Improved detection of tuberculosis by Gene Xpert Ultra

Although Gene Xpert has shown high overall sensitivity and specificity with pulmonary samples, its sensitivity has been lower with smear-negative pulmonary samples and extrapulmonary samples. In addition, the prediction of rifampin resistance (RIF-R) in paucibacillary samples and for a few rpoB mutations has resulted in both false-positive and false-negative results.

An advanced version of the Gene Xpert MTB/RIF assay with better TB detection capabilities and more definitive identification of RIF susceptibility and resistance was developed.
This Gene Xpert Ultra had the following changes:

i) Inclusion of two new PCR assays that target two different multicopy genes,
ii) Conversion of the rpoB and IS6110 assays into fully nested PCRs, and
iii) Use of a larger PCR tube that doubles the amount of sample DNA that can be tested.

These changes have allowed for an almost 10-fold increase in analytical sensitivity for the detection of *M. tuberculosis* H37Rv. Importantly, this increase in sensitivity was not restricted only to H37Rv, which contains 16 copies of the IS6110 assay target. Sensitivity was also more than doubled for the detection of *BCG*, which contains only one IS6110 copy, suggesting that the additional sensitivity gained by Ultra is expected to be true for different clinical *M. tuberculosis* strains containing both high and low numbers of copies of IS6110.

When Gene Xpert Ultra was used on 277 sputum samples from TB suspects, the enhanced sensitivity of Ultra led to identification of more cases of smear negative and culture-positive TB and more TB cases overall. Levels of RIF resistance detection were comparable between Xpert and Ultra, although Ultra detected a hetero-resistant sample that was missed by both phenotypic susceptibility testing and Xpert. Analytic results have confirmed that Ultra has an improved ability to detect resistance in mixed samples, an observation that likely explains why Ultra detected RIF-R in the hetero-resistant clinical sample.

Importantly, the manual steps required to perform Xpert and Ultra are identical. Furthermore, the two assays can run in identical Gene Xpert instruments after a software upgrade. Thus, it is expected that Ultra can be implemented with little additional training in sites that already use the current Xpert assay.

Results suggest that Ultra will result in greater TB case detection rates are not only in subject to paucibacillary TB, such as those with HIV coinfection or tubercular meningitis, but also in pediatric patients with TB and those with extrapulmonary TB, which are known to have lower mycobacterial loads. As TB elimination programs achieve initial successes, rates of paucibacillary TB should increase compared to rates of smear-positive disease.

In this setting, assays with increased sensitivity, such as Ultra, may be instrumental in identifying the remaining cases of TB, and these assays are likely to prove valuable in furthering WHO goals to eradicate this disease.

References:

1. Chakravorty S et al. The new Xpert MTB/RIF Ultra: improving detection of Mycobacterium tuberculosis and resistance to rifampin in an assay suitable for point-of-care testing. 2017; mBio8:e00812-17

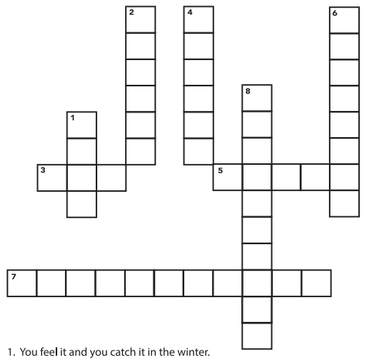
2. WHO Meeting Report of a Technical Expert Consultation: Non-inferiority analysis of Xpert MTB/RIF Ultra compared to Xpert MTB/RIF. Geneva: World Health Organization; 2017 (WHO/HTM/TB/2017.04). Licence: CC BY-NCSA 3.0 IGO.

3. Cousins Sophie: India rolls out new TB diagnosis aimed at catching child case. Devex. Sept 2017

> *"Doing what you like is freedom. Liking what you do is happiness. Let's be free and happy."*

PathCross

The crossword where fun and knowledge cross paths



1. You feel it and you catch it in the winter.
2. This might take your breath away.
3. The common cold is also known as _____.
4. Winters have people making tea with this vegetable, for cough and sore throats.
5. It's full of Vitamin C and makes a great summer drink.
6. Fruits and vegetables are full of these nutrients.
7. A doctor uses this to make sure you don't skip a heartbeat.
8. You use this to provide vaccinations.

