Evaluating light-emitting diode fluorescent microscopy for the diagnosis of active tuberculosis

Jessica Minion

Department of Epidemiology, Biostatistics & Occupational Health

McGill University, Montreal, Canada

April 2010

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

Page

List of Figures

List of Tables

List of Appendices

Abbreviations and Acronyms

AFB – Acid Fast Bacilli
BAL – Bronchioalveolar Lavage
C – Celsius
CFM – Conventional Fluorescent Microscopy (Mercury Vapour Lamp)
CI – Confidence Interval
Cx – Culture
EGM – Expert Group Meeting
CXR – Chest X-Ray
EQA – External Quality Assurance
FIND – Foundation for Innovative New Diagnostics
FM – Fluorescent Microscopy
FN – False Negative
FP – False Positive
FRG – Refrigerator
GLI – Global Laboratory Initiative
HIV – Human Immunodeficiency Virus
INC – Incubator
JGH – Jewish General Hospital
LED – Light Emitting Diode
LR- – Negative Likelihood Ratio
LR+ – Positive Likelihood Ratio
MTB – Mycobacterium tuberculosis
NPV – Negative Predictive Value
PPV – Positive Predictive Value
Ref – Reference
RT – Room Temperature
RVH – Royal Victoria Hospital
STAG-TB – Strategic Advisory Group – Tuberculosis
TB – Tuberculosis
TN – True Negative
TP – True Positive
UV – Ultraviolet
WHO – World Health Organization
ZN – Ziehl-Neelsen

Abstract (English)

First, through a systematic review and meta-analysis, we summarize the current evidence pertaining to LED microscopy used for TB diagnosis and discuss its potential for widespread implementation. Next, we evaluated the accuracy and reading efficiency of two LED microscopes and compared them to a conventional fluorescent microscope (CFM). Finally, recognizing that global expansion of FM poses a potential challenge for current TB laboratory external quality assurance (EQA) programs, we evaluated the effect of storage duration, storage conditions and frequency of reading on the rate of fading of auramine stained smears. We conclude that LED microscopy is a feasible technology for implementation in TB laboratories. LED microscopy offers improved sensitivity and reading efficiency over light microscopy, and appears equivalent in diagnostic accuracy and reading efficiency, while featuring significant practical benefits over CFM. The use of fluorescent stains will necessitate a re-evaluation of EQA programs used in many TB laboratories at this time.

Abstract (French)

Nous avons tout d'abord, via une revue systématique de la littérature et une méta-analyse, résumé l'état actuel des connaissances concernant l'utilisation de la microscopie *LED* dans le diagnostic de la tuberculose et considéré la faisabilité d'une utilisation de celle-ci à grande échelle. Nous avons ensuite évalué la fiabilité et la facilité d'interprétation de deux microscopes *LED* comparés à un microscope à fluorescence traditionnel. Nous avons finalement évalué l'impact de la durée d'entreposage, des conditions d'entreposage, et de la fréquence des lectures sur la vitesse de disparition de la coloration à l'auramine, afin de mieux juger du fardeau supplémentaire engendré par la généralisation de la microscopie à fluorescence pour les programmes actuels de contrôle de qualité externe des laboratoires de tuberculose. Nous concluons que la microscopie *LED* est une technologie qui pourrait être implanté dans les laboratoires de tuberculose. Elle offre une meilleure sensibilité et spécificité que la microscopie ordinaire et semble être aussi fiable et efficace que la microscopie à fluorescence traditionnelle tout en offrant des avantages pratiques non négligeables. L'utilisation de colorations fluorescentes demandera néanmoins une réévaluation des programmes de contrôle de qualité actuellement en place dans plusieurs laboratoires de tuberculose.

Acknowledgments

Jessica Minion is supported by a fellowship from the Quebec Respiratory Health Training Program (QRHTP). Research studies in this thesis were conducted with resources from a CIHR operating grant (CIHR MOP-89918).

The systematic review was supported by funding from the World Health Organization, Geneva, Switzerland. I am grateful to Karin Weyer (World Health Organization, Switzerland) for helpful input and feedback. I would also like to thank Catharina Boehme (Foundation for Innovative New Diagnostics, Switzerland), Rick O'Brien (Foundation for Innovative New Diagnostics, Switzerland), Andrew Ramsay (Special Programme for Research and Training in Tropical Diseases, Switzerland), Luis Cuevas (Liverpool School of Tropical Medicine, UK), Armand Van Deun (Institute of Tropical Medicine, Belgium), Alexander Trusov (USA), Ben Fourie (Medicine in Need, South Africa), and Walter Kuhn (Medical College of Georgia, USA) for providing me with unpublished data.

I am grateful to CN Paramasivan (Foundation for Innovative New Diagnostics, Switzerland), Ernie Tai (LW Scientific, USA), Warren Sanborn (Portable Medical Laboratories, USA), Thomas Hanschied (Hospital Santa Maria, Portugal), Richard Bumgarner (USA), Richard Anthony (Royal Tropical Institute, Netherlands), Ben Marais (Stellenbosch University, South Africa), Howard Shapiro (Center for Microbial Cytometry, USA), Maryline Bonnet (Medicine Sans Frontiers, Switzerland), Chris Graham (QBC Diagnostics, USA), and Craig Stout (QBC Diagnostics, USA) for helping me to identify relevant publications and studies.

For the LED evaluation study I would like to gratefully acknowledge the work and assistance of our laboratory technologists Kathy Kyriazopoulos and Jennifer Glasgow. We are thankful to the microbiology laboratories of the Royal Victoria Hospital and the Jewish General Hospital for their participation, and to all of the mycobacteriology technologists who participated in slide collection.

For the Auramine Fading study I would like to thank the Microbiology Department at the Hinduja Hospital and Medical Research Centre and its technologists working in mycobacteriology for their participation in the study.

Co-Authors

In all 3 manuscripts contained in this thesis, I (Jessica Minion) am the primary author. I was the lead investigator in protocol development and study execution, performed the analyses, wrote the first drafts of all manuscripts and conducted all revisions.

LED Systematic Review: Laurence Brunet was the second reviewer; Madhukar Pai was the supervisor and senior author who also contributed to protocol development, analysis and writing of the manuscript.

LED Microscopy Evaluation: Andrew Ramsay contributed to protocol development and data interpretation; Dick Menzies and Madhukar Pai contributed to protocol development, analysis and writing of the manuscript; Christina Greenaway was the senior author who also contributed to protocol development, aided in managing laboratory access and laboratory staff, analysis and writing of the manuscript.

Auramine Fading Evaluation: Shubhada Shenai and Viral Vadwai performed slide staining and the LED microscopy readings of slides; Tejashree Tipnis performed the collection of data and organized the storage and blinding of slides; Christina Greenaway, Dick Menzies and Andrew Ramsay contributed to protocol development, data analysis and writing of the manuscript; Camilla Rodrigues provided supervision and guidance for the laboratory work; Madhukar Pai was the senior author who also contributed to protocol development, analysis and writing of the manuscript.

Chapter 1 – Background on TB Diagnosis and Smear Microscopy

Tuberculosis (TB) continues to be one of the world's most important infectious causes of morbidity and mortality among adults. An estimated 9.3 million people develop TB disease each year and approximately 1.7 million[1] die from the disease (1). Of these deaths, nearly 500,000 occur in HIV infected individuals, equating to 33% of HIV-positive incident TB cases and accounting for 23% of all HIV deaths. The preponderance of TB infection is borne by nations in Asia and Africa (55% and 31% of all estimated cases respectively), which often lack the resources to mount effective TB control initiatives.

One of the important deficiencies in global TB control is the detection of active TB cases. Despite the enormous global burden of TB, only 5.3 million new TB cases (57% of the total estimated) were diagnosed and notified in 2007, which falls well short of the global target set by the World Health Organization (WHO) of 70% case detection (1). Without this crucial first step of case detection, improvements in treatment, case management and infection control cannot realize their full impact on the TB epidemic.

The diagnosis of active TB has become reliant on the microbiologic detection of *Mycobacterium tuberculosis* (MTB) bacilli in a patient specimen. This is because while there are numerous clinical symptoms and signs associated with TB disease, none of them provide the sensitivity or specificity needed to differentiate TB from other common ailments (2). Likewise, chest x-ray (CXR) is a common tool used for TB diagnosis, however, even in the hands of experienced radiologists and pulmonologists the accuracy of TB diagnosis using CXR is non-specific and unreliable (3). While TB infection of the respiratory tract remains the most common manifestation and the most important with respect to public health and infection control, between 15 – 30% of active TB cases are considered

[1] Estimates cited are from 2007, the most current year available from the World Health Organization

extrapulmonary (i.e. infection outside the respiratory tract) (4). The diagnosis of extrapulmonary TB using clinical signs and symptoms is even more imprecise, and radiology is generally unable to add diagnostic value. The advent of the HIV epidemic has only added to the challenge of accurate TB diagnosis (5-6). HIV infection predisposes individuals to developing active TB and TB is in turn a major cause of morbidity and mortality in HIV positive individuals. Unfortunately, the attenuated immunity associated with HIV both alters the presentation of TB disease and broadens the number of differential diagnoses needing to be considered in a patient suspected of TB, thus making it even more important to secure microbiologic evidence of TB disease.

Currently, the gold standard for the diagnosis of active TB is the isolation of MTB through culture of a patient specimen (2). Since MTB is a strict pathogen, any isolation of it from any patient specimen is a clear indication of active disease requiring treatment. Mycobacterial culture provides the most sensitive method of detection and allows for both species confirmation and drug susceptibility testing.

However, there are several draw-backs to using routine culture for diagnosis. The first being the significant biohazard risk that is posed by culturing this highly infectious, airborne pathogen (7). The danger posed to laboratory technologists working with TB cultures necessitates a number of biosafety procedures be in place and strictly followed, along with relatively expensive investments in equipment and infrastructure. Mycobacterial culture is an expensive multi-step process which requires a high level of expertise from laboratory technologists along with ongoing monitoring and quality control to ensure its proper implementation. Finally, and most importantly, MTB is a slow growing bacterium and mycobacterial culture often takes weeks to months before results are available to aid in clinical decision making. While the development of liquid culture media, the addition of growth supplements and the use of more sensitive techniques to detect positive cultures have all served to decrease the

turnaround time of TB cultures, the delay still leads to many patients being diagnosed and treated empirically or sent home and lost to follow-up before final culture results are available (8-9).

As a result, significant investments in the development of simple, rapid, accurate and affordable diagnostics have been made in recent years (10). Advancements in molecular diagnostics, antigen and antibody detection, identification of volatile organic compound signatures, and nanotechnology have all opened exciting new areas of investigation. Yet despite many promising candidate tests, nothing has emerged as a clear improvement upon traditional means of microbiologic detection.

Hence, in most of the world, the diagnosis of TB relies on smear microscopy. This is the direct visualization of MTB bacilli or acid fast bacilli (AFB) in patient specimens, usually using traditional light microscopy and the Ziehl-Neelsen stain. This century-old technology is able to quickly diagnose the sickest TB patients and to identify the most contagious individuals. It has maintained its usefulness because smear microscopy is simple, easy to implement in a wide variety of settings, and affordable. Smear microscopy does not require the same level of technical expertise or infrastructure as mycobacterial culture and does not pose any significant biosafety concerns for laboratory workers. While smear microscopy is not able to provide speciation (i.e. a microscopist cannot differentiate between MTB and other acid fast organisms including non-tuberculous mycobacteria), in high incidence populations this probably has only a small negative impact on its diagnostic specificity. An increasingly important disadvantage is the inability of smear microscopy to provide drug susceptibility testing in order to identify multidrug resistant and extremely drug resistant TB (MDR- and XDR-TB). Arguably the most important drawback to using smear microscopy for the diagnosis of TB is its poor sensitivity. The sensitivity of smear microscopy is less than 60% compared to mycobacterial culture and this drops dramatically in certain subgroups (11-12). In the detection of HIV-related TB,

paediatric TB, and extrapulmonary TB the sensitivity of smear microscopy is unacceptably low. Smear microscopy is operator-dependent and reliant on the motivation of microscopists and the time they spend searching for rare AFB (13). This translates into over-worked and over-burdened laboratories in resource-poor high incidence areas missing many more positive smears compared to more ideal research-like settings.

Efforts to maximize the yield and sensitivity of smear microscopy have led to recent changes in specimen collection procedures, specimen processing, and microscopy techniques (14-17). One improvement over traditional smear microscopy has been the use of fluorescent microscopy (FM). Traditional smear microscopy has used light microscopes equipped with incandescent light sources that enable the visualization of characteristic bacilli after staining with an acid fast stain such as Ziehl-Neelsen (ZN). Fluorescent microscopes have, until recently, relied on the production of light using a mercury vapour light source combined with filters to stimulate fluorescence from bacilli stained with a fluorochrome-containing dye such as auramine. Due to the relative ease of detecting these brightly fluorescing bacilli couched on a dark background, FM has been shown to be both more sensitive and more efficient than light microscopy for the detection of AFB (17). The efficiency[2] of reading slides with FM is further enhanced due to the lower magnification needed to screen slides, thus allowing a microscopist to examine a larger area of a smear in less time (17-18). This not only translates into valuable time savings for busy TB laboratories, but allows them to offer higher quality service through more thorough smear examinations (19-20). In most of the developed world where the cost of labour is very high FM has now been widely adopted and is used routinely either in addition to or in replacement of ZN staining and traditional light microscopy.

[2] Slide reading 'efficiency' refers to the average reading time per smear; more efficient readings are synonymous with less time spent per slide and result in time savings for technologists and laboratories.

However, the expansion of FM for TB diagnosis to low-resource settings has not been successful to date. The primary barrier to global FM implementation has been the impractical features of conventional mercury vapour fluorescent microscopes themselves (21-23). These microscopes have relatively high initial capital costs, and also high recurrent costs. Their life-span is further shortened in dusty conditions or where the power supply is unpredictable. Their mercury vapour lamps require frequent replacement and can release potentially toxic products if they are broken. The fear of UV light production has contributed to poor acceptance from technologists. Finally, the use of conventional fluorescent microscopes requires working in an enclosed darkroom, which in addition to adding to infrastructure costs also hinders user acceptance, especially in tropical countries without the benefit of air conditioning.

Light emitting diode (LED) microscopy is a novel diagnostic tool developed primarily to provide resource-poor parts of the world access to the benefits of FM (24). Compared to conventional FM, LED microscopes are less expensive and require minimal maintenance. They are very robust and function well in remote areas with minimal to no infrastructure. Indeed, they require very little power and are able to run on batteries, making them portable on mobile clinics and suitable for areas with unreliable electricity. The bulbs have a very long half-life and do not pose any health risks to users. Lastly, LED devices seem to perform equally well without a darkroom obviating many users objections against their replacement of traditional light microscopes (21, 25). These qualities make them feasible for use in low resource settings and have the potential to bring the benefits of FM to areas where their improved sensitivity and reading efficiency are needed most.

While the first use of LED technology was seen as people began converting existing fluorescent microscopes for use with LED light sources (21, 26), there are now several commercial LED microscopy products on the market (27). Table 1 compares the major commercial LED microscopes for TB detection. This includes

a stand-alone light-fluorescent hybrid model developed by Zeiss in collaboration with the Foundation for Innovative New Diagnostics (FIND), the Primo Star iLED. This is being sold as a high quality all-in-one solution for laboratories looking to upgrade their microscopy capabilities, and special pricing has been negotiated for high-burden countries. The CyScope (Partec, Germany) and the FieldLab (Cytoscience, Switzerland) are stand-alone LED microscopes which are smaller and built for maximum portability. Two products, the Lumin (LW Scientific, USA) and the ParaLens (QBC Diagnostics, USA), are LED enabled objective lenses which can be swapped for a regular objective lens on an existing light microscope to confer fluorescent capability without the full purchase of a new microscope. The FluoLED attachment (Fraen, Italy) requires installation onto an existing light microscope and then also provides full 2-in-1 light and fluorescent functionality.

If FM is to be implemented widely in low resource settings, it will need to demonstrate more than accuracy and reading efficiency. The feasibility of diagnostic tools hinges on many factors unrelated to their performance (28). Some of these factors are easily identifiable such as cost-effectiveness, additional equipment and infrastructure required. Other considerations which are often overlooked include the availability of supply chains to remote areas, training requirements and established quality assurance procedures. An issue specific to the implementation of FM concerns the current system of external quality assurance (EQA) used by global TB laboratories. In this system, a sample of smears which have been stained and examined by the laboratory is saved and sent to a centralized reference laboratory approximately every 3 months (i.e. quarterly) (29). The reference laboratory then re-examines the smears and results are compared with the original readings reported. A high degree of correlation between these two readings is an indication of reliability in the smear microscopy results, while significant differences can trigger trouble-shooting strategies to investigate causes of the discrepancies. This system of storage and blinded re-reading has been promoted in busy low resource laboratories because

it serves to motivate lab technologists to maintain high standards of smear examination during routine work due to the knowledge that someone will be re-checking some of their work.

The problem which arises for this EQA system when FM is implemented is due to the impermanent nature of fluorescent stains such as auramine (the most common fluorescent stain used for mycobacteria) (30). Once a smear is stained with auramine, its fluorescence will fade over time until the bacilli are no longer visible against the dark background. What is not clear, however, is how quickly this fading process takes. Conventional protocols will insist that fluorescent smears should be kept away from light and read the same day they are stained (31). In contrast, anecdotal reports from many microbiologists suggest that auramine stained smears are easily re-read after many months of storage. Depending on how long these fluorescent smears can be kept and reliably re-read, modifications to current EQA programs may need to be made and implemented simultaneously with changes from light microscopy to LED fluorescent microscopy.

There is now much impetus for the evaluation of these new LED microscopes to assess their potential to replace both traditional light microscopy as well as conventional FM. If LED microscopy is shown to demonstrate improvements in sensitivity and reading efficiency compared to light microscopy in TB diagnosis, they may finally provide a long-overdue upgrade to microscopy services globally. Additionally, if they prove to be equivalent to conventional fluorescent microscopy, developed countries such as Canada are likely to take advantage of their practical benefits and cost savings as well, potentially replacing conventional fluorescent microscopes in many settings. If LED microscopes do enable the expansion of FM globally, the next steps will be to address practical implementation issues such as potential effects on EQA programs.

The objectives of this manuscript-based thesis research were to conduct 3 studies with the following aims:

1) systematically review evidence related to the performance of LED microscopy, including not only estimates of diagnostic accuracy but also assessments of factors important to the implementation of LED microscopy globally for the diagnosis of TB;
2) evaluate the performance of promising LED devices in a Canadian setting, specifically measuring their diagnostic accuracy and reading efficiency compared to the current standard of conventional fluorescent microscopy;
3) measure the fading of auramine stained smears over time in order to assess its potential impact on current EQA programs considering the implementation of LED microscopy for TB diagnosis.

Chapter 2 – LED Microscopy for Detection of Tuberculosis: a Systematic Review and Meta-Analysis

2.1 INTRODUCTION

LED microscopy has been developed for use in the diagnosis of active TB and has many benefits which could allow expanded access to FM and its associated improved sensitivity and reading efficiency. In order for LED microscopy to be a worthwhile investment for global TB control programs, it must not only demonstrate increased case detection and decreased time to read slides but also to show feasibility for wide spread implementation. Important issues related to programmatic feasibility include cost, required training, effects on current external quality control (EQA) programs, and user acceptability.

Cost-effectiveness is the heart of one of the key benefits to FM: increased reading efficiency. Currently, laboratories in areas with a high incidence of TB are often over-burdened and understaffed. Proper examination of a ZN stained smear with light microscopy requires at least 5 minutes (13), whereas examination of an auramine stained slide with fluorescence takes significantly less time because screening can be performed at a lower magnification (17).

When considering a new laboratory method such as FM for introduction on a large scale, it is important to estimate the training requirements needed before test results are reliable enough to be used for clinical decision making. All procedures and technologies have learning curves for users and implementation of a new test without proper training and validation can lead to poor initial performance. Not only does this risk clinical mismanagement, but also can lead to user frustration and rejection of the test itself for reasons unrelated to its true potential.

Current EQA programs for TB smear microscopy involve saving and storing a selection of routinely read stained slides which are submitted to a centralized reference laboratory for re-reading. While this works well for ZN stained slides, the switch to FM may make this unfeasible. The fluorescent auramine stain fades over time, which could invalidate the results of the second slide reading. However, the rate at which auramine staining fades is not clear. If the fluorescence fades significantly within 3 months (the usual period of time for storing slides for quarterly re-reading) then alternative EQA programs will need to be developed in advance of large scale implementation of LED microscopy.

Finally, user acceptability has been a key issue in previous attempts to implement FM in low-resource settings. Concerns over UV light production, dark room requirements and maintenance requirements are among the reasons many laboratories and technologists have elected not to implement FM to date. While manufacturers of LED microscopes claim to have eliminated many of the objections raised with respect to CFM, field demonstration trials which collect user assessments are critical to affirm these benefits.

We undertook a systematic review of the literature concerning the use of LED microscopy for the detection of *Mycobacterium tuberculosis* and performed meta-analyses of data examining its diagnostic accuracy. Additionally, we reviewed publications providing evidence concerning qualitative aspects of LED microscopy and its feasibility for large scale implementation.

2.2 METHODS

To conduct this systematic review and meta-analysis, we used a standard protocol [Appendix 1](32).

Search Strategy

We systematically searched 3 databases for relevant citations: PubMed, EMBASE and BIOSIS (January 1990 – February 2009 inclusive). The search strategy used for PubMed is shown in Appendix 2. All searches were performed with the help of an experienced librarian. Publications in English, French or Spanish were considered. Reference lists from included studies were hand searched. Additionally, experts and manufacturers were contacted to identify additional studies. Unpublished studies were considered eligible if detailed methods and results were provided in manuscript format by August 2009.

Eligibility Criteria

Predetermined eligibility criteria for the primary analysis were: assessment of the diagnostic accuracy or performance characteristics of LED microscopy for the detection of mycobacteria in patient specimens, use of culture as a reference standard, and adequate information to populate a diagnostic 2 by 2 table. Studies using alternate reference standards, such as expert rechecking of smears, were included and evaluated separately. Studies evaluating other characteristics such as time to read slides, cost-effectiveness, user assessments, or implementation issues such as training, staining or smear fading with respect to LED microscopes were also reviewed and their results incorporated into this report even if they did not report estimates of sensitivity and specificity.

Study Selection

Titles and abstracts were screened for relevance by one reviewer (JM) and obtained for full text review. Full text review was performed by one reviewer (JM). Articles retrieved for full text review along with reasons for exclusion are available from the authors.

Data Extraction

We created and piloted a data extraction form with a subset of eligible studies. Based on experience gained in the pilot study, the extraction form was finalized

[Appendix 3]. All studies included in the final review were extracted independently by 2 reviewers (JM and LB) and any disagreements were resolved by consensus.

