time-consuming mycobacterial identification tests. These probes can be used to identify isolates growing on conventional or radiometric media and are available for the detection of the *M. tuberculosis* complex. In this regard, they compare favourably with the BACTEC 460 NAP radiometric inhibition test. Turnaround time for test results once the sample has been cultured is 24 hours.

Because conventional methods for identifying mycobacteria are cumbersome, rapid methods for identifying many species of *Mycobacterium*, such as nucleic acid sequencing, have been developed. One of these methods is based on the sequencing of 16S ribosomal RNA (rRNA), also referred to as ribotyping. This form of rRNA has minor variations in its base sequence, which appear to correspond closely with established mycobacterial species. Preliminary results¹² have shown good correlation with conventional testing for some well-defined reference and test isolates, but the method fails to distinguish between the members of the *M. tuberculosis* complex, between *M. kansasii* and *M. gastri*, and between *M. marinum* and *M. ulcerans*. Attempts to identify problematic, intermediate-type mycobacterial isolates have so far yielded disappointing results.

New genetic tests for drug resistance based on the sequencing of DNA have been developed following the recent identification of mutations leading to either the modification of the streptomycin, rifampin, isoniazid or ethambutol target molecules or to the loss of activation of pyrazinamide or isoniazid. Because the targets for each

Table 1: Detection time and isolation rate for various media used to culture *Mycobacterium tuberculosis**

Culture medium	Mean time to detection, d	Isolation rate, %†
BACTEC 460, 7H12	14	72
Loewenstein-Jensen	28	62
Middlebrook 7H10	21	56
Middlebrook 7H11	21	52
Loewenstein-Jensen + 7H10 + 7H11	26	89

*Source: Tuberculosis case finding and chemotherapy.⁵

†Isolation rates were calculated by dividing the number of isolates detected with a particular media by the total number of isolates detected with all 4 media.



drug are different, a separate assay is required for each, and as many as 3 tests might be needed to detect resistance to a single drug. No gene has been found that accounts for all resistance to any of the main antituberculosis drugs. Current genetic tests typically detect only 50% to 95% of mutations, depending on the drug.

The technique of DNA fingerprinting,¹³ also referred to as RFLP (restriction fragment length polymorphism), has found a niche in the investigation of TB outbreaks, in other epidemiologic studies in which it is of interest to distinguish between exogenous reinfection and reactivation, and in investigations of laboratory cross-contamination. The analysis of results is difficult if the isolate has fewer than 5 bands and, in view of the occurrence of clustering of strains in some TB outbreaks, the basic assumption of the stability of this genetic feature remains to be demonstrated convincingly. The time required for standard IS 6110-based RFLP analysis is 6 to 7 days from the beginning of a work week, which includes the time for DNA extraction from heat-killed cultures. (IS 6110 is insertion sequence 6110, a se-

quence on the mycobacterial genome that allows the insertion of a piece of DNA.)

Turnaround time for analysis

The turnaround time from receipt of specimen to reporting of results to the physician and the public health authorities ranges from 1 day to as long as 4 weeks, depending on the method (Table 2).

Location and availability of TB diagnostic services

The diagnostic services available differ from one area of the country to another (Table 3). For the convenience of readers, a list of the members of the Canadian Tuberculosis Laboratory Network is given in the box on page 1729. Physicians should contact their provincial or territorial public health laboratory. If need be, these laboratories will contact the National Reference Centre for Tuberculosis.

Case resolution

The patient described at the beginning of this article should be told that, in North America, detection of acid-fast bacilli in a sputum sample is not diagnostic for TB. Further testing, such as culture of sputum, identification of the mycobacterial isolate and testing of the isolate for drug susceptibility, should be requested. In this situation, culture of *M. tuberculosis* from a sputum sample would be diagnos-

Table 2: Turnaround time (from receipt of specimen to reporting of results) for analysis of specimens in cases of suspected tuberculosis

Method of analysis	Turnaround time
Acid-fast smear	1 d
Culture report	1-2 wk
Identification of <i>M. tuberculosis</i>	2-3 wk
Drug susceptibility testing	3-4 wk

Table 3: Services provided in national and provincial tuberculosis laboratories of the Canadian Tuberculosis Laboratory Network, as of January 1998

Province/ territory*	Isolation					Identification							Special testing		
	Microscopy		Specimen			Tuberculosis		MAC		MOTT		Special testing			
	AUR	ZN-K	BACTEC	Solid media	Primary AMPL	Biochem	NA probes	Drug sus.†	Ident'n	Drug sus.	Ident'n	Drug sus.	RFLP	Drug levels	Other
Nfld.	R		R	R	X	R	R	1	R		R				
PEI	R	R	R	R											
NS	R	R	R	R			R	R-1	R						
NB	R	R	R	R											
Que.						R (HPLC)		2	R (HPLC)	R	R (HPLC)	R			
Ont.	R	R	R	R	R	R	R	2	R (HPLC)	R	R (HPLC)	R	R	R	PCR-X
Man.	R	R	R	R	R	R	R	R-2	R	R	R	R	R	X (urine)	
Sask. (N)	R	R	R	R	X	R	R	1	R		R				
Sask. (S)	R	R	R	R			R	R-1	R		X				
Alta.‡	R	R	R	R	X	R	R	R-2	R	R	R	R	R		MG/T
BC§	R	R	R	R	R	R	R	2	R	X	R	X	X		
NWT	R	R	R	R											
LCDC‡	X	X	X	X		R	R	R-2	R	R	R	R	X	R	PCR

Note: MAC = *Mycobacterium avium* complex, MOTT = mycobacteria other than tuberculosis, AUR = auramine fluorescence staining, ZN-K = Ziehl-Neelsen and Kinyoun staining, BACTEC = BACTEC 460TB system (Becton-Dickinson Diagnostic Instruments Systems, Maryland), AMPL = amplification, Biochem = biochemical testing, NA = nucleic acids, Ident'n = identification, Sus. = susceptibility, RFLP = DNA fingerprinting, R = test performed routinely, available at all times, X = laboratory can perform the test, if not routinely, HPLC = high-performance liquid chromatography, PCR = polymerase chain reaction, LCDC = Laboratory Centre for Disease Control, Health Canada.

*For addresses of laboratories, see listing elsewhere in this paper.

†1 = first-line agents, 2 = first- or second-line agents.

‡Certified level 3 laboratory.

§Pending level 3 certification.



tic for TB. In the meantime, the patient should be kept under observation for other symptoms of TB such as fever or sweating, and she should undergo skin testing and chest radiography. If the results of chest radiography are suggestive of active TB and if the results of a Mantoux test are positive, she should be started on antituberculosis chemotherapy until the laboratory results became available and the situation can be re-assessed in light of those results.

Competing interests: None declared.

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