DIAGNOSTIC MODALITIES EMPLOYED FOR THE DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS

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Abstract: Tuberculosis (TB) is the second most deadly disease around the globe. Because of the higher mortality rate, this disease has been given worldwide attention, killing over 2 million individuals every year. Tuberculosis has the highest prevalence in sub-Saharan Africa, whereas China, Bangladesh, Pakistan, India, and Indonesia globally account for nearly half of the tuberculosis load. Mycobacterium tuberculosis, whose coating is impervious, makes gram-staining difficult, therefore, there are other processes to identify this pathogen. This pathogen causes intense cold-like symptoms and thrives in the lungs, although it can also spread throughout the body. The main aspect of tuberculosis control is the quick diagnosis and efficient treatment of patients. In order to isolate and identify Mycobacteria, culture on slant media such as the Lowenstein-Jensen slant media is used. Biochemical testing is now surpassed by molecular or immune-chromatographic tests. The other common and accepted protocols for detection of active tuberculosis are nucleic acid amplification tests, antigen detection tests, Patho-TB kit, anti-tuberculosis antibodies and XPERT MTB/RIF molecular assays for quick recognition and simultaneous identification of rifampin resistance of M. tuberculosis right from patient samples. When screening for active tuberculosis, chest X-ray imaging is the most commonly used. There are also molecular diagnostic tools that use the growth of the desired organism in culture to aid in the diagnosis of a patient suspected of having active tuberculosis, such as nucleic acid probes, line probe assays, and DNA sequencing. An insight of major in-practice diagnostic modalities for M. tuberculosis has been presented in present review.

Keywords: Diagnostic modalities, mycobacterium tuberculosis, nucleic acid probes

INTRODUCTION

Active tuberculosis is a disease that is found around the world and has been in the spotlight for health care providers more so in recent years, especially in low and middle-income countries (1). Tuberculosis is an infectious and somewhat contagious disease caused by *Mycobacterium tuberculosis*. This pathogen affects the lungs primarily; leading to pulmonary tuberculosis, and can affect the liver, brain, joints, intestines, lymph nodes, and other tissues of the body. Tuberculosis is deadly and persistent, with a wide range of signs and symptoms (2). Moreover, it can also cause bovine tuberculosis in animals like cattle, which can sometimes be transmitted to a person (3). Rapidly emerging problems include the multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis*. CDC claims that MDR-tuberculosis is resistant to two of the most powerful drugs (isoniazid and rifampin); a scary thought (CDC, 2009). XDR-tuberculosis is also resistant to many potent first-line antibiotics, including rifampin and fluoroquinolones, but also many other secondary drugs, such as amikacin (4). Death rates for individuals with XDR-

tuberculosis are like those individuals from the pre-antibiotic era, a frightening thought.

GENERAL CHARACTERISTICS – *MYCOBACTERIUM TUBERCULOSIS*

It is a slender, slightly curved rod, having complex peptidoglycan–arabinogalactanmycolate cell wall that is consisted mostly of lipids and no endotoxins (5). An acid-fast bacillus, Mycobacterium tuberculosis captures the carbol fuchsin when acid alcohol decolorizes. This is because of mycolic acids in the cell wall, which are long fatty-acid chains. The bacteria, however, stains poorly with a gram stain due to the impermeable coating around the bacteria.

Mycobacterium tuberculosis spreads by coughing and sneezing by infected individuals, dust droplets covered with the pathogen that can be inhaled after sweeping, or even through food handled by a patient where the patient sneezed or coughed on the food or the utensils being used. Moreover, the pathogen can be spread through common Hookah smoking, contaminated milk, or even flies carrying infection from sputum to food (6). Incubation period consists of three to six weeks, but it can be months or even years in rarer cases.

SIGNS AND SYMPTOMS

The beginning of tuberculosis feels like a normal cold, with consistent coughing and sinus like symptoms. This disease even spreads the same way that a normal cold does, person to person. A simple cough or sneeze or inefficient hand washing can result in spreading of the disease.

There are two phases of infection, latent and disease. The condition of latent tuberculosis is where the vicious bacteria can remain in the body and not make an individual sick. Essentially, the bacteria are transferred to an individual when he or she breathes it in, but the body's immune system can fight off the bacteria through adaptive immunity. Individuals with a latent phase of tuberculosis do not feel sick or have any symptoms and therefore are unable to spread the bacteria to anyone else.

