AN EVALUATION OF CURRENT DIAGNOSTIC METHODS FOR TUBERCLOSIS IN RESOURCE-POOR AREAS AND A PROPOSAL FOR A MORE SENSITIVE TEST

by

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ABSTRACT

Tuberculosis is a uniquely tough disease to diagnose and treat, mainly due to its unique cell membrane, the environment, and other socio-cultural factors. As HIV and TB rates rise in endemic countries, more and more people are becoming susceptible to an incurable TB infection, and more are being missed in the diagnostic process. Now, the need for a more sensitive diagnostic test that can be easily used in resource-poor settings is more necessary than ever. In this essay, a review of current TB diagnostic techniques used in resource-poor settings is discussed, as well as their drawbacks for detecting TB in the various forms of TB disease. To illustrate this point, one TB diagnostic clinic located in South India, is used as an example of how current techniques are used in resource-poor settings. Finally, the gold standard of TB diagnostics is compared to an already existing method adapted with the cheap and easy-to-use TB Beads. If TB Beads are in fact able to detect mycobacterium better, faster, and cheaper than other methods available, its importance in global public health and the control of TB disease is beyond measurable.

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1.0 INTRODUCTION

1.1 GLOBAL HISTORY OF THE TUBERCULOSIS EPIDEMIC

Mycobacterium tuberculosis is by no means a new public health threat; it has remained silently endemic in our world since the existence of mankind. The genius *Mycobacterium* is hypothesized to have originated more than 150 million year ago during the Jurassic Period; and an early progenitor of *Mycobacterium tuberculosis* infected hominids in East Africa as early as 3 million years ago[1, 2]. However, it wasn't until the Renaissance that mankind developed written documentation of TB diagnosis. René Théophile Hyacinthe Laennec, inventor of the stethoscope, first described the pathology of tuberculosis as well as the symptomology associated with pulmonary TB in terms that are still used in diagnosis today[3]. Presently, there were an estimated 8.6 million new cases of TB worldwide in 2012 alone (Figure 1), and 1.3 million deaths due to the disease. And TB disease is the second leading cause of death by infectious disease, after HIV[4].

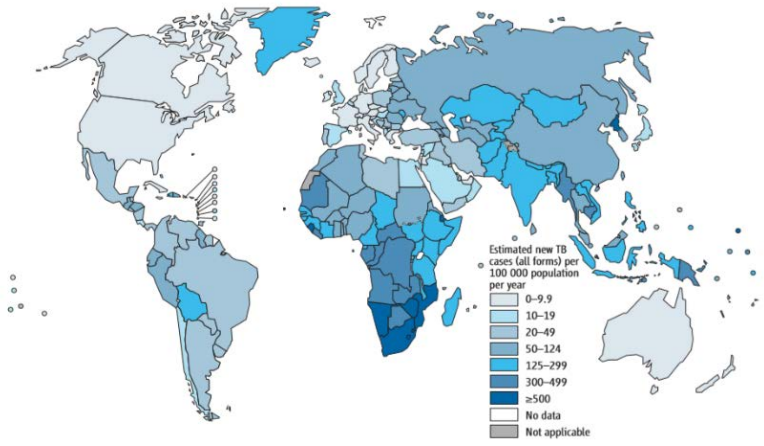

Figure 1. Estimated new cases of TB per 100,000 population in 2012 (WHO)

The driving forces behind resurgence of TB epidemics and the reasons why many countries hold TB endemic within their population are due to the lack of rapid point-of-care diagnostics. Less than 30% of the estimated number of people with TB were actually diagnosed in 2012 with a proven efficient diagnostic method. This is due to the fact that early TB diagnosis is dependent on test accuracy, accessibility, cost, and complexity, but also depends on the political will and funder investment. We now understand all too well that immediate and accurate diagnosis of TB is crucial in interrupting the transmission of *Mycobacterium tuberculosis* (*Mtb)*[4, 5].

As TB infections have waxed and waned throughout the centuries humankind is constantly struggling to stay one step ahead of the disease. Now, confounding factors like HIV, over-population, and multi-drug resistance (MDR) continue to threaten control of the disease[6]. The emergence of TB-HIV co-infections are quickly growing out of control; nearly one quarter of TB deaths in 2012 occurred in people co-infected with HIV[4]. The rapid increase in co-infection rates is partially due to the presenting atypical clinical symptoms, compared to those HIV-negative patients, causing TB detection rate to drop, and *Mtb* to therefore continuously spread throughout the community. Figure 2 illustrates that in TB- and HIV-endemic countries, less than 40% of TB cases were detected in 2010, emphasizing that optimal detection of *Mtb* infection in HIV-infected individuals remains a major challenge in resource-poor settings[5].

Figure 2. Estimated global TB case detection rates, 2010 (WHO)

In the 1990s new strains of *Mtb* have emerged that are much more expensive, time-consuming, and toxic to treat[7]. Multidrug-resistant tuberculosis (MDR-TB) is resistant to at least isoniazid and rifampicin, two first-line drugs for TB treatment[8]. The WHO estimated that there were approximately 450,000 new cases of MDR-TB worldwide in 2012, encompassing 3.6% of new TB cases and 20.2% of previously treated cases. Most of the highest rates of MDR-TB are located in central Asia, where MDR-TB accounts for more than 20% of new infections and more than 50% of previously treated cases[4]. Furthermore, only 7% of the estimated 500,000 new MDR-TB patients are detected each year, resulting not only in inappropriate treatment and continual transmission of MDR-TB, but also facilitates amplification of drug susceptibility and resistance[5].

1.2 TUBERCULOSIS IN INDIA

Over half of the estimated number of TB cases in 2012 occurred in South East Asia and Western Pacific, and India is one of 22 countries that accounts for approximately 80% of incident cases for these diseases. In 2012, India had the world's largest number of incident cases, totaling approximately 2.0 to 2.4 million people. Compared with China, which had the second highest number of global incident infections that year, 0.9 to 1.1 million people became infected with TB. Figure 3 shows the enormity of the situation, where India's TB incidence vastly outnumbers the other nine high burden countries. India alone accounted for 26% of the total global cases in 2012[4].

In 2012, the WHO designated India as a high TB, HIV, and MDR-TB burden country. TB-HIV co-infections is a fast growing concern for the population of India. Only 56% of TB patients

in India knew their HIV status in 2012, 5% of which were HIV positive. TB has become a huge barrier to economic and social development. Every year in India, an estimated 100 million workdays are lost, and every three minutes two Indians will die from TB. Unfortunately, TB support has recently decreased in the past year and the economic burden weighs heavily on the population. In 2012, TB incurred nearly $3 billion in indirect costs due in part to loss of job productivity and financial burden left on family members[4, 9].

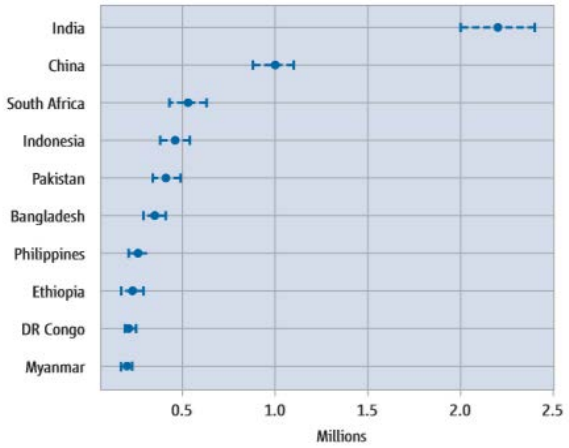

Figure 3. Estimated TB incidence (absolute numbers): top-ten countries, 2012 (WHO)

The Revised National Tuberculosis Control Program (RNTCP) is a government-run program created for the control of TB in India. The RNTCP provides completely free TB diagnosis and treatment for government and privately-run health clinics throughout the country, as well as incorporates principles of DOTS (Directly Observed Treatment/Therapy, Shortcourse), and the Stop TB Partnership developed by the WHO for comprehensive TB control[9]. Patients are able to access this free treatment at Designated Microscopy Centers (DMCs) located throughout the country. The DOTS strategy is currently used in 182 countries, and has been used in India since early 1993. Nine years after the program's national launch in 1997 the RNTCP declared the entire population of India covered by DOTS. The program has five core components which ensure

quality management of TB throughout the country: 1) political and administrative commitment; 2) good quality diagnosis; 3) an uninterrupted supply of good quality anti-TB drugs; 4) supervised treatment (DOTS); and 5) systematic monitoring and accountability.

The RNTCP estimates that more than 15,000 TB suspects are screened for TB daily in India, 3,500 of which are started on treatment. Diagnosis and follow-up with at least six months of treatment is established with over 50,000 sputum smears and laboratory specimens. Since the RNTCP first started, they have trained more than 600,000 health care workers and lab technicians in 11,500 DMCs. The RNTCP contributes their higher sputum-positive confirmed diagnosis, and their tripling of the treatment success rate to 86% (compared to the previous program NTP), as a result of their rapid expansion of DMCs and trained professionals[9].

However, Table 1 displays the shockingly low number of laboratories available for sputum smear, culture, and drug susceptibility testing for the continuously growing population of India. With culturing being the WHO recommended gold standard for TB diagnosis, there is less than one laboratory that is able to perform this technique per 15 million people. Compare these numbers to China and South Africa, with 11.1 and 4.2 labs available to do culturing per 15 million people, and we begin to understand where the mismanagement of TB diagnostics has gone so wrong in India. There are even fewer drug susceptibility clinics in India, with one clinic available for MDR-TB screening per 25 million population. Regardless of this, in 2012 India had the highest number of laboratory-confirmed cases infected with MDR-TB, totaling 16,588, 14,143 of which were actually started on treatment (Table 1)[4].

Table 1. Number of laboratories that perform smear, culture, and drug susceptibility testing in India, 2012 (WHO)

Laboratories	**2012**
Smear (per 100,000 population)	1.1
Culture (per 5 million population)	0.3
Drug susceptibility testing (per 5 million population)	0.2
Is second-line drug susceptibility testing available?	Yes, in country

1.2.1 LEPRA India and UMEED DMC, Hyderabad India

Hyderabad is the state capital of Andhra Pradesh, located along the southeastern coast of India. As the fourth most populous city in India, Hyderabad is a bustling metropolis where the vibrant cultures of India mix with internationally rated schools, tech corporations, and a rich history. According to recent 2014 estimates, the city of Hyderabad has a population of approximately 8.7 million, which has increased 28% since the last official census in 2011. 1.7 million people are estimated to live in 1,476 slums located across Hyderabad. Additionally, 24% of the city's population are considered migrants. Due to Hyderabad's large migrant community, overpopulated slums, and inadequate access to cutting-edge medical technology, Hyderabad represents an ideal city for evaluating the natural course of TB among its population in South India[10].