Ans. 1.Cold, 2.Asthma, 3.Flu, 4.Ginger, 5.Lemon, 6.Vitamins, 7.Stethoscope, 8.Injections

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THE MICROSCOPE Newsletter

ISSUE NO. 1 | Vol. 1 | Jan-Mar'18

INDEX

RECENT ADVANCES IN DIAGNOSIS OF ACTIVE TUBERCULOSIS

Recent Advances in diagnosis of active tuberculosis

The *Mycobacterium Tuberculosis Complex comprises of* eight species which include *M. tuberculosis, M. bovis, M. bovis Bacillus Calmette-Guerin (BCG), M. africanum, M. caprae, M. microti, M. can- nettii, 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.

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 (C60 - C90) fatty acids called mycolic acids. 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.

Specimen Collection:

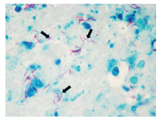
Depending on the clinical manifestation of disease, virtually any specimen type may be processed for the presence of mycobacteria. The most common sources are respiratory specimens including sputum, bronchial aspirates, and bronchoalveolar lavage fluid. However tissues, normally sterile body fluids, 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 re-frigerated 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.

Sputum is the most common specimen obtained for the diagnosis of pulmonary infection with MTBC and nontuberculous mycobacteria (NTM). Since shedding of pathogen may be intermittent in early stages of disease, most authorities suggest processing multiple samples, Revised National Tuberculosis Control Program (RNTCP) recommends at least two: one spot and the other early morning. Mycobacteria become more concentrated in the sputum as patients sleep, so smear sensitivity increases with the use of early morning sputum. 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. 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 Non respiratory samples, clean-catch urine specimens may be collected on 3 consecutive days for culture. 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.

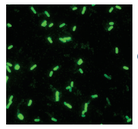
Blood may be collected in tubes containing SPS, heparin, or citrate. EDTA tubes should not be used for blood collection. The majority of disseminated mycobacterial infections occur in immunocompromised hosts and are due to M. avium complex (MAC), however, blood-stream infections can also occur with MTBC 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. 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 (AFB):



Microscopic evaluation of stained smears is a rapid and inexpensive screening method for mycobacteria within clinical specimens. 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. However, under certain conditions aryl methane 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 counter stain. Since these cell wall dye complexes are resistant to destaining with mineral acids, mycobacteria are referred toas `acid-fast bacilli' o `AFB'. Fluorescent staining is more

Number of AFB seen	Report
0 in 300 OIF	Smear is Negative for AFB
1 – 2 / 300	Doubtful, please repeat
1 – 9 / 100	AFB positive, 1 +
1 – 9 / 10	2+
1 – 9 / 1	3+
> 10	4+



sensitive and allows for more rapid reading of slides. 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. 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%. 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 about 104 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. Turn around Time for AFB smear should be 24 hours and if the report is in semiquantitative format, it could be of use in follow-up.

Specimen processing and decontamination:

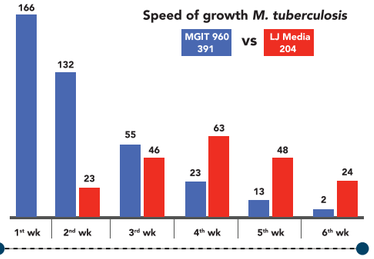
Since mycobacteria are slowly-growing organisms, the contamination of specimens with more rapidly growing bacteria may prevent their detection by culture. Non-sterile respiratory specimens typically contain bacteria that will overgrow any mycobacteria potentially present. Therefore, it is important to process specimens prior to culture in a way that will reduce the burden of contaminating bacteria without adversely affecting mycobacterial viability. Nonsterile specimens can be pretreated with a variety of agents, but the most common are N-acetyl-L-cysteine (NALC) and sodium hydroxide (NaOH). NALC is a mucolytic agent that helps to disrupt mucus present in respiratory specimens and releases bacteria so they can access nutrients provided by the culture medium. NaOH, often used at a final concentration of 2%, is a decontaminating agent that kills contaminating bacteria while leaving any mycobacteria viable for culture. Maintaining strict time limits for exposure to NaOH is important because mycobacteria are generally more hardy than other bacteria due to their waxy cell walls, but will also be rendered non-viable if exposed to NaOH for too long. Culture contamination rates must be monitored to ensure that the optimum balance of NaOH concentration and incubation times are being used. Contamination rates with the BACTEC MGIT 960 system suggests that an overall rate of 6-8% may be appropriate benchmark. Contamination of solid media should be monitored separately from broth cultures with 3–5% as an acceptable contamination rate. Conversely, contamination rates approaching 0% suggest that decontamination conditions are too harsh and may be adversely affecting mycobacterial recovery. After NALC-NaOH treatment, the specimen is centrifuged to pellet any mycobacteria followed by rehydration with a minimal volume of sterile phosphate buffered saline before being plated onto culture medium. Specimens may also be treated with various antibiotics to suppress the growth of bacterial and fungal contaminants. PANTA, a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin is often added prior to broth culture.

