Title
Mobile Digital Imaging-based Microscopy for Tuberculosis Diagnosis

Author
Tapley, Asa L

Publication Date
2012-04-01

Mobile Digital Imaging-based Microscopy for Tuberculosis Diagnosis

By

Asa Lyman Tapley

A thesis submitted in partial satisfaction of the

requirements for the degree of

Master of Science

in

Health and Medical Sciences

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

John Balmes, MD, Chair
Adithya Cattamanchi, MD, MAS
Daniel Fletcher, PhD
John Swartzberg, MD

Spring 2012

Dedicated to my grandfather,
Lyman Tapley

Lyman Tapley was born in the small town of Brooksville, Maine, in 1918. A welder at the Pearl Harbor Navy shipyard during World War II, he contracted tuberculosis (TB) in Hawaii.

After the war, Lyman entered a TB sanatorium in Saranac Lake, NY, in the Adirondacks. At the time it was believed that the mountain air was therapeutic for TB patients. Lyman spent two years there and had half of a lung removed during that time.

Returning to Maine, he became an X-ray technician and his first job was in the sanatorium – or the "san," as my father called it – on the top of the biggest hill in the town of Fairfield. Lyman worked there only briefly before moving on to a hospital in nearby Waterville, where he became the chief technician and an instructor in the X-ray technician school there. But he continued smoking, and he also drank heavily. All during his childhood, my father remembers him being short-winded. In 1969, Lyman fell ill with a relapse of TB. He was cared for in the same sanatorium where he had worked previously and where my grandmother worked as a nurse.

Thanks to antibiotics, Lyman was able to return home after three weeks of treatment. But he worked little after this episode. Having moved to the town of Ellsworth in the early 1970s, Lyman died of emphysema in the hospital there in 1979. My father was there with his arms around him when he died. I was three months old.

ACKNOWLEDGEMENTS

First and foremost I want to thank my research mentors Adithya Cattamanchi and Luke Davis of the Division of Pulmonary and Critical Care Medicine in the Department of Medicine at San Francisco General Hospital/UCSF. They both taught me how good clinical and operational research is done. They also entrusted me with responsibilities rare for a medical or Master's degree student, which has pushed my learning and allowed me greater personal growth.

I thank Daniel Fletcher of the Department of Bioengineering at UC Berkeley, who enthusiastically invited me into his lab and the CellScope project and immediately made me feel like part of the team. I am tremendously grateful for the opportunity to collaborate with Neil Switz, Clay Reber, and Lina Nilsson, who have been a source of friendship and good humor. I also thank Amy Shen and Erik Douglas, co-founders of CellScope, Inc.

I feel lucky to have had throughout the thesis research and writing process the reassuring guidance of John Balmes, my mentor at the UC Berkeley School of Public Health and a faculty member with Adithya and Luke at SFGH. He is a true role model. I am also grateful for the thoughtful support of John Swartzberg, who generously agreed to be on my committee; Ron Dahl, who led my thesis working group seminar; and Doug Jutte, who co-directed the Master's curriculum.

In addition, I want to acknowledge Cecily Miller, who led the data analysis and was always good natured and responsive to our sometimes last-minute requests.

I thank my family, who has supported my pursuits no matter how challenging and has instilled in me the values that define so much of who I am today. I especially am grateful to my best friend, my twin brother Adam.

I want to acknowledge all of my Ugandan colleagues – only a few of whom I have met – who are part of the Uganda Tuberculosis Surveillance Project and are responsible for much of the original data collection. Finally, I wish to thank the many patients without whom this research would have been impossible and to whom I hope this work may eventually provide benefit.

TABLE OF CONTENTS

SECTION 1, PART 1:

TUBERCULOSIS: FROM EGYPTIAN MUMMIES TO MODERN CHEMOTHERAPY

A dread disease in which the struggle between soul and body is so gradual, quiet and solemn, and the result so sure that day by day, and grain by grain, the mortal part wastes and withers away. A disease . . . which sometimes moves in giant strides and sometimes at a tardy sluggish pace, but, slow or quick, is ever sure and certain.