LEPRA India is a non-governmental organization that provides community-based services for TB, HIVAIDS, leprosy, malaria, lymphatic filiriasis, and basic eye care. UMEED designated microscopy center (DMC) is a collaborative health clinic shared between the Indian government and LEPRA India; one of the clinics run collaboratively by LEPRA and RNTCP, is located in the neighborhood of Gaddiannaram. The DMC serves a population of approximately 94,000 within

an area of 5km, including 20 slums with a population of 22,000. UMEED employs 34 DOTS providers who work at the dispensary, and 30 private medical practitioners who work at the adjacent 17-ward hospital. Most residents of Gaddiannaram neighborhood live within the catchment area of UMEED, meaning that they have legitimate and free access to the health clinic. UMEED is also well-known and respected in the community for its approach to the TB-HIV co-infection epidemic, allowing co-infected patients who live outside the catchment area to access the clinic for treatment, education, and support.

UMEED DMC recorded statistics of their diagnostic record ranging from 2006-2012. Over that timespan they tested 3,519 TB suspects, of which they found 457 to be positive for TB. During the same time they tested 1,823 of their follow-up patients at their two month treatment mark, 77 of which tested positive for TB (Figure 4). No data was found regarding prevalence of MDR-TB concerning the 77 patients who were positive after two months of treatment, however this does not necessarily mean that there was no follow-up conducted.

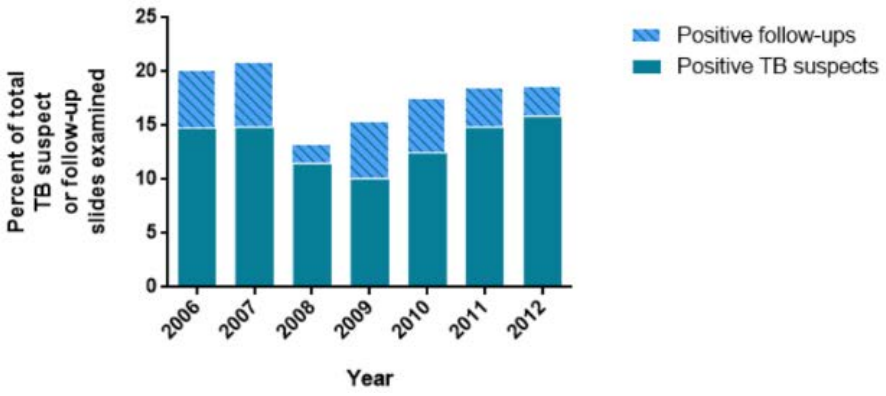

Figure 4. TB suspect and follow-up patients determined positive for TB by SSM, represented as percent of total slides examined for TB suspects and follow-ups individually, UMEED DMC, 2013 (McGuirk).

The Blue Peter Public Health & Research Center (BPHRC) is a clinical research facility funded by LEPRA India, as well as other NGO and the Indian government. The labs are divided into three divisions: clinical and epidemiological, immunology, and, microbiology and molecular biology. Their main research interests are leprosy, TB, malaria, HIV/AIDS, lymphatic filiriasis, and basic eye care.

1.3 CLINICAL MANIFESTATIONS OF TUBERCULOSIS

The type of diagnostic algorithms used for TB detection is directly related to the type of TB disease the patient presents with. Therefore, this section will elaborate on the different types of TB disease and the pathophysiology of the hosts' immune response attributed to *Mtb* infection. The risk factors for susceptibility towards *Mtb* infection may be different from the risk factors involved in progression to active TB disease[11]. Much of what happens after *Mtb* infection is determined by geographical location, strain type, host genetic background, and immunosuppression; however, a lot is still unknown[5].

1.3.1 Active TB

Active TB infection causes a variety of symptoms, due to individual hosts' immune response to various triggers[11]. As opposed to latent TB, active TB disease can take many forms, which are typically diagnosed by their symptomology and tests detecting the presence of *Mtb*. The most common form of active TB is pulmonary TB. Pulmonary TB is classified as an active state of TB disease, and is often characterized by a cough lasting two to three weeks, becoming more

productive throughout the course of infection as more tissue is destroyed. Other symptoms may include, but are not limited to: unexplained weight loss, loss of appetite, night sweats, fever, fatigue, chills, coughing up blood, and chest pain[12]. Although these symptoms are the most obvious ways to tell someone is suffering from TB disease, unfortunately only a small percentage of people with active TB will develop these symptoms, making cough and fever insensitive predictors for TB disease[13]. Pulmonary TB can be confirmed by the detection of *Mtb* in the sputum, using sputum smear microscopy as the diagnostic tool.

1.3.2 Latent TB

Often dubbed “the hidden epidemic”, it is estimated that one third of the world is latently infected with *Mtb*[14]. Latent TB infection is defined as a homeostasis, creating an equilibrium between host resistance and bacterial persistence[15]. Granulomas, a characteristic of latent TB, are a complex and organized collection of host inflammatory cells which have surrounded the *Mtb* infection, limiting the bacilli’s’ multiplication and dissemination throughout the body[16]. Due to granuloma formation, latent TB is not a form of active TB disease, therefore there are no clinical symptoms, nor is the patient infectious[17]. However, disruption of the granuloma structure, is likely to lead to reactivation of latent *Mtb*, and active TB disease. Factors leading to granuloma disruption include: chronic diseases, HIV, malnutrition, tobacco smoke, indoor air pollution, alcoholism, silicosis, insulin-dependent diabetes, renal failure, malignancy, and immune suppressive treatment, [5, 15, 16]. Approximately 10% of latently infected people will develop active tuberculosis in their lifetime[14]. However, it has been shown that *Mtb* can persist within a human host for decades[18].

1.3.3 Extra-pulmonary TB

Extra-pulmonary TB is a type of active TB disease in which *Mtb* can be found in a multitude of tissues and organs within the host. This state of TB disease is especially common in the immunocompromised host, and is often present in HIV positive individuals[19]. In addition, symptoms of extra-pulmonary TB are often confused with other diseases, making extra-pulmonary TB difficult to diagnose and treat[20]. The WHO estimated there were 0.8 million incident cases of extra-pulmonary TB reported in 2013. However, the global presence of extra-pulmonary TB is difficult to assess because the disease is not yet considered a credible threat to public health by those who are still struggling to control the more evident pulmonary TB. Extra-pulmonary TB may also not be considered the culprit as often as pulmonary TB due to that fact that it is extremely difficult to diagnose, especially in resource-poor settings[4].

In the early 1980's before the HIV epidemic, approximately 85% of reported TB cases were pulmonary, with the remaining 15% classified as either extra-pulmonary or a mixture of the two. A recent retrospective study of people co-infected with TB-HIV reported that 38% had pulmonary TB, 30% had extra-pulmonary TB, and 32% had both pulmonary and extra-pulmonary disease, detailing a significant increase in the number of extra-pulmonary TB cases[21]. Sputum smear-negative and extra-pulmonary TB now account for as much as 60% of all TB reports, especially in high HIV prevalence areas[22]. Because extra-pulmonary TB occurs at sites that are not readily available or dangerous for most diagnostic clinics to access, the patient will need to be transferred to a hospital, incurring a much higher expense or increasing the risk of patient-loss. In addition, because of the nature of the sites involved, fewer bacilli are necessary for a pronounced infection. This combined with poor accessibility to proper healthcare make bacteriologic diagnosis difficult in resource-poor areas[21].

1.3.4 TB-HIV co-infection

In 2012, 1.1 million people worldwide who became infected with *Mtb* were HIV positive; TB is the most common cause of mortality in HIV infected adults living in resource-poor countries [4]. The risk of an HIV positive person developing TB disease is between 20 to 37 times greater as compared to those who are HIV negative[23]. The emergence of the HIV epidemic has drastically changed the detection, epidemiology, and treatment outcomes for TB[24]. It is well-known that HIV destroys CD_4 T cells, which is theorized to contribute to the susceptibility of HIV positive persons to *Mtb* infection. HIV also affects macrophages, and has influence on cytokine production, which may prevent the host from progressing to latent TB and contain the *Mtb* infection in a granuloma[15]. HIV infection has been shown to increase the risk of reactivating latent TB, as well as rapidly progressing the disease, as compared to those not infected with HIV[22]. Several studies in TB-HIV endemic areas have shown that HIV leads to an increased risk of developing TB shortly after HIV infection. For example, HIV positive South African miners were two to three times more likely to develop TB than their HIV negative counterparts within two years of HIV infection[25].

1.4 STATISTICAL CALCULATIONS FOR DIAGNOSTIC TESTS

The sensitivity of a diagnostic test is the probability that the test in question is positive given that the patient has the disease. The specificity of a diagnostic test is the probability that the test in question is negative given that the patient does not have the disease. For a diagnostic test to be effective in predicting disease, it is important that both the sensitivity and specificity be high[26].

Table 2 has more detail in calculating sensitivity and specificity for a new diagnostic test when compared a gold standard. The positive predictive value (PPV) of a diagnostic test is the probability that the patient has the disease given that the test in question is positive. The negative predictive value (NPV) of a diagnostic test is the probability that the patient does not have the disease given that the test in question is negative[26]. See Table 2 for an example of calculating PPV and NPV. A false positive, also known as type I error, is defined as a positive test result when the disease being tested for is not actually present. A false negative, also known as type II error, is defined as a negative test result when the disease being tested for is actually present (Table 2)[26].

Table 2. Example of sensitivity, specificity, PPV, and NPV calculations for new diagnostic tests compared with the gold standard, 2014 (McGuirk)

Test outcome

Gold standard	*Test positive*	*Test negative*	
Disease present	True positive	False negative	**Sensitivity** = Σ true positive / Σ disease positive
Disease absent	False positive	True negative	**Specificity** = Σ true negative / Σ disease negative
	PPV = Σ true positive / Σ test outcome positive	**NPV** = Σ true negative / Σ test outcome negative	

where Σ = the sum of all

2.0 A REVIEW OF CURRENT TB DIAGNOSTIC METHODS IN RESOURCE-POOR SETTINGS

Despite the fact that more accurate methods are available for TB diagnosis, many laboratories around the world use the same methods today that were used nearly half a century ago. One possible reason for continuing use of these conventional methods is simply due to the lack of technical expertise and equipment[14]. When considering the dynamics of TB disease, using one diagnostic method alone may not be sensitive enough to accurately diagnose TB [21]. Therefore, the methods presented in this section are listed in order of their preference and simplicity, which is typically (but not exclusively) correlated with lower cost and less expertise needed. In addition, not all TB diagnostic methods are mentioned in this essay, only ones commonly used in resources-poor settings.