If an individual's immune system is unable to fight off the foreign invader, the bacteria can weaken the immune system, therefore multiply and divide, causing the individual to become sick. It is possible for a person with latent tuberculosis to go from being normal to becoming sick if the bacteria at any point become active. Because the immune system is not perfect and some conditions may cause the bacteria to survive, patients with latent tuberculosis are sometimes given treatment to save them from having the diseased phase of tuberculosis. It is estimated that about 2 million individuals carry a latent phase of tuberculosis (Houben and dodd, 2016).

Like the bacteria and viruses responsible for the common cold, M. Tuberculosis grows mostly in the lungs. For this reason, main symptoms include a rough cough that can last three weeks or longer, tightness and pain in the chest and abdomen, and/or output of blood and sputum with the rough cough. Associated symptoms include fatigue, weakness, and loss of appetite, chills, fever, and sweating. In more rarity, it is possible the bacteria can spread to the liver, kidneys, and brain, also known as extrapulmonary tuberculosis. For this reason, this infection may spread to the spine, causing back pain or can even spread to the kidneys, causing the output of blood in the urine. These earlier symptoms are similar to those of a common cold and can be misleading, so it is crucial to get a check-up because there is a possibility that *M. Tuberculosis* might be in the body. With any infection or disease, it is vital to take the proper measures to treat the disease. Visiting the doctor even when a common cold is suspected, getting regular check-ups to

avoid having latent tuberculosis become diseased tuberculosis, and taking medication to the fullest extent of its prescription helps eliminate the bacteria quickly and safely.

GLOBAL EPIDEMIOLOGY OF TUBERCULOSIS

NCBI has reported that tuberculosis is a disease that affects millions worldwide (NCBI, 2018). In 2016, about 10.4 million cases arose worldwide, a number that has been frustrating for public health experts who are trying to reduce this number. WHO reports that there are 22 countries that are at a higher risk of contracting tuberculosis, where Africa is the most burdensome, followed by countries such as China, Bangladesh, and Pakistan (World Health Organization, 2009). Pakistan ranks sixth in the world in terms of tuberculosis burden, as reported by the World Health Organization (WHO). Although it is thought that new infections and deaths have most likely been falling at a global level over the past few years of research. It shows that new tuberculosis cases have been gradually rising due to overall human population growth (3).

DIAGNOSIS OF *M. TUBERCULOSIS*

A core aspect of tuberculosis control is to identify the presence of the pathogen as quickly as possible. Even though in several settings delayed diagnosis or nonconfirmatory results of active tuberculosis cases have been observed due to unavailability of reliable diagnostic tools, molecular or immune-chromatography lateral flow testing is more efficient than biochemical testing, which was widely used in the past. Researchers have developed monoclonal antibodies against the pathogen, which have been shown to be extremely sensitive, specific, and inexpensive when observing M. tuberculosis. Other tests used for the identification of tuberculosis are nucleic acid amplification tests, slant media cultures, antigen detection tests and chest X-ray imaging (7). Culture isolates are conventional means for antimicrobial susceptibility testing, which is important for the accurate and precise clinical management of this disease. Table 1 displays the diagnostic tools that are reviewed by the WHO.

SMEAR STAINING AND MICROSCOPY

Traditionally, the diagnosis of tuberculosis was done by analysing sputa under the microscope and viewing the acid-fast bacilli, which is followed by a confirmation of diagnosis done by cultures (8). AFB, also known as acid-fast bacillus smear microscopy, is the first diagnostic protocol used to detect tuberculosis in the lungs, whereas DST, also known as drug susceptibility testing, isn't executed as commonly because these protocols are performed in areas where laboratories have up-to-date and time-efficient technologies. Managing and maintaining a reliable laboratory is costly and difficult in countries where tuberculosis kills a big portion of the population. Since AFB smear microscopy is time-effective for the clinician to diagnose his or her patient, all health related institutions consented that diagnosis needs to be confirmed with AFB microscopy in tuberculosis-endemic populations (9, 10). In most low-income populations, Ziehl-Neelsen-stained slides are the most commonly used tool and are seen under light microscopy. However, diagnosis with the use of fluorescent microscopy is 8-10% more responsive to change and displays faster screening of larger sample sizes at lower magnification (11).

Lack of quick responsiveness is a hindrance to smear microscopy, with a varying range from 20% to 80%. A researcher or lab technician would need a range of 5,000 to 10,000 bacilli in a sample

of sputum per millimeter for an unconcentrated smear, otherwise known as a positive direct smear. Recommendations by WHO for smear microscopy are:

- Fluorescent and light microscopy must be replaced by LED microscopy
- There must be evidence that microscopy of concentrated sputa that shows the results to be of exceeding superiority to smear microscopy.