- Charles Dickens, *Nicholas Nickleby*, 1838-39

ORIGINS OF TUBERCULOSIS

Tuberculosis (TB) has plagued humankind since our prehistory. In fact, *Mycobacterium tuberculosis* has likely killed more people than any other microbial pathogen, even malaria.[1] "If the number of victims which a disease claims is the measure of its significance," remarked the great microbiologist Robert Koch, "then all diseases, particularly the most dreaded infectious diseases, such as bubonic plague, Asiatic cholera, et cetera, must rank far behind tuberculosis."

The *Mycobacterium tuberculosis* complex (MTBC) consists of seven species and subspecies that can cause TB disease: *M. tuberculosis, M. canettii, M. africanum, M. bovis, M. microti, M. pinnipedii,* and *M. caprae*.[2] While phenotypic characteristics and the range of potential hosts vary among the different members of MTBC, there is extremely little genetic variability between species.[3]

Indeed, members of MTBC represent one of the most extreme examples of intraspecies genetic homogeneity observed in the bacterial world.[3] This very low level of genetic variation suggests that the entire population of current MTBC members evolved from one common ancestor following an evolutionary bottleneck about 35,000 years ago.[4,5] Gene sequencing of several MTBC species done in recent years has led researchers to conclude that an ancestral species of *M. tuberculosis* existed in East Africa about 3 million years ago and likely infected the earliest hominids.[3]

Thus, human hosts and tubercle bacilli arose in East Africa together. While archeological evidence of disease in this region is scarce, indications of TB can be documented in Egypt as far back as 5,500 years ago. Among Egyptian mummies, paleopathologists have found skeletal signs of TB, such as Pott's deformities, indicating spinal TB, as well as depictions of such abnormalities in Egyptian art.[6-9] The earliest known medical papyri describe diseases that may be TB.[8] A 3,500-year-old Egyptian papyrus, for example, seems to document cases of TB infection of the cervical lymph nodes. But TB – known as "the great imitator" – has a wide range of possible clinical manifestations, making possible ancient written and artistic depictions of TB difficult to interpret.[10] Nevertheless, recent amplification and analysis of mycobacterial DNA recovered from tissues of

Egyptian mummies has allowed molecular-level confirmation that *M. tuberculosis* was indeed the cause of their skeletal deformities.[7,11-14] (Figure 1.[15])

Populations of *H. erectus* began moving out of Africa and dispersing to Europe and Southeast Asia about 1.7 million years ago,[16-18] and these groups were later replaced by subsequent waves of human migration out of Africa between 35,000 and 89,000 years ago.[17] From the beginning, it is presumed that these wandering humans brought TB along with them.[1] Texts dating back as far as 3,500 years ago or earlier that have been found in India, China and Mesopotamia seem to depict human TB.[1,10]

Figure 1. Opened thorax of Egyptian mummy. Large arrow = pleural adhesions of right lung; small arrow = destruction of boney elements of lumbar vertebral bodies L4 and L5.

Although the Bering Straight delayed human migration to the Western Hemisphere, evidence from the Monte Verde, Schaefer, and Hebior archeological sites suggests that humans inhabited the Americas by at least 15,000 years ago.[19] Archeological evidence clearly identifies the presence of TB among these early peoples long before European colonizers arrived on the scene.[20] As in Egypt, Andean mummies provide paleopathological signs of skeletal TB disease and these findings have been confirmed by analysis of mycobacterial DNA recovered from the preserved tissues. Paleologic evidence indicates that TB existed at low levels among the early populations of the Americas, probably because they lived in small, dispersed groups – a level of social organization that does not favor infectious diseases.[20] As larger, settled communities developed, however, TB flourished and an epidemic of the disease appears to have spread from Andean areas of South America about 1,500 years ago.[20] However, the initial TB epidemic seems to have peaked and dissipated early on. When European colonizers later reintroduced TB, the indigenous peoples were largely immunologically naïve to the disease and were deeply impacted.[6]