Outcome Measures

Data were extracted to construct 2 by 2 tables of true positive (TP), false positive (FP), false negative (FN), and true negative (TN) values. True positives were defined as specimens found smear positive by LED microscopy and positive by the reference method. False positives were defined as specimens found smear positive by LED microscopy, but negative by the reference method. False negatives were defined as specimens found smear negative by LED microscopy, but positive by the reference method. True negatives were defined as specimens found smear negative by LED microscopy and negative by the reference method. From these data we calculated the sensitivity and specificity of LED compared to culture and compared to microscopic reference standards.

Studies which provided head to head comparisons between LED and CFM or ZN microscopy had these data extracted and the differences in sensitivity and specificity were calculated and pooled (see Analysis Methods below). Time to read smears was extracted and summarized; the relative time to read smears using LED compared to ZN was calculated, if available. Studies which performed costing analyses were reviewed and summarized narratively. When described, studies' approaches to training were compiled and described. Studies which provided head to head evaluations of different LED devices are elaborated on separately. Other outcomes of interest that were reviewed and are presented include mycobacterial fluorochrome staining methods, fluorochrome stain fading, and user reviews. Studies which reported on alternative or novel evaluation methods of LED microscopy are described narratively.

Assessment of Study Quality

Using the QUADAS criteria (33) for assessment of quality of diagnostic studies, we assessed quality characteristics that were considered important for this particular review: (i) blinded interpretation of the test results with reference standard results and vice-versa, (ii) complete verification of test results with the same reference standard, (iii) recruitment of patients/specimens either consecutively or randomly, and (iv) study design (cross-sectional vs. case-control; prospective vs. retrospective). The full QUADAS instrument is reproduced within Appendix 3.

Analysis

Data were analyzed using STATA/IC 11.0 (Stata Corp. Texas, USA). Forest plots visually displaying sensitivity and specificity estimates and their exact 95% confidence intervals (CIs) from each study were constructed using MetaDiSc software (34). Since these measures tend to be correlated and vary according to thresholds (either explicit or implicit cut-off values determining positive vs. negative results), hierarchical summary receiver operating characteristic (HSROC) curves were analyzed to explore the influence of those thresholds (32, 35). In HSROC curves a hierarchical model is used to account for variation both within and between studies contributing to the overall summary estimate and curve shape, analogous to random effects meta-analysis methods. The HSROC curve displays each study's sensitivity and specificity estimates within the ROC space. The area under the HSROC curve (AUC) provides an estimate of the overall accuracy. An AUC of 50% would indicate poor discriminatory ability, while an AUC of 100% means that the test discriminates perfectly.

Accuracy measures were pooled using bivariate random effects regression models (36), using the user-written program "metandi" in STATA (37). Bivariate models take into consideration the fact that sensitivity and specificity are not independent of one another, but are generally inversely correlated. Random effects models assume that individual studies are estimating non-identical

effects and expects variability to arise both within and between studies. As described by Reitsma and colleagues, the bivariate regression method assumes that the sensitivity values from individual studies (after logit transformation) within a meta-analysis are approximately normally distributed around a mean value with a certain amount of variability around this mean (36). This is a random effects approach. This variation in underlying sensitivity estimates between studies can be related to unmeasured differences in study population, differences in implicit threshold (cut-off), or unnoticed variations in index test protocol. These considerations also apply to specificity estimates. The potential presence of a (negative) correlation between sensitivity and specificity within studies is addressed by explicitly incorporating this correlation into the analysis. The combination of two normally distributed outcomes, the logit transformed sensitivity and specificity values, while acknowledging the possible correlation between them, leads to the bivariate normal distribution (36). The bivariate approach overcomes the problems associated with simple pooling (i.e. weighted average) of sensitivity and specificity estimates.

Sensitivity and specificity differences between LED and ZN or CFM were pooled using “metan, rd”, a random effects regression model for differences in proportions. Heterogeneity of accuracy estimates was assessed using the I^2 statistic and explored through subgroup analysis. The I^2 statistic measures the proportion of variation in study estimates that is attributable to heterogeneity as opposed to random chance (38). This is supplemented by p-values arising from the chi-squared distribution of a Cochran’s Q test, a classical measure of heterogeneity which is calculated as the weighted sum of squared differences between individual study effects and the pooled effect across studies (38). If fewer than 4 studies were available, their results were pooled using fixed effects models because bivariate random effects models do not converge with small numbers of studies. These estimates are clearly indicated and note is made of their relatively narrower confidence intervals.

Results from studies with multiple sites using the same study protocol were pooled directly and considered a single study for the purpose of this meta-analysis (i.e. these studies were treated as multi-centric studies). Studies which evaluated more than 1 LED device (e.g. Lumin and Zeiss) were treated as separate study arms and entered as multiple studies for the purpose of this meta-analysis. Studies which reported results separately for significantly different subgroups (concentrated and direct smears; high and low screening magnifications) were not pooled and considered as multiple arms entered individually into the meta-analysis.

Subgroup Analyses

Studies using culture as a reference standard were analyzed and pooled separately from studies using a microscopic reference standard. Subgroup analysis by smear type (direct vs. concentrated) and screening magnification was performed. Subgroup analysis by study design was done to explore its effect on heterogeneity.

2.3 RESULTS

Diagnostic Accuracy

The selection of included studies is summarized in Figure 1. We identified 2489 citations from the initial searches, and 2102 unique articles were left after excluding duplicate articles. After screening titles and abstracts, 34 articles were eligible for full-text review. Of these, 2 studies were included in the review. An additional 10 studies were identified by contacting experts, manufacturers and subsequent hand searches. Nine of the 12 included studies were unpublished.

Characteristics of Included Studies

Studies that met our selection criteria are described in Table 2. We identified 3 published (23, 39-40) and 9 unpublished (41-46) studies. Evaluations performed by the Foundation for Innovative Diagnostics (FIND) were reported in a single document (43), but describe 4 separate studies which we will refer to as: FIND – Feasibility Study, FIND – Evaluation Study, FIND – Demonstration Study, and FIND – Comparative Study.

Four of the commercial LED products were represented in these evaluations: Lumin (6 studies), Primo Star iLED (4 studies), FluoLED (3 studies), ParaLens (1 study). Included in these were 2 head-to-head evaluations: FluoLED vs. Lumin; Primo Star iLED vs. FluoLED vs. Lumin. A single study used a conventional fluorescent microscope adapted for used with LED illumination.

There were 8 studies which used mycobacterial culture as a reference standard. The remaining 4 studies used a microscopic reference standard: 2 used a pre-specified expert rechecking mechanism and 2 used CFM in parallel. Five studies each used direct smears and concentrated smears for their evaluations; 2 studies reported data on both direct and concentrated smears separately. Different magnifications were used for the screening of smears with 400x being the most common (6 studies) and 200x being the most common alterative magnification (4 studies). A single study used 600x and one study compared 200x with 400x.

Direct comparisons of LED with ZN (7 studies) and LED with CFM (6 studies) were also available for analysis.

Quality of Included Studies

Table 3 provides an overview of the key quality indicators found in the included studies. All of the included studies reported blinding their evaluation of slides using the LED microscopes. Nine studies reported complete verification using their respective reference standards (3 used partial or differential verification). Specimen recruitment was reported as prospective in all but 1 study, and

sampling was reported as either consecutive or random in 8 of the 12 studies (the remaining 4 were unclear). Seven of the evaluations used a case-control study design and the remaining 5 used a cross-sectional design.

Compared to the included unpublished studies, those which were published were more likely to be clearly reported: all 3 published studies reported prospective recruitment and all 3 published studies reported consecutive or random sampling. All 3 published studies used complete verification (compared to 6 out of the 9 unpublished), but only 1 of the published studies used a cross-sectional design (compared to 5 out of the 9 unpublished).

Accuracy of LED in Comparison to a Reference Standard

Figures 2 and 3 display sensitivity and specificity estimates from individual studies, using culture and microscopic reference standards respectively. Estimates from reading direct smears are shown as closed squares and estimates from reading concentrated or processed smears are shown as open squares. Using a culture reference standard, sensitivity estimates ranged from 67 – 96% and specificity estimates ranged from 89 – 100%. Not surprisingly, studies which compared LED to a microscopic reference standard yielded generally higher estimates of sensitivity (73 – 100%) and specificity (range 98 – 100%).

Pooled estimates of sensitivity and specificity, along with I^2 measures of heterogeneity are shown in Table 4. Overall, when culture was used as a reference standard, LED achieved 83.6% sensitivity (95% CI: 76.3, 89.0) and 98.2% specificity (95% CI: 96.6, 99.0). When a microscopic reference standard was used, overall sensitivity was 92.7% (95% CI: 84.9, 96.7) and overall specificity was 98.5% (95% CI: 98.2, 98.8).

Hierarchical Summary Receiver Operating Characteristic (HSROC) Curves

Figures 4 and 5 plot the sensitivity (or true positive rate) and 1-specificity (or false positive rate) in an HSROC curve for each of the reference definitions. The

curves show greater variation in sensitivity than specificity, with minimal specificity variation when a microscopic reference standard was employed.

Head-to-head Comparisons of LED with ZN and CFM

Tables 5 and 6 summarize studies with head-to-head comparisons between LED and ZN or LED and CFM respectively. When compared to ZN microscopy, LED sensitivity ranged from being 9% less sensitive to 24% more sensitive and LED specificity ranged from being 7% less specific to 1% more specific. When compared to CFM, LED sensitivity ranged from being 4% less sensitive to 16% more sensitive and LED specificity ranged from being 1% less specific to 5% more specific.

Pooled differences in sensitivity and specificity using random effects regression estimated LED sensitivity to be 6% (95% CI: 0.1, 13) greater than ZN and 5% (95% CI: 0, 11) greater than CFM. Pooling of specificity differences find LED to be 1% (95% CI: -3, 1) less specific than ZN and 1% (95% CI: -0.7, 3) more specific than CFM.

Subgroup Analyses

Concentrated vs. Direct Smears

Subgroup analysis was performed depending on whether direct or concentrated smears were used (Table 4). For subgroups with at least 4 studies, bivariate random effects pooling was performed; for subgroups with less than 4 studies available, a fixed effects model was used. Based on non-overlapping confidence intervals, there was a significant increase in sensitivity when direct smears were used (88.9%, 95% CI: 81.1, 93.7) compared to concentrated smears (72.7%, 95% CI: 69.2, 76.0) in the studies using culture as a reference standard. This difference was even more pronounced in those studies using a microscopic

reference standard (direct smear sensitivity=93.6%, 95% CI: 99.8, 96.4 vs. concentrated smear sensitivity=78.0%, 95% CI: 69.0, 85.0), although this was based on only 2 studies using concentrated smears and this estimate was derived from a fixed effects model that would result in narrower confidence intervals. One of the studies which included a head to head evaluation of direct and concentrated smears found improved sensitivity and specificity using direct smears. The second study which compared direct and concentrated smears found no difference in sensitivity or specificity overall, but noted that 2 of their 4 participating sites did find concentrated smears to have a lower sensitivity than direct smears.

Screening Magnification

Subgroup analysis was also performed depending on whether the screening magnification used was 200x or higher (400x or 600x were combined) (Table 4). Within the group of studies using culture as a reference standard, those which used a lower screening magnification had a significantly lower pooled specificity compared to studies using a higher screening magnification, based on non-overlapping confidence intervals: 94.4% (95% CI: 91.5, 96.4) with 200x screening vs. 99.0% (95% CI: 98.0, 99.5) with 400x/600x screening. This difference in specificity was not seen in pooled estimates of studies using a microscopic reference standard, but was observed in the single head to head evaluation comparing 200x vs. 400x readings (96.4%, 95% CI: 93.6, 98.0 vs. 100%, 95% CI: 98.6, 100). A difference in sensitivity was also detected between studies using higher vs. lower screening magnifications (+5% sensitivity using 400x/600x), however these pooled estimates were both calculated using fixed effects models which results in narrower confidence intervals.

Effect of Study Design

As there was significant heterogeneity in our pooled estimates, post-hoc subgroup analysis was performed according to the study design used to help

identify whether this was a contributing factor. Pooling studies that used a cross-sectional design and a culture reference standard (n=5), sensitivity was estimated to be 72.6% (95% CI: 69.2. 75.8) and specificity 96.9% (92.1, 98.8). This was a significantly lower sensitivity compared to studies using a case-control selection design and a culture reference standard (n=8): sensitivity 88.7% (81.4, 93.4), and specificity 98.6% (97.3. 99.3). There were 4 studies using a case-control design and a microscopic reference standard and their estimates of accuracy (sensitivity = 94.7%, 95% CI: 85.7, 98.2; specificity = 98.9%, 95% CI: 98.2, 99.3) were not significantly different from the single study using a cross-sectional design and similar reference standard (sensitivity = 95.6%, 95% CI: 95.0, 96.1; specificity = 98.4%, 95% CI: 98.2, 98.5).

Outcomes Important to Implementation

Time to Read Slides

Six studies provided measures of the time needed for readers to examine smears using LED (Table 7). There were a total of 14 comparisons made to ZN and 7 comparisons made to CFM, with varying proportions of smear +/-, smear type, and screening magnification used. Using simple averages (with equal weighting given to each study arm) the mean time saved compared to ZN was 46%. When only considering studies which provided estimates by smear result, LED was 48% more efficient than ZN in reading smear + slides, and 59% more efficient in reading smear - slides. Compared to CFM, the time to read slides was approximately equal (LED examination taking 4% more time).

The FIND Demonstration study measured the time to read slides 1 month after introduction of the iLED, and again 3 months after its introduction (43). Although after 1 month a 20% reduction in reading time was seen, this increased to 45% after 3 months showing that reading efficiency continued to increase after the

first month of use and full benefits were not realized immediately following introduction.

In another measure of smear reading efficiency, the FIND Evaluation study recorded the sensitivity of reading smears for 30 sec, 1 min, 3 min and 5 min (43). They found that when using either CFM or LED >80% of positive slides were correctly identified within 30 sec and increasing the reading time from 3 min to 5 min did not result in significant additional yield. In contrast, when using ZN <50% of positive slides were correctly identified within 30 sec and the full 5 min was required to maximize yield.

Cost Evaluations

The equipment costs of the major commercial LED devices, as obtained from their respective companies, are summarized in Table 1 (27).

In conjunction with the demonstration studies performed by FIND, costing data were collected for three participating settings: India, Lesotho, and Peru (43). Taking into account equipment costs, staffing costs, chemicals and reagents, consumables, building and overhead costs, they estimated that the average unit costs (cost per test) would be 10 – 12% lower for iLED compared to ZN. An important factor in this analysis was the time savings of using iLED (estimated to require 55% less reading time), which resulted in significant savings in staff costs. Assuming an overall iLED sensitivity of 96.3% and ZN sensitivity of 90.5%, both with 100% specificity, the cost per new case diagnosed was lower using iLED across all settings and across a wide range of TB prevalence. They concluded that implementation of this technology would not require significant modifications to the budgets of current TB programs, except for the initial capital investment for equipment purchase.

Using retrospective data collected from an urban centre in Malawi, Ramsay et al (47) modeled the effects of implementing LED microscopy along with the

adoption of a 2-specimen diagnostic strategy (from 3-specimen) and changes to the threshold for definition of a positive smear and a smear-positive case. Not only would these combined approaches significantly increase case detection (nearly doubling the detection of smear-positive cases), but the workload and time savings would enable a currently overburdened diagnostic laboratory to meet recommended smear examination times using existing human resources and minimal additional equipment.

Previous cost analyses have shown that despite higher upfront equipment costs, CFM can be a cost-effective alternative compared to ZN given the savings in labour and reagents (19-20). Considering the lower equipment costs for LED devices compared to CFM, their lower maintenance and their lack of a need for a dark room, LED technology can be considered a more cost effective option compared to both ZN and CFM.

Training

During the FIND Demonstration study, personnel with experience in ZN microscopy (but no experience with FM) were given between 1 – 5 days of training before entering the first phase of the study (43). Accuracy estimates were calculated separately for three distinct phases: Validation phase (1 month post initial training), Implementation phase (3 months following validation), and Continuation phase (6 months following implementation). Overall accuracy was strong even during the validation phase (sensitivity 94.2%, 95% CI: 92.2, 94.6; specificity 98.2%, 95% CI: 97.9, 98.5), and remained consistent throughout implementation (sensitivity 96.7%, 95% CI: 95.6, 97.2; specificity 98.4%, 95% CI: 97.8, 98.5) and continuation (sensitivity 96.7%, 95% CI: 92.2, 98.6; specificity 97.3%, 95% CI: 95.2, 98.4)(continuation phase estimates based on data collected to date).

Standardized proficiency testing post-training was performed, and repeated at 1 month and 3 months. Target specifications were required to be met at the end of

the validation phase, before continuing on to the implementation phase. These targets (>95% accuracy, 100% acceptable staining quality, >80% proficiency) were met by 27/28 of the study sites, with the remaining site achieving the targets 1 month later. Feedback from the microscopists undergoing this training emphasized the importance of practical hands-on training, with the availability of a supervisor to help distinguish AFB from artefacts. Most microscopists thought that 5 days of training was optimal for those experienced with ZN microscopy, and at least 13 days would be required for those without.

Training issues were not addressed in most of the other reports. However, three studies which did not include such extensive training and standardized proficiency testing noted the possible underperformance of LED upon its introduction due to insufficient training of staff (23, 42, 46).

Head-to-head LED Device Comparisons

Two studies included head-to-head evaluations of different LED models. Affolabi et al compared the LW Scientific Lumin and the Fraen FluoLED (41). When using 200X magnification, significantly more positive smears were detected using the Fraen module compared to the Lumin. This difference did not persist when comparing the two using 400X magnification in a secondary analysis. Additionally, the technologists unanimously preferred using the Fraen module, citing easier focusing and better image quality.

In the FIND Comparison study, all three LED devices (Zeiss iLED, Fraen FluoLED, LW Scientific Lumin) resulted in improved sensitivity over ZN and received positive feedback from users (43). Estimates of sensitivity gains compared to ZN resulted in +5.7% for iLED, +7.7% for FluoLED, and +3.8% for Lumin (statistically significant increase for FluoLED). Estimates of specificity gains compared to ZN resulted in +0.8% for iLED, -3.1% for FluoLED, and +0.8% for Lumin (statistically significant decrease for FluoLED). The time to examine slides was significantly less for all models compared to ZN; however, the Lumin examination times were

higher than the other 2 models (2.94 min/slide Lumin vs. 2.3 min/slide iLED and 2.38 min/slide FluoLED). User assessments of the 3 models indicated a preference for the iLED citing its high quality optics, operational characteristics and ease if viewing in full light as significant advantages.

Staining Methods

Another sub-study reported by FIND compared the performance and suitability of different commercial and in-house stains for use with FM (43). Users reported that all of the fluorochrome staining methods were easier to perform than the ZN stain (likely due to the absence of a heating step). Although experienced users preferred the Auramine O/$KMnO_4$ stain (and the reading time was significantly shorter for both Auramine/$KMnO_4$ and Auramine-Rhodamine/$KMnO_4$), less experienced users found it easier to focus and less tiring to read the stains with coloured backgrounds (Auramine/Methylene Blue and Auramine/Thiazine Red). In a separate evaluation of staining preference done in conjunction with the FIND Feasibility study, users preferred the Auramine/KMnO4 stain over Auramine-Rhodamine/KMnO4 and Auramine/Methylene Blue.

All studies included in this review used auramine O/$KMnO_4$ staining.

Fading of Fluorochrome-Stained Slides

Given the current reliance of many External Quality Assurance (EQA) programs on quarterly rechecking of a selection of stored slides, the potential for fading of the fluorescent stained smears was evaluated by 2 studies.

In a sub-study reported by FIND, 6 microscopy centers kept a set of 10 positive smears at room temperature (without air conditioning) and re-read them on a monthly basis for 4 months (43). None of the monthly readings changed from the initial positivity grading at months 1, 2 or 3 and a single center reported misclassifying a single positive slide as negative during the month 4 reading.

Qualitative assessments from all sites reported no impairment of reading at month 1 or 2, 1 site reported mild fading at month 3, and 3 sites reported impairment ranging from mild to significant by week 4.

A study performed by Minion et al in Mumbai, India, stored sets of slides in different environments (air conditioned room temperature, 22^{o}C; humidified incubator, 30^{o}C; refrigerator, 4^{o}C – all in slide boxes sealed against light) and re-read them on a monthly basis for up to 5 months (48). A mixture of negative, low positive and high positive smears were included and reading was done in conjunction with a larger LED evaluation study to ensure technologist blinding (i.e. readers did not know they were reading stored smears, but read them as if they were routine). Overall, the proportion of positive slides read on a monthly basis that remained positive decreased to 63% at month 1, 43% at month 2, 26% at month 3, 15% at month 4, and 11% at month 5 for slides stored at air conditioned room temperature. Slides stored in a humidified incubator faded faster than those at room temperature, and surprisingly, slides stored in a refrigerator experienced the fastest fading.

Other Assessments

The study published by Van Deun et al also reports on a field evaluation in 2 high throughput Tanzanian laboratories (23). The field evaluation centres did not have access to culture references, and thus historical comparisons were made to the yield of positive specimens using ZN staining in previous years. In addition to overwhelming user-acceptance and approval of the new LED microscopes, they found a 20% proportional increase in yield of positive smears (from 10 – 12% positives during years of using ZN, to 13 – 16% positives during 2 years of LED use at the two laboratories)(23).

Kuhn et al undertook a field evaluation of the Lumin attachment to assess portability, durability and ease of use (44). All of these characteristics were assessed favourably. It was noted that although viewing was best achieved in

completely dark conditions, use was adequate even in bright ambient lighting. This ability to use LED FM without a special dark room was confirmed by several of the other studies (39-40, 43, 46).

Omar et al also conducted standardized laboratory experiments to compare the Lumin LED with CFM (45). Using smears prepared from known concentrations of MTB suspension, slides were read using standardized protocols in order to quantify their bacillary burden. Concordance between the two microscopes was very high and the technologists preferred the contrast achieved with the LED enabled microscope.

Criticisms of the LED FMs included reports by technologists working with Omar et al that using the lower magnification of FM resulted in difficulty differentiating artefacts from bacilli (45). Additionally, when using the attachments it is not possible to easily switch to other lenses (requires removing the adapter).

User assessments were also collected during all of the FIND studies (43). Participants in the Feasibility study rated the iLED 3/3 on contrast, resolution, depths of focus, signal-to-noise ratio, and homogeneity of illumination. Evaluation and Demonstration studies asked users to judge the iLED on a number of characteristics including ease of installation, training required, overall handling and features, use of switching between ZN and FM, light intensity/background/contrast, resolution/depth of focus, need for a darkroom, magnification objectives, preferred bulbs for light microscopy, gain in speed, and recommendations for implementation. Overall, reviews were very positive and 94 – 100% of respondents would recommend implementing the iLED system over ZN (43).

