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Diagnosis of active tuberculosis disease: From microscopy to molecular techniques

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ABSTRACT

Methods used for the laboratory diagnosis of tuberculosis are continually evolving in order to achieve more rapid, less expensive, and accurate results. Acid-fast staining and culture for mycobacteria remain at the core of any diagnostic algorithm. Following growth in culture, molecular technologies such as nucleic acid hybridization probes, MALDI-TOF MS, and DNA sequencing may be used for definitive species identification. Nucleic acid amplification methods allow for the direct detection of *Mycobacterium tuberculosis* complex within respiratory specimens without relying on culture growth, leading to more rapid diagnoses and appropriate patient care.

Introduction

Mycobacterium tuberculosis is the causative agent of tuberculosis, a disease causing significant worldwide morbidity and mortality. Tuberculosis remains a major public health problem with approximately one-third of the world's population infected [1]. In 2014, tuberculosis was responsible for the death of nearly 1.5 million people, representing a global mortality impact larger than any other infectious disease. The emergence of multi-drug resistant (MDR) strains has reduced viable treatment options and threatens to make tuberculosis both an untreatable and highly fatal disease. MDR tuberculosis elicits great economic and quality of life burdens due to costly and time-consuming therapeutic interventions. Reducing the burden of tuberculosis depends in part on the implementation of proper laboratory systems for the accurate and rapid diagnosis of active tuberculosis disease. Of the 5.2 million patients with pulmonary tuberculosis reported to the World Health Organization (WHO) in 2014, only 58% of cases were confirmed by laboratory methods such as smear or culture [1]. The remaining 42% of patients were diagnosed using clinical criteria alone (symptom history or chest X-ray), highlighting the need for increased availability of diagnostics in resource limited settings.

The *M. tuberculosis* complex (MTBC) is comprised of eight species which include *M. tuberculosis, M. bovis, M. bovis Bacillus Calmette-Guerin* (BCG), *M. africanum, M. caprae, M. microti, M. cannettii,* and *M. pinnipedii*. The majority of pulmonary tuberculosis cases are caused by *M. tuberculosis*, however, it may be clinically meaningful to identify members of the *M. tuberculosis* complex to the species level. For example, *M. bovis* is intrinsically resistant to the first-line drug pyrazinamide, and disseminated *M. bovis* BCG may be found as a complication following vaccination or intravesical instillation as treatment for bladder cancer [2,3]. Mycobacteria are obligately aerobic, nonmotile, rod-shaped bacilli. Members of the genus *Mycobacterium* have several unique characteristics as compared to other genera of bacteria, largely due to structural differences in cell wall composition. The cell wall of mycobacteria contains a higher content of complex lipids (>60% as opposed to approximately 5% and 20% in gram-positive and gram-negative organisms respectively) including long chain (C_{60}–C_{90}) fatty acids called mycolic acids [4,5]. Mycolic acids make the cell wall extremely hydrophobic and enhance resistance to desiccation, killing by disinfectants, staining with basic aniline dyes, and penetration by many of the drugs that are used to treat infections caused by other bacteria. These unique features of mycobacterial cell wall structure provide the basis for special laboratory considerations when performing direct stains from specimens, growing organisms in culture, and determining species identification by molecular methods.

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Table 1
Acid-fast smears and cultures prepared from early morning sputum specimens have better sensitivity.

Study	Random specimen positive (%)	Early morning specimen positive (%)
Abraham et al. [10] (smear positivity)	21/49 (43)	32/49 (65)
Ssengooba et al. [11] (MGIT culture positivity)	12/21 (57)	21/21 (100)

Specimen collection

Depending on the clinical manifestation of disease, virtually any specimen type may be processed for the presence of mycobacteria [6]. The most common sources are respiratory specimens including sputum, bronchial aspirates, and bronchoalveolar lavage fluid, however tissues, normally sterile body fluids, blood, and urine are also commonly submitted for analysis. Specimens should be collected in sterile, leak-proof containers and do not generally require transport media for preserving viability due to the hardy nature of mycobacterial organisms. Tissue may be placed in a small amount of sterile saline to avoid dehydration, while non-sterile water should be avoided due to the possibility of confounding contamination with environmental mycobacteria. Most specimens should be refrigerated during transport to the laboratory and up until the time of processing to maintain the viability of any mycobacteria present while preventing overgrowth of contaminating bacterial organisms [7].