2.1 PASSIVE CASE-FINDINGS

Passive case-finding is usually the first technique used to detect active TB in resource-poor settings. It is defined as identifying pulmonary TB among people who are actively seeking care, or in the context of TB-endemic areas, those who are aware they have been exposed to *Mtb* and/or know they are exhibiting the symptoms of TB and understand that they need medical care. Theoretically, passive case-finding would be the best scenario for pulmonary TB detection due wholly to the fact that it is free of cost for the patient and the healthcare provider. However, the majority of people who have been exposed to *Mtb* are either unaware that they are infected,

attribute their infection to another disease, are deterred by costs they might incur for visiting a clinic, or simply do not exhibit the typical symptoms of TB, and therefore do not seek treatment. Symptom screening is typically performed by a health care worker at a clinic. Their training and patient care history can vary from volunteer community health workers to possession of a medical degree. These patients are usually exhibiting the traditional symptoms of pulmonary TB, and are therefore known as “TB suspects” until their case can be confirmed by culture, smear, and/or other methods. The sensitivity of using symptom screening as a diagnostic tool is quite low, ranging from 35-84%, depending on the specific symptom being used[13]. In addition, with symptom screening the higher the PPV the more valuable the test is. Although clinicians cannot directly measure the PPV of symptoms, they can measure how often specific symptoms occur using sensitivity and specificity[26].

2.2 SPUTUM SMEAR MICROSCOPY

Sputum smear microscopy (SSM) is the most common method for diagnosing TB in resource-poor settings, as well as one of the most unpredictable. A sample must be produced before SSM can be performed; the sample is usually sputum (for pulmonary TB diagnosis), but SSM can be used with other (extra pulmonary) samples as well. There are several crucial steps that must be understood and followed by both the patient and the technician when collecting and processing the samples, which leaves room for multiple errors. It is recommended that personnel observe the sputum collection to avoid accepting samples such as nasal discharge and saliva, which are not sputum (material brought up from the lungs after a productive cough). The RNTCP has recommended that two sputum specimens are to be collected over one, or two consecutive days, in order to improve

the chance of detecting *Mtb* by SSM. In addition, it is recommended that one of the samples be collected on the spot and the other collected in the early morning by the patient at home[21, 27]. However, providing two samples on two separate days is often a practical challenge for many patients who may travel far to reach a clinic that specializes in TB diagnosis and SSM[28].

The detection of acid-fast bacilli (AFB) in stained sputum smears from clinical samples is the easiest and quickest bacteriologic method to prove the presence of *Mtb* infection. It can also give a quantitative estimation of the number of bacilli being produced and the infectiousness of the patient. SSM was developed based on mycobacteria's unique ability to retain fuchsin dye within its mycolic acids in the presence of a strong acid or alcohol. Ziehl–Neelsen (a type of carbolfuchsin staining) is the most common method used for acid–fast staining in resource-poor areas due to its low cost and quick methodology. However, studies have shown that there must be 5,000 to 10,000 bacilli per milliliter of sputum sample in order to visualize the mycobacteria in the stained smear[29]. Unfortunately this bacterial load is not typical for many of the presentation that TB disease can take, therefore the sputum smear is far too insensitive for much of *Mycobacterium* detection.

The Stop TB Partnership and DOTS recommends using oil immersion light microscopy and scanning the stained smear from left to right, covering approximately 100-150 microscopic 100x fields. This should take about five minutes for a trained technician to complete. Since *Mtb* can be variable in shape and size, it is important for technicians to count even one bacilli as a positive result. The WHO recommends scanning up to 300 fields of view before reporting a negative result, which can be both time consuming (15 minutes per slide) and exhausting. Table 3 shows the most common way laboratories record AFB quantifications during SSM in resource-poor areas[30].

Table 3. Recording results of AFB sputum smear quantification (The Stop TB Partnership)

Finding	Recording
No AFB found in at least 100 fields	negative
1-9 AFB per 100 fields	exact figure/100
10-99 AFB per 100 fields	+
1-10 AFB per field (count at least 50 fields)	++
More than 10 AFB per field (count at least 20 fields)	+++

Table 4 displays the sensitivity and specificity of SSM as compared to the gold standard of culturing. Factors influencing the sensitivity of SSM include, sample collection, disease state or type, technicians' experience in staining and reading, and the prevalence of TB in the population[21]. If given the choice between symptom screening or SSM, it is not surprising that most clinicians would be able to give a more accurate diagnosis given the patients symptoms[5, 13].

Table 4. Sensitivity and specificity of SSM as a diagnostic tool for pulmonary TB, using culture-confirmed pulmonary TB as the gold standard, 2013 (WHO)

Diagnostic test	Sensitivity % (95% confidence interval)	Specificity % (95% confidence interval)
Culture (gold standard)	100	100
SSM	61 (31-89)	98 (93-100)

Although smear-positive cases are the most infectious, approximately half of pulmonary TB case overall are sputum smear-negative, and are typically those who are immunosuppressed[17, 24]. In sputum smear-negative pulmonary TB, the *Mtb* bacterial load is so low in the specimen that it cannot be detected by sputum smear microscopy, which has a low sensitivity compared to other diagnostic methods. The Stop TB Partnership and DOTS has developed an algorithm (Figure 5) for effectively diagnosing pulmonary TB in resource-poor

settings. Due to the necessary time requirements and cost associated with these methods, sputum smear-negative patients are more likely to be admitted to a hospital, lost to follow-up, or simply diagnosed as TB negative[27].

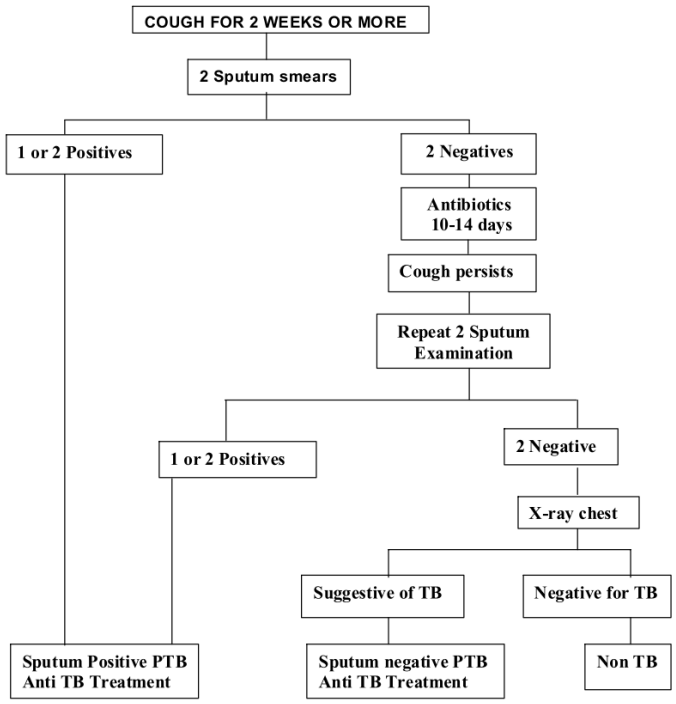

Figure 5. Diagnostic algorithm for pulmonary TB in resource-poor settings, 2009 (DOTS)

With so many personnel, equipment, and technology invested in SSM over the decades, the WHO estimates that only 57% of the 4.6 million incident pulmonary TB patients in 2012 were bacteriologically confirmed [4]. Furthermore, the arrival of TB-HIV co-infection has completely changed everything we know about SSM. It is theorized that If SSM is used for diagnosis in HIV-endemic areas, about one in five people co-infected with TB-HIV will have a negative result[21]. As many public health systems still struggle with finding, detecting, and treating typical TB patients, those co-infected with HIV presenting with abnormal symptoms are often overlooked.

2.3 CULTURING OF MYCOBACTERIA

The WHO recommends that all clinical samples suspected of *Mtb* infection be inoculated onto culture media for four reasons: (1) culture is much more sensitive than microscopy; (2) culturing is necessary for precise identification of mycobacterial species; (3) culture is required for drug susceptibility testing; and (4) genotyping of cultured organisms may be useful to identify epidemiological links between patients. The specimens may be very viscous, holding suspected *Mtb* organisms within the sputum matrix of mucous. Therefore, before the gold standard of culturing can begin, it is necessary to clarify (liquefy) the sputum samples so decontaminating agents may kill contaminating bacteria and surviving *Mtb* may easily access nutrients of the culture medium[21]. Samples are typically clarified with N-acetyl-L-cysteine (NaLC--a mucolytic agent) and then decontaminated with a 1 to 2% sodium hydroxide (NaOH) solution to ensure that the fast-growing contaminating organisms are killed, allowing the *Mtb* to grow at their slower rate (henceforth referred to as "NaLC-NaOH"). The WHO recommends that sputum processing take place in a biosafety cabinet (BSC) and in a minimum biosafety level two (BSL2) laboratory[31].

Due to their unique cell membrane, *Mtb* is considered to be very robust organism that is able to survive in harsh environments. It was once thought that the chemicals used to decontaminate specimens do not affect *Mtb* nearly as much as the contaminating organisms. However, more studies are suggesting that the decontamination procedure may kill up to 90% of *Mtb* in the specimen [21, 32, 33]. Although culturing is a more sensitive technique compared to SSM, the significance of *Mtb* killing during clarification cannot be overlooked. With more immunosuppressed patients coming to DMCs for TB diagnosis, there is a great demand for more specific *Mtb* detection at all stages of disease, something that the culturing may not be able to offer as long as NaLC-NaOH is being used.

The sensitivity of culture is 80–85% with a specificity of approximately 98%. Three different types of traditional culture media are available: egg based (Löwenstein–Jensen), agar based (Middlebrook 7H10 or 7H11 medium), and liquid (Middlebrook 7H12). *Mtb* grow more rapidly on the agar medium (approximately 3-8 weeks) compared to Löwenstein–Jensen slants, and even faster in liquid media (1-3 weeks). A drawback to liquid media is the inability to isolate specific *Mycobacterium* species. Diagnostic labs in resource-poor areas tend to use Löwenstein–Jensen (LJ) slants because *Mtb* tend to grow slightly better on it (although it takes 3-8 weeks), and it is also a cheaper alternative. Löwenstein–Jensen slants are also used for detecting rare *Mycobacterium* strains that may not grow on the other media. Table 5 shows a commonly used scale for quantitating growth on Löwenstein–Jensen slants[21].