2.4 DISCUSSION

In this meta-analysis, we performed an extensive literature search and identified 12 studies that evaluated the performance of LED microscopy, 9 of which are currently unpublished. A single evaluation was done using a non-commercial LED adapted microscope, 6 study arms used LED objective lens attachments (5 Lumin, LW Scientific; 1 ParaLens, QBC Diagnostics), 3 study arms used the trans-fluorescent module FluoLED (Fraen) and 4 study arms using the Primo Star iLED (Zeiss). Eight evaluations used culture as a reference standard and 4 used a microscopic reference standard. Pooled estimates of accuracy found LED to have 83.6% sensitivity and 98.2% specificity when compared to culture, and 92.7% sensitivity and 98.5% specificity when compared to a microscopic reference. Direct comparisons, using culture as a reference standard, estimated LED to have 6% greater sensitivity than ZN and 5% greater sensitivity than CFM, with no appreciable difference in specificity.

Timing data show that compared to ZN, LED microscopy has similar gains in reading efficiency to CFM, requiring approximately 46% less time than ZN for smear examination. Cost assessments predict improved cost-effectiveness compared to ZN microscopy, with the improved reading efficiency being a key quality of both LED and CFM. Qualitative assessments of LED microscopes confirmed many touted advantages, including the ability to use without a darkroom, durability and (in the case of the attachment models) portability. User acceptance in all field trials was reported as excellent.

The use of direct vs. concentrated smears was identified as a potential source of heterogeneity when comparing the performance of microscopy methods to a culture reference standard. A previous review found that sputum processing through concentration generally leads to an increase in sensitivity (15). In the studies included in this review, those that used concentrated smears had an overall pooled sensitivity lower than those using direct smears. However, only two studies included in this review directly compared direct vs. concentrated smears and a variety of important confounding factors including prevalence of

HIV, prevalence of extrapulmonary TB, severity of TB disease and laboratory methods used were not balanced between studies reporting results using direct smears and those using concentrated smears.

We also found that using a lower magnification when screening smears was associated with a lower specificity. This would be expected if microscopists used only the lower magnification to declare a smear positive, without confirming its morphology with a higher magnification. In this way, fluorescing artefacts could easily become confused with AFB. It should be ensured that technologists who are comfortable using lower magnifications to rapidly screen smears take the time to confirm the morphology of positive smears with higher magnifications. With LED objective lens attachments, such as the Lumin and ParaLens, this change in magnification involves unplugging the power source from the first lens attachment and reattaching it to the second lens attachment before use. This could influence technologists to avoid changing magnifications during screening, and result in lower specificity when low magnification screening is being performed.

Barriers that remain with respect to implementing wide spread use of LED FM include training of laboratory staff unfamiliar with FM and a proposed mechanism of EQA for the inherently impermanent auramine stain used with FM. Insufficient training was cited as a potential limiting factor in several studies, however, with the use of standardized training and assessment, it appears that LED performance can be maximized within a period 1 month. Evidence regarding the effect of fluorochrome fading on the reproducibility of slide reading over time is inconclusive, but suggests that current EQA programs may not be able to reliably use stored slides to evaluate a laboratory's performance.

Our SR had several strengths. First, we used a standard protocol for doing the systematic review, including a comprehensive search strategy to retrieve both published and unpublished relevant studies. By contacting several experts and

manufacturers, we were able to identify and include several unpublished studies. In addition to assessing accuracy, multiple other important outcomes were reviewed and summarized including time to read slides, costing, stain fading, training and user assessments. Lastly, we used rigorous methods for data analysis, including bivariate random effects models, HSROC analyses, and tests for statistical heterogeneity. Subgroup analyses using type of smear, magnification used and study design were performed in an attempt to explain some of the observed heterogeneity in estimates of accuracy.

Our SR was limited by the lack of a common reference standard (some studies using culture and others using a microscopic reference), and lack of well-accepted and consistently applied microscopy methods such as smear processing and screening magnification. Considerable heterogeneity was found in many of the pooled estimates, which was not unexpected given the different products, diverse settings and study designs used. Other factors which likely contributed to the heterogeneity of study estimates include the prevalence of HIV infection in the study populations, severity of disease, proportion of non-tuberculous mycobacteria and extra-pulmonary specimens included in individual studies. These hypothesized sources of heterogeneity should be confirmed by future studies to further characterize their impact on LED microscopy performance.

The decision to leave subgroup comparisons of smear type and screening magnification un-pooled, and thus essentially counted as separate studies, may have over-weighted the results from those studies (FIND Feasibility , FIND Evaluation (43), and Shenai et al (46)). However, given the differences between these subgroups it was felt that using the random effects model during meta-analysis would be more appropriate than simple pooling of these data. The other alternative would have been to exclude one of these subgroups from the analysis, however, since neither smear type (direct or concentrated) and neither screening magnification (200x or >/=400x) could be considered ‘standard’ this was also felt to be inappropriate. The majority of studies included in this review

are unpublished and thus are not peer reviewed. This decreases the confidence we have in the primary study results and subsequently the meta-analyzed estimates. It also is likely to reduce the reproducibility of this meta-analysis, if some of these studies remain unpublished or are published with revised results. Nevertheless, being a new technology we felt it was important to summarize the most up to date evidence available regardless of publication status. A significant amount of data included in the meta-analysis came from studies performed by FIND, a group which was involved in the development of the LED device they were evaluating (Zeiss Primo Star iLED). It is possible that these studies were subject to conflict of interest given their close relationship with the manufacturer. While this cannot be ruled out, FIND has an impressive record as a non-profit foundation dedicated to the promotion of high quality new diagnostics for neglected diseases and the performance of objective implementation research.

Conclusions

LED microscopy has comparable diagnostic accuracy to CFM while using a more durable, safer, and less expensive technology than mercury vapour fluorescent microscopes. The benefits associated with using CFM have been previously established, and current LED evaluations are consistent with the improved sensitivity, simplicity and reading efficiency of FM compared to ZN light microscopy. The barriers to widespread implementation of FM in many low-income settings have been largely practical and several may be overcome with the introduction of LED fluorescent microscopy. Remaining issues are likely to involve the implementation of an effective EQA system for use with the impermanent fluorescence of the auramine stain, and training for technologists new to using fluorescent microscopy.

This report was submitted to an Expert Group Meeting (EGM) assembled by the World Health Organization (WHO) in September 2009 to assess approaches to

improve sputum smear microscopy for TB diagnosis. Based on recommendations from this EGM, the WHO Strategic and Technical Advisory Group for Tuberculosis (STAG-TB) issued a policy recommending the replacement of CFM with LED microscopy and the phased implementation of LED in place of ZN microscopy in TB laboratories (Appendix 4)(49).

Chapter 3 – Research Gaps and Need for LED Evaluation

Based on our systematic review and meta-analysis on LED microscopy for the diagnosis of active TB, we identified several areas of interest where further research was warranted. These include the development of evidence-based training standards for laboratories without prior experience in FM; the assessment of auramine stain fading during storage and its effects on current EQA protocols; confirmation of optimal standard operating procedures for slide examination (including screening magnification, specimen processing techniques, alternative staining methods); performance of LED microscopy in important clinical subgroups (i.e. HIV positive individuals, paediatrics, extra-pulmonary TB); comparative performance of the different commercially available LED devices; assessment of LED feasibility and benefits in low-incidence, high-resource settings.

The final two research questions were identified for investigation in Montreal, Canada. Montreal has a low incidence of active TB (11.2 per 100,000) and laboratories performing TB diagnostics on specimens from TB suspects receive approximately 50 negative specimens for every specimen that is found to be culture positive (50). The benefits of improved sensitivity and reading efficiency of FM compared to ZN microscopy have long been realized in high resource settings such as Canada through the use of CFM, and Canadian technologists have ample expertise using FM. Nevertheless, the operational benefits of LED microscopy over CFM would be of interest for Canadian laboratories and technologists. The ability for technologists to work without a dark room using LED microscopes could significantly improve workflow and maximize space utilization in the lab. Lower purchase price and maintenance costs, longer bulb life, absence of toxic components, the lack of warm up time required between turning on a CFM and its use, and the lack of any need to record time spent with the microscope on in order to judge its bulb life are all factors that would

influence Canadian laboratory managers to switch from CFM to LED microscopy for TB diagnosis.

However, taking advantage of these benefits in Canada would only be considered feasible if the more important aspects associated with CFM, namely sensitivity and reading efficiency, were maintained with LED microscopy.

Another consideration for laboratory managers considering the implementation of LED microscopy in either a low- or high-resource setting is the choice of LED device. There are several commercial manufacturers now marketing LED microscopes, but very few studies comparing their head-to-head performance. Given the very different types of devices available, with different touted benefits and potential roles, it is important to compare them with respect to a specific setting or situation. For instance, in Canadian laboratories portability and the ability to withstand power fluctuations and dusty environments are less important considerations. However, technologist acceptance and confirmation that use without a darkroom is feasible are relatively more important, specifically when compared to current high quality mercury vapour fluorescent microscopes.

The LW Scientific Lumin attachment (LW Scientific Inc., Atlanta, USA) had the largest number of product evaluations identified in our systematic review. This very affordable LED option has many appealing characteristics, particularly for laboratories currently using ZN microscopy. The Lumin is an objective lens attachment that is used with an existing standard light microscope. Thus laboratories which have already invested in light microscopes are able to continue using their existing equipment without losing those investments. The Lumin can readily be attached and removed again to restore the original light microscope capabilities, which would appeal to small laboratories whose workload consists of multiple tasks outside of TB diagnosis. Its simple, portable

design is well suited to many low resource settings and its ability to function on battery power addresses concerns of laboratories without a stable power supply.

The Zeiss Primo Star iLED microscope was designed through collaboration with the Foundation for Innovative New Diagnostics (FIND) to be a high quality all-in-one light and fluorescent device. It converts from light to fluorescence by flicking a switch and is also capable of running on batteries for a short time. It is not meant to be a portable unit, and its high quality optics make it the most expensive LED option currently marketed for TB diagnostics (although special pricing is available for high-burden countries). Despite the comparatively higher initial cost, the Zeiss LED Primo Star iLED maintains the significant cost-savings in bulb replacement and maintenance costs associated with CFM.

In this context, we designed and conducted a head-to-head evaluation of the commercially available Lumin and Zeiss LED units, and compared their performance to a conventional fluorescent microscope using mycobacterial culture as a reference standard in Montreal, Canada.

Chapter 4 – Evaluation and Comparison of LED Microscopy Devices in a Canadian Setting

4.1 INTRODUCTION

The diagnosis of tuberculosis (TB) continues to rely on traditional microscopy and culture to detect *Mycobacterium tuberculosis* bacteria in patient specimens. Efforts to maximize the performance and sensitivity of these procedures have led to changes in specimen collection, processing and culture techniques (14-15, 51). For direct detection, the development of fluorescent microscopy (FM) using auramine staining has been a major improvement from Ziehl-Neelsen (ZN) staining for use with light microscopy. FM has been shown to have 10% higher sensitivity compared to routine light microscopy, with no significant compromise in specificity (17). FM is also more time efficient, with one large study finding FM to take only 25% of the time required for ZN examination (18). In most of the developed world, FM has now been widely adopted and is used routinely either in addition to or in replacement of ZN staining.

Light emitting diode (LED) microscopy is a novel diagnostic tool developed primarily to allow resource-poor parts of the world access to the benefits of FM (24, 27). Compared to mercury vapour fluorescent microscopes, LED microscopes are less expensive, have lower maintenance requirements, require less power and are able to run on batteries. The bulbs have a very long half-life and do not pose the risk of releasing potentially toxic products if they are broken, there is no UV light production and they are reported to perform equally well without a darkroom. These qualities make them feasible for use in low resource settings, and they have performed well in evaluations in high burden countries. Many of the benefits of LED technology would also be appealing to developed nations, if LED microscopy is found to perform equivalently to mercury vapour lamp FM.

There are now multiple LED manufacturers marketing their products for use. The Lumin Portable Fluorescent Kit (LW Scientific, Atlanta, Georgia, USA) is an objective lens attachment with an integrated LED illuminator, which comes with a universal power supply in a portable carrying case. The attachment can be used with most light microscopes, is able to run on battery power, and the company advertises a 50,000hr bulb life. The Zeiss Primo Star iLED microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany) was developed in collaboration with FIND (Foundation for Innovative New Diagnostics) and can be used as a bright-field or fluorescent microscope; the two modes are changed by the flick of a switch. It also is able to run on battery power and the company estimates at least a 10yr bulb life.

The objectives of this study were to determine and compare the sensitivity and specificity of fluorescent smear microscopy using the LW Scientific Lumin, the Zeiss Primo Star iLED microscope and a conventional mercury vapour fluorescent microscope using mycobacterial culture as a gold standard. Additionally, we measured and compared the time required to read fluorescent smears with the three FM devices and collected feedback from independent microscopists regarding user-important characteristics.

We hypothesized that there would be no significant difference in diagnostic accuracy or reading efficiency between the conventional fluorescent microscope, the LW Scientific Lumin, or the Zeiss Primo Star iLED.

4.2 METHODS

Study Setting

The study was conducted in Montreal, Canada using specimens submitted for mycobacterial culture to either the Montreal Jewish General Hospital (JGH) or the Royal Victoria Hospital (RVH). All readings were performed by technologists

with expertise in mycobacteriology and fluorescent microscopy, at the JGH. The JGH microbiology laboratory receives approximately 5 mycobacterial culture positive specimens per month. The RVH microbiology laboratory receives approximately 30 mycobacterial culture positive specimens per month. Overall culture positivity is approximately 2% in both laboratories.

Specimen Processing:

Respiratory specimens (including sputum, BAL, BW, lung aspirates) were digested/decontaminated with NALC-NaOH for 15 minutes before neutralization with phosphate buffer. Specimens were then centrifuged at 4 degrees C for 15 minutes at 3000 x g and supernatant was removed. Sediments were re-suspended in phosphate buffer before slide preparation and culture inoculation.

Sterile body fluids including cerebrospinal fluid (CSF) were centrifuged 10 minutes at 3000xg before smear preparation and culture inoculation. Prior to decontamination, urine was centrifuged for 10 minutes at 3000xg. The sediment was re-suspended in 10mL of supernatant after the excess was removed. Swabs were vortexed in 10mL sterile water and soaked for 2 hours. Swabs were subsequently discarded and the solution decontaminated and processed.

3 drops of each specimen were inoculated onto Lowenstein-Jensen media and 0.5mL was added to a MGIT tube. Specimens were incubated on an inclined rack at 37 degrees C or in a BACTEC MGIT 960 (Becton Dickenson, Sparks, MD, USA) respectively for 8 weeks. LJ cultures were inspected weekly for growth. Positive growth was confirmed by Kinyoun staining, and mycobacterial isolates were sent to the Provincial Laboratory for species confirmation.

Smears were fixed with heat before storage. Staining was performed immediately before the first smear examination. Smears were flooded with auramine O for 15 minutes, then rinsed with sterile water; decolourized with acid-alcohol for 2 minutes, then rinsed with sterile water; counterstained with

potassium permanganate for 2-4 minutes, then rinsed with sterile water and allowed to air dry.

Specimen Selection

Given the low culture positivity of our setting, we elected to use a nested case control design in order to include all culture positive specimens and an equal number of culture negative specimens. All consecutive specimens submitted for mycobacterial culture to either the JGH or the RVH laboratories had an additional smear prepared and heat fixed. These unstained smears were stored in dry, dark smear boxes. When culture results were available, all smears originating from culture positive specimens were selected for study inclusion and examination by the three fluorescent microscopes. An equal number of smears originating from culture negative specimens were randomly selected (using random number generators) for study inclusion and examination by the three fluorescent microscopes.

Fluorescent Microscopy Comparison

Selected smears were examined by one of two experienced technologists, blinded to the culture results and any patient details. Smear readings were done in parallel on the LW Scientific Lumin LED attachment, the Zeiss Primo Star iLED, and a conventional mercury vapour microscope (Leica DMLS). Between readings slides were randomized (with the aid of random number lists) to maintain technologist blinding. The time required to read slides was estimated by logging the time at the start and at the end of reading a group of 25-27 slides. This time was then averaged for the number of slides read to calculate a time per slide estimate.

Fluorescent Smear Examination:

Negative smears had 300 fields examined before being declared negative.

Slides were reported according to the following scale at 400X (52):

1+ = 1-18 AFB / 50 fields

2+ = 4 - 36 AFB / 10 fields

3+ = 4 - 36 AFB / 1 field

4+ = >36 AFB / 1 field

Sample Size Calculation

We used a two-sided hypothesis of equivalence, where we estimated a sensitivity of all the microscopes to be 40% and non-equivalence was defined as a change in sensitivity of more than 10%. In order to achieve 80% power with a significance level of 0.05, the number of samples read by each microscope needed to be 397. We included a total of 400 specimens to be read by each microscope, 200 culture positive and 200 culture negative.

Based on the volume of specimens received and the culture positivity of the participating laboratories (see Study Setting above), it was predicted that in order to collect 200 culture positive specimens for the study, specimen collection would require approximately 6 months. Thus the preparation and collection of duplicate slides for use in the study was initiated in April 2009 and continued until the end of September 2009.

Analysis

Sensitivity, specificity, positive and negative predictive values, and likelihood ratios were calculated for the LW Scientific Lumin LED attachment, the Zeiss Primo Star iLED and conventional mercury vapour (Leica DMLS) microscope using mycobacterial culture as the reference standard. Confidence intervals were constructed using exact methods for proportions. A second reference standard with acknowledged incorporation bias was defined assuming 100% specificity,

where any slide read as positive by any microscope was considered positive. The yield of positive specimens detected by each microscope was then calculated. Inter-rater agreement between the three microscopy readings was estimated using kappa statistics considering dichotomous results (where 1+, 2+, 3+, and 4+ are pooled as positive) as well as weighted kappa statistics with linear weighting of 5 categories: negative, 1+, 2+, 3+, and 4+. Kappa statistics measure the agreement between two readers (in this case two microscopes) evaluating the same items (in this case mycobacterial smears). Kappa takes into account the agreement occurring by chance and can be roughly interpreted according to the following table (53):

≤0: no agreement

0.0 – 0.20: slight agreement

0.21 – 0.40: fair agreement

0.41 – 0.60: moderate agreement

0.61 – 0.80: substantial agreement

0.81 – 1.00: almost perfect agreement

Subgroup analysis was done by specimen type (sputum, non-sputum respiratory, extrapulmonary) and mycobacterial species isolated (*M. tuberculosis* complex, non-tuberculous mycobacteria and acid fast non-mycobacteria).

The analysis was performed by specimen and not by patient. While this is most consistent with other studies in this field, we recognize that the lack of independence between specimens arising from the same patient may artificially elevate estimates of precision.

4.3 RESULTS

A total of 200 culture positive specimens were included in the study, with 200 randomly selected culture-negative controls. 296 specimens were submitted as sputum (74.0%), 64 originated from the respiratory system but not classified as sputum (16.0%; includes specimens such as bronchioalveolar lavage (BAL) fluid and lung biopsies), and 40 specimens were categorized as extrapulmonary (10.0%). The highest rate of culture positivity was seen in sputum specimens (57.1%), followed by extra-pulmonary specimens (35.0%) and non-sputum respiratory (26.6%).

There were 87 specimens which were read as smear positive by at least 1 of the 3 microscopes. The CFM identified 75 specimens as smear positive, the Zeiss identified 83 and the Lumin identified 76. Using a microscopic reference standard where any positive reading was considered accurate (i.e. 100% specificity), this resulted in sensitivities of 86.2% (95% CI: 77.1, 92.7), 95.4% (95% CI: 88.6, 98.7), and 87.4% (95% CI: 78.5, 93.5) for CFM, Zeiss and Lumin respectively (Table 8).

Using mycobacterial culture as a reference standard, the accuracy of the 3 microscopes is shown in Table 9. Zeiss achieved the highest sensitivity with 40.5% (95% CI: 33.6, 47.7), followed by Lumin with 37.5% (95% CI: 30.8, 44.6) and CFM with 36.5% (95% CI: 29.8, 43.6). None of the differences in sensitivity were significantly different based on overlapping confidence intervals. Specificity was very similar between all 3 microscopes (CFM and Zeiss were equal: 99.0% [95% CI: 96.4, 99.9]; and Lumin: 99.5% [95% CI: 97.2, 100]).

Inter-rater agreement was measured with the kappa statistic using dichotomized results (where 1+, 2+, 3+ and 4+ were pooled as positive). Agreement was high between all three microscopes: unweighted kappa = 0.91 (95% CI: 0.85, 0.96) between CFM and Zeiss; 0.89 (95% CI: 0.84, 0.95) between CFM and Lumin; and 0.91 (95% CI: 0.86, 0.96) between Zeiss and Lumin. Kappa values remained high if linear weights for categories of smear positivity (negative, +1, +2, +3, +4) were

used: weighted kappa = 0.92 (95% CI: 0.89, 0.96) between CFM and Zeiss; 0.92 (95% CI: 0.88, 0.96) between CFM and Lumin; and 0.93 (95% CI: 0.90, 0.97) between Zeiss and Lumin. The distribution of all positive smear readings is displayed in Figure 6.

Accuracy was also calculated depending on the category of specimens examined and the species isolated. Table 10 shows sensitivity and specificity of all 3 microscopes stratified by sputum specimens, non-sputum respiratory specimens, and extra-pulmonary specimens. There were no significant differences between the microscopes for any of these subgroups, based on non-overlapping confidence intervals.

Of the 200 culture positive specimens, 115 isolated MTB Complex organisms (106 *M. tuberculosis*, 9 *M. africanum*). The remaining 85 culture positive specimens isolated a wide range of non-tuberculous mycobacteria (NTM) as well as acid-fast organisms capable of surviving mycobacterial decontamination and growing in mycobacterial growth media (2 *Streptomyces* species, 1 *Nocardia puris*, 1 *Tsukamurella tyrosinosolvens*). These were considered true positives since smears are read as positive for “acid fast bacilli” which include organisms from Streptomyces, Nocardia and Tsukamurella genera. The sensitivity of all 3 microscopes was higher in detecting MTB Complex organisms compared to NTM or other acid fast organisms; however, there was no difference between the 3 devices (Table 11).

The time required for the experienced fluorescent microscopists to read slides was measured for each microscope. On average, reading slides using the CFM took 1.51 mins/slide (95% CI: 1.47, 1.55). This was identical to the time required using the Lumin (1.51 mins/slide; [95% CI: 1.48, 1.54]), but longer than the time required using the Zeiss (1.12 mins/slide; [95% CI: 1.09, 1.15]). The time savings using the Zeiss microscope was statistically significant compared to the other 2 microscopes.

4.4 DISCUSSION

We found no difference in the accuracy of smear reading either between the two LED microscopes or compared to the conventional fluorescent microscope. The inter-rater agreement was high for all three microscopes assessed (kappa >0.88 for all comparisons). When smears were stratified by their specimen type or organism isolated, their diagnostic accuracy remained equivalent.