ANTI-TUBERCULOSIS ANTIBODIES ANDELISA

In order to identify active tuberculosis, rapid and economically priced tests have been invented that use anti-tuberculosis antibodies. However, some of these quick methods showcase good responsiveness to change but are not specific enough to be functional in most situations (12). Antituberculosis antibodies test is performed on a sample of blood through an enzyme-linked immunosorbent assay (ELISA). Certain antigens are attached to a well, where antibodies for tuberculosis are added to the sample and will attach. Then, a second antibody conjugated with an enzyme marker is added. If there a positive signal, the marker will change color after the second antibody attaches to the first, indicating there are antibodies in the sample.

The tests are a great source in distinguishing a true tuberculosis infection from those caused by bacilli that don't cause tuberculosis, but the overall responsiveness to change is quite low. Low sensitivity can be detrimental, showing positive results when the result is actually negative, is both costly and time consuming to perform, and the materials used makes these tests poor alternatives to replace microscopy.

MYCOBACTERIUM TUBERCULOSIS CULTURE TEST

This culture is a test that helps in identifying bacteria that cause tuberculosis and other similar bacteria. The mycobacterial culture test is considered as the best test in the diagnosis of tuberculosis. Lowenstein-Jensen slant media, which is a solid agar, is used for separation, recognition and identification of mycobacteria from liquefied sputum samples, recommended by both CDC and IUATLD (Kent and Kubica, 1985 and Rieder et al., 1998). After eight weeks of isolation, the results are recorded by looking at biochemical and morphological characteristics.

Use of solid culture is economical compared to liquid culture and isn't contaminated as much as liquid broth can be. However, the liquid broth is more rapid and sensitive (nearly 15% more detection), and is more efficient since growth is recognized by monitoring fluorescence (13). Samples contaminated with normal flora are decontaminated by using a mixture of both NaOH and NaCl, which rapidly kills developing microorganisms, but has a narrow effect on the Mycobacterial activity. Recommendations provided by WHO for mycobacterial cultures are (13):

- Liquid broth must be economical and practical in low-income settings
- Liquid broth must have a shorter detection time and a higher rate of accurate isolation than solid cultures.
- It is crucial to quickly differentiate many different acid-fast microorganisms and identify mycobacterial growth.

PATHO-TB KIT

A tool based on filtration; Patho-TB kit is an uncomplicated method to detect tuberculosis. The filtration is followed by locating the bacteria on the filter using a gold conjugate, a biochemical method used commonly in immuno-chromatography. This test consists of

three main aspects: filtration, decontamination, and rapid test.

The kit platform, otherwise known as the filtration system, has a plastic cartridge with glass fiber and funnel. There is an empty space in the shape of a cube in the cartridge, with separate layers for straining and soaking, as well as a hole located in the middle. There is a thick porous pad at the bottom and a nitrocellulose membrane made up of 0.45 micron on the top. When the cartridge fills both layers, the funnel application enhances the responsiveness of the platform with chemical solutions. Many glass fibers are located in the filtration system that has applications in the first stage of the platform, which are responsible for removing debris and large particles from clinical or patient samples that can potentially obstruct the main membrane. In the aspect of decontamination, this next stage includes materials that are required to produce Kubica solution. This part of PathoTB Kit is used to absorb and decontaminate extremely polluted samples. This is an essential step because clinical samples almost always have unwanted microorganisms and debris. Kubica solution kills rapidly growing microorganisms and liquefies organic particles that are contaminating the mycobacteria. To further isolate the bacilli, centrifugation of the liquefied sample further increases the likelihood of accurate results later on.

The last system, the rapid test, has both negative and positive controls, dissolving and washing solutions, gold-conjugated antibodies, and a purified antibody (collected through affinity chromatography) against antigens made up of the lysed proteins from the mycobacterium (8).