Table 5. Quantification scale for mycobacterial growth on Löwenstein–Jensen slants, 2000 (CDC)

No. of Colonies Seen	**Quantity Reported**
No colonies seen	Negative
Fewer than 50 colonies	Report actual number seen
50-100 colonies	1+
100-200 colonies	2+
200-500 colonies (almost confluence)	3+
>500 colonies (confluence)	4+

A major improvement in *Mtb* diagnostics has been the development of commercial broth systems for mycobacterial growth detection. Automated culture systems such as BACTEC 460, Mycobacterial Growth Indicator Tube (MGIT) systems, Extra Sensing Power (ESP) Myco-ESPculture System II, and BacT/ALERT MB Susceptibility Kit, use Middlebrook 7H12 media

with added radiometric or colorimetric material for detection of *Mycobacterium*. These systems allow for rapid growth in liquid culture with drug susceptibility testing, whereas agar and egg-based media allow detection of mixed cultures. Therefore, it is recommended that at least one Löwenstein–Jensen slant or agar plate should be inoculated when using the automated broth culture systems[21]. The automated broth systems are indeed faster and more sensitive in detecting *Mtb*; but, due to the initial cost of the machines, difficulty in working with radioactive materials, and the cost of necessary supplies for culturing, the materials needed for commercial culturing machines are currently too high for most resource-poor health systems[34].

Culturing is considered the gold standard of *Mtb* diagnostics, and despite several hindrances it is still the most precise and inexpensive way to determine the species and drug susceptibility of *Mtb*. Delay in TB diagnosis due to the lengthy culturing time is still a large problem globally, resulting in increased morbidity and prolonged transmission[34]. Unfortunately, it is known that by the time an active TB case is detected, they have already infected many of their close contacts[35]. With up to eight weeks turnover time many patients may be lost to follow-up by the time their results come in. In addition, significant killing of *Mtb* during the clarification and decontamination process necessary for culturing may lead to more false negatives and untreated patients. One of the major drawbacks to culturing is that clinicians must have access to a laboratory with trained professionals running at a biosafety level three (BSL3) level capacity. This is an incredibly huge expense to incur in many areas, developed or not. The current gold standard for *Mtb* diagnosis in resource-poor areas is beyond ideal.

2.4 NUCLEIC ACID AMPLIFICATION TESTS

The relatively recent development of nucleic acid amplification tests (NAATs) has been one of the most important diagnostic advances for TB control in the past few decades. PCR-based methods can be used for quick diagnosis of TB through amplifying specific areas in the *Mtb* genome, typically with IS6110 and16S ribosomal DNA primers. In addition, the NaLC-NaOH method is not required Studies have found PCR to be more sensitive than smear, but less sensitive than culture as a diagnostic tool for TB. To improve NAAT diagnosis, real-time PCR (RT-PCR) was introduced to quantitate and better detect *Mtb* infection with a higher sensitivity compared to traditional PCR. One study conducted with Iranian TB patients compared conventional methods with RT-PCR for the detection of *Mtb* from clinical samples. They found that when compared to culturing, the sensitivity and specificity of RT-PCR were 90.2% and 97.8%, respectively. In this specific study RT-PCR was able to detect 1.5×10^2 to 4.3×10^3 copies of *Mtb* genomic DNA from a sample that was previously determined to be smear-negative. The sensitivity of the RT-PCR for culture-positive smear-positive samples was 95.4%, and its sensitivity for culture-positive smear-negative samples was 70.8%. This is especially important when comparing this assay to culturing, which is incredibly time-consuming and expensive (for at least a BSL2), as well as the Xpert MTB/RIF (discussed later), which is a more expensive test that can basically do the same thing as RT-PCR[34, 36]. One drawback for RT-PCR is the machine and reagents are both more expensive than traditional PCR, and should not be considered unless the laboratory intends to do high-throughput testing.

It is worth discussing the variability of results obtained in when working with all types of NAATs, especially those not commercially purchased. Keeping in mind that test performance in routine clinical use is not expected to be as accurate as results obtained in controlled study

conditions, a recent evaluation of six experienced Latin American diagnostic labs showed poor and inconsistent performance when using non-commercial PCR reagents[34]. In addition, regardless of how sensitive the diagnostic technique is, it is heavily reliant on the method of DNA extraction. Therefore, inconsistencies in NAAT diagnostic results are not only restricted by mistakes made during the amplification process, but also in the extraction of genomic DNA from the specimen. Some studies using PCR as a diagnostic test have shown different sensitivity results, varying from 11-81%[37]. Although this variability depends on a number of factors, studies have suggested that PCR results depend majorly on different DNA extraction techniques available to the clinical technician or researcher[38]. In addition, when considering all NAATs, the laboratory must have a reliable power supply connected to a back-up power source, as well as a biosafety cabinet for safe specimen handling. A molecular microbiology laboratory is necessary for any type of NAAT, where technicians need to have access to a sterilization instrument (like an autoclave or pressure cooker), polymerase enzyme, as well as clean, DNAse-free space for the preparation of PCR.

2.5 DRUG SUSCEPTIBILITY TESTING

Drug resistance in *Mtb* is mostly due to point mutations and deletions in the chromosome, and the variability of genes conferring drug resistance is a vast, yet slow process. Rifampicin resistance is due to mutations in the *rpoB* gene, while isoniazid resistance is due to changes in the *inhA* and *katG* genes. MDR-TB has been linked to treatment, clinical, and programmatic problems, where poor or inadequate treatment has allowed drug-resistant mutants to become the dominant circulating strain in some areas. It is important to note that MDR-TB is man-made, in that we

exposed *Mtb* to either wrong or mismanaged treatment methods with poor drug-adherence outcomes[34, 39].

It is recommended by the WHO that DST be performed on all clinical isolates for optimum treatment, although it is well-known that the majority of clinics that test for TB do not have the capacity to culture their samples. The WHO also recommends that DST be done for every repeat, failed, and/or reinfection treatment attempt in order to accurately create a personalized drug regimen[8]. DST should also be performed if the patient continues to produce sputum-positive samples after three months of treatment at their follow-up appointment[21]. Many new diagnostic techniques have capitalized on molecular methods that can detect mutations in these genes, however culturing in media with antibiotic is still the most widely accepted test today for drug susceptibility[34].

Culture-based methods of DST can be complicated by the varying levels of susceptibility *Mtb* may express, which allows the bacterium to respond to a variety of drug concentrations[40]. The agar or egg-based methods allow for quantifying the number of organisms that are susceptible to a certain drug at a known critical concentration at which wild-type strains are killed, yet mutant strains survive. This method, however, takes an average time of three to four months for results, during which the time patient is at risk for being lost to follow-up. The automated liquid and radiometric systems like BACTEC 460 and BacT/ALERT MB were also designed for rapid DST. These systems test for all primary drug susceptibility, including: isoniazid, rifampin, pyrazinamide, and ethambutol. However, BACTEC and some other systems can only test for susceptibility within one drug concentration, so in order to determine a critical concentration or if the strain is completely resistant to a drug, more tests will be needed. This is especially important for areas that have access to several treatment programs in which drug concentrations can be

personalized for the benefit of the patient and immediate recovery. Although the automated methods return results faster than agar or egg-based (several weeks), they are still slower and more expensive than other molecular-based DST methods available[21, 41].

In contrast to culture-based DST, there are some NAATs that offer fast and accurate detection of resistance mutations. Line Probe Assay (LPA) is currently the most preferred method for DST[39, 40]. LPA is a basic amplification and reverse hybridization diagnostic technique for detecting MDR-TB. It was endorsed by the WHO in 2008 and has been commercially available for several years. Before LPA is started, the sputum is traditionally processed by the same clarification and decontamination procedure used for culturing; however studies have reported successful isolating of *Mtb* DNA for LPA directly from sputum samples[31, 41]. LPA can also be performed directly from an agar or liquid culture. Following clarification procedure (if necessary) DNA is extracted from the *Mtb*-suspected samples. Polymerase chain reaction (PCR) is performed to amplify the drug resistant gene under question using specifically labeled primers. After PCR, the labelled products are hybridized with specific oligonucleotide probes immobilized on a strip. Depending on how they are designed, the probes can adhere to specific regions of interest, like wild-type, or mutations. The labeled hybrids that have been captured on the strip are detected by colorimetric development and observed by eye. If a mutation is present in one of the probe specific regions, the amplified DNA will not hybridize with the relevant wild-type probe. Mutations are therefore detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations[31].

The GenoType MTBDR*plus* assay is one type of commercially available LPA that is designed to simultaneously detect mutations in *rpoB* and both *inhA,* and *katG* genes. A 2008 meta-analysis found that the GenoType MTBDR*plus* assay has a sensitivity of 98% for detecting

rifampicin (R) resistance, and 89% for detecting isoniazid (H) resistance, and specificity of 99% for both R and H[41]. This assay was recently extended for the detection of XDR-TB (Genotype MTBDRsl)[31]. INNO-LiPA RIFTB is another LPA system which can only detection *rpoB* mutations, however it is able to detect the emergence of rifampin-resistant populations due to the presence of both wild-type and mutant probes[40].

Studies have shown that the reduction in cost of LPAs under routine diagnosis ranged from 30 to 50% when compared to culture-based DST. However, it is cautioned that LPA should not be used as a replacement for culture and DST, as culture is still required in some labs for confirmation of smear-negative patients. Manual LPA can be used in labs processing small numbers of samples. Larger labs wanting to do high-throughput will require a larger initial investment, but can process up to 48 samples per run, with each run taking between 2 and 3.5 hours[31]. LPA systems have great potential for quick drug resistant detection within a variety of countries[34].

Xpert MTB/RIF is the first fully automated cartridge-based NAAT that enables rifampicin resistant detection. It was also designed for clinics with basic laboratory infrastructure and personnel with minimum technical skills. The assay is able to extract, concentrate, and amplify DNA, as well as identify targeted genes in the *Mtb* genome, all from unprocessed sputum samples in less than two hours[42]. Although Xpert MTB/RIF was originally intended for analyzing sputum samples, studies have shown extra-pulmonary TB samples, like biopsies, urine, pus and cerebrospinal fluids, have been diagnosed with rifampicin resistant *Mtb* using the Xpert MTB/RIF assay[14]. One study used culture-positive patients as a comparison and found that Xpert MTB/RIF had a specificity of 99.2% and a sensitivity of 90.2%. When compared with DST the assay correctly identified 97.6% of the samples with rifampin-resistant bacteria and 98.1% of the samples with rifampin-sensitive bacteria[43].