Mycobacterial culture-Solid media:

Following decontamination, specimens may be cultured for the growth of MTBC and NTM. Culture is traditionally performed on solid egg-based media, such as Lowenstein-Jensen (L-J) media, which is composed of egg proteins, potato flour, salts, and glycerol. L-J media supports good growth of MTBC, but is not as reliable for all mycobacterial species including *M. bovis* and *M. genavense*. Most Mycobacterium species, including MTBC, grow best at a temperature of 35 - 37°C. Select species such as *M. haemophilum, M. marinum, M. paratuberculosis, and M. ulcerans* have an optimum growth temperature of 30°C and may be cultured in a separate incubator. Like many aerobic bacteria, mycobacterial growth is stimulated by incubation in air containing 5-10% CO2. It takes upto 8 weeks for the colonies to grow. Despite the slow growth rate, sensitivity of culture is about 100x more than that of sputum and if the sample contained between 100 to 1000 bacteria, they will grow on solid medium.

Mycobacterial culture-Liquid media:

Optimal recovery of mycobacteria from clinical specimens is achieved through the use of a combination of solid and liquid media. In general, mycobacteria grow faster in broth than on solid media plates, which allows for improved patient management and clinical outcomes. Growth of MTBC from clinical specimens takes an average of 10 days by automated broth systems versus 20-25 days on solid media. The MGIT system is named for its use of Mycobacterial Growth Indicator Tubes. Each tube contains a modified Middlebrook 7H9 broth and a fluorescent indicator that is quenched by the presence of oxygen within the tube. Growth of mycobacteria in the medium consumes oxygen over time and allows the fluorescent indicator to signal as positive once a certain growth threshold has been met. The instrument continuously monitors tube fluorescence allowing lab staff to quickly identify positive tubes and begin the task of identifying any mycobacteria present. The MGIT system is an improvement over past BACTEC platforms that utilized radiometric assessment of growth and required manual intervention to place bottles on the machine once or twice per day for reading. In addition to faster growth, many studies have documented the improved sensitivity of the MGIT broth system as compared to solid culture.



In a study, 500 consecutive samples received in the laboratory for culture were processed by both MGIT and LJ methods and following results were obtained. Of the 500 cultured, 391 were positive by MGIT and only 204 by LJ. Majority of MGIT cultures gave positive signal by the first and second week while growth on LJ was visible atleast two weeks after it had been flagged positive by MGIT.

So MGIT was not only more sensitive for growth of *M. tuberculosis* by also more rapid as compared to growth on LJ. Once growth has occurred in solid medium, the species of mycobacteria should be identified by either a combination of biochemical reactions, mycolic acid analysis by HPLC, MALDI TOF or molecular methods. Recently a lateral flow assay doe detection of MTb 64 antigen has become available and it can identify *M. tuberculosis* from culture is 15 minutes. Growth from liquid media should also be similarly identified, in addition, it should be sub-cultured onto solid media to ensure purity and to rule out contamination or mixed growth.

Molecular detection directly from patient specimens:

Early and rapid diagnosis of a patient suffering from tuberculosis is required for patient's own benefit and to prevent spread of infection to other susceptible persons. Currently, there are a considerable number of commercial polymerase chain reactions (PCR) based assays available for the detection of *M. tuberculosis*, some of which incorporate testing for the presence of drug resistance genes, including the COBAS TaqMan MTB (Roche Diagnostics), the ProbeTee ET Direct TB (Becton Dickinson), the FluoroType MTB (Hain Life science), the m2000 Real Time MTB (Abbott Labs) and Gene Xpert MTB/RIF assay (Cepheid).