XPERT MTB/RIF

This test is a molecular assay that is cartridgebased, which allows for quick recognition of *M. tuberculosis* and simultaneous identification of resistance to a drug called rifampin. Sputum or other clinical samples are liquefied and deactivated with a solution of NaOH and a reagent with isopropanol. To capture the bacilli causing tuberculosis, the liquefied broth filtered inside the cartridge where the sample resides. To export the bacterial DNA, sonication is done and right after, a polymerase chain reaction (PCR) is performed. The PCR employs a primer and DNA polymerase to target the rpoB gene, an 81 base-pair region of the bacilli, where more than 90% of mutations responsible for rifampin resistance occur (14). Five different probes are used to bind to the rpoB gene (wild-type) and the use of fluorescent signals showcase when binding has occurred. When there are at least two signals from at any two of the probes, the researcher or technician knows there is a presence of *M. tuberculosis*. However, a delay or failure in fluorescent signals indicates rifampin resistance (14). This test also has hindrances, the most important including the inability to differentiate between living and dead mycobacteria. Xpert MTB/RIF can still showcase as positive after the patient has been completely treated and definitely must not be used to evaluate drugs chosen to treat tuberculosis (15). Another hindrance of the Xpert MTB/RIF assay is that sputa samples are studied at around 130 bacilli per milliliter of the sample (16). For extra-pulmonary samples, the sample type indicates the range of sensitivity. The lowest sensitivity is in pleural fluid, whereas sensitivity is highest in lymph node fluid (17, 18). Limitations also include high cost, a requirement for continuous power supply, and sensitivity to higher temperatures. Xpert MTB/RIF Ultra is a more responsive molecular assay (like a liquid broth), one that is being clinically reviewed.

Recommendations by WHO for the Xpert MTB/RIF method are:

- In individuals suspected of having MDR-tuberculosis or tuberculosis associated with HIV, Xpert MTB/RIF should be used as the first diagnostic test (19)
- In patients suspected of having tuberculosis meningitis or extrapulmonary tuberculosis, this test must be used for cerebrospinal fluid and non-respiratory specimens (19)

LOOP-MEDIATED AMPLIFICATION TEST This assay, otherwise known as LAMP, is a method that employs isothermal amplification with the use of a heating device. This result is easily seen with the naked eye with the addition of a UV light and it can be performed in less than an hour. It, therefore, is advantageous because it's cost-friendly and easily implemented in low-income areas (20). WHO reported the sensitivity of LAMP to be over 95% and efficient for the positive sample, but lower for negative samples, ranging from 50-62% (19)? WHO also recommends that TB-LAMP can replace microscopy for diagnosing individuals suspected of pulmonary tuberculosis, or as a confirmatory test following microscopy.

URINE LIPOARABINOMANNAN RAPID TEST

This test is an alternative, based on the identification of secreted M. tuberculosis biochemical molecules. LAM, also known as lipoarabinomannan, is part of the outer cell wall of this pathogen that is detected in the urine of patients with tuberculosis. Although it's not certain that LAM in the blood circulation goes through filtration in the glomerulus, it is known that this is an unlikely explanation since LAM usually will circulate within a complex of immune system molecules or with a lipoprotein carrier molecule of high density (21).

Initially, the enzyme-linked immunosorbent assay-based (ELISA) test for LAM was used but is replaced with a lateral flow assay because there is inconsistency in distinguishing between the pathogen that causes tuberculosis and other Mycobacterium species. Therefore, a positive test is sufficient grounds to start treatment, but a negative result cannot be confirmatory to claim the illness isn't tuberculosis. WHO also has recommendations on LAM assays, which are:

- This test should be used as a confirmatory or screening test for tuberculosis (4)
- LAM assays must only be used to accompany in the diagnosis of tuberculosis in individuals with HIV who have very low amounts of CD4 biomolecules or individuals with HIV who are extremely ill (4)

MOLECULAR DIAGNOSTICS FROM CULTURE

GROWTH

Like many diseases, recognition of positive mycobacterial cultures was based on colonial morphology and selective chemical reactions, but these inferences were not definitive in identifying most mycobacterial cultures. Molecular methods permit quick species detection, more so than previously accepted biochemical tests (22). There are many molecular technologies that are accepted by several clinical diagnostic laboratories to identify mycobacterial cultures. Some examples include nucleic acid hybridization probes, line probe protocols, and sequencing of DNA.

NUCLEIC ACID HYBRIDIZATION PROBES

These devices are economical and costfriendly and allow for quick detection of Mycobacterium species. These FDA approved devices are available for identification of clinically relevant species

such as M. tuberculosis, M. avium, M. gordonae, and M. kansasii (22-25). The Figure 3 below shows how nucleic acids are exported during a sterilizing heating treatment, followed by sonication. These hybridization probes are species-specific and are labeled with chemiluminescence. The probe is designed to be quantifiable and complementary to the 16S ribosomal RNA of the bacilli, and if there is a match, the chemiluminescence from the DNA and RNA complement hybrid is easily detected. A larger amount of target nucleic acids is required more than other tests because there is no PCR or other amplification steps prior to probing.

Since these hybridization probes are only utilized with mycobacterial species or complexes, it is a limitation to researchers and lab technicians. This is because additional biochemical and molecular tools are required to confirm that identification of the mycobacteria that cause deadly tuberculosis in the clinical laboratory.