Xpert MTB/RIF is a relatively low-throughput technology made for clinics which are geographically distant from any DST center. Therefore Xpert MTB/RIF may not be worth the investment of time and money for a laboratory that tests several hundred specimens a day. Studies have shown that the minimal training requirements needed to operate the assay and manage the software reporting system are often more difficult to achieve than expected. As with any NAAT developed for low-income settings, the error rate is expected to rise once the test is in the field due to a multitude of conditions, some listed previously. The results from one 11 month trial testing the feasibility of Xpert MTB/RIF in lower-level health care systems in India, show that after the "grace period" of about seven months the sub-optimal performance rate increased dramatically[42]. Regardless, Xpert MTB/RIF represents a huge leap in *Mtb* diagnosis and MDR-TB detection. Endorsed by the WHO in 2008, Xpert MTB/RIF is the product of years of research and several excellent assays combined, which opened new doors for technologically advanced *Mtb* diagnosis in resource-poor settings[14].

3.0 EXPERIENCE WITH CURRENT TB CLINICAL DIAGNOSTIC TECHNIQUES USED AT A DMC IN HYDERABAD, INDIA

As previously discussed, the methods of data and sample collection are intrinsically important for the diagnostic process. Therefore, a detailed discussion about the collection and handling methods of data and samples at LEPRA's UMEED designated microscopy center (DMC) is necessary to gain a better understanding of current diagnostic methods in South India. IRB approval for sample collection (discussed in Chapter 4) was met by the Blue Peter Health & Research Center's pre-approved study cleared by the Institutional Ethical Committee, entitled "Clinical, genetic, and phenotypic analysis of *Mycobacterium tuberculosis* strains for TB-HIV co-infected prevalent area in South India". Informed consent was taken from all patients who gave samples.

3.1 PROCESSING PATIENTS

The typical patients who sought care at UMEED were either passive-case findings or referrals from another DMC; their demographics ranged from teenagers at boarding school, HIV positive patients, and those living in slums. The DMC typically tested new patients on Mondays, Wednesday, and Fridays, leaving Tuesdays and Thursday for follow-ups and re-checking of sputum smear slides. Upon arrival at the clinic patients were given a sheet of paper (herein called a patient TB card) which had their contact information, medical history, treatment information, and sputum smear/HIV test results. The attending doctor privately met with each patient to collect their symptomology, medical history, and conduct an examination. Patients were then sent to the

TB treatment room with their patient TB card where they continued the TB diagnostic process by giving a sputum sample. Figure 6 shows an example of the settings at UMEED in which patient TB cards are filed. The cards were organized by date of treatment started and type of TB disease.

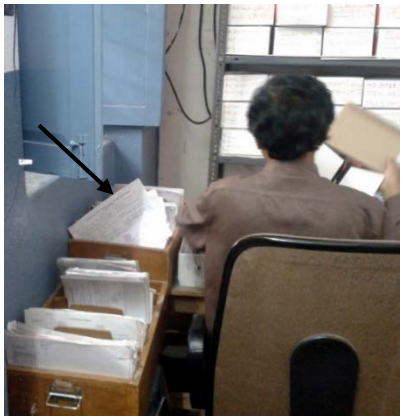

Figure 6. A photo of the patient TB cards and the filing system used at UMEED DMC, 2013 (McGuirk).

3.2 COLLECTION OF SAMPLES

The DMC began seeing patents around 9am Monday through Saturday, and the clinic generally stopped accepting samples around 1pm, or before lunch. Those patients who knew they were TB positive and/or were on DOTs used a tissue or a piece of cloth to cover their mouths upon entering the clinic, to prevent any further infections. Patients were instructed by the trained technician on the proper process for giving sputum samples in the language they were most fluent in. Each patient was given two sterile collection cups with screw-on lids, each with a capacity of about 50 mL. The technician wrote their names, TB number, and date of birth on stickers that were attached to each cup, as well as the letters “A” and “B” which designated the first and second sample given. Depending on the time of day the patient was instructed to either come back later in

the day or the following morning to give their second sputum sample. If the patient did not have trouble producing sputum they were allowed to take the second cup home to produce their second sample the following day when they felt prepared, and return it the same day the sample was produced. Patients who were unable to produce adequate samples usually stayed on the clinics compound. Approximately one sample per day was unusable due to the sample consisting of spit or nasal discharge instead of sputum. Although this situation significantly lowers the sensitivity of the test, the second sample would usually consist of sputum and therefore would be the only sample use to test for *Mtb* infection. Samples were produced by patients on the DMC compound, outside, and a bit away from the entrance to the building, which ensured adequate ventilation and prevention of infecting other patients. The sputum samples were stored on an open windowsill leading to the lab with the caps tightly snapped/screwed on. Since the patient number would typically dwindle after 12 PM, SSM was performed by the technician after lunch, at approximately 2pm.

All patients were asked when (if at all) they had last been tested for HIV. If the patient did not know their HIV status they would be referred to the HIV counselor, in the same DMC building, for counseling and testing. Counselors gave a private session lasting about 10-15 minutes, during which the patient could decide if they wanted to take a voluntary HIV test; patients almost always decided to take the test. The patient was given a piece of paper with their TB number and a new HIV number with no personal identifying information like their name, date of birth, or national ID number. After counseling, the patient was referred to the lab where approximately 2-5 mL of blood was drawn by a certified technician. Blood samples labeled with identifying HIV numbers were left at room temperature to let the plasma separate by gravity. All blood samples collected in the

morning were processed around 2pm, after the patient had left. If a patient tested positive, they were told at their follow-up appointment along with their TB diagnosis.

3.3 SPUTUM SMEAR MICROSCOPY

Ziehl-Neelsen staining is recommended by The Stop TB Partnership for AFB staining and detection of *Mtb*[30]. Two microscopy slides were labeled with each patients' four digit TB number as well as "A" or "B", referring to samples one and two given by each patient. A spirit lamp was lit for maintaining sterility during the smear process. A small broom stick or twig was used to mix and select purulent or muco-purulent material from the sputum sample and transferred to the slide. This material from the sample was spread with the twig over an equal area to about 2-3 cm x 1-2 cm, using circular movements without reaching the edge of the slide. Care was taken to make sure the smear was as even as possible and no thick mucosal portions remained. The smears were air-dried at room temperature for about 30 minutes; the overhead fan was often turned on at this point if electricity was available to speed-up the process. The specimens on the slides were heat fixed by slowly passing them through the spirit lamp flame three to four times. Slides were then placed upwards on a staining rack over the sink, making sure that they were not touching other slides. A new filter paper was placed in a small funnel, which was then filled with 1% carbol-fuchsin. As the dye ran through the filter paper and funnel it completely covered the first slide before it was moved over the next slide, repeating the process until all the slides were covered. A flame was prepared by dipping cotton in surgical spirit and lighting it with a match. Forceps were used to move the flame under the slides allowing each slide to be heated for about 15 seconds, or until steam rose from the carbol-fuchsin. After 10 minutes the slides were rinsed with tap water

and turned to let as much liquid as possible drain off. The slides were then de-stained with 25% sulfuric acid, poured completely over the slides and allowing them to sit for three minutes. The slides were washed again with tap water, and if any pink color remained the sulfuric acid was used again for another 30 seconds to one minute at most. After rinsing and allowing all the remaining water to drain off, the slides were flooded with 0.1% methylene blue, and sat for one minute. They were then rinsed and tipped to allow all the excess water to drain off. The smears were left to air dry overnight.

Figure 7 shows a photo of the area where sputum samples were left by patients on the windowsill as well as the area where SSM was performed at UMEED DMC. Note the broomstick used for the smear process. The slides were generally collected over a few days and examined twice weekly (Tuesday and Thursday) by a technician using light microscopy with oil immersion at 100x magnification[30]. Results were counted and recorded as recommended by the WHO and shown in Table 7. When a patient was determined to be TB positive they were encouraged to name their contacts and bring them in for testing.

Figure 7. A photo of the area where sputum samples were left by patients and SSM was performed at UMEED DMC, 2013 (McGuirk).

3.3 PATIENTS WHO GAVE SAMPLES

37 sputum samples were collected from patients whose sputum was tested for *Mtb* at the UMEED DMC from May 16th to July 4th 2013. Their ages ranged from 11 to 70 years with a mean age of 30. There were 17 females and 20 males. Figure 8 and Table 6 summarizes the following patient disease classification, patient type, and TB/HIV test results, in terms of percent of total samples collected. There were three extra-pulmonary cases, two of which were in the cervical lymph nodes, the third being a man whose site of infection was not noted. One of the three extra-pulmonary cases was HIV positive. The remaining 34 patients were diagnosed with pulmonary TB. Six of the patients were contact cases, 10 were follow-ups, nine were new patients referred from another DMC, and 12 were TB suspects. Of the pulmonary TB patients, three were reinfection cases, two of which were co-infected with HIV. 11 of the patients were considered sputum positive, and 26 were sputum negative based on the SSM done at the DMC. None of the contact cases were sputum positive, and none of the follow-ups were sputum positive, however three of them (all HIV positive) were referred from another DMC for their treatment and were therefore not retested. Six of the 12 TB suspects were sputum positive. Three patients were serologically confirmed HIV positive, 16 were confirmed HIV negative, and 18 had unknown HIV status when the data was collected.

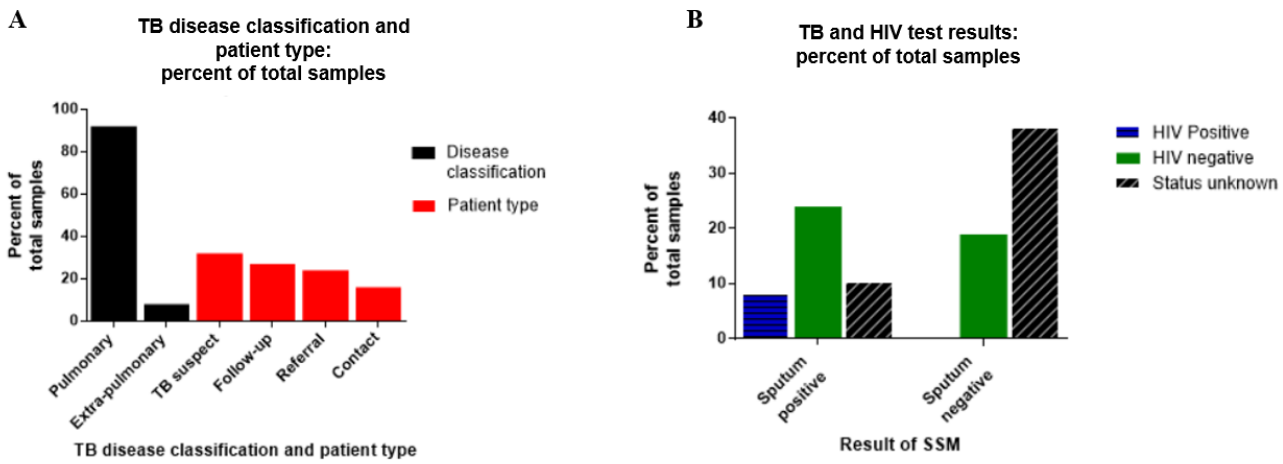

Figure 8. Patient statistics summarized by: A. TB disease classification, patient type, and B. SSM and HIV test results, in terms of percent of total samples collected, 2013 (McGuirk).