The time required to examine slides was identical for the CFM and the Lumin LED attachment. However, the average time spent examining slides with the Zeiss Primo Star iLED was less than with the other two microscopes. Subjective reports from the technologists confirmed that the Zeiss device was easier to use, facilitated focusing and provided a pleasant viewing contrast. Importantly, the technologists confirmed that the Zeiss microscope was easily used without a dark room, while the fluorescence provided by the Lumin was not adequate for use in a lighted room.

This study was conducted in an idealized research setting with experienced mycobacterial technologists familiar with the use of FM. While the lack of training requirements and controlled reading environment does not provide a demonstration of LED microscopy implementation, it does enable the control of important study design factors such as blinding to previous results and clinical patient status and the dedicated measurement of time required to read the same slides on all three microscopes.

Study Limitations

Our evaluation was limited by the small number of culture positive and smear positive specimens available for inclusion, resulting in wide confidence intervals around estimates of diagnostic accuracy. Due to the low incidence of TB in Montreal and the limited time frame of the study we opted to enrich the

population of included slides by using nested case control selection. This allowed us to select all of the culture-positive specimens during our data collection phase, while minimizing the number of culture-negative controls. Furthermore, despite sample size calculations to ensure adequate power to detect statistically significant differences in accuracy, this study may still have been underpowered since the actual rate of smear positive slides was slightly lower than what was predicted.

Conclusions

We did not find evidence of any difference in the diagnostic accuracy using the Zeiss Primo Star iLED, LW Scientific Lumin, or a CFM (Leica DMLS) for the detection of AFB in patient specimens. While both LED microscopes were able to maintain the reading efficiency of the CFM, the Zeiss required significantly less time for smear examination compared to either the CFM or the Lumin. Given the practical benefits of LED microscopes for TB diagnosis, and absence of inferior performance compared to the current standard of CFM, we conclude that LED microscopy should be considered by all TB diagnostic laboratories, including those in Canada, as a replacement for CFM. Our findings provide support to the recent WHO policy which recommended that CFM be replaced by LED microscopy using auramine staining in all settings where fluorescent microscopy is currently used (49).

Chapter 5 – Research Gaps and Need for Fading Study

Based on our systematic review of LED microscopy, it became apparent that the switch from traditional light microscopy to fluorescent microscopy for the diagnosis of active TB, as recommended by the WHO, would face several challenges in implementation. One of the more pragmatic issues identified relates to the fading of auramine stained smears and the impact that may have on current EQA programs.

With the expansion of laboratory services and increased access to diagnostics, ensuring quality control and quality assurance programs are put in place simultaneously has been an important initiative of the World Health Organization (WHO) and Global Laboratory Initiative (GLI) (54). Smear microscopy remains the backbone of TB diagnosis in much of the world and expansion of dependable smear diagnostics into peripheral health centres has been a generally successful means of increasing TB case detection in low resource areas. The need for quality assured smear microscopy has been highlighted in these situations due to the operator-dependent nature of smear microscopy. In order to ensure ongoing high quality performance of smear examinations during routine working conditions, the rechecking of previously reported saved smears has become the mechanism of choice for global EQA programs (29).

The switch from light microscopy to fluorescent microscopy is accompanied by a change in stains used to visualize AFB. While ZN staining, used with light microscopy, is generally considered quite stable over time under appropriate storage conditions (i.e. dry and dark), auramine staining used with fluorescent microscopy is known to fade over time (30). However, the length of time that auramine stained slides remain readable is not clear. If auramine stained slides cannot be reliably re-read after storage of approximately 3 months, the common practice of quarterly re-reading of slides for EQA will need to be adapted.

With this background, we designed and conducted a study in Mumbai, India to evaluate the rate of fading of auramine stained smears. It was decided to perform the study in India because of the high rate of smear and culture positivity in the specimens they receive, thus allowing a larger number of specimens to be collected during the time frame of the study. Additionally, considering the potential for unforeseen and uncontrolled differences between laboratory practices in high and low incidence settings, conducting the study in India allowed us to better reflect the target setting where these EQA programs will need to be implemented.

Chapter 6 – Fading of Auramine-Stained Mycobacterial Smears and its Effect on LED Microscopy Implementation

6.1 INTRODUCTION

Fluorescent microscopy (FM) has become an important tool for the diagnosis of smear positive tuberculosis (TB). The use of FM has increased the sensitivity of sputum smear examination by 10% compared to Ziehl-Neelsen (ZN) staining and examination under light microscopy, and takes up to 75% less time to read smears (17). FM using auramine O stained smears has now become standard practice in developed countries with access to traditional mercury vapour fluorescent microscopes and the introduction of low-cost, robust LED fluorescent microscopes will facilitate expansion of FM into low resource settings (24, 27). With infrequent access to culture facilities, the introduction of FM into high burden countries could have a significant impact on TB case detection rates and the increased reading efficiency of smear reading will provide important time savings in many over-burdened laboratories. With these goals in mind, the Strategic and Technical Advisory Group for Tuberculosis (STAG-TB) of the World Health Organization (WHO) recently recommended that fluorescent LED microscopy be phased in to replace ZN microscopy in TB laboratories (49).

As part of the global expansion of FM for TB diagnostics, current systems for external quality assurance (EQA) of smear microscopy may need to be adapted. In the ongoing effort to improve TB diagnosis, laboratory quality assurance and quality improvement programs are a vital component to expanding diagnostic services. Saving a sample of ZN stained smears for blinded re-reading by a centralized reference laboratory has become a mainstay of many EQA programs (29, 55). The introduction of FM, and the replacement of ZN with auramine staining, creates a potential problem with the current method of re-reading

stained slides for quality control monitoring by controllers that do not routinely re-stain slides before reading.

Fluorochrome-based stains are subject to fading over time (30). This is a function of the fluorochrome molecule itself which has a limited number of photons available for emission during its chemical lifespan. The rate of fluorochrome fading (also called photobleaching) is unique to the particular fluorochrome used and is affected by multiple factors including exposure to light, oxygen, temperature and pH.

There is a wide spectrum of opinion regarding the rate of fading and its effect on the readability of fluorochrome-stained smears. Some experts report anecdotally that stained slides kept away from light and moisture can be reliably re-read after many months. However, with the proposed wide-scale expansion of FM to global TB laboratories there is concern that sensitivity of detection will decrease over the time of storage, especially in paucibacillary smears, leading to the inappropriate appearance of high rates of false positive fluorescent smear results being reported from peripheral laboratories.

The conditions under which slides are stored are likely to affect the rate of fading. When looking at the fading of ZN stained smears, Van Deun et al found that exposure to light, heat and humidity contributed to more rapid fading (56). They also found that processed (or concentrated) smears faded more quickly than direct smears, and hypothesized that this was likely because they were thinner and more watery in consistency. Since exposure of fluorescent stains to light is an important factor contributing to their fading, there is also the concern that in a research situation where smears are re-read multiple times, this re-reading and brief exposure to light may itself contribute to the fading of smears.

Our objectives were to measure the effect of storing auramine-stained slides at different temperatures and environments, and the effect of multiple re-readings on the concordance of smear readings over time compared with those

performed originally on the day of staining. We hypothesized that slides being stored at room temperature in a darkened environment would fade significantly (<50% originally positive remaining positive) within 3 months. Further, we expected to see relatively more fading in slides stored exposed to light or stored in a humidified incubator, and relatively less fading in slides stored in a refrigerator. We expected little to no difference between the fading of slides read weekly, monthly or quarterly and little to no difference between the fading of concentrated and direct smears.

6.2 METHODS

Setting

The study took place at the Hinduja National Hospital and Medical Research Centre clinical microbiology laboratory in Mumbai, India. The study was conducted in parallel with an evaluation of LED microscopy for mycobacterial smear diagnosis (Shenai et al, unpublished). The laboratory is certified by the College of American Pathologists and takes part in routine quality assurance procedures, which were unaffected by this protocol.

Slide Selection

All specimens submitted for routine mycobacterial smear (ZN) and culture had an extra slide prepared and stained with auramine O – rhodamine. These smears were examined by two independent microscopists using a Lumin LED objective lens attachment (LW Scientific, USA) as part of an LED microscopy evaluation project. A subset of these slides was then selected by an un-blinded researcher for storage and inclusion in this study. Thirty auramine-stained slides per week were selected to achieve a sample enriched with scanty and 1+ smears, and approximately 10% negatives. The approximate distribution of slides was to be 10% negatives, 25% scanty, 25% 1+, 25% 2+, and 15% 3+. We aimed for

approximately half of the slides selected to be direct smears and half to be concentrated smears. Only smears whose initial reading was identical between the two microscopists were included.

Slide Storage Environments

Weekly batches of 30 slides were allocated to one of four storage environments: Room Temperature (closed smear boxes; RT), Humidified Incubator (closed smear boxes; INC), Refrigerator (closed smear boxes; FRG), and Room Temperature (open smear boxes; RT-open). These storage environments were selected to reflect the range of conditions likely to be encountered or available in countries implementing blinded rechecking EQA programs.

Slides stored at RT were kept at approximately 22 degrees C (temperature controlled) and humidity varied depending on local conditions (monthly averages during study period 61 – 79% relative humidity). Slides stored in an INC were kept at 30 degrees C and humidity was increased using open water inside the incubator. Slides stored in a FRG were kept at 4 degrees C. Slides stored at RT, INC or FRG were all kept in sealed smear boxes to ensure a dark environment and were only exposed to light (or temperature changes) during re-reading. Slides stored at RT-open were kept in the same environment as RT, except the smear boxes were left open to both natural and artificial light in the laboratory.

Slide Re-Reading Schedules

Batches of slides were allocated to be re-read on either a weekly basis, a monthly basis, or once only at 3 months. Twice as many slides were allocated to monthly readings because this was our pre-specified primary outcome measure. All slides were re-read by both original microscopists during routine daily work and recorded as negative, scant, 1+, 2+, 3+ according to the scale recommended by the WHO (57). Between readings the slides were relabelled to conceal their previous ID numbers and maintain blinding over time. Blinding, re-assortment

and re-storage was performed by an un-blinded researcher not involved in smear reading. Original slide readings performed on the day that slides were stained are considered the reference standard and subsequent re-readings are compared to these Day 0 readings.

Auramine Staining

Smears were fixed with heat and staining was performed on the day of the original slide reading. Smears were flooded with auramine O (HiMedia K021-Kit) for 15 minutes, then rinsed with sterile water; decolourized with acid-alcohol for 2 minutes, then rinsed with sterile water; counterstained with potassium permanganate for 3-4 minutes, then rinsed with sterile water and allowed to air dry.

Sample Size Calculation

We used a one-sided hypothesis of equivalence, where the known proportion of positive slides at each reading was 100% and non-equivalence was defined as a drop in the proportion detected of more than 10%. In order to achieve 80% power with a significance level of 0.05, the number of slides per group needed to be 25. We included 27 positive slides in each batch (plus 3 negative slides for the purposes of blinding).

Analysis

Data were collected and analyzed using MS Excel, Stata/IC 11.0 and WinPepi 9.9 software. Smear examination results were dichotomized and smears read as scant, 1+, 2+ or 3+ were considered positive. Using only slides which were read as positive on the day of staining, the proportion which continued to be read as positive was calculated for each re-reading. Tests of significance use 2-tailed Fisher's exact tests (paired 2-tailed Fisher's exact tests were used when comparing the same slides read at different times). Subgroup analysis was

performed according to storage environment, reading schedule and specimens processing (direct vs. concentrated smears).

6.3 RESULTS

A total of 330 slides were included in this study (Table 12). There were 180 read at monthly intervals, 120 read at weekly intervals and 30 read once at 3 months. Of the slides being read monthly, there were equal numbers allocated to the 3 dark storage environments (90 slides each at RT, INC, FRG). Of the slides being read weekly, there was equal allocation to the 3 dark storage environments as well as to storage with exposure to light at room temperature (30 slides each at RT, INC, FRG, RT-open). Additionally, 30 slides were kept in a dark environment at room temperature and read once at 3 months. Overall, 54% of the included slides were direct smears and 46% were concentrated. The only groups which deviated noticeably from the desired 50:50 division were those read weekly and stored at either INC (67% direct, p=0.295) or FRG (63% direct, p=0.435). Table 13 shows the distribution of original slide readings on the day of staining for smears included in the study.

The distribution of slide readings over time for each of the different storage environments, read weekly and monthly, is shown in Figures 7 and 8. In all of the different storage environments there was a rapid increase in the proportion of slides being read as negative. Weekly readings were more variable; however, they exhibited the same trend towards an increasing proportion of negative smears.

When considering the slides originally read as positive (combining scant, 1+, 2+, 3+), the proportion of slides continuing to be read as positive was significantly lower for all storage environments by the first re-reading, regardless of reading schedule (based on paired 2-tailed Fisher's exact tests, 0.05 level of significance).

In other words, even slides read 1 week after staining had faded significantly: out of 27 originally positive slides kept at room temperature and read at 1 week, only 21 remained positive (p=0.03). The proportion of originally positive slides read as positive dropped to less than 50% by 2 months (or 8 weeks) for all storage environments and both monthly and weekly reading schedules (Figure 9). Slides which were exposed to light faded the most rapidly with only 24% of originally positive slides remaining positive by 4 weeks and none remaining positive by 10 weeks. Contrary to expectations, storage in a cold environment (FRG) did not seem to prevent or delay fading. In fact, positive slides stored at 4 degrees C faded to negative quicker than positive slides stored at 30 degrees C in a humidity-enriched environment. Slides stored at temperature controlled (22 degrees C) room temperature in a dark environment remained positive the longest, regardless of reading schedule.

When slides stored at RT and read at different intervals (weekly, monthly, quarterly) were compared there were no statistically significant differences in the overall rate of fading (Table 14), suggesting that the process of reading slides and exposing them to light intermittently did not have a large effect on their rate of fading compared to the duration of storage itself. We did not find any statistically significant difference between the overall proportion of faded slides between those originating from direct specimens compared to those originating from concentrated specimens (statistical testing performed at all times for smears read monthly and at 4, 8, 12, 16 and 20 weeks for smears read weekly).

Slides which were originally read as having higher positivity (2+ or 3+) tended to maintain their positive status for a longer time compared to slides originally read as having lower positivity (scant or 1+) (Table 15). Nevertheless, even when only slides originally read as 3+ were considered, less than 35% of all included slides (combining monthly and weekly readings and all storage environments) remained positive at 3 months.

6.4 DISCUSSION

The introduction of FM to a wide range of low resource settings is likely to require a number of adaptations from TB laboratories. Issues that still need to be resolved include the duration and type of training of technologists unfamiliar with FM, the most efficient screening magnification to be used, and the best stains and counterstains to be used. The need to adapt current EQA practices will depend on the rate of fading of the fluorescent stain used and whether that fading adversely affects the ability to detect AFB in low positive smears.

In this study, we used a sample enriched with low positives in order to target those most at risk of being missed during re-reading. The technologists performed the re-readings as part of routine work, not aware that they were potentially 'faded slides', and thus the duration of smear examination may not reflect the attention given by reference laboratories performing EQA. Nevertheless, we show quite clearly that even high positive slides stained with auramine fade quite quickly, making the current program of saving slides to be sent intermittently to reference centers for blinded rechecking infeasible.

Fading of ZN stained smears has been evaluated by Van Deun et al who found that exposure to direct sunlight, high temperatures and high humidity increased their rate of fading (56, 58). We also found that auramine stained slides exposed to light faded more rapidly than those stored in dark, sealed smear boxes. Given the mechanism of light production from fluorochrome molecules, this is not surprising (30). However, we also found that storage in a cold environment did not prevent or delay fading and indeed slides kept at 4 degrees C appeared to fade more quickly than those at 22 degrees C or even in a humidified incubator at 30 degrees C. The explanation for this is not entirely clear. It may be the case that condensation accumulated on the cold smears after removal from the refrigerator, and this direct contact with moisture accelerated fluorescent fading

even more than constant increased environmental humidity. It is also possible that the fluorochrome stain itself is less efficient at light production at a lower temperature, although the smears were allowed to warm to room temperature before being examined.

We used different reading schedules (weekly, monthly, and quarterly) for this evaluation because it was uncertain whether the process of re-reading the slides itself would contribute to smear fading. For slides kept in darkened environments, this would be the only exposure to light they would have and depending on how sensitive the stains were to this brief light exposure it was unclear whether more frequent readings would hasten fading. Comparing slides kept at 22 degrees C, those read weekly did appear to fade slightly more than those read monthly and slides read only once after 3 months experienced the least fading (Table 14), although these differences were not statistically significant. The amount of fading seen even with slides read only once at 3 months, shows that the duration of storage was a far more important factor in stain fading compared to the brief light exposure associated with the readings themselves.

The simplest solution for TB programs switching from light microscopy to FM is to implement routine re-staining of slides undergoing EQA rechecking before the controller's examination. Indeed it has been advocated as preferable even for ZN EQA programs due to the (less significant) fading of ZN stained slides, especially in warm, humid environments (29, 58). Many programs have not adopted the practice of re-staining slides before re-reading, however, citing the increased costs and resources required for already strained centralized laboratories. Additionally, there is the potential risk of environmental contamination of stored slides (either during storage and transport or during the re-staining process itself) with acid fast organisms that upon re-staining could lead to false positive readings on the part of the controllers (56). It is also possible that paucibacillary smears could experience loss of small numbers of AFB during one of the several

rinsing steps of re-staining, leading to occasional false negative readings on the part of the controllers (56).

Further research is warranted into potential staining or storage strategies that could extend the useful life of fluorescent stained smears, although our results suggest that significant potentiation would be needed to make rechecking without re-staining feasible. The re-staining of smears for EQA rechecking will need to be carefully implemented to minimize contamination with environmental mycobacteria as well as loss of fixed bacilli. Finally, these issues may warrant the development and investigation of alternative quality assurance initiatives for TB smear microscopy services.

Study Limitations

As discussed above, this study may be criticized for including a high proportion of scanty slides and for performing the rechecking in a way that blinded the microscopists from knowing they were reading 'faded' slides. Both of these factors would contribute to a less realistic setting and could have led to an exaggeration of the fading effect. Additionally, while blinded rechecking reflects the actual practice of many EQA programs, it is inherently somewhat subjective in that the readings performed by microscopists will vary depending on the fields examined and the care taken to quantify AFB even without a fading effect. However, while this is likely to add noise and variability to the readings over time, it should not affect the overall trend.

Conclusions

Auramine-stained smears fade quickly during storage, making subsequent re-checking of results without routine re-staining unreliable. TB laboratories planning to implement FM for smear diagnosis need to ensure appropriate EQA procedures are in place before moving forward with the provision of FM

services. Recommendations for both EQA and internal quality assurance processes are currently being developed by the WHO to accompany their recommendations to implement LED fluorescent microscopy (49).

Chapter 7 – Discussion and Conclusions

In our systematic review of LED microscopy, we were able to identify numerous evaluations, mostly unpublished, of LED devices. While the estimates of diagnostic accuracy were heterogeneous, overall assessments were unanimously positive both when comparing the LED microscopes to traditional light microscopes and when comparing them to conventional fluorescent microscopes. Head to head evaluations of ZN microscopy to LED microscopy showed improved sensitivity and reading efficiency, while head to evaluations of CFM to LED microscopy showed equivalence in both of these important performance outcomes. Importantly, repeated reports from technologists in different countries and settings confirmed many of the practical, user-friendly advantages promised by LED microscopy including the ability to view fluorescent smears without requiring a dark room.

While our review and research focused on the use of LED microscopy for the diagnosis of TB, there are likely to be additional benefits to laboratories performing services other than TB diagnostics. Similar to CFM, LED fluorescent microscopy can be used for the detection of malaria, parasites, other bacteria, as well as environmental monitoring, food safety inspections, and immunofluorescent microscopy (24, 30, 59-60).

From the information we acquired in our extensive literature review performed as part of the systematic review and meta-analysis, we identified 2 areas of research towards which we aimed to contribute.

In our first study, conducted in Montreal, Canada, we evaluated the performance of 2 of the more popular LED devices and compared them to our current standard of practice, a conventional fluorescent microscope. The goals of this study were twofold: to validate the performance of LED microscopy in a Canadian setting and to compare the two LED devices for suitability in a Canadian laboratory. There are several important differences between low

resource laboratories (where the majority of previous LED evaluations were conducted) and high resource laboratories, such as those in Canada. With respect to FM, Canadian laboratory technologists are very familiar with this technology and have significant experience using CFM. Not only does this obviate the need for significant training for use of LED microscopes, it sets high expectations of device quality and reading efficiency. While equivalent diagnostic accuracy to CFM is necessary for Canadian laboratories to consider adopting LED microscopy, this would not be sufficient for user acceptance.

Another important difference between our Canadian setting and those with a high incidence of TB is the spectrum of specimens our laboratories receive. The proportion of positive specimens that we receive is far lower than that seen in other areas, which results in proportionately more time screening negative smears. Additionally, those smears which are positive are often very low positive or paucibacillary. There are multiple factors that contribute to this situation. Many specimens are received from symptom-free individuals undergoing screening for the purposes of immigration, active case finding through contact investigation, or follow up of incidental chest x-ray findings. Due to broader access to healthcare, patients generally have less severe disease when they present to physicians for investigation. Our population also tends to have a higher proportion of extrapulmonary TB which is commonly smear negative and significant numbers of non-tuberculous mycobacterial infections which are also commonly smear negative. Therefore in the Canadian setting, while sensitivity and reading efficiency remain important, the factors upon which they are determined may vary and device performance cannot be assumed to be equivalent to that in high incidence settings.

In our LED evaluation, we found no difference in diagnostic accuracy between the Zeiss Primo Star iLED, LW Scientific Lumin and conventional fluorescent microscope (Leica DMSL). This was regardless of whether we used sensitivity, specificity or kappa measurements to compare the 3 devices. However, this

study may have been underpowered to detect statistically significant differences because the actual rate of smear positive specimens was slightly lower than predicted when the required sample size was calculated.

It should be noted that the diagnostic sensitivity we found in our study for all 3 FM modalities was much lower than the pooled estimates of sensitivity found in the systematic review and meta-analysis. This illustrates the discussion above describing how our setting is unique from the majority which contributed to evaluations included in the review and underscores the importance of setting diversity when evaluating a new diagnostic. The fact that we have a much higher proportion of smear negative, culture positive specimens will lead to all types of microscopic TB diagnostics appearing to underperform when compared to culture. This is one reason why, despite the inherent incorporation bias introduced by assuming 100% specificity, some people advocate for the use of a microscopic reference standard that assumes any smear positive detected is correct. While these analyses are not ideal, they tend to be more consistent between settings. This is true of our LED evaluation where sensitivity estimates using this “any positive” reference standard are comparable to pooled estimates from our systematic review and meta-analysis for microscopic reference standards.