Sputum is the most common specimen obtained for the diagnosis of pulmonary infection with MTBC and nontuberculous mycobacteria (NTM). To enhance sensitivity by smear, current guidelines recommend the collection of early morning sputum specimens on 3 consecutive days with a minimum of 8 hours between collections [8,9]. Mycobacteria become more concentrated in the sputum as patients sleep, so smear sensitivity increases with the use of early morning sputum (Table 1) [10,11]. Though supported by guidelines, this data is somewhat controversial [12]. Since infants and young children may have difficulty producing expectorated sputum, swallowed sputum may be aspirated from the stomach by gastric lavage. Since lengthy exposure to acidic gastric washings may decrease the viability of mycobacteria, specimens must be neutralized with sodium bicarbonate if not processed within 4 hours of collection. For patients that are unable to expectorate sputum, alternatives include sputum induction and collection of bronchoalveolar lavage (BAL) fluid. The induction of sputum using hypertonic saline with an ultrasonic nebulizer is a non-invasive method, while BAL fluid may be invasively collected during bronchoscopy [13,14]. Bronchoscopes should be decontaminated according to manufacturer's instructions between uses, and cleaning procedures should not utilize tap water which may contain environmental mycobacteria.

Non-respiratory specimens may also be collected for the testing of MTBC and other mycobacteria. As for sputum samples, clean-catch urine specimens may be collected on 3 consecutive days for culture [8]. Early morning collection provides the greatest sensitivity by culture since organisms accumulate in the bladder overnight. Normally sterile body fluids such as cerebrospinal fluid, pleural fluid, pericardial fluid, and synovial fluid may all be useful for culture of mycobacteria, however, these specimens are often paucibacillary and may require processing additional volume to achieve adequate sensitivity. In general, swabs are discouraged since they only are able to transfer a minimal volume of specimen onto culture media. Stool culture may be useful for the detection of disseminated *M. avium* complex (MAC) from AIDS patients, while lymph node, skin, and other biopsy tissue specimens may be processed under an appropriate clinical context.

Blood may be collected in tubes containing SPS, heparin, or citrate. EDTA tubes should not be used for blood collection [8,15]. The majority of disseminated mycobacterial infections occur in immunocompromised hosts and are due to MAC, however, bloodstream infections can also occur with MTBC and other NTM species. Blood for mycobacterial culture can be processed using either the Isolator tube system (Wampole Laboratories), BACTEC Myco/F Lytic bottles (Becton Dickinson), or BacT/ALERT MP bottles [16,17]. Isolator tubes undergo a lysis centrifugation method to recover intracellular organisms from whole blood specimens followed by inoculation of appropriate media plates. Myco/F Lytic and BacT/ALERT MP bottles are inoculated with whole blood and are optimized to promote the growth of mycobacterial and fungal organisms, which is monitored using automated blood culture instruments.

Acid-fast stains for mycobacteria

Microscopic evaluation of stained smears is a rapid and inexpensive screening method for mycobacteria within clinical specimens. While related to gram-positive bacteria based on peptidoglycan composition within the cell wall, mycobacteria are not reliably detected with the traditional Gram stain. Their hydrophobic cell wall resists penetration of aniline dyes such as crystal violet, so mycobacteria are either not visible with the Gram stain or may appear as bacilli-shaped clear zones or "ghosts" when direct specimens are stained [18]. However, under certain conditions arylmethane dyes are able to form stable complexes with the mycolic acids within mycobacterial cell walls. In the presence of phenol and applied heat, carbol fuchsin dye can be used as performed during Ziehl–Neelsen staining, which also utilizes methylene blue as a counterstain [19,20]. Since these cell wall dye complexes are resistant to destaining with mineral acids, mycobacteria are referred to as "acid-fast bacilli" or "AFB". The Fite stain is a modified acid-fast stain that uses weaker acid decolorization conditions to allow for the visualization of *M. leprae* and partially acid-fast organisms such as *Rhodococcus* spp. and *Nocardia* spp. [21]. Each of these stains use conventional light microscopy, while alternative stains utilizing fluorescence detection provide distinct advantages. Fluorescent stain consisting of a mixture of auramine O and rhodamine B dyes binds to the nucleic acids within acid-fast organisms [22]. Fluorescent staining is more sensitive and allows for more rapid reading of slides [23]. For these reasons, the WHO has endorsed the global phase out of conventional Ziehl–Neelsen light microscopy in favor of auramine-rhodamine AFB staining even though acquisition of fluorescent light-emitting diode (LED) microscopes remains challenging in resource-limited settings. In 2014, only 7% of laboratories had the capability of performing fluorescent AFB smears, up from 2% in 2012 [1].

Due to the small size of mycobacterial cells, sufficient training is required to reliably differentiate AFB from debris present in specimens that may be non-specifically stained. In addition to cell size (1–10 μm in length), a beaded staining appearance is suggestive of AFB (Fig. 1). The overall clinical sensitivity of sputum AFB smear is 22–80% depending on the burden of mycobacteria, the type of AFB stain used, and experience of the laboratory technician, while the positive predictive value for mycobacteria is > 95% [24]. However, acid-fast stains are not specific for MTBC as they cannot differentiate between mycobacteria species. Smear sensitivity varies greatly based on AFB burden within sputum with 1000–10,000 CFU/ml required for reliable detection. These higher AFB concentrations correlate with the severity of infection and positive sputum smears suggest a higher likelihood of infectivity for patients with pulmonary tuberculosis.