Table 6. Patients summarized by disease classification, patient type, and SSM and HIV test results, 2013 (McGuirk).

Patient number	Disease Classification	Type of Patient	DMC SSM Result	HIV Status
4421	Pulmonary	New Referral	Neg	Unknown
4422	Pulmonary	New Referral	1+ (Malkapet DMC) Neg (UMEED)	Neg
4423	Pulmonary	Follow-up	Neg	Neg
4424	Pulmonary	Follow-up	Neg	Neg
4425	Pulmonary	Follow-up	Neg	Neg
4433	Extra pulmonary (cervical lymph nodes)	Follow-up	Neg	Neg
4434	Pulmonary	Follow-up	Neg	Neg
4435	Pulmonary	TB suspect	Neg	Unknown
4436	Pulmonary	Follow-up	Neg	Neg
4437	Pulmonary	TB suspect	1+	Neg
4438	Extra pulmonary (cervical lymph nodes)	New Referral	not done at UMEED	Neg
4447	Pulmonary	Follow-up	not done at UMEED	Pos
4448	Pulmonary	TB suspect	2+	Neg
4450	Pulmonary	New referral	1+ (Malkapet DMC) not done (UMEED)	Unknown
4451	Pulmonary	TB suspect	3+	Neg
4453	Pulmonary	New Referral	1+ (Malkapet DMC)	Neg
4454	Pulmonary	New Referral	2+	Neg
4466	Pulmonary	Follow-up	Neg	Unknown
4467	Pulmonary	TB suspect	Neg	Unknown
4468	Pulmonary	Contact	Neg	Unknown
4470	Pulmonary	Contact	Neg	Unknown
4471	Pulmonary	Contact	Neg	Unknown
4472	Pulmonary	Follow-up	not done at UMEED	Pos
4473	Pulmonary	New referral	2+ (at DTC)	Neg
4474	Extra pulmonary	Follow-up	not done at UMEED	Pos
4475	Pulmonary	New referral	not done at UMEED	Neg
4476	Pulmonary	Contact	Neg	Unknown
4477	Pulmonary	Contact	Neg	Unknown
4478	Pulmonary	Contact	Neg	Unknown
4479	Pulmonary	TB suspect	Neg	Unknown
4480	Pulmonary	TB suspect	2+	Unknown
4502	Pulmonary	TB suspect	1+	Unknown
4503	Pulmonary	TB suspect	3+	Unknown
4504	Pulmonary	TB suspect	Neg	Unknown
4505	Pulmonary	TB suspect	Neg	Unknown
4506	Pulmonary	New Referral	1+ (Malkapet DMC)	Neg
4507	Pulmonary	TB suspect	Neg	Unknown

4.0 COMPARING CURRENT LABORATORY DIAGNOSTIC TECHNIQUES USED IN RESOURCE-POOR SETTINGS WITH NEWLY AVAILABLE DIAGNOSTIC METHODS

In the process of evaluating TB diagnostic techniques used in resource-poor areas, it is important to assess and understand how modern clinical laboratories are using current methods and developing new diagnostic technology for *Mtb* detection. This chapter will examine the commonly used laboratory method of *Mtb* concentration from samples, by using the NaLC-NaOH method practiced at LEPRAs Blue Peter Public Health & Research Center (BPHRC). BPHRC has a BSL3 which serves as a laboratory for culture-based diagnosis of TB and MDR-TB. LEPRA employs three technicians in the microbiology department at BPHRC to perform NaLC-NaOH clarification and decontamination of sputum samples, and inoculation for diagnosis by culture. However, as discussed previously, the NaLC-NaOH method is known to kill up to 90% of *mycobacterium* in samples[33], making culturing diagnostics not as sensitive as previously thought. Therefore, in addition to evaluating the use of the NaLC-NaOH method for concentration of *Mtb*, it will be compared with a new method of capturing *Mtb* with specialized microparticles called TB Beads™, which is a much simpler and sensitive technique for *Mtb* detection. The two methods will be compared with the samples that were previously collected and tested by SSM at UMEED DMC. In addition the two methods will also be compared to the gold standard of culturing.

TB Beads™ (Microsens Medtech Ltd., London, United Kingdom), was developed to provide an alternative method to capture many *mycobacterial* species from patient specimens, and concentrate the *Mtb* into a smaller volume size, effectively increasing the specificity for *Mtb* detection. TB-Beads are small magnetic beads coated with a polymeric ligand that binds to several

mycobacterium species, with minimal binding of contaminating bacteria present in the specimen. The ligand is made of hydrophobic chemical groups that are known to have a high affinity for specific materials on the mycobacterial cell surface. TB Beads are stable at room temperature and at high pH, which makes for an easy-to-use method for resource-poor labs to concentrate mycobacteria from specimens [44, 45]. In addition, the staff in the microbiology department of BPHRC had previously worked with TB Beads after being given a sample by the production company, making introduction of the technique very simple.

4.1 NALC-NAOH METHOD

All samples used were first tested by technicians with SSM at the UMEED DMC before they were transported to BPHRC. At BPHRC both sputum samples produced by the same patient (A and B) were mixed together and homogenized before dividing them evenly into two separate bottles in order to maintain even numbers of *Mtb* for each method of concentration. Care was used to divide mucosal materials evenly for even homogenization.

This protocol was written and is currently being used by LEPRA: The following reagent stocks were prepared individually with distilled water and autoclaved at 121 ºC for 15 minutes: 0.1M trisodium citrate ($Na_3C_6H_5O_7$), 1M sodium hydroxide (NaOH), 70 mM monopotassium phosphate (KH_2PO_4), and 65 mM disodium phosphate (Na_2HPO_4). Following sterilization, each solution was made fresh for the day. Solution 1 was made: 100 ml of 0.1M $Na_3C_6H_5O_7$ was mixed with 100 ml of 1M NaOH; as well as solution 2: 125 ml 70 mM KH_2PO_4 was mixed with 125 ml of 65 mM Na_2HPO_4. 0.5g of N-acetyl-L-cysteine was added to the 200 ml of solution 1 directly before clarification started. Twice the volume of solution 1 was added to one of each divided

sample in the McCartney bottle, and mixed thoroughly. This was incubated at 37 ºC for 15 minutes rotating. After incubation solution 2 was added up to the neck level of the bottle, which was then closed tightly, and mixed by inversion. The sample was then centrifuged at 3000 x g for 15 minutes in a sealed-bucked swing-out centrifuge. Supernatant was then poured off carefully into the discard container with 5% phenol. 1 ml of solution 2 was then added to the sediment and mixed well by pipette. The sample was split into two aliquots (0.5 ml each), one of which was stored at -20 ºC for back-up.

4.2 TB BEADS™

The TB Beads method recommended protocol, which suggests that the specimens first be processed by the NaLC-NaOH method before using the beads[44], was not used. Instead, the TB Beads bottle was inverted several times to homogenize the solution before use. 300 µl of the beads was added to the second sputum aliquot and mixed by rotation at room temperature for five minutes. The beads were then collected on the bottom of the bottle using a magnet. The supernatant was removed from the top without disturbing the beads. The beads were then washed with 3 ml of 1x phosphate buffer solution (PBS). The solution was inverted occasionally and incubated at room temperature for no more than three minutes. The magnet was again used to collect the bead as the supernatant was removed. The beads were finally resuspended in 300 µl of 1x PBS, half of which was stored at -20 ºC for back-up. For simplicity, *Mtb* was not eluted off the beads, which was also an adaptation from the original protocol.

4.3 CULTURING

The gold standard of culturing was used to compare the *Mtb* detection rates among the two concentration methods. After concentration by the NaLC-NaOH, an inoculating loop was used to pick-up the freshly decontaminated and clarified sputum, which was inoculated onto two separate LJ slants, incubated at 37 ºC for four to six weeks. Samples from the TB Beads method could not be cultured because they were not decontaminated and therefore contained common flora found in the airways and mouths of humans, which would overgrow and interfere with the culturing of slow-growing *Mtb*.

4.4 ZIEHL–NEELSEN AFB STAINING

Each sample processed by both methods (NaLC-NaOH and TB Beads) was smeared onto a glass slide and was prepared for Ziehl–Neelsen AFB staining, the same staining method that was used in SSM. AFB staining was chosen because it is the fastest and most commonly used method for *Mtb* detection in clinical diagnostics. In addition, most clinics are likely to know Ziehl–Neelsen staining, and the method would theoretically be easier to teach and adapt to. AFB staining was done as described previously; 20 fields of view were examined per slide, and *Mtb* bacilli were counted and recorded.

4.5 RESULTS

Figure 9 consists of microscopic images of two randomly chosen samples, both processed by NaLC-NaOH and TB Beads individually, and after AFB staining. *Mtb* bacilli are circled. The black areas in the TB Beads samples are aggregates of the magnetic beads. Interestingly, no bacilli were recorded in sample 4480 after NaLC-NaOH processing; also note the complex matrix (blue) of the remaining intact sputum. Table 7 summarizes the patients results from the DMC SSM, HIV status, and NaLC-NaOH and TB Beads concentration methods compared to the gold standard of culturing. 11 of the samples that were tested by SSM at the DMC were *Mtb* positive. Of the samples prepared by the NaLC-NaOH method, nine were *Mtb* positive, four of which were considered scanty (defined as an average of 0.5 bacilli per field of view). Of the samples concentrated by the TB Beads method, nine were *Mtb* positive, three of which were considered scanty. Only six of the samples that were concentrated with the NaLC-NaOH method and cultured were positive for *Mtb*. It is important to note that a few of the samples that were positive in both the TB Beads and the NaLC-NaOH method were negative in SSM and culturing. In addition, one sample that was positive by the TB Beads method was negative in the NaLC-NaOH method, and vice versa.