These differences arising from setting diversity also demonstrate an important source of heterogeneity in the meta-analysis. Since no two laboratory settings are exactly the same, it would be unrealistic to assume that a diagnostic evaluated in one setting will perform identically in another. Considerations such as patient population, HIV prevalence, disease severity, proportion of non-tuberculous mycobacteria and type of specimens received all decrease the external validity of any TB diagnostic evaluation. Even when equipment, supplies and laboratory procedures are carefully standardized, these types of variables which are generally uncontrolled in diagnostic evaluations will often demonstrate the inaccuracy of the commonly held belief that measures of

sensitivity and specificity are fixed values associated with a test (compared to PPV and NPV which obviously depend on disease prevalence) (61-62). This makes a strong argument for the need to perform lab-specific validation studies before introducing new tests for patient management decisions.

In our LED evaluation, we found that while the Lumin LED device was equally efficient to the conventional fluorescent microscope, the Zeiss Primo Star iLED was more efficient than both. Subjective reports from our technologists suggest that the Zeiss was generally the easiest of the 3 to read with and provided the most convenient focusing and brightest viewing fields when screening slides. The spectrum of light produced by LED devices is narrower than that provided by mercury vapour conventional fluorescent microscopes and its wavelength is produced to match specifically the peak absorbance of auramine stains (24). This is the likely explanation for the increased brightness produced by LED microscopes and why they can be used without a darkroom. However, the Lumin attachment did not demonstrate the same superior reading efficiency as the Zeiss. Our technologists reported that not only was the Lumin more difficult to focus, the resulting fluorescence of the auramine stained bacilli was very dim and they stated they would not recommend its use without a dark room. Another practical characteristic of the Lumin (and other similar objective lens attachments) is the fact that the light source needs to be plugged in directly to the objective lens being used. Not only does this occasionally create a small obstruction while the technologist is working, but it also makes it inconvenient to switch between different objective lenses and thus different viewing magnifications. While the Lumin was reviewed favourably by most users identified in our systematic review, many of its benefits (including low upfront cost and portability) are not as important in high resource settings. Thus we would not recommend its implementation in Canadian laboratories.

In the final study, we focused on a very pragmatic problem identified as a potential hurdle for the successful implementation of LED microscopy in low

resource settings. While it is well accepted that fluorescent stains fade over time, it was debated whether this would significantly affect TB programs' ability to use blinded re-reading of smears as a mechanism for EQA. In our study conducted in Mumbai, India we found marked fading of stored smears over even short periods of time. While we expected to find some fading, the extent of the unreliability of readings after even 1 week was surprising. This was especially unexpected when compared to a simultaneous unpublished evaluation conducted by FIND which found minimal detrimental effects of storage on technologists' ability to accurately re-read smears after several months (43). Differences between the two studies may have included the adequacy of blinding on the part of the FIND study, where their technologists knew that they were reading stored slides and thus may have looked more closely for faded or morphologically abnormal bacilli. The technologists performing our re-readings examined the stored slides in concert with daily work, which in a busy Indian laboratory may have led to less thorough readings. We do not know what commercial brand of auramine stain was used in the FIND evaluations; however, it may have been different than that used in our study. If this is the case it will be interesting to see whether this effect is replicated in future evaluations, as it would have important implications to recommendations to labs newly introducing LED microscopy.

We purposely enriched our sample of stored slides with scant and low positive smears. This was done in an effort to detect small amounts of fading since these paucibacillary slides would be the first to show noticeable effects. In hind sight this may have allowed for a more prominent role of random chance in the re-reading of smears as the technologists could have missed those very occasional bacilli simply by not reading the same fields as the first reading.

The effect of storage conditions on slide fading was also unexpected. Slides stored in a refrigerated environment faded surprisingly fast and this was seen in both the group of slides read weekly and those read monthly. While we are not aware of previous studies looking specifically at the effects of refrigeration on

fluorochrome stain fading, previous observations that high heat increased the rate of ZN stain fading led to the hypothesis that cooler conditions may prevent or delay it (56). It is possible that any protective effect of the cool temperature was more than counteracted by the frequent changes in temperature which the slides would have experienced during the repeated re-readings. If this was the case, however, we would expect to see significantly more fading in the slides read weekly compared to those read monthly. While there appeared to be a small effect of re-reading on slide fading, this was not large enough to account for the observed effect of storage conditions.

While the FIND study did not systematically study the effect of storage conditions on fading, they repeated their protocol at multiple sites with different weather conditions. Most of the sites included were warm or hot (26 – 38 degrees C) without the availability of air conditioning or temperature controls. Only one site was judged to be humid (Vietnam, with ambient temperatures around 32 degrees C) and this did not appear to affect the rate of slide fading. We attempted to recreate the atmosphere of many low resource laboratories by including a group of slides stored in a humidified incubator at 30 degrees C. While these slides faded faster than those kept at air conditioned room temperature (22 degrees C), the effect was less than that of storage duration.

Given the recently released recommendation from the WHO to implement LED microscopy in TB laboratories, plans for corresponding EQA programs are needed urgently. One suggestion is that global TB laboratories switching to FM adopt EQA procedures similar to some high resource countries, where instead of routine smear rechecking they often use panel testing. This involves sending standardized panels of pre-made smears to participating laboratories to have them read and then send back their results. This effectively tests the competence of the technologists and would identify gross deficiencies in skill. In low burden countries, where a technologist from a small lab may only rarely see a positive TB smear, the ability to recognize AFB morphology and differentiate it

from artefacts is indeed a concern. This is much less of a problem for technologists in high burden settings though, where the greater concern is discordance between skill capability and daily performance. These panel tests are not treated as part of routine work and are commonly read by multiple senior technologists for extended times before consensus results are sent back (29). This does not give an accurate reflection of daily performance in the laboratory where due to labour constraints and high workloads it is common for capable and skilled technologists to cut corners (13).

If EQA programs using blinded smear rechecking procedures are to be continued, in light of the concerns regarding fluorescent smear fading, there will need to be modifications to current procedures. Re-staining smears before they are re-read is one option which is supported by the TB Control Assistance Program (TB CAP)(63). Critics of this approach site the increased work and expense that this involves for the central rechecking sites, as well as the unknown error introduced by the re-staining process itself. There is a risk of washing off small numbers of bacilli during restaining, which could lead to missing originally paucibacillary smears, and there is a risk of staining contaminant mycobacteria accumulated during the storage and transportation process. The second concern can be somewhat alleviated by cleaning the stored smears with xylene before restaining and taking care to use only sterile water in the staining process. The increased risk of missing scant positive slides may be unavoidable, but compared to the same risk posed when rereading slides without restaining it is likely less significant.

There are other areas of investigation which may lead to potentiation of fluorescence. It is clear that keeping slides away from light is imperative, and avoidance of humidity and temperature extremes also seems to be important. Are there components of the staining process itself which contribute to the impermanence of fluorescence production? Using different concentrations of auramine or different concentrations of phenol in the stain itself may influence

the strength of the original fluorescence and allow for longer storage. Are there alternative acid fast fluorochrome stains available which may allow longer fluorescence production? Acridine orange is a commonly used microbiologic stain that has been demonstrated as feasible for use for mycobacterial FM (64) and numerous different counterstains are available which may help or hinder the readability of fluorescent smears over time (30).

Given its current advantages, smear microscopy itself is likely to continue to inspire research to further optimize its performance. The same Expert Group Meeting of the World Health Organization held in the fall of 2009 which evaluated LED microscopy, also examined 2 other strategies for optimizing smear microscopy.

The first looked at the schedule of specimen collection for TB diagnosis. Currently it is recommended that patients suspected of pulmonary TB submit 2 sputum specimens over 2 days (1 spot sputum specimen and 1 morning sputum specimen) (14, 65). The 'Same-day Diagnosis' strategy (also called 'Front-loading') uses 2 specimens submitted on the same day (2 spot specimens). This has the potential of decreasing the turnaround time of smear microscopy results and allowing patients to complete their diagnostic work-up within a single clinic visit. This could lead to fewer patients defaulting during the TB diagnostic process, however, it risks decreasing the diagnostic yield since spot sputum specimens are generally less sensitive than morning sputum specimens (66-68). Evidence presented for this strategy was positive and final decisions regarding policy recommendations are awaited.

The final strategy for optimizing smear microscopy for TB diagnosis examined by the World Health Organization in the fall of 2009 involved specimen processing. Specimen processing for smear microscopy is a controversial topic for global laboratories (15-16). Most developed country laboratories perform some form of specimen processing including sputum liquefaction and concentration using

centrifugation. A previous systematic review of these procedures has shown this to improve sensitivity at a small cost to specificity (15). These procedures slow down the turnaround time of smear microscopy results though, and most pose increased biosafety concerns. These concerns have prevented the WHO from recommending specimen processing for smear microscopy, in light of the admittedly mild improvements in sensitivity.

Novel technologies are increasingly being applied to the improvement of smear microscopy. The use of automated image analysis, similar to face recognition software, has been developed to detect AFB in digital images of stained mycobacterial smears (69-71). The automation of the smear reading process has the potential to alleviate significant human resource deficiencies in global TB laboratories. Another area identified which could benefit from automated smear microscopy would be blinded rechecking EQA programs. These programs currently demand significant resources from centralized reference centres performing the re-readings, and even if the automated detection were not equivalent in sensitivity to technologists' readings it could be used to detect a threshold of correlation below which more intensive attention would be needed. Using this technology could also alleviate concerns detailed above regarding fluorochrome stain fading since digital images could be taken by the peripheral laboratory on the day of staining without any storage and delay in reading. While promising, this technology has not yet been validated in prospective diagnostic studies. A practical limitation to its development into a usable diagnostic tool is the digitalization of smears: currently available devices able to automatically digitalize slides are prohibitively expensive and manual digitalization would essentially obviate the potential benefits of an automated slide reading technology.

Another new technology developed by researchers in California, USA involves using a camera-equipped mobile phone for LED microscopy (72). This involves fitting an external lens system onto a compatible camera and using an LED light

source to stimulate auramine stained AFB. Images can then be viewed either on the phone's screen or on a connecting computer. Alternatively, digital images could be analyzed by automated slide reading algorithms described above or sent wirelessly to a distant laboratory for examination by a trained technologist. The image capture mechanism of this technology would not digitalize an entire smear, however, so screening and focusing on AFB-like objects would be the responsibility of the operator. Clinicians on mobile clinics or caring for remote communities without the benefit of a trained TB technologist are one identifiable use for this type of technology.

CONCLUSIONS

TB diagnosis continues to be one of the important hurdles in the control of TB globally. Smear microscopy is a simple, low cost diagnostic that provides the backbone of TB diagnostic services in most high incidence areas. Fluorescent microscopy has long been known to improve the performance of smear microscopy services by increasing sensitivity and reading efficiency. Despite this, practical aspects of fluorescent microscopes and their use have prevented their widespread use. LED microscopy aims to overcome these feasibility issues and expand access to the benefits of FM.

Through a systematic review of the literature and meta-analysis of LED diagnostic accuracy, we found that LED microscopy provides improved sensitivity and reading efficiency over commonly used light microscopy and ZN staining. Additionally, LED microscopy appears to perform equivalently to conventional FM with respect to diagnostic accuracy and reading efficiency, making it a possible alternative to the mercury vapour conventional fluorescent microscopes used in high resource countries as well. While there is evidence to support many of the practical advantages promised by LED microscopy, we identified several implementation issues that need to be addressed.

In a head-to-head diagnostic evaluation in Montreal, Canada we evaluated the diagnostic accuracy and reading efficiency of two competing LED microscopes and compared them to a conventional fluorescent microscope. We found no difference in the diagnostic accuracy of any of the microscopes studied; however, there was significantly improved reading efficiency when using the Zeiss Primo Star iLED. Combined with favourable user reviews and the ability for this device to be used without the use of a darkroom, we would recommend the use of this device over either a conventional fluorescent microscope or the competing Lumin LED attachment in similar Canadian laboratories.

One of the important implementation issues identified by the systematic review was the effect of fluorescent stain fading on current EQA programs. In a study conducted in Mumbai, India we looked at the rate of auramine stain fading under different storage conditions and using different reading schedules. We found rapid fading of slides, regardless of storage conditions or reading schedules, and conclude that the current system of storage and rereading is not feasible for fluorescent microscopy without modifications. Further research is needed to develop and evaluate alternative EQA programs, but based on our findings we would not recommend rereading stored auramine stained slides without restaining them at this time.

In conclusion, LED microscopy is likely to provide a welcome opportunity to optimize and improve TB diagnostic services globally. While not a revolutionary technology, the implementation of this simple, low-cost equipment is an excellent example of how thoughtful research and design can make a positive difference in the control of one of the world's most important infectious diseases.

REFERENCES

1. World Health Organization. Global Tuberculosis Control 2009: Epidemiology, Strategy, Financing. Geneva, Switzerland: World Health Organization. http://www.who.int/tb/publications/global_report/2009/pdf/full_report.pdf.
2. Diagnostic standards and classification of tuberculosis in adults and children: official statement of the American Thoracic Society and the Centers for Disease Control and Prevention. Am J Respir Crit Care Med. 2000;161(4):1376.
3. Toman's tuberculosis: case detection, treatment, and monitoring - questions and answers. Frieden T, editor. Geneva: World Health Organization; 2004.
4. Canadian tuberculosis standards. 6 ed. Long R, Ellis E, editors: Tuberculosis Prevention and Control, Public Health Agency of Canada & Canadian Lung Association/Canadian Thoracic Society; 2007.
5. TB/HIV: a clinical manual. Geneva: Stop TB Department, Department of HIV/AIDS & Department of Child and Adolescent Health and Development, World Health Organization 2004.
6. Improving the diagnosis and treatment of smear-negative pulmonary and extrapulmonary tuberculosis among adults and adolescents: recommendations for HIV-prevalent and resource constrained settings: Stop TB Department & Department of HIV/AIDS, World Health Organization2007.
7. Laboratory biosafety manual. Geneva: World Health Organization 2004 http://www.who.int/bulletin/volumes/85/5/06-039081/en/index.html.
8. Millen SJ, Uys PW, Hargrove J, van Helden PD, Williams BG. The effect of diagnostic delays on the drop-out rate and the total delay to diagnosis of tuberculosis. PLoS ONE. 2008;3(4):e1933.
9. Sherman LF, Fujiwara PI, Cook SV, Bazerman LB, Frieden TR. Patient and health care system delays in the diagnosis and treatment of tuberculosis. Int J Tuberc Lung Dis. 1999 Dec;3(12):1088-95.
10. Pai M, Minion J, Sohn H, Zwerling A, Perkins MD. Novel and improved technologies for tuberculosis diagnosis: progress and challenges. Clin Chest Med. 2009 Dec;30(4):701-16, viii.
11. Aber VR, Allen BW, Mitchison DA, Ayuma P, Edwards EA, Keyes AB. Quality control in tuberculosis bacteriology. 1. Laboratory studies on isolated positive cultures and the efficiency of direct smear examination. Tubercle. 1980 Sep;61(3):123-33.
12. Urbanczik R. Present position of microscopy and of culture in diagnostic mycobacteriology. Zentralbl Bakteriol Mikrobiol Hyg A. 1985 Aug;260(1):81-7.
13. Cambanis A, Ramsay A, Wirkom V, Tata E, Cuevas LE. Investing time in microscopy: an opportunity to optimise smear-based case detection of tuberculosis. Int J Tuberc Lung Dis. 2007 Jan;11(1):40-5.
14. Mase SR, Ramsay A, Ng V, Henry M, Hopewell PC, Cunningham J, et al. Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. Int J Tuberc Lung Dis. 2007;11(5):485-95.
15. Steingart KR, Ng V, Henry M, Hopewell PC, Ramsay A, Cunningham J, et al. Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006;6(10):664-74.
16. Steingart KR, Ramsay A, Pai M. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis. Expert review of anti-infective therapy. 2007 Jun;5(3):327-31.

17. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 2006 Sep;6(9):570-81.
18. Bennedsen J, Larsen SO. Examination for tubercle bacili by fluorescence microscopy. Scand J Respir Dis. 1966;47(2):114-20.
19. Kivihya-Ndugga LE, van Cleeff MR, Githui WA, Nganga LW, Kibuga DK, Odhiambo JA, et al. A comprehensive comparison of Ziehl-Neelsen and fluorescence microscopy for the diagnosis of tuberculosis in a resource-poor urban setting. Int J Tuberc Lung Dis. 2003 Dec;7(12):1163-71.
20. Sohn H, Sinthuwattanawibool C, Rienthong S, Varma JK. Fluorescence microscopy is less expensive than Ziehl-Neelsen microscopy in Thailand. Int J Tuberc Lung Dis. 2009 Feb;13(2):266-8.
21. Anthony RM, Kolk AH, Kuijper S, Klatser PR. Light emitting diodes for auramine O fluorescence microscopic screening of Mycobacterium tuberculosis. Int J Tuberc Lung Dis. 2006 Sep;10(9):1060-2.
22. Rieder H, Van Deun A, Kam K, Kim S, Chonde T, Trebecq A, et al. Priorities for tuberculosis bacteriology services in low-income countries. 2nd ed. Paris, France: International Union Against Tuberculosis and Lung Disease; 2007.
23. Van Deun A, Chonde TM, Gumusboga M, Rienthong S. Performance and acceptability of the FluoLED Easy module for tuberculosis fluorescence microscopy. Int J Tuberc Lung Dis. 2008 Sep;12(9):1009-14.
24. Hanscheid T. The future looks bright: low-cost fluorescent microscopes for detection of Mycobacterium tuberculosis and Coccidiae. Trans R Soc Trop Med Hyg. 2008 Jun;102(6):520-1.
25. Van Hung N, Sy DN, Anthony RM, Cobelens FG, van Soolingen D. Fluorescence microscopy for tuberculosis diagnosis. Lancet Infectious Diseases. [Letter]. 2007 Apr;7(4):238-9.
26. Martin G, Agostini HT, Hansen LL. Light emitting diode microscope illumination for green fluorescent protein or fluorescein isothiocyanate epifluorescence. Biotechniques. 2005 Feb;38(2):204, 6.
27. Minion J, Sohn H, Pai M. Light-emitting diode technologies for TB diagnosis: what is on the market? Expert Rev Med Devices. 2009 Jul;6(4):341-5.
28. Pai M, Minion J, Steingart K, Ramsay A. New and improved tuberculosis diagnostics: evidence, policy, practice, and impact. Curr Opin Pulm Med. Mar 10.
29. Van Deun A, Portaels F. Limitations and requirements for quality control of sputum smear microscopy for acid-fast bacilli. Int J Tuberc Lung Dis. 1998 Sep;2(9):756-65.
30. Sanborn WR, Heuck CC, El Aouad R, Storch WB. Fluorescence microscopy for disease diagnosis and environmental monitoring. Cairo: World Health Organization, Regional Office for the Eastern Mediterranean; 2005.
31. Winn W, Allen S, Janda W, Koneman E, Schreckenberger P, Woods G. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th ed. Baltimore, MD, USA: Lippincott Williams & Wilkins; 2006.
32. Pai M, McCulloch M, Enanoria W, Colford JM, Jr. Systematic reviews of diagnostic test evaluations: What's behind the scenes? ACP J Club. [Instructive]. 2004 Jul-Aug;141(1):A11-3.
33. Whiting P, Rutjes A, Reitsma J, Bossuyt P, Kleijnen J. The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in

systematic reviews. BMC Medical Research Methodology. [diagnostic methods]. 2003;3(1):25.
34. Zamora J, Abraira V, Muriel A, Khan K, Coomarasamy A. Meta-DiSc: a software for meta-analysis of test accuracy data. BMC Med Res Methodol. 2006;6:31.
35. Littenberg B, Moses LE. Estimating diagnostic accuracy from multiple conflicting reports: a new meta-analytic method. Med Decis Making. 1993 Oct-Dec;13(4):313-21.
36. Reitsma JB, Glas AS, Rutjes AW, Scholten RJ, Bossuyt PM, Zwinderman AH. Bivariate analysis of sensitivity and specificity produces informative summary measures in diagnostic reviews. J Clin Epidemiol. 2005 Oct;58(10):982-90.
37. Harbord R, Whiting P. metandi: Meta-analysis of diagnostic accuracy using hierarchical logistic regression. In: Sterne J, editor. Meta-Analysis in Stata: An Unpdated Collection from the Stata Journal. College Station, Texas, USA: Stata Press; 2009. p. 181 - 99.
38. Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ. 2003 Sep 6;327(7414):557-60.
39. Marais BJ, Brittle W, Painczyk K, Hesseling AC, Beyers N, Wasserman E, et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. Clin Infect Dis. 2008 Jul 15;47(2):203-7.
40. Trusov A, Bumgarner R, Valijev R, Chestnova R, Talevski S, Vragoterova C, et al. Comparison of Lumin LED fluorescent attachment, fluorescent microscopy and Ziehl-Neelsen for AFB diagnosis. Int J Tuberc Lung Dis. 2009 Jul;13(7):836-41.
41. Affolabi D, Torrea G, Odoun M, Senou N, Ali Ligali M, Anagonou S, et al. Comparison of two LED fluorescence microscopy build-on modules for acid-fast smear microscopy.unpublished.
42. Cuevas LE, Yassin MA, Lawson L, Al-Sonboli N, Arbide I, Al-Aghbari N, et al. A multi-country randomized controlled trial of LED-based fluorescent microscopy for the diagnosis of pulmonary tuberculosis in the context of frontlaoded and standard specimen collection.unpublished.
43. Foundation for Innovative New Diagnostics (FIND). Primo Star iLED evaluation and demonstration: use of reflected light LED-based fluorescent microscopy for detection of tuberculosis in low and medium income settings.Report for WHO, unpublished.
44. Kuhn W, Armstrong D, Atteberry S, Dewbrey E, Smith D, Hooper N. Testing the ParaLens fluorescent microscope adaptor in field and laboratory setings.unpublished.
45. Omar SV, Germishuizen WA, Mudau FC, Muthivhi T, Said HM, Osman AG, et al. The Lumin fluorescent microscopy attachment for low-cost diagnosis of TB using an ordinary light-microscope.unpublished.
46. Shenai S, Minion J, Vadwai V, Tipnis T, Pai M, Rodrigues C. Evaluation of LED microscopy for detection of Mycobacterium tuberculosis in a high incidence setting.unpublished.
47. Ramsay A, Munthali S, Mundy C, Nathanson C-M, Chirambo P, Dacombe R, et al. New policies, new technologies: modeling the potential for improved smear microscopy services in Malawi.unpublished.
48. Minion J, Shenai S, Vadwai V, Tipnis T, Rodrigues C, Pai M. Fading of auramine-stained slides of Mycobacterium tuberculosis in different storage environments.unpublished.