LINE PROBE ASSAYS

These assays also use hybridization-based probes but are an alternative method in detecting mycobacteria. With the usage of strips made of nitrocellulose membranes, DNA from isolates from lysed cultures hybridizes with probes that are species specific. These devices produce colorful strips when complementary DNA binds, indicating the presence of Mycobacterium. Three costeffective line probe assays are INNO-LiPA Mycobacteria assay, the GenoType MTBC test, and the Speed Oligo-Mycobacteria. The hybridization probes target distinctions that reside in the 16S or 23S RNA domains. Each probe is able to recognize M. tuberculosis members in complex, as well as many common non-tuberculosis mycobacteria species, species that are commonly mistaken morphologically for the bacilli that cause the disease. Sensitivity and specificity are usually more than 85% for line probe hybridization methods and within 3-5 hours the results are ready for analysis (26, 27). These probes are not currently approved by the FDA in the United States for clinical diagnosis, which is a hindrance.

SEQUENCING

Another standard method in detecting mycobacteria species is the method of Sanger sequencing. There are many completely

sequenced genomes for over 35 species, including M. tuberculosis, but it is more cost-friendly and time efficient to sequence certain conserved regions such as the 16S or 23S RNA sequences, heat shock proteins, or the rpoBgene responsible for encoding a subunit of the bacteria's RNA polymerase (22). Processes such as PCR are designed to bind to conserved regions, which are amplified. PCR also assists in flanking variable regions that are used to compare, contrast and differentiate among the mycobacterial species. Then, with the use of NCBI GenBank, a DNA database, the amplified DNA is then further sequenced to be compared to the database (28, 29). DNA sequencing is a valid tool for objective identification for a wide range of mycobacteria species including the pathogen of interest in this paper. After an organism is grown in the desired culture, the organism is sequenced and results are readily available within 8-24 hours.

CONCLUSION

As seen throughout this research paper, Mycobacterium Tuberculosis is deadly because it's easily spread, has evolved rapid drug resistance, and is most harmful in low-income areas. Organizations like WHO, report annually on the progression of the disease at levels that range locally to globally. That is why the tests and laboratory techniques discussed in this

paper are crucial to the diagnosis and treatment of tuberculosis. There is a collective effort to control tuberculosis within and among nations to provide the best clinical treatment to reduce drug-resistant forms of the disease and soon eliminate tuberculosis altogether as a public health issue.

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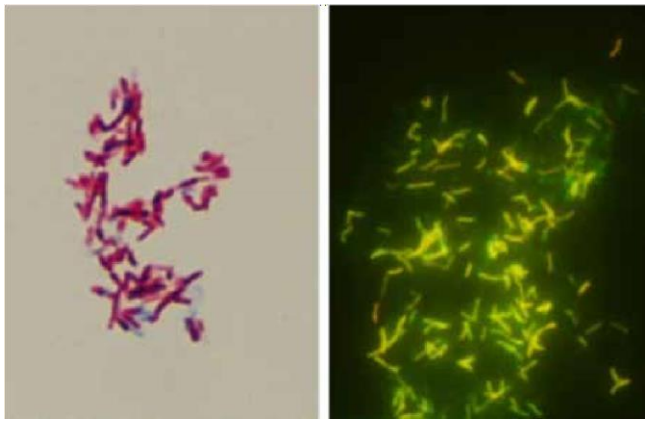

Figure 1: M. tuberculosis stains (acid-fast). Carbol fuchsin stain on the left and fluorescentauramine-rhodamine stain on the right

Figure 2: The BACTEC TM MGIT TM 960 instrument and culture tubes used to isolate

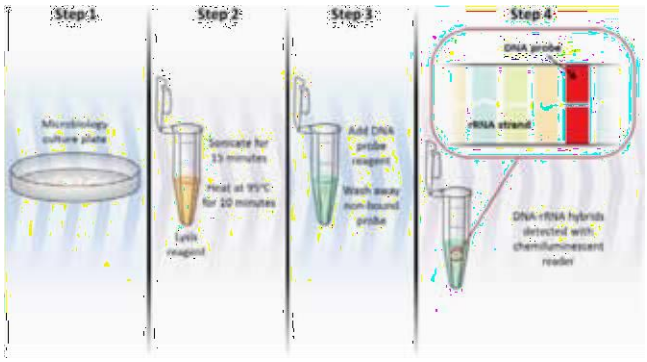

Figure 3: Nucleic acid hybridization probe steps