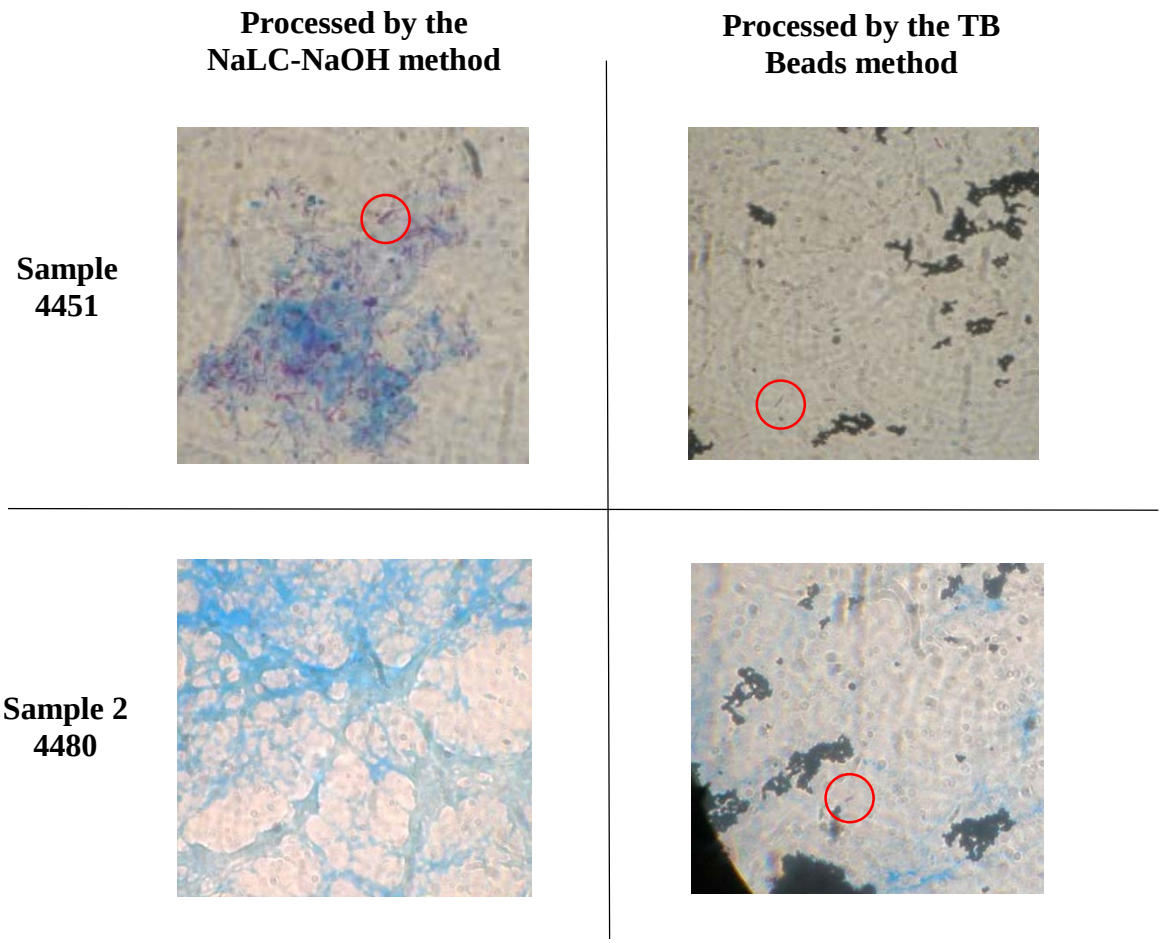

Figure 9. Microscopic examination of two samples treated with NaLC-NaOH and TB Beads methods after AFB staining, 100x magnification oil immersion, 2013 (McGuirk).

Table 7. Patients summarized by DMC SSM result, HIV status, and NaLC-NaOH and TB Beads concentration methods compared to culturing diagnostic results, 2013 (McGuirk).

Patient number	DMC SSM Result	HIV Status	NaLC-NaOH AFB stain result	TB Beads AFB stain result	Culture
4421	Neg	Unknown	Neg	Neg	Neg
4422	1+ (Malkapet DMC) Neg (UMEED)	Neg	Neg	Neg	Neg
4423	Neg	Neg	Neg	Neg	Neg
4424	Neg	Neg	Neg	Neg	Neg
4425	Neg	Neg	Neg	Neg	Neg
4433	Neg	Neg	Neg	N/A	Neg
4434	Neg	Neg	Neg	N/A	Neg
4435	Neg	Unknown	Neg	N/A	Neg
4436	Neg	Neg	Neg	N/A	Neg
4437	1+	Neg	Scanty	Pos	Pos
4438	not done at UMEED	Neg	Neg	Sample missing	Neg
4447	not done at UMEED	Pos	Neg	Pos	Neg
4448	2+	Neg	Pos	Pos	Pos
4450	1+ (Malkapet DMC) not done (UMEED)	Unknown	Neg	Neg	Neg
4451	3+	Neg	Pos	Pos	Pos
4453	1+ (Malkapet DMC)	Neg	Neg	Neg	Neg
4454	2+	Neg	Pos	Pos	Pos
4466	Neg	Unknown	Neg	Neg	Neg
4467	Neg	Unknown	Neg	Neg	Neg
4468	Neg	Unknown	Neg	Scanty	Neg
4470	Neg	Unknown	Neg	Neg	Neg
4471	Neg	Unknown	Neg	Neg	Neg
4472	not done at UMEED	Pos	Sample missing	Neg	Neg
4473	2+ (at DTC)	Neg	Scanty	Neg	Neg
4474	not done at UMEED	Pos	Scanty	Neg	Neg
4475	not done at UMEED	Neg	Scanty	Scanty	Neg
4476	Neg	Unknown	Neg	Neg	Neg
4477	Neg	Unknown	Neg	Neg	Neg
4478	Neg	Unknown	Neg	Neg	Neg
4479	Neg	Unknown	Neg	Neg	Neg
4480	2+	Unknown	Neg	Scanty	Pos
4502	1+	Unknown	Pos	Pos	Pos
4503	3+	Unknown	Pos	Neg	Neg
4504	Neg	Unknown	Neg	Neg	Neg
4505	Neg	Unknown	Neg	Neg	Neg
4506	1+ (Malkapet DMC)	Neg	Neg	Neg	Neg
4507	Neg	Unknown	Neg	Neg	Neg

Table 8 displays the sensitivity, specificity, PPV (positive predictive value), and NPV (negative predictive value), of each test comparison. Compared to the NaLC-NaOH and culturing

method, samples tested by SSM had a 100% sensitivity and an 84% specificity; SSM had a 54% PPV and a 100% NPV. Compared to the SSM method done at the DMC, samples tested by the NaLC-NaOH method had a 63% sensitivity and a 92% specificity; NaLC-NaOH had a 78% PPV and an 86% NPV. Compared to the NaLC-NaOH and culturing method, samples tested by the NaLC-NaOH method alone had an 83% sensitivity and an 87% specificity; NaLC-NaOH had a 55% PPV and a 96% NPV. Compared to SSM, TB Beads samples had a 54% sensitivity and an 88% specificity; TB beads had a 67% PPV and an 82% NPV. Compared to NaLC-NaOH method, samples processed with TB Beads had a 67% sensitivity and an 89% specificity; here TB Beads had a 67% PPV and 89% NPV. When samples that were prepared by the NaLC-NaOH method followed by culturing, were compared to the TB Beads concentration method, TB Beads samples had a 100% sensitivity and a 90% specificity; TB Beads had a 67% PPV and a 90% NPV.

Table 8. Results of comparing sensitivity, specificity, PPV, and NPV among three Mtb detection methods with the gold standard of culturing, using a denominator of 37 total samples, 2014 (McGuirk).

Diagnostic methods	Preparation methods	N positive*	Sensitivity	Specificity	PPV	NPV
Culture vs. SSM	NaLC-NaOH, culture vs. SSM	6 vs. 11	100%	84%	54%	100%
SSM vs. NaLC-NaOH	SSM vs. NaLC-NaOH, AFB stained	11 vs. 9	63%	92%	78%	86%
Culture vs. NaLC-NaOH	NaLC-NaOH, culture vs. NaLC-NaOH, AFB stained	6 vs. 9	83%	87%	55%	96%
SSM vs. TB Beads	SSM vs. TB Beads, AFB stained	11 vs. 9	54%	88%	67%	82%
NaLC-NaOH vs. TB Beads	NaLC-NaOH, AFB stained vs. TB Beads, AFB stained	9 vs. 9	67%	89%	67%	89%
Culture vs. TB Beads	NaLC-NaOH, culture vs. TB Beads, AFB stained	6 vs. 9	100%	90%	67%	90%

***The number of positive results from each of the two tests compared.**

Of the three patients that were HIV positive, none of them had their sputum tested by SSM during this analysis, therefore a complete comparison of the two concentration methods cannot be made between SSM and HIV positive patients. After NaLC-NaOH concentration, one patient was negative for *Mtb*, the second was missing, and the third was scanty for *Mtb*. In comparison, after TB Beads concentration and AFB staining of the three HIV positive patients' samples: one sample was positive for *Mtb* with at least two bacilli per field of view, and the last two were negative for *Mtb*. Interestingly, the one that was positive was not the same sample as the positive scanty with the NaLC-NaOH method. All three of the samples were negative by LJ slant culture.

It is important to note that because some TB positive patients were referred from another DMC, UMEED did not collect sputum to re-test them. Therefore, only two of the nine new referral patients were re-checked by SSM, since they were assumed to be sputum-positive. In addition, some of these patients had started their anti-TB therapy, which could have also altered the chances for *Mtb* detection. Most of the referred patients only gave one sputum sample (opposed to the WHO recommended two) due to miscommunication and incomplete direction given by technicians and myself; this could have also significantly reduce the detection rate of *Mtb*. Keeping all this in mind, only one of the nine samples from referral patients was culture positive. Interestingly, three of nine samples were positive by the NaLC-NaOH concentration method, two of which were considered scanty; two of eight (one sample was missing) samples were positive by the TB Beads method, one of which was scanty. Both of the positive samples that were not scanty from each concentration method were from the culture positive sample.

One drawback to the TB Beads method is the samples are not clarified, and many of them remained viscous and difficult to manipulate. NaLC-NaOH clarification used to be a necessary component for diagnostic culturing, due to N-acetyl-L-cysteine's mucolytic activity on the sputum

matrix. However, there are now commercial products available with dithiothreitol (DTT), which has been shown to significantly decrease the viscosity of the sputum as compared to NaLC methods[46]. In addition, products with DTT like Sputagest Selectavial^{TM}, has also been found to yield a higher number of organisms after processing and culturing the sample as compared to NaLC methods[47]. If this study were to be repeated, it is hypothesized that the use of Sputagest with TB Beads could drastically increase the *Mtb* detection rate.