49. World Health Organization (WHO). Strategic and technical advisory group for tuberculosis: report of the ninth meeting. Geneva, Switzerland. 2009 November 9-11. http://www.who.int/tb/advisory_bodies/stag_tb_report_2009.pdf.
50. Rivest P, Tannenbaum T, Bedard L. Epidemiology of tuberculosis in Montreal. CMAJ. 1998 Mar 10;158(5):605-9.
51. World Health Organization. The use of liquid medium for culture and DST. 2007; Available from: http://www.who.int/tb/dots/laboratory/policy/en/index3.html.
52. Isenberg HD. Clinical Microbiology Procedures Handbook. Washington, D.C.: American Society for Microbiology; 2004.
53. Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics. 1977 Mar;33(1):159-74.
54. Aziz M, Ryszewska K, Laszlo A, Blanc L. Strategic approach for the strengthening of laboratory services for tuberculosis control, 2006-2009. Geneva: World Health Organization 2006.
http://whqlibdoc.who.int/hq/2006/WHO_HTM_TB_2006.364_eng.pdf.
55. Gilpin C, Kim SJ, Lumb R, Rieder HL, Van Deun A. Critical appraisal of current recommendations and practices for tuberculosis sputum smear microscopy. Int J Tuberc Lung Dis. 2007 Sep;11(9):946-52.
56. Van Deun A, Roorda FA, Chambugonj N, Hye A, Hossain A. Reproducibility of sputum smear examination for acid-fast bacilli: practical problems met during cross-checking. Int J Tuberc Lung Dis. 1999 Sep;3(9):823-9.
57. De Kantor IN, Kim SJ, Frieden T, Laszlo A, Luelmo F, Norval PY, et al. Laboratory Services in Tuberculosis Control. Geneva, Switzerland: Global Tuberculosis Program, World Health Organization. 1998.
58. Van Deun A, Chambugonj N, Hye A, Hossain A. Rapid fading of carbolfuchsin stained AFB smears under extreme conditions of temperature and humidity. Int J Tuberc Lung Dis. 1997 Aug;1(4):384-5.
59. Hanscheid T, Valadas E. Diagnosis of cryptosporidiosis using PCR or auramine O with LED fluorescent microscopy: which end of the stick? Acta Trop. 2009 Mar;109(3):247-8; author reply 9.
60. Hanscheid T, Cristino JM, Salgado MJ. Screening of auramine-stained smears of all fecal samples is a rapid and inexpensive way to increase the detection of coccidial infections. Int J Infect Dis. 2008 Jan;12(1):47-50.
61. Whiting P, Rutjes AW, Reitsma JB, Glas AS, Bossuyt PM, Kleijnen J. Sources of variation and bias in studies of diagnostic accuracy: a systematic review. Ann Intern Med. 2004 Feb 3;140(3):189-202.
62. Moons KG, van Es GA, Deckers JW, Habbema JD, Grobbee DE. Limitations of sensitivity, specificity, likelihood ratio, and bayes' theorem in assessing diagnostic probabilities: a clinical example. Epidemiology. 1997 Jan;8(1):12-7.
63. Tuberculosis Coalition for Technical Assistance. Lab Tools - Microscopy & EQA Part 2. Netherlands: KNCV Tuberculosis FoundationTB CAP (2005 - 2010) http://www.tbcta.org/Library/#186.
64. Smithwick RW, Bigbie MR, Jr., Ferguson RB, Karlix MA, Wallis CK. Phenolic acridine orange fluorescent stain for mycobacteria. J Clin Microbiol. 1995 Oct;33(10):2763-4.
65. World Health Organization (WHO). Reduction of number of smears for the diagnosis of pulmonary TB. [April 11, 2010]; Available from: http://www.who.int/tb/dots/laboratory/policy/en/index2.html.

66. Van Deun A, Salim AH, Cooreman E, Hossain MA, Rema A, Chambugonj N, et al. Optimal tuberculosis case detection by direct sputum smear microscopy: how much better is more? Int J Tuberc Lung Dis. 2002 Mar;6(3):222-30.
67. Schoch OD, Rieder P, Tueller C, Altpeter E, Zellweger JP, Rieder HL, et al. Diagnostic yield of sputum, induced sputum, and bronchoscopy after radiologic tuberculosis screening. Am J Respir Crit Care Med. 2007 Jan 1;175(1):80-6.
68. Gopi PG, Subramani R, Selvakumar N, Santha T, Eusuff SI, Narayanan PR. Smear examination of two specimens for diagnosis of pulmonary tuberculosis in Tiruvallur District, south India. Int J Tuberc Lung Dis. 2004 Jul;8(7):824-8.
69. Sadaphal P, Rao J, Comstock GW, Beg MF. Image processing techniques for identifying Mycobacterium tuberculosis in Ziehl-Neelsen stains. Int J Tuberc Lung Dis. 2008 May;12(5):579-82.
70. Veropoulos K, Learmonth G, Campbell C, Knight B, Simpson J. Automated identification of tubercle bacilli in sputum. A preliminary investigation. Anal Quant Cytol Histol. 1999 Aug;21(4):277-82.
71. Classification of Mycobacterium Tuberculosis in Images of ZN-Stained Sputum Smears. IEEE Trans Inf Technol Biomed. 2009 Sep 1.
72. Breslauer DN, Maamari RN, Switz NA, Lam WA, Fletcher DA. Mobile phone based clinical microscopy for global health applications. PLoS ONE. 2009;4(7):e6320.

Figure 1. Systematic Review: Study Selection

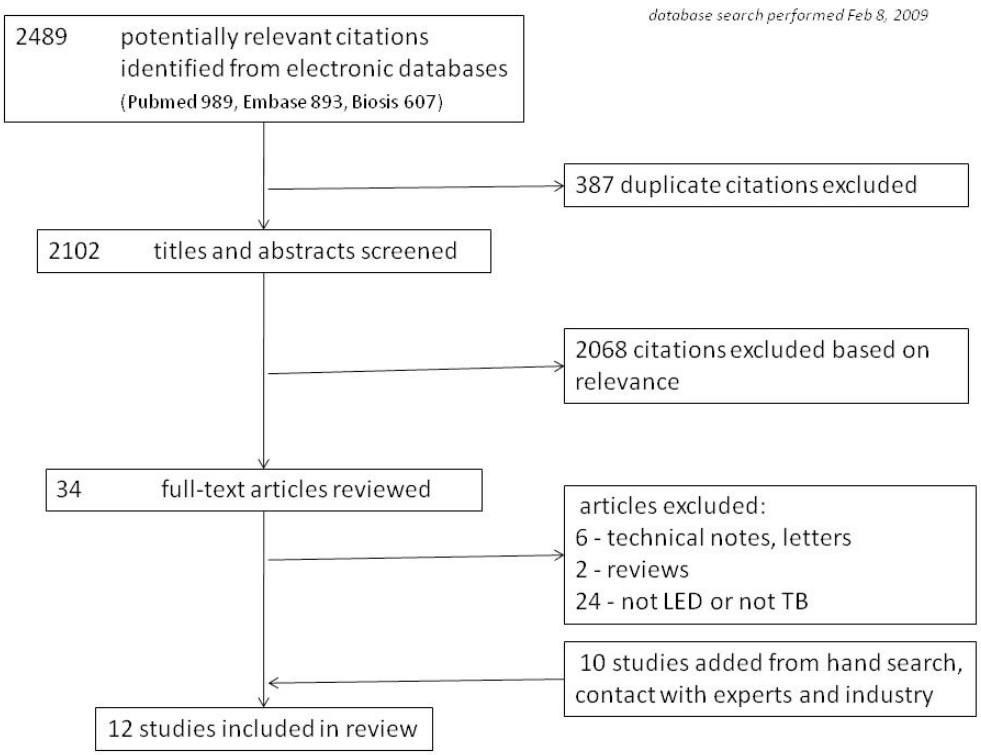
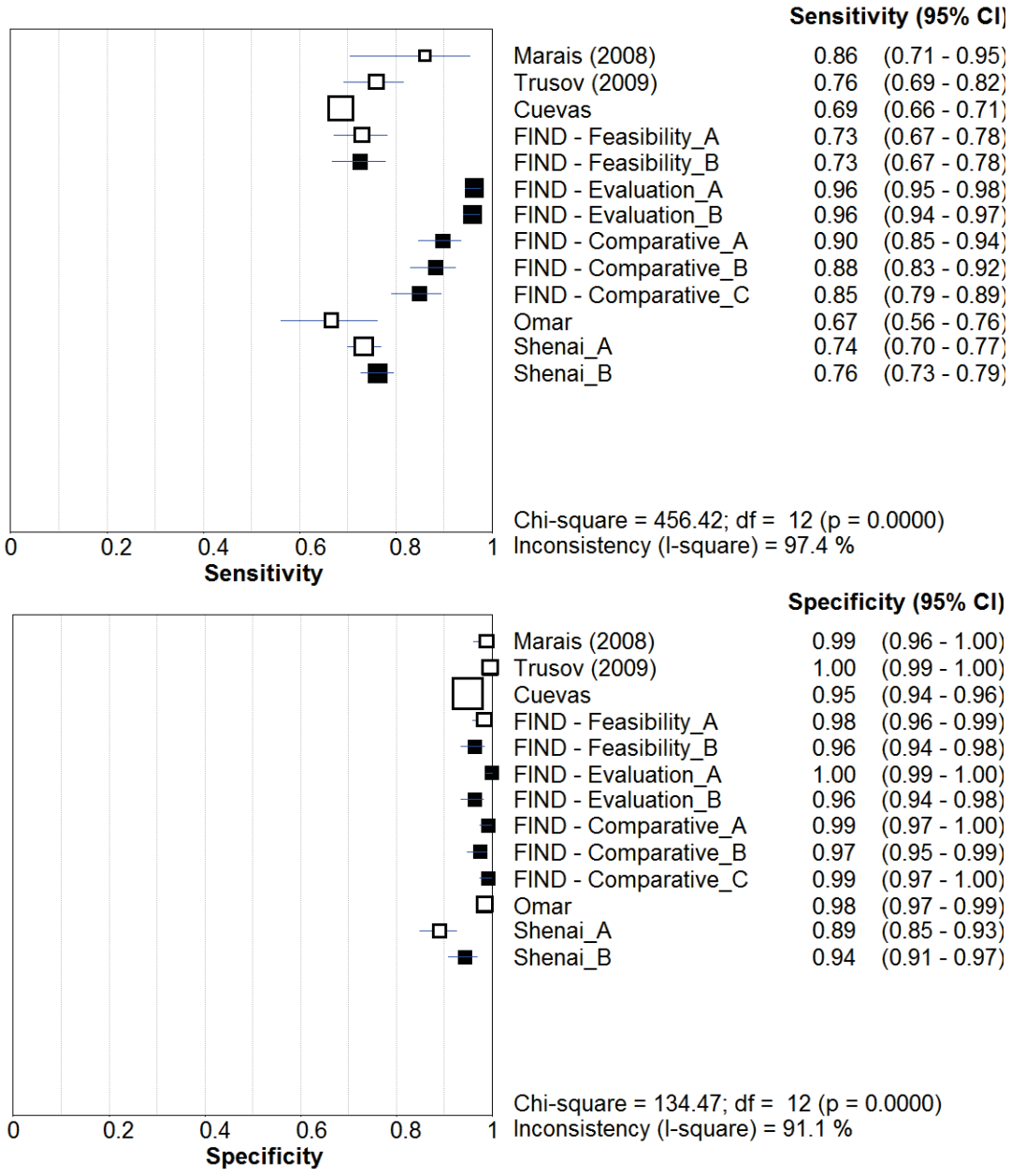
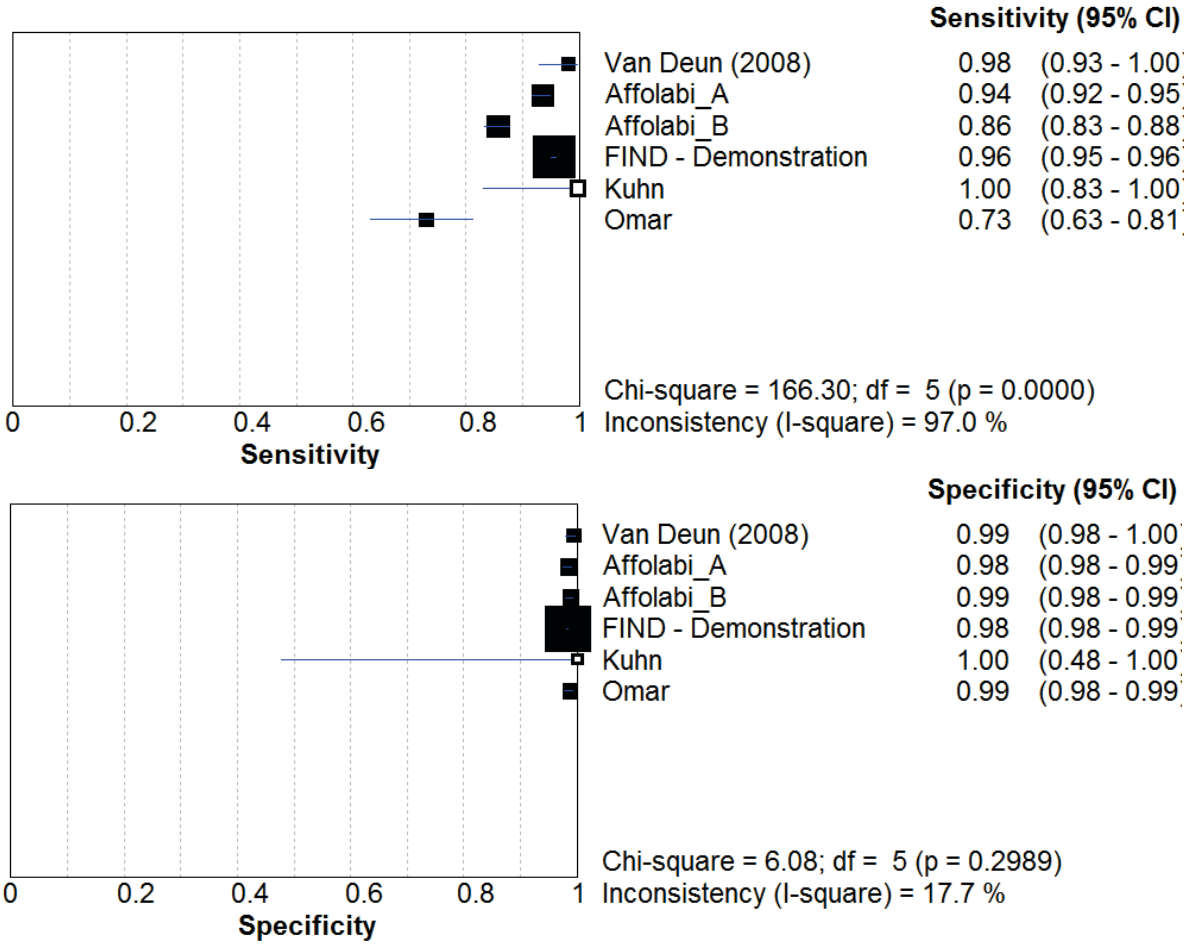


Figure 2. Systematic Review: Forest Plots of LED Studies Using Culture as a Reference Standard (n=13)



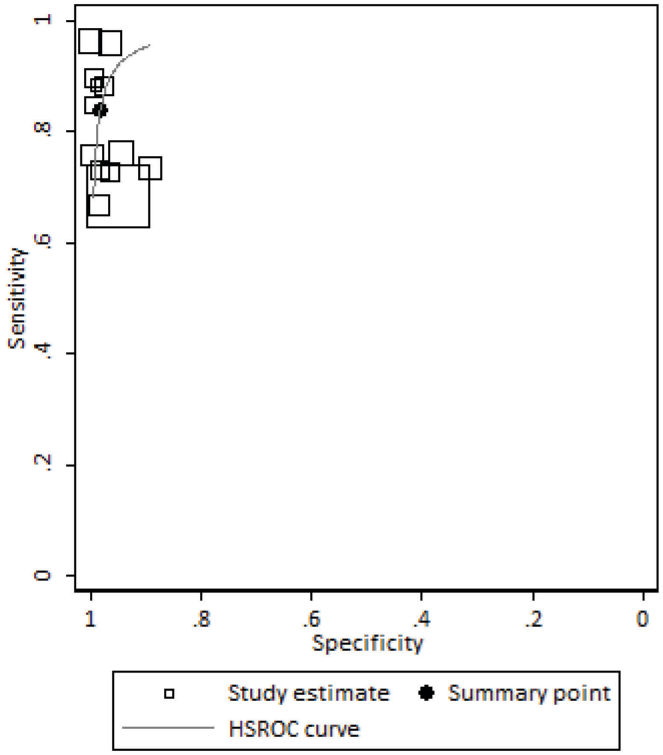
Published studies have year of publication in brackets; remaining studies are unpublished. Point estimates of sensitivity and specificity from each study are shown as solid squares (if direct smears were used) or open squares (if processed/concentrated smears were used). Size of the square is proportionate to the size of the study. Solid lines represent 95% confidence intervals.
FIND – Feasibility_A used concentrated smears; FIND – Feasibility_B used direct smears
FIND – Evaluation_A used 400x screening magnification; FIND – Evaluation_B used 200x screening magnification
FIND – Comparative_A used iLED; FIND – Comparative_B used FluoLED; FIND – Comparative_C used Lumin
Shenai_A used concentrated smears; Shenai_B used direct smears

Figure 3. Systematic Review: Forest Plots of LED Studies using Microscopy as a Reference Standard (n=6)



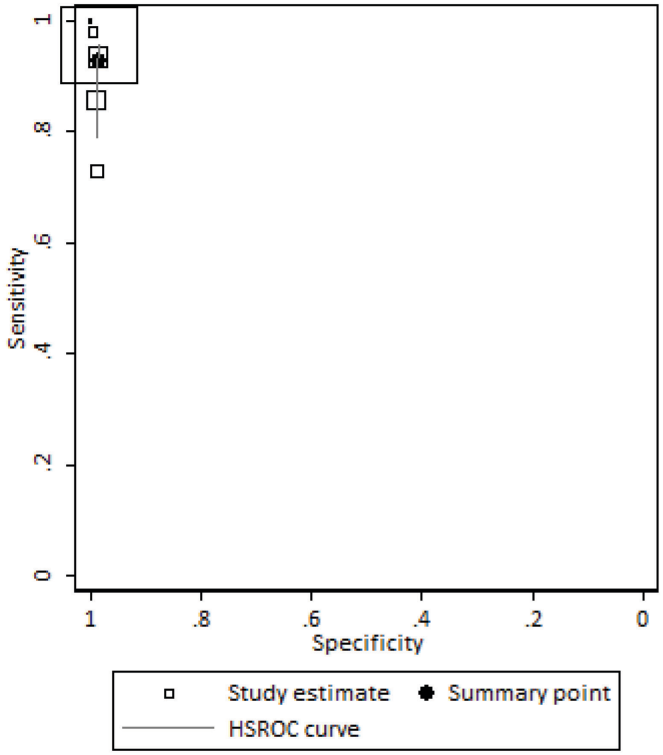
Published studies have year of publication in brackets; remaining studies are unpublished. Point estimates of sensitivity and specificity from each study are shown as solid squares (if direct smears were used) or open squares (if processed/concentrated smears were used). Size of the square is proportionate to the size of the study. Solid lines represent 95% confidence intervals. Affolabi_A used FluoLED; Affolabi_B used Lumin

Figure 4. Systematic Review: HSROC Plot for LED Studies Using Culture as a Reference Standard (n=13)



Individual studies are shown as open squares whose size is proportionate to the size of the study. Summary point is shown as a closed circle, representing sensitivity and specificity estimates pooled using bivariate random effects model. HSROC curve is truncated outside of the area for which data exist.

Figure 5. Systematic Review: HSROC Plot for LED Studies Using Microscopy as a Reference Standard (n=6)



Individual studies are shown as open squares whose size is proportionate to the size of the study. Summary point is shown as a closed circle, representing sensitivity and specificity estimates pooled using bivariate random effects model. HSROC curve is truncated outside of the area for which data exist.