Following the official endorsement by the WHO in 2010, attention has focused on Xpert assay. This assay is based on a qualitative, nested real time PCR that allows *M. tuberculosis* complex to be directly detected in clinical samples. It targets MTBC specific rRNA and DNA sequences by utilizing transcription mediated amplification. Xpert MTB/RIF test is an automated, cartridge-based system that benefits from ease of use and a closed amplification system that reduces the potential for cross-contamination between specimens. The assay has a number of advantages: it can be operated by personnel with minimal training, the sample preparation required is minimal and the test result is available within 2 hours once the assay is initiated.

Xpert MTB/RIF assay has a sensitivity of 88–98% from smear-positive respiratory specimens as compared to culture as the gold-standard test. Sensitivity decreases to 66–74% from smear-negative specimens. Despite the reduction in sensitivity when AFB smear negative, Gene Xpert testing is still recommended for patients with suspected tuberculosis due to superior performance as compared to AFB staining. For this reason, culture must always be ordered in conjunction with more rapid smear and Gene Xpert tests. In addition, Gene Xpert tests cannot differentiate between live and non-viable MTBC, so they cannot be used to monitor response to treatment. The Xpert MTB/RIF assay has the added benefit of providing information about potential rifampin resistance, by detecting mutations in an 81-base pair region of the rpoB gene that are responsible for conferring approximately 96% of rifampin resistance in MTBC. Rifampin resistance is also a predictor of multidrug-resistant tuberculosis since the majority of rifampin-resistant isolates will also be isoniazid-resistant.

Sputum sample

Sensitivity of Xpert MTB/RIF assay in Smear Positive samples

Author	True positive	False positive	False negatives	True negatives	Sensitivity %
Bates	11	0	1	0	92
Vadwai	87	0	5	0	95
Miller	4	0	0	0	100
Malbruny	7	0	0	1	100
Armand	8	0	0	0	100

Sputum sample

Sensitivity of Xpert MTB/RIF assay in Smear Negative samples

Author	True positive	False positive	False negatives	True negatives	Sensitivity %
Moure	63	0	45	39	58
Bates	22	0	14	0	61
Zeka	17	5	10	140	63
Vadwai	38	0	20	0	66
Mulbruny	10	2	2	100	83
Al Ateah	11	0	1	49	92

Tissue Samples

Sensitivity of Xpert MTB/RIF assay

Author	True positive	False positive	False negatives	True negatives	Sensitivity %
Moure	29	0	17	12	63
Hillemann	20	3	9	204	69
Vadwai	54	51	16	157	77
Tortoii	71	13	11	274	87
Van Rio	139	23	10	172	93
Ligthelm	28	3	1	16	97

Lymph Node Samples

Sensitivity of Xpert MTB/RIF assay

Author	True positive	False positive	False negatives	True negatives	Sensitivity %
Van Rio	139	23	10	172	93
Ligthelm	28	3	1	16	97

CSF

Sensitivity of Xpert MTB/RIF assay

Author	True positive	False positive	False negatives	True negatives	Sensitivity %
Nhu	103	6	18	252	85
Syed Beenish	38	2	14	223	55

Miscellaneous tests for active infection

Miscellaneous tests for active infection: Assays based on the detection of lipoarabinomannan (LAM) in sputum or in the urine have been extensively evaluated in the research setting. A commercial test, the Determine TB LAM Ag (Alere) has become available. LAM based urine tests generally have insufficient sensitivity (< 50%) to be used as a rule out tests, but perform marginally better in HIV infected patients with low CD 4 cell counts. Adenosine deaminase based assays can only be used for the diagnosis of certain forms of extrapulmonary TB (TB meningitis, pleuritis, pericarditis and ascites) and have been shown to have limited sensitivity (typically < 50 to 70%). The recent detection of characteristic volatile organic compounds in the breadth of patients with pulmonary TB has opened promising new avenues. Its non-invasiveness makes this approach attractive, but it is likely that this method will require relatively expensive instruments. How it performs in paucibacillary disease (in children) and in extrapulmonary tuberculosis will need to be explored.

References:

1. Caulfield AJ & Wengenack NL. Diagnosis of active tuberculosis disease: From microscopy to molecular techniques. J clin tuber other myco dises 2016; 4:33
2. Maynard Smith L et al. Diagnostic accuracy of the Xpert MTB/FIR assay for extrapulmonary and pulmonary tuberculosis when testing non-respiratory samples: A systematic review. BMC Infect Disease 2014; 14: 709