In terms of processing time, TB Beads took approximately 10 minutes per sample, and is capable of being scaled up if necessary to anywhere from 10-20 samples per run. In comparison, the NaLC-NaOH method takes approximately 45 minutes per sample, accommodating about 16 samples per run. In addition, TB Beads does not need a centrifuge for processing as compared to the NaLC-NaOH method. Additionally, the WHO does recommend that a BSL3 and a BSC be used when working with specimens to avoid possible infection. However, as discussed earlier, SSM (which also involves manipulation of specimens) can be done in the DMC. A BSL3 is definitely important for safety of the laboratory technicians in terms of infection prevention; however, the TB Beads method involves such little processing and no centrifugation of the sample, a BSC could be added to a DMC laboratory for an improved diagnostic method using TB Beads and SSM. This may reduce the cost of setting up a BSL3 and make a more sensitive diagnostic technique easily available for many resource-poor clinics globally.

5.0 CONCLUSION

5.1 DISCUSSION

In evaluating diagnostic methods for TB disease in resource-poor areas, it is important to understand not only the methods that are available, but also how they are used, and whether this affects the outcome of the tests results. Chapter two reviewed current diagnostic techniques of TB used in resource-poor settings. Each technique had its advantages and disadvantages, and it was ultimately concluded that although we are getting closer to a more sensitive method of TB diagnosis, there are still many barriers that need to be overcome. The issue of poor TB diagnostics is just the tip of the iceberg. When evaluating this public health issue it is important to understand the impact that unavailable resources make when comparing TB diagnosis globally in resource-poor countries. A health network is bound to fail when the infrastructure of public health systems are compromised, either through lack of funding, supplies, qualified personnel, or inadequate government involvement. Ultimately what is needed in every community is a stable testing center with highly trained professionals and reliable equipment with a steady stream of supplies. Only when those issues are addressed can we begin to develop a more sensitive test. Many countries, like India, have developed strong programs (The Stop TB Partnership, for example) that offer great promise for the control of TB. Unfortunately, a lot of these plans do not address the social and cultural factors surrounding TB and TB-HIV co-infections, and therefore the execution of these plans are ineffective. As previously discussed, every community is different and every patient responds differently to their infection based on many biological, cultural, and environmental factors.

The goal of chapter three was to give an objective view of current TB diagnostics and the challenges encountered, and not to single-out one DMC. Additionally, how this DMC functions cannot be generalized throughout the world, as TB, HIV, funding, infrastructure, and social factors are presented differently in various locations globally. Keeping this in mind, UMEED DMC is, in my opinion, a shining light in the shadow of the TB epidemic. Having worked with several clinics in developing countries, UMEED DMC is by far the most organized and well-staffed that I have ever worked with. Their collaborative efforts with the government and their intelligent and highly driven workers gives hope that they will reach the people most in need of help. Therefore, the most important note I took away from my time at UMEED is that every clinic operates as an adaptation to best meet their patient's needs. To an outsider it may look like UMEED had an unorganized process for TB diagnosis; however, they were doing what best worked for them, and nothing in their TB diagnostic process was glaringly wrong. In addition, UMEED was the clinic that difficult TB cases were sent to, especially those who were co-infected, re-infection, or re-treatment patients.

Chapter four consisted of comparing different types of laboratory-based diagnostic methods that are either currently being used in resource-poor settings, or new methods that can be easily added to a laboratory's protocol. The most important comparisons that can be made are between the three different methods of *Mtb* detection (SSM; NaLC-NaOH and AFB staining; TB Beads and AFB staining) and the gold standard of culturing. When SSM was compared to culturing, SSM had an expected sensitivity and specificity, with 100% and 84% respectively, which are acceptable numbers for *Mtb* diagnostics. However, when the NaLC-NaOH concentration method was compared to culturing, NaLC-NaOH samples had lower percentages, with an 83% sensitivity and an 87% specificity. The difference between SSM and NaLC-NaOH could be due to many environmental and processing errors. It is important to note that the gold

standard for *Mtb* diagnosis is not a perfect test. As previously discussed, culturing can only be done after the NaLC-NaOH method, which is now known to affect *Mtb*. This may be another explanation as to why the sensitivities and specificities varied among the tests. Regardless of these reasons, when the TB Beads concentration method wass compared to the gold standard of culturing, TB Beads had a 100% sensitivity and a 90% specificity, both as high as or higher than any other test used.

The number of bacilli per field of view varied depending on the mucosal consistency, time of day the sample was produced, how many samples given by the patient, if the patient had started treatment, and if the samples were produce properly by the patient. Obviously a lot of these variables are intrinsically linked with patient presentation of their individual TB disease, and therefore un-controllable. The sample size was also too small to make any significant conclusion about the sensitivity of TB Beads in comparison to the NaLC-NaOH concentration method and culturing. However, this was the first noted occurrence of TB Beads being used to concentrate *Mtb* from a whole, un-processed sputum sample, which shows promise for more trials.

Although TB Beads have great promise for a more sensitive detection of *Mtb* infection, the idea was not met with great enthusiasm at BPHRC. Despite recent evidence, the NaLC-NaOH clarification and decontamination procedure followed by culturing is still considered by some to be a flawless and ultimate test for *Mtb* detection. However, the fact remains that several samples processed by both the NaLC-NaOH method and TB Beads were positive after AFB staining that were later declared negative by culture. Based on all the presented information, a concentration method that involves TB Beads and whole sputum samples while circumventing the NaLC-NaOH process has the potential to be much faster, cheaper, easier, and more sensitive/specific as compared to culturing.

The TB Beads concentration method clearly has great potential for increasing *Mtb* detection in DMCs globally; however, it is still unknown if TB Beads are sensitive-enough for SSM detection of low-load *Mtb* cases, commonly seen in the immunocompromised, children, the elderly, and newly infected cases. One of the major difficulties encountered when working with *mycobacterium* is lysing the cell. As discussed previously, some of the most accurate diagnostic tests amplify *Mtb* genomic DNA extracted from the specimen. A few enzymes are currently being studied at the University of Pittsburgh, in Pittsburgh Pennsylvania. TDMH (Trehalose 6,6'-dimycolate hydrolase) and mycobacteriophage lysins both target different parts of the mycobacterial cell wall and ultimately lyse the cell[48, 49]. They are hypothesized to help the release of genomic DNA during the lytic process, and are therefore candidates to be part of the future of *Mtb* diagnostics.

5.2 PUBLIC HEALTH SIGNIFICANCE

There are public health systems currently in India that have a strong promise for the control of TB. If given the correct tools, like a more sensitive test for *Mtb* infection that is easy to use and store, they have the potential to help relieve the burden of TB in a population, allowing the community to gain control of the disease and manage it in an organized fashion. More investigations will be needed before TB Beads can be used routinely in clinics globally. However, there is great promise that TB Beads can be used in whole, unprocessed sputum samples for the quick and cheap concentration and detection of *Mtb*, as compared to the NaLC-NaOH method and culturing. In addition, because the materials needed for TB Beads are commonly available in most clinics even in resource-poor areas, few adaptations will be needed to prepare for this new method. As of now

TB beads are not endorsed by the WHO to be used as a diagnostic tool in clinics, and it may take years for this to take place, if ever. In conclusion, if the world's burden of TB diagnosis is to be overcome, the public health infrastructure will need to adapt for better diagnosis of TB disease. Only when we recognize and fix the incomplete state of TB response will we be able to address the diagnostic issues surrounding the disease.

APPENDIX A: GLOSSARY OF TERMS

BSL-3 Laboratory: This level is applicable to clinical, diagnostic, teaching, research, or production facilities in which work is done with indigenous or exotic agents which may cause serious or potentially lethal disease after inhalation. It includes various bacteria, parasites, and viruses that can cause severe to fatal disease in humans, but for which treatments exist. All procedures involving the manipulation of infectious materials are conducted within biological safety cabinets, specially designed hoods, or other physical containment devices, or by personnel wearing appropriate personal protective clothing and equipment.

Clarification: The process by which the sputum matrix is broken down to allow access to *Mtb* for diagnostic detection.

DST: The testing of a strain of *Mtb* for its susceptibility or resistance to one or more anti-TB drugs.

Follow-up: The term for testing a TB patient for MDR-TB with SSM who has been on anti-tubercular drugs for two months.

Gold standard: Any standardized clinical assessment, method, procedure, or measurement of known validity and reliability which is generally taken to be the best available, with which new tests are compared with.

Lysis: The disintegration of a cell by rupture of the cell wall or membrane.

Referral case: A type of TB patient who is sent to another DMC, most-likely due to the specialties of that DMC or location in association with the patients' home.

Re-infection case: A patient who was previously declared cured or treatment completed and is diagnosed with bacteriologically TB; also known as "relapse". A type of retreatment patient.

RT- PCR: A laboratory technique based on PCR, which is used to simultaneously amplify and quantify a targeted DNA molecule. For one or more specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification.

Sputagest Selectavial™: A reagent for clarifying sputum that contains DTT, an alternative to NaLC used in the NaLC-NaOH decontamination and clarification method of concentration *Mtb* from specimens.

TB Beads™: (Microsens Medtech Ltd., London, United Kingdom) Small magnetic beads coated with a polymeric ligand that binds to several *mycobacterium* species. Commercially used as a concentration method for *Mtb* detection and diagnosis.

TB suspect: An individual with any symptom of TB infection or who tested positive on sputum culture during screening.

Treatment failure: A patient who was initially smear-positive and who remained smear-positive at month five or later during treatment. A type of retreatment patient.

APPENDIX B: ACRONYMS

AFB: Acid fast bacilli

BSC: Biosafety cabinet

BSL3: Biosafety level three laboratory

DMC: Designated microscopy center

DOTS: Directly Observed Treatment, Short-course

DST: Drug susceptibility test

DTT: Dithiothreitol

LJ: Löwenstein–Jensen

MDR-TB: Multiple drug resistant tuberculosis

Mtb: *Mycobacterium tuberculosis*

NAAT: Nucleic acid amplification test

NaLC: N-acetyl-L-cysteine

NPV: Negative predictive value

PBS: Phosphate buffer solution

PCR: Polymerase chain reaction

PPV: Positive predictive value

RNTCP: Revised National Tuberculosis Control Program

RT-PCR: Real-time polymerase chain reaction

SSM: Sputum smear microscopy

TDMH: Trehalose 6,6’-dimycolate hydrolase

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