Figure 6. LED Evaluation: Distribution of Positive Smear Readings

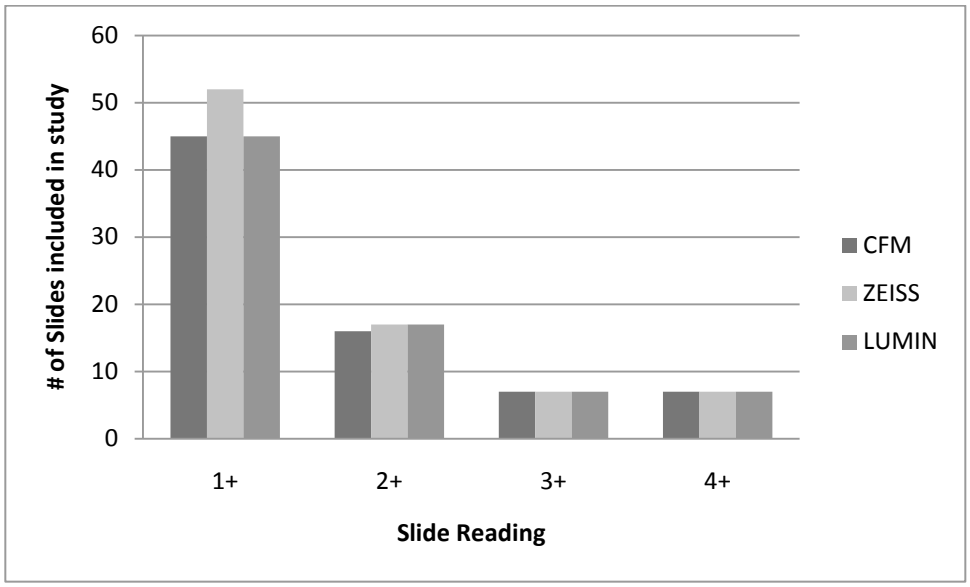
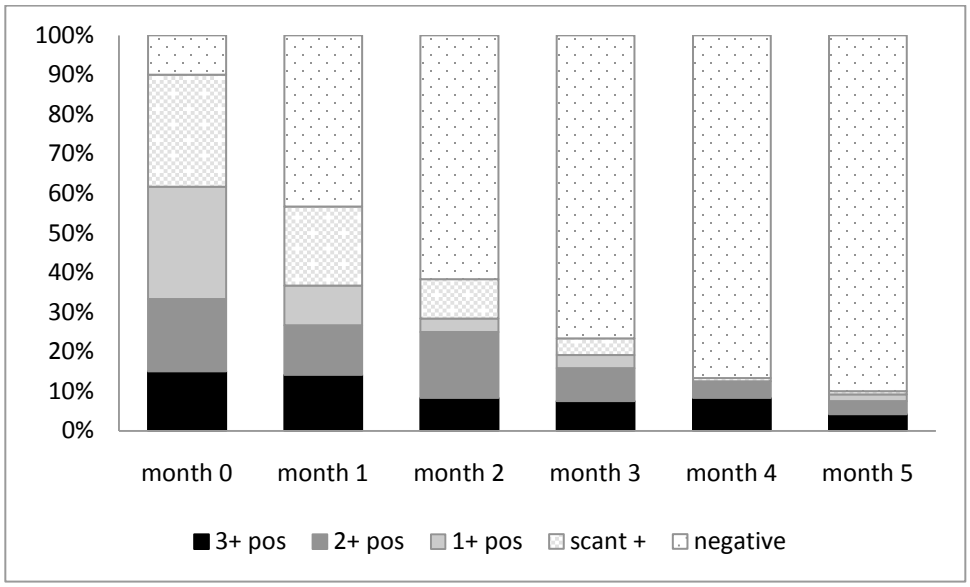
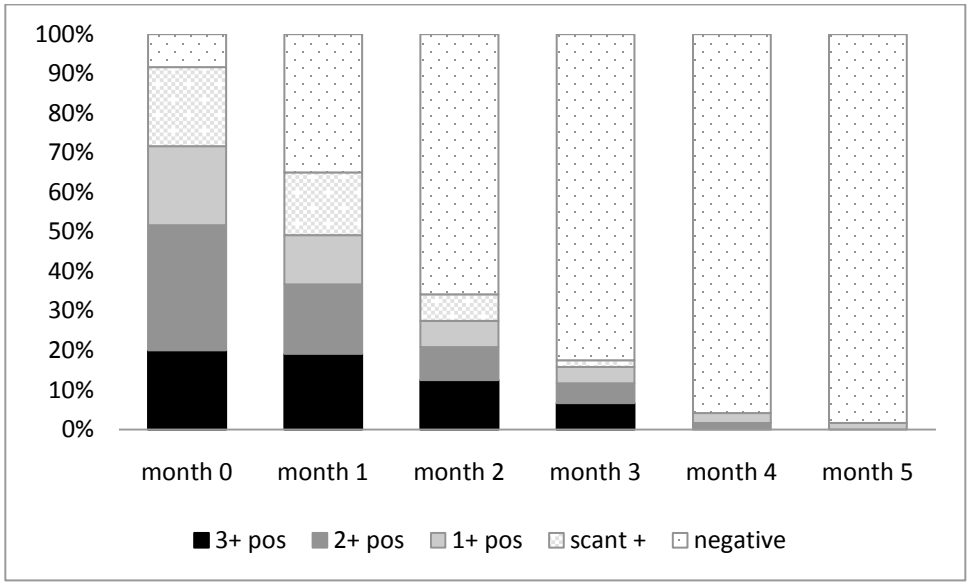


Figure 7. Fading Evaluation: Distribution of Slide Readings Evaluated Monthly

a) Room Temperature



b) Incubator



c) Refrigerator

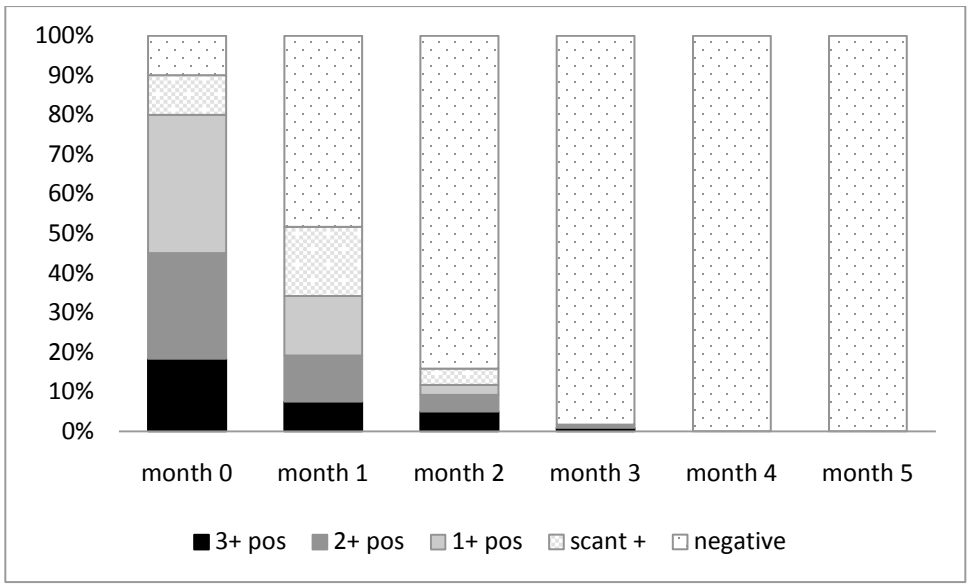
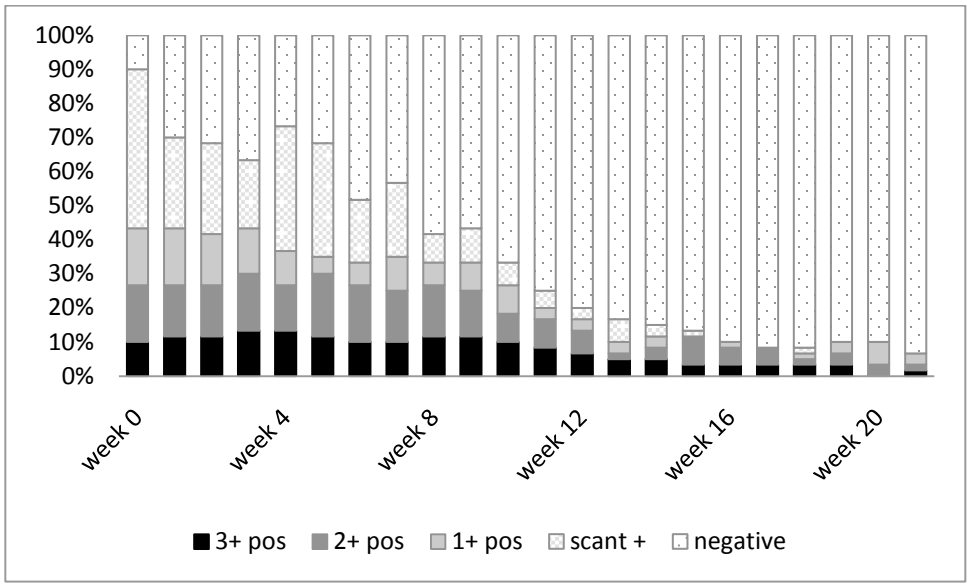
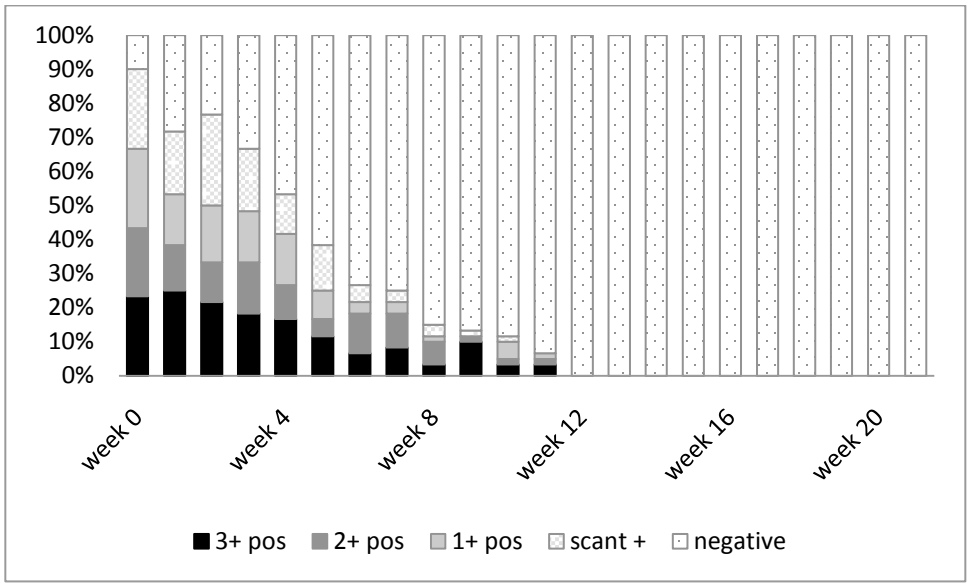

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
month 0
month 1
month 2
month 3
month 4
month 5
3+ pos
2+ pos
1+ pos
scant +
negative

Figure 8. Fading Evaluation: Distribution of Slide Readings Evaluated Weekly

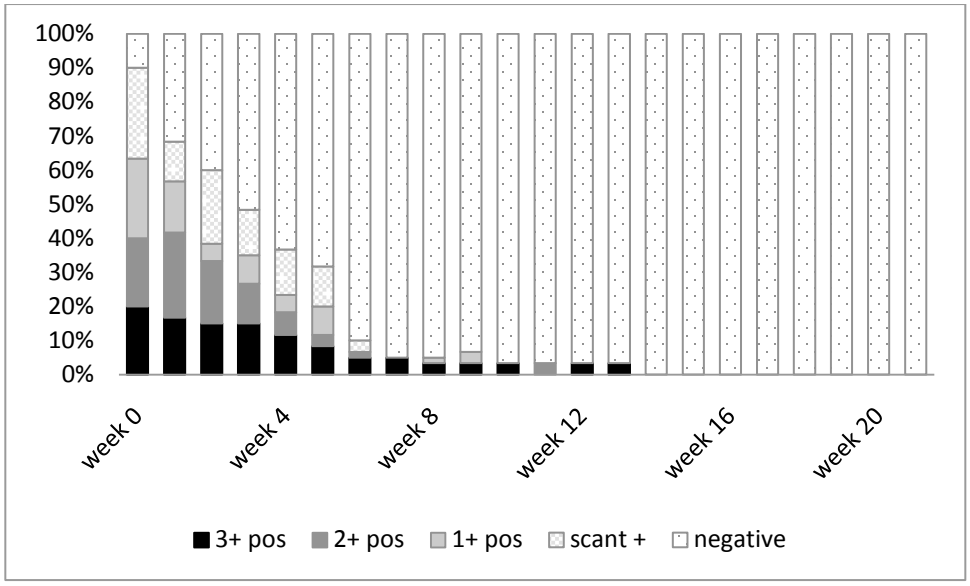
a) Room Temperature



b) Incubator



c) Refrigerator



d) Room Temperature – open to light

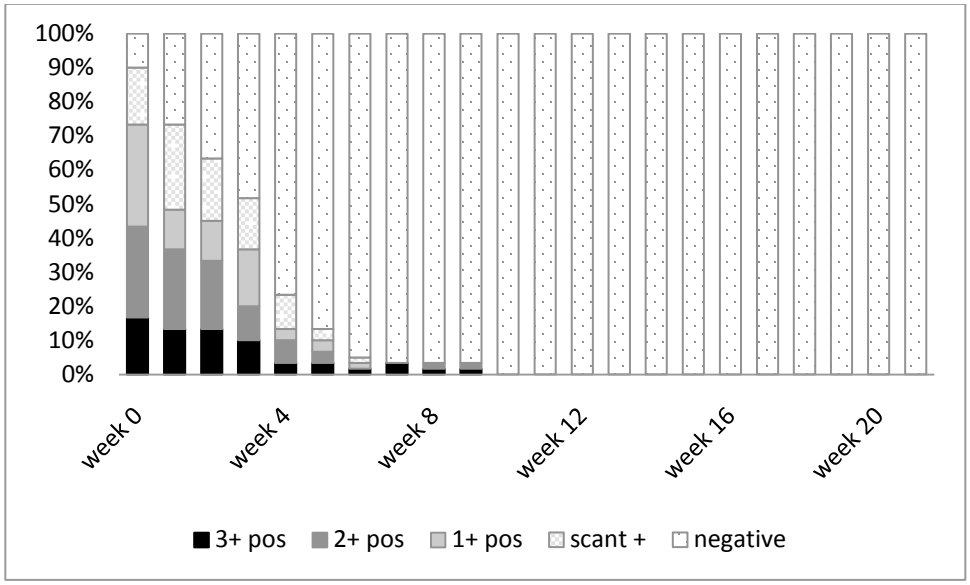
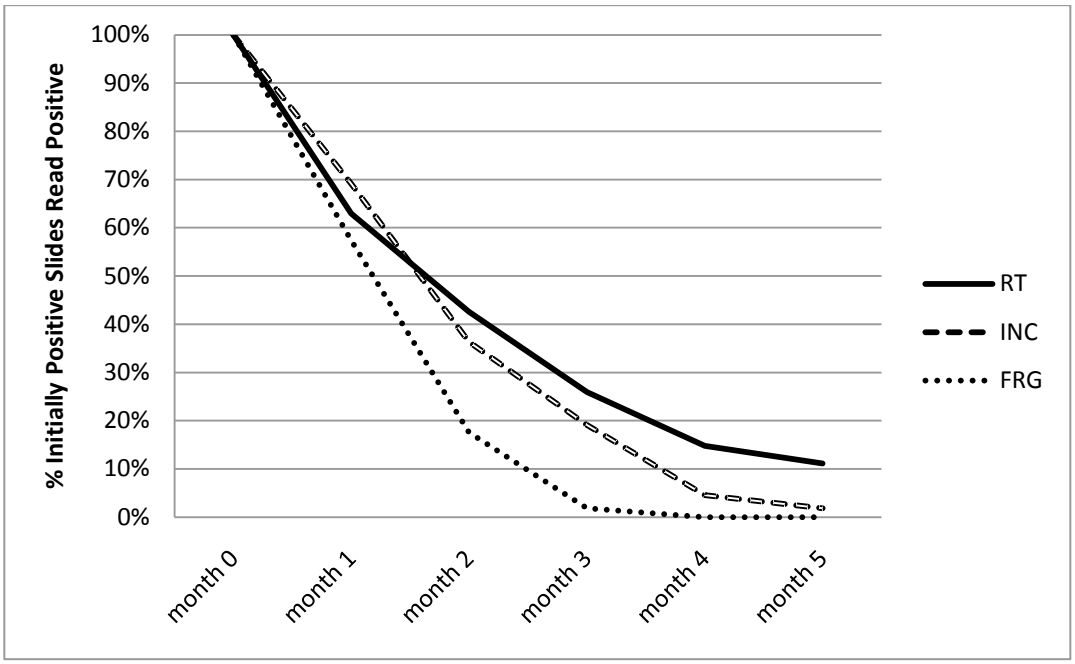


Figure 9. Fading Evaluation: Fading of Auramine-Stained Smears Examined Monthly

a) Examined Monthly



b) Examined Weekly

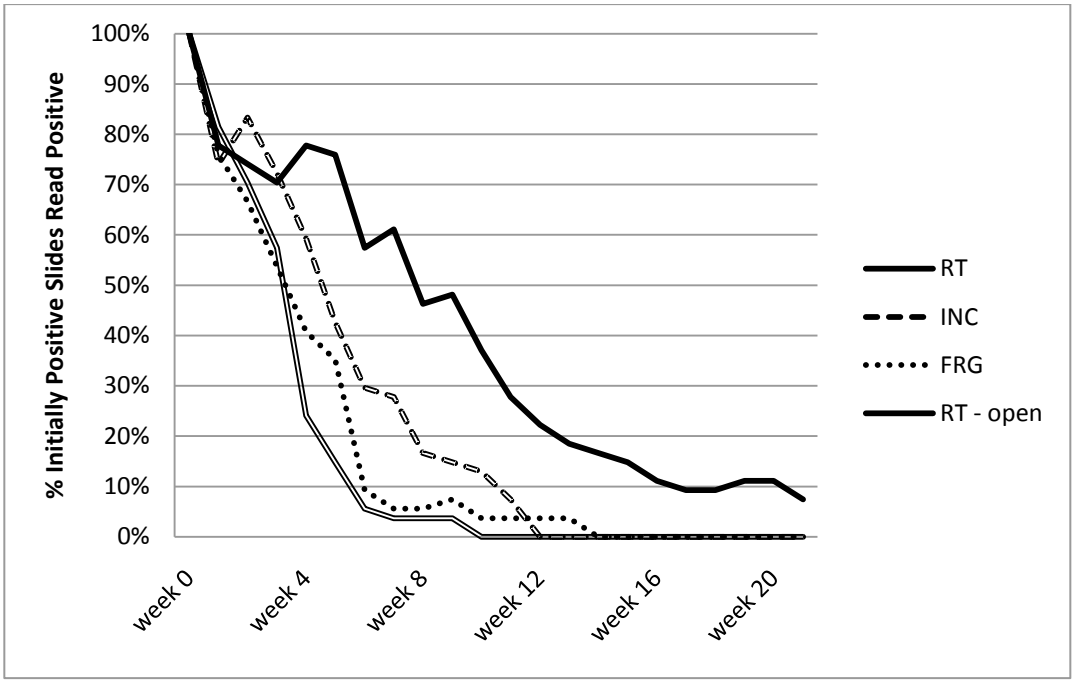


Table 1. Comparison of Commercial LED Products Currently Available for TB Diagnostics

Table 1. Comparison of commercial light-emitting diode products currently available for TB diagnostics.

Device	Manufacturer	Standalone microscope	Attachment	Light transmission	Battery powered	Weight (kg)	Cost (US$)	Ref.
Primo Star iLED™ 	Carl Zeiss, Oberkochen, Germany	Yes	NA	Epifluorescent	Yes	9.5	4825*	[101]
Lumin™ 	LW Scientific, Lawrenceville, GA, USA	No	Objective lens replacement (20, 40, 60 and 100× oil)	Epifluorescent	Yes	0.448	700–2000‡	[102]
ParaLens™ 	QBC™ Diagnostics, Philipsburg, PA, USA	No	Objective lens replacement (40, 60 and 100× oil)	Epifluorescent	Yes	1.27	995§	[103]
FluoLED™ 	Fraen Corporation Srl, Settimo Milanese, Italy	No	Adaptor attached to base and filter installed on head of microscope	Transfluorescent	Yes	5	1977–3530¶	[104]
CyScope® 	Partec, Gorlitz, Germany	Yes	NA	Epifluorescent	Yes	2.7	2372–3699#	[105]

Quotes in currencies other than US dollars were converted using rates published 11 June 2009.
*Special pricing available for high-burden countries: €1250.
‡Depending on options.
§When purchased in quantity.
¶Depending on model and quantity of order.
#Special pricing available for high-burden countries: US$1398.
NA: Not applicable.
Images have been reproduced with the permission of the respective companies.

From: Minion et al. 2009. *Expert Rev Med Devices* 6(4):341.

Table 2. Systematic Review: Study Characteristics (n=12)

Ref	Author	Year	Total N (Ref+/Ref-)	Country	LED Device	Reference	Comparison	Smear	Screening Magnification
(41)	Affolabi	unpublished	941/996	Benin	Lumin, FluoLED	rechecking	-	direct	200x
(42)	Cuevas	unpublished	1513/4999	Ethiopia, Nepal, Nigeria, Yemen*	Lumin	LJ	ZN	conc	200x
(43)	FIND – Feasibility	unpublished	263/282	Thailand, Germany, Peru, Gambia*	iLED	LJ	CFM	direct, conc	400x
(43)	FIND – Evaluation	unpublished	600/280	Thailand, Vietnam, India, Germany, Peru*	iLED	LJ	ZN, CFM	direct	200x, 400x
(43)	FIND – Demonstration†	unpublished	1317/8229	multiple‡*	iLED	rechecking	ZN	direct	400x
(43)	FIND – Comparative	unpublished	205/277	Zambia, Uganda*	iLED, FluoLED, Lumin	LJ	ZN, CFM	direct	400x
(44)	Kuhn	unpublished	20/5	USA/Bangladesh	ParaLens	CFM	-	conc	600x
(39)	Marais	2008	36/185	South Africa	Adapted CFM	MGIT, LJ	ZN, CFM	conc	400x
(45)	Omar	unpublished	93/616	South Africa	Lumin	MGIT	CFM	conc	400x
(46)	Shenai	unpublished	635/267	India	Lumin	MGIT, LJ	ZN	direct, conc	200x
(40)	Trusov	2009	199/508	Russia, Macedonia*	Lumin	LJ	ZN, CFM	conc	400x
(23)	Van Deun	2008	100/361	Tanzania/ Thailand	FluoLED	CFM	-	direct	200x

†available data from all phases pooled (validation, implementation, continuation)
‡study sites: India, Vietnam, Cambodia, Thailand, Peru, Russia, Lesotho, Ethiopia, South Africa
*study sites pooled
Direct = smear made directly from patient specimen
Conc = concentrated smear made after specimen liquefaction and centrifugation

Table 3. Systematic Review: Study Quality

Characteristic	Frequency (n = 12 studies)
Specimen Selection	
- Prospective	11
- Unclear	1
Study Design	
- Cross-Sectional	5
- Case-Control	7
Sampling	
- Consecutive or Random	8
- Unclear	4
Verification	
- Complete	9
- Partial	3
Blinded Interpretation	
- Yes	12

Table 4. Systematic Review: Pooled Estimates of LED Accuracy Using Bivariate Random Effects Models

Test (# arms)	Pooled Sensitivity (95% CI)	I^2 (p-value) ‡	Pooled Specificity (95% CI)	I^2 (p-value) ‡
Culture Reference (n=13)	83.6% (76.3, 89.0)	97.4% (p<0.0001)	98.2% (96.6, 99.0)	91.1% (p<0.0001)
Direct Smears only (n=7)	88.9%* (81.1, 93.7)	96.5% (p<0.0001)	98.3% (96.2, 99.3)	82.6% (p<0.0001)
Concentrated Smears only (n=6)	72.7%* (69.2, 76.0)	69.3% (p=0.006)	97.9% (94.8, 99.2)	93.9% (p<0.0001)
400x/600x Magnification (n=9)	84.1% (76.0, 89.8)	95.3% (p<0.0001)	99.0%* (98.0, 99.5)	67.4% (p=0.002)
200x Magnification (n=4)	82.1% (64.4, 92.1)	98.7% (p<0.0001)	94.4%* (91.5, 96.4)	80.3% (p=0.002)
Microscopy Reference (n=6)	92.7% (84.9, 96.7)	97.0% (p<0.0001)	98.5% (98.2, 98.8)	17.7% (p=0.30)
Direct Smears only (n=4)	93.6%* (88.8, 96.4)	97.5% (p<0.0001)	98.5% (98.1, 98.9)	42.4% (p=0.16)
Concentrated Smears only (n=2)†	78.0%* (69.0, 85.0)	91.2% (p=0.0008)	99.0% (98.0, 99.0)	0% (p=0.73)
400x/600x Magnification (n=3)†	95.0%* (95.0, 96.0)	96.6% (p<0.0001)	98.0% (98.0, 99.0)	0.0% (p=0.6)
200x Magnification (n=3)†	90.0%* (89.0, 91.0)	95.3% (p<0.0001)	99.0% (98.0, 99.0)	16.4% (p=0.3)

*non-overlapping confidence intervals by subgroup

†too few studies to perform bivariate random effects pooling; univariate random effects pooling performed
‡ I^2 describes the percentage of variation across studies that is due to heterogeneity rather than chance. P-value tests the null hypothesis that there is no heterogeneity between studies. Statistical significance indicates heterogeneity of results greater than that expected by random chance.

Table 5. Systematic Review: Head to Head Comparisons of LED with ZN (n=8)

Ref	Author	Smear	Reference	Sample Size	LED		ZN		LED – ZN	
					Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
(42)	Cuevas	conc	culture	6512	0.69	0.95	0.60	0.98	+9%	-3%
(43)	FIND – Evaluation†	direct	culture	880	0.96	1.00	0.91	1.00	+6%	0%
(43)	FIND – Demonstration‡	direct	microscopy	9546	0.93	0.99	0.78	0.99	+15%	0%
(43)	FIND – Comparison§	direct	culture	482	0.90	0.99	0.79	0.99	+11%	0%
(39)	Marais	conc	culture	221	0.85	0.99	0.61	0.99	+28%	0%
(46)	Shenai	conc	culture	903	0.74	0.89	0.74	0.96	1%	-7%
(46)	Shenai	direct	culture	904	0.76	0.94	0.85	0.93	-9%	+1%
(40)	Trusov	conc	culture	707	0.76	1.00	0.60	1.00	+16%	0%
	Pooled Differences Using Random Effects Models				**84.7% (73.6, 90.4)**	**98.8% (94.7, 99.7)**	**77.2% (67.6, 84.6)**	**98.8% (97.0, 99.5)**	**+6% (+0.1, +13)**	**-1% (-3, +1)**
	I^2 (p-value)*				97.7% (p<0.0001)	94.1% (p<0.0001)	98.7% (p<0.0001)	89.2% (p<0.0001)	90.8% (p<0.001)	96.0% (p<0.001)

†iLED used for LED data

‡subset of sites with head-to-head ZN comparison, not pooled with culture reference studies

§400x magnification used for LED data

* I^2 describes the percentage of variation across studies that is due to heterogeneity rather than chance. P-value tests the null hypothesis that there is no heterogeneity between studies. Statistical significance indicates heterogeneity of results greater than that expected by random chance.

Table 6. Systematic Review: Head to Head Comparisons of LED with CFM (n=7)

Ref	Author	Smear	Reference	Sample Size	LED		CFM		LED – CFM	
					Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
(43)	FIND - Feasibility	conc	culture	545	0.73	0.98	0.68	0.99	+5%	-1%
(43)	FIND – Feasibility	direct	culture	545	0.73	0.96	0.68	0.96	+5%	0%
(43)	FIND – Evaluation†	direct	culture	880	0.96	1.00	0.96	0.95	0%	+5%
(43)	FIND – Comparison‡	direct	culture	482	0.90	0.99	0.84	0.97	+6%	+2%
(39)	Marais	conc	culture	221	0.85	0.99	0.72	0.99	+13%	0%
(45)	Omar	conc	culture	709	0.67	0.98	0.71	0.99	-4%	-1%
(40)	Trusov	conc	culture	707	0.76	1.00	0.60	1.00	+16%	0%
	Pooled Estimates Using Random Effects Models*				**83.2% (72.3, 90.3)**	**99.2% (97.9, 99.7)**	**77.5% (64.2, 86.9)**	**98.5% (96.0, 99.4)**	**+5% (0, +11)**	**+1% (-0.7, +3)**
	I^2 (p-value)**				96.4% (p<0.0001)	71.7% (p=0.002)	97.3% (p<0.0001)	87.4% (p<0.0001)	73.5% (p=0.001)	85.2% (p<0.001)

†iLED used for LED data

‡400x magnification used for LED data

*Positive values indicate increased sensitivity of LED over CFM

** I^2 describes the percentage of variation across studies that is due to heterogeneity rather than chance. P-value tests the null hypothesis that there is no heterogeneity between studies. Statistical significance indicates heterogeneity of results greater than that expected by random chance.

Table 7. Systematic Review: Time to Read Slides (n=14)

Ref	Author	% Smear+	Smear	Screening Magnification	LED	Time/Slide (LED)	Comparison	Time/Slide (Comparison)	Relative Time (LED/Comparison)
(43)	FIND – Feasibility	100%	direct	400x	iLED	0.6 min	ZN	1.1 min	0.55
(43)	FIND – Feasibility	0%	direct	400x	iLED	1.4 min	ZN	3.2 min	0.44
(43)	FIND – Evaluation	100%	direct	400x	iLED	0.6 min	ZN	1.1 min	0.55
							CFM	0.6 min	1.00
(43)	FIND – Evaluation	0%	direct	400x	iLED	1.4 min	ZN	3.3 min	0.42
							CFM	1.2 min	1.17
(43)	FIND – Evaluation	100%	direct	200x	iLED	0.5 min	ZN	1.1 min	0.45
							CFM	0.6 min	0.83
(43)	FIND – Evaluation	0%	direct	200x	iLED	1.3 min	ZN	3.3 min	0.39
							CFM	1.2 min	1.08
(43)	FIND – Demonstration†	50%	direct	400x	iLED	1.62 min	ZN	2.02 min	0.80
(43)	FIND – Demonstration‡	50%	direct	400x	iLED	1.18 min	ZN	2.13 min	0.55
(43)	FIND – Comparison	50%	direct	400x	iLED	2.55 min	ZN	4.17 min	0.61
							CFM	2.51 min	1.02
(43)	FIND – Comparison	50%	direct	400x	FluoLED	2.59 min	ZN	4.17 min	0.62
							CFM	2.51 min	1.03
(43)	FIND – Comparison	50%	direct	400x	Lumin	2.92 min	ZN	4.17 min	0.70
							CFM	2.51 min	1.16
(39)	Marais	0%	conc	400x	adapted	1.4 min*	ZN	3.6 min	0.39
(46)	Shenai	58%	direct	200x	Lumin	1.4 min	ZN	2.5 min	0.56
(46)	Shenai	71%	conc	200x	Lumin	0.6 min	ZN	1.1 min	0.55

*mean of LED and CFM
†after 1 month experience with LED
‡after 3 months experience with LED

Table 8. LED Evaluation: Sensitivity Using a Microscopic Reference Standard

	Smear + / "Any Smear Positive"	Sensitivity (95% CI)
CFM	75/87	86.2% (77.1, 92.7)
Zeiss	83/87	95.4% (88.6, 98.7)
Lumin	76/87	87.4% (78.5, 93.5)

Table 9. LED Evaluation: Accuracy Using a Culture Reference Standard

	TP/Cx+	Sensitivity (95% CI)	TN/Cx-	Specificity (95% CI)
CFM	73/200	36.5% (29.8, 43.6)	198/200	99.0% (96.4, 99.9)
Zeiss	81/200	40.5% (33.6, 47.7)	198/200	99.0% (96.4, 99.9)
Lumin	75/200	37.5% (30.8, 44.6)	199/200	99.5% (97.2, 100)

	PPV*	NPV*	LR+	LR-
CFM	0.97 (0.91, 1.00)	0.61 (0.55, 0.66)	36.50 (9.08, 146.69)	0.64 (0.58, 0.71)
Zeiss	0.98 (0.92, 1.00)	0.62 (0.57, 0.68)	40.50 (10.10, 162.46)	0.60 (0.54, 0.67)
Lumin	0.99 (0.93, 1.00)	0.61 (0.56, 0.67)	75.00 (10.53, 534.17)	0.63 (0.56, 0.70)

TP = true positive, TN = true negative, Cx + = culture positive, Cx - = culture negative, PPV = positive predictive value, NPV = negative predictive value, LR+ = positive likelihood ratio, LR- = negative likelihood ratio

*PPV and NPV calculated for fixed prevalence of 50% due to case-control study design

Table 10. LED Evaluation: Accuracy by Specimen Type

a) Sputum

	TP/Cx+	Sensitivity (95% CI)	TN/Cx-	Specificity (95% CI)
CFM	63/169	37.3% (30.0, 45.0)	125/127	98.4% (94.4, 99.8)
Zeiss	68/169	40.2% (32.8, 48.0)	125/127	98.4% (94.4, 99.8)
Lumin	64/169	37.9% (30.5, 45.6)	126/127	99.2% (95.7, 100)

b) Other Respiratory*

	TP/Cx+	Sensitivity (95% CI)	TN/Cx-	Specificity (95% CI)
CFM	5/17	29.4% (10.3, 56.0)	47/47	100% (92.5, 100)
Zeiss	6/17	35.3% (14.2, 61.7)	47/47	100% (92.5, 100)
Lumin	6/17	35.3% (14.2, 61.7)	47/47	100% (92.5, 100)

c) Extrapulmonary

	TP/Cx+	Sensitivity (95% CI)	TN/Cx-	Specificity (95% CI)
CFM	5/14	35.7% (12.8, 64.9)	26/26	100% (86.8, 100)
Zeiss	7/14	50.0% (23.0, 77.0)	26/26	100% (86.8, 100)
Lumin	5/14	35.7% (12.8, 64.9)	26/26	100% (86.8, 100)

*includes specimens from respiratory system other than sputum (e.g. BAL, lung biopsy)
TP = true positive, TN = true negative, Cx + = culture positive, Cx - = culture negative

Table 11. LED Evaluation: Sensitivity by Species Isolated

	MTB Complex[1]		NTM[2] & others[3]	
	TP/Cx+	Sensitivity (95% CI)	TP/Cx+	Sensitivity (95% CI)
CFM	57/115	49.6% (40.1, 59.0)	16/85	18.8% (11.2, 28.8)
Zeiss	61/115	53.0% (43.5, 62.4)	20/85	23.5% (15.0, 34.0)
Lumin	58/115	50.4% (41.0, 59.9)	17/85	20.0% (12.1, 30.1)

[1]includes 106 *M. tuberculosis*, 9 *M. africanum*
[2]NTM includes 26 *M. avium*, 16 *M. gordonae*, 7 *M. kansasii*, 7 *M. chimaera*, 6 *M. intracellulaire*, 3 *M. conceptionense*, 2 *M. abscessus*, 2 *M. xenopi*, 2 *M. porcinum*, 2 *M. simiae* grp, *1 M. fortuitum*, *1 M. shimoidei*, 1 *M. terrae*, 1 *M. celatum*, 1 *M. lentifalvum*, 3 *Mycobacterium* spp (undetermined)
[3]others include 2 *Streptomyces* spp., 1 *Norcardia puris*, 1 *Tsukamurella tyrosinosolvens*
TP = true positive
Cx+ = culture positive

Table 12. Fading Evaluation: Summary of Slides Kept in Different Storage Environments

Slides Read Weekly	# Slides	Proportion Direct:Concentrated
Room Temperature (dark)[1]	30	50:50
Incubator (humidified, dark)[2]	30	67:33
Refrigerator (dark)[3]	30	63:37
Open weekly (light)[4]	30	50:50
Total = 120		
Slides Read Monthly		
Room Temperature (dark)[1]	60	52:48
Incubator (humidified, dark)[2]	60	53:47
Refrigerator (dark)[3]	60	52:48
Total = 180		
Slides Read Once at 3 Months		
Room Temperature (dark)[1]	30	53:47
Total = 30		

[1]stored at 22 degrees C in a sealed smear box
[2]stored at 30 degrees C in a sealed smear box
[3]stored at 4 degrees C in a sealed smear box
[4]stored at 22 degrees C in an open smear box

Table 13. Fading Evaluation: Distribution of Initial Slide Readings

Read Weekly (n=120)	Number of Slides (%)	p-value*
negative	12 (10)	0.78
scant	34 (28)	
1+	28 (23)	
2+	25 (21)	
3+	21 (18)	
Read Monthly (n=180)		
negative	17 (9)	0.64
scant	35 (19)	
1+	50 (28)	
2+	46 (26)	
3+	32 (18)	
Read Once at 3 Months (n=30)		
negative	3 (10)	0.56
scant	7 (23)	
1+	9 (30)	
2+	8 (27)	
3+	3 (10)	

*Pearson's chi-square goodness-of-fit test of the null hypothesis that the distribution for each group equals: 10% negative, 25% scant, 25% 1+, 25% 2+, 15% 3+

Table 14. Fading Evaluation: Comparison of Reading Schedules for Slides Stored at Room Temperature

Time*	Proportion +**			p-value†
	Weekly (n=27)	Monthly (n=54)	Quarterly (n=27)	
1 month	78%	63%		0.21
2 months	46%	43%		0.64
3 months	22%	26%	35%	0.79[a], 0.54[b], 0.60[c]
4 months	11%	15%		0.74
5 months	11%	11%		1.0

*1 month = 4 weeks

**Proportion + refers to the number of slides read as positive after storage in a closed smear box at temperature controlled room temperature (22 degrees C) for the indicated length of time compared to the total number of slides read as positive on the day of staining

†Fisher's exact test, 2-tailed testing null hypothesis that the proportions of positive slides between weekly, monthly and/or quarterly readings are equal

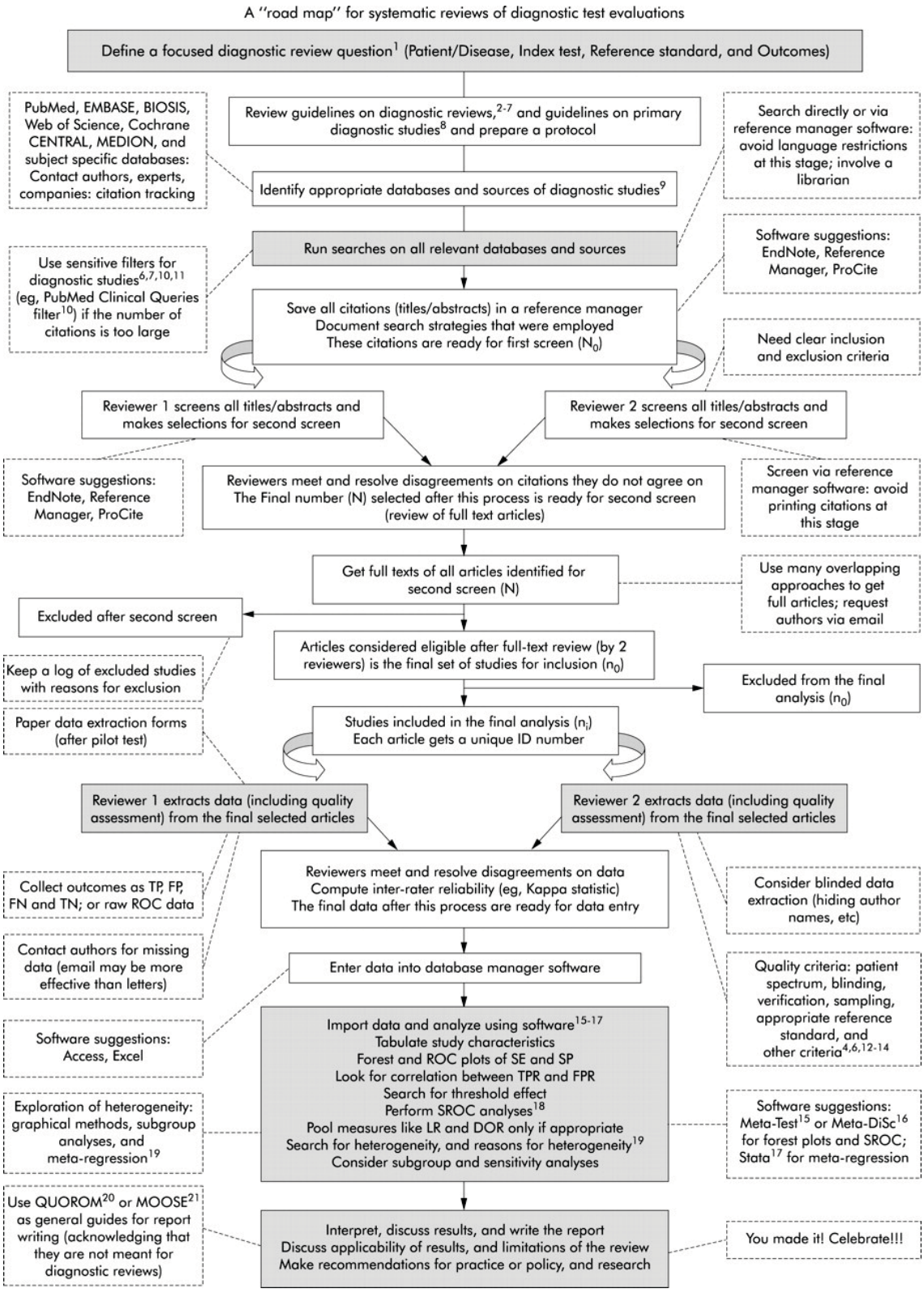
[a]comparing weekly reading with monthly reading; [b]comparing weekly reading with quarterly reading; [c]comparing monthly reading with quarterly reading

Table 15. Fading Evaluation: Proportion of All Slides Remaining Positive by Original Slide Reading

	Original Reading (n=# slides)			
	3+ n=53	**2+ n=71**	**1+ n=78**	**scant n=69**
T=0	100.0%	100.0%	100.0%	100.0%
1 month	83.0%	63.6%	45.2%	42.1%
2 month	61.2%	27.0%	13.8%	14.1%
3 month	35.7%	10.7%	6.0%	1.7%
4 month	16.9%	2.8%	1.2%	2.6%
5 month	14.1%	2.3%	1.2%	0.0%

All storage environments combined; monthly readings combined with respective weekly readings at 4 weeks, 8 weeks, etc.

APPENDIX 1. Protocol for a Diagnostic Meta-Analysis



From: Pai et al. 2004. *ACP J Club* 141(1):A11.

APPENDIX 2. Search String Used in PubMed for LED Systematic Review

Search #1: tuberculosis[mesh] OR tuberculosis[tiab] OR mycobacter*[ti] OR acid-fast[ti] OR tuberculous[ti]

Search #2: (fluorescen*[ti] AND microscop*[ti]) OR Auramine[tiab] OR "light-emitting"[ti] OR LED[tiab] OR (LED[tiab] AND microscopy[tiab]) OR (light[tiab] AND emitting[tiab] AND diode[tiab]) OR diode[tiab] OR lumin[tiab] OR FluoLED[tiab] OR Zeiss[tiab]

Search #3: #1 AND #2

Restricted to papers published from Jan 2000 to Feb 2009

APPENDIX 3. Data Extraction Form Used In LED Systematic Review

Study #: _____ Reviewer: _____ Author: _____ Year: _____
Language: _____ Published: Y / N Country: _____ Sponsor: _____

LED model: ☐ standalone ☐ attachment used with: _____ Bulb/Spectra:_____	 Magnification used Screening:_____ Confirmation:_____ ☐ not specified	Smear: ☐ direct ☐ concentrated ☐ other processing _____ Dark Room Y / N / ?	Stain/Counterstain: _____ Criteria for +: _____ Criteria for –: _____
Comparison 1: ☐ different slide used ☐ same slide restained ☐ same slide: Time b/w readings____ Order read: 1st 2nd 3rd	 Magnification used Screening:_____ Confirmation:_____ _ ☐ not specified	Smear: ☐ direct ☐ concentrated ☐ other processing: _____ Dark Room Y / N / ?	Stain/Counterstain: _____ Criteria for +: _____ Criteria for –: _____
Comparison 2: ☐ different slide used ☐ same slide restained ☐ same slide: Time b/w readings____ Order read: 1st 2nd 3rd	 Magnification used Screening:_____ Confirmation:_____ ☐ not specified	Smear: ☐ direct ☐ concentrated ☐ other processing: _____ Dark Room Y / N / ?	Stain/Counterstain: _____ Criteria for +: _____ Criteria for –: _____:
Reference:	☐ culture method: _____ duration: _____	☐ other specify:	QC described / ref'd: Y / N
Specimens: ☐ unit of analysis	☐ pulmonary only ☐ extrapulmonary only ☐ mixed ☐ not specified	☐ case control selection ☐ consecutive selection ☐ random selection ☐ not specified	☐ prospective ☐ retrospective ☐ not specified
Patients: ☐ unit of analysis	☐ adults only ☐ peds only ☐ mixed ☐ not specified	☐ inpatients only ☐ outpatients only ☐ mixed ☐ not specified	☐ HIV included % _____ ☐ HIV not included ☐ not specified
Time per slide LED:_____ Comp1:_____ Comp2:_____ ☐ not given Turnaround LED:_____ Comp1:_____ Comp2:_____ ☐ not given	Cost Info LED unit: Labour: Reagents: Other: ☐ not given	User Identified Pros: User Identified Cons: Implementation Issues: Training Described: QC discussed:	

QUADAS CHECKLIST

1. Was the spectrum of patients representative of the patients who will receive the test in practice?	❑ YES	❑ NO	❑ UNCLEAR
2. Were selection criteria clearly described?	❑ YES	❑ NO	❑ UNCLEAR
3. Is the reference standard likely to correctly classify the target condition?	❑ YES	❑ NO	❑ UNCLEAR
4. Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests?	❑ YES	❑ NO	❑ UNCLEAR
5. Did the whole sample or a random selection of the sample, receive verification using a reference standard of diagnosis?	❑ YES	❑ NO	❑ UNCLEAR
6. Did patients receive the same reference standard regardless of the index test result?	❑ YES	❑ NO	❑ UNCLEAR
7. Was the reference standard independent of the index test (i.e. the index test did not form part of the reference standard)?	❑ YES	❑ NO	❑ UNCLEAR
8. Was the execution of the index test described in sufficient detail to permit replication of the test?	❑ YES	❑ NO	❑ UNCLEAR
9. Was the execution of the reference standard described in sufficient detail to permit its replication?	❑ YES	❑ NO	❑ UNCLEAR
10. Were the index test results interpreted without knowledge of the results of the reference standard?	❑ YES	❑ NO	❑ UNCLEAR
11. Were the reference standard results interpreted without knowledge of the results of the index test?	❑ YES	❑ NO	❑ UNCLEAR
12. Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?	❑ YES	❑ NO	❑ UNCLEAR
13. Were uninterpretable/intermediate test results reported?	❑ YES	❑ NO	❑ UNCLEAR
14. Were withdrawals from the study explained?	❑ YES	❑ NO	❑ UNCLEAR

RESULTS:

Strata	Test	Ref	Specimen	Patient	Time	TP	FP	TN	FN

APPENDIX 4: STAG-TB Recommendations Concerning LED-based Microscopy

1. Recommend conventional fluorescence microscopy be replaced by LED microscopy in all settings where fluorescence microscopy is now used, and that LED microscopy be phased in as an alternative for conventional ZN microscopy in both high- and low volume laboratories;

   The switch to LED microscopy should be carried out through a carefully phased implementation plan, using LED technologies that meet WHO specifications;

   Countries implementing LED should address the following issues:

- Training requirements, especially for laboratory staff unfamiliar with FM techniques;
- Validation during the introductory phase;
- Monitoring of trends in case-detection and treatment outcomes;
- Introduction of adapted systems for internal quality control and external quality assurance.

2. Develop and disseminate technical specifications for LED devices (including stand-alone LED microscopes and LED attachments to light microscopes) to guide countries, technical and funding agencies to purchase high-quality equipment;

3. Develop and disseminate standard operating procedures and a programme for external and internal quality assurance of LED microscopy;

4. Facilitate, with partners and technical agencies, a coordinated approach to standardised training on LED technology at country level;

[From: Strategic and technical advisory group for tuberculosis: report of the ninth meeting. World Health Organization (WHO), Geneva, Switzerland. 2009 November 9 – 11. page 9. http://www.who.int/tb/advisory_bodies/stag_tb_report_2009.pdf]