Known as *phthisis*, arising from the Greek word for 'to decay,'[6,21] TB was well-known in classical times. Graeco-Roman physicians could commonly recognize trademark symptoms of TB such as night sweats, spitting blood, and shortness of breath.[10] Hippocrates, perhaps the first to use the term phthisis, speculated that TB originated from growths that he observed in autopsies of the lungs and he seemed to associate the pulmonary TB with that of the spine.[22] In his famous collection of aphorisms, Hippocrates noted some 2,500 years ago that:

> In persons affected with phthisis, if the sputa which they cough up have a heavy smell when poured upon coals, and if the hairs of the head fall off, the case will prove fatal.[23]

The Greek-born physician Clarissimus Galen – who would become the personal physician to Roman Emperor Marcus Aurelius – frequently encountered TB and prescribed his patients treatments involving fresh milk, pure air, maritime activities, horseback riding and rest in dry, high-altitude areas.

Although there are few written records from Europe's middle ages – the roughly 1,000-year period following the fall of the Roman Empire – there is widespread archeological evidence of skeletal TB infections from this period.[6,24,25] Moreover, there is ample evidence that TB of the cervical lymph nodes, called scrofula, was widespread in medieval Europe. Early monarchs, beginning with the Frankish King Clovis in 496, claimed to have the power to heal by touch those afflicted by scrofula and charged their subjects for these professed cures.[6,22] Widespread scrofula may have been the result of ingestion of milk contaminated with bovine TB.[6]

TUBERCULOSIS IN THE MODERN ERA

As the Renaissance began in Europe, the written record of TB reemerged along with the increased interest in literature, art and science. Increasing urbanization and appalling living conditions led to an epidemic of TB starting in the 1600s that continued through the next two centuries.[1,6,22] By the 1800s, annual death rates from TB in cities such as London, Stockholm and Hamburg had reached as high as one out of 100 people per year.[26,27] American cities at the time faced similar death rates from TB. More than one quarter of all deaths in New York City between 1810 and 1815 were attributed to TB.[28]

However, in the face of such devastating disease and the need to make sense of it, North American and European society's fear of TB in the 1600s gave way to romanticism in the eighteen and nineteenth centuries. "I should like to die of consumption," the poet Lord George Byron was said to have remarked to a friend. When asked why, he said "Because the ladies would all say, 'Look at that poor Byron, how interesting he looks in dying!'"[29] Indeed, the dying young person was often perceived to have romantic and creative gifts, especially among those in the arts and literature. The famous composer Camille Saint-Säens wrote in 1913 that "it was fashionable to be pale and drained."[22] Almost a century earlier, the poet Percy Shelly wrote to John Keats, "this consumption is a disease particularly fond of people who write such good verses as you have done." A doctor and poet, Keats was well familiar with TB: His mother died of TB, his brother also contracted the disease, and in 1830 Keats himself passed away from TB – only a year after first noticing symptoms.[22]

Tuberculosis, wrote Charles Dickens in *Nickolas Nickleby*, is the "disease in which death and life are so strangely blended, that death takes the glow and hue of life, and life the

gaunt and grisly form of death; disease which medicine never cured, wealth never warded off, or poverty could boast exemption from...."[30] (Figure 2.[31])

The epidemic of TB in Europe and North America peaked in the middle of the nineteenth century and has declined ever since. The decline in TB mortality was more rapid than for all-cause mortality[32] and preceded major discoveries related to the cause of the disease and effective medications for its treatment. Researchers have debated the exact reasons for the dramatic decline, including improved socioeconomic conditions that led to better nutrition and working and living conditions, enhanced herd immunity, sequestering of infectious individuals in hospitals and sanatoriums, and the application of basic public health interventions.[6,27,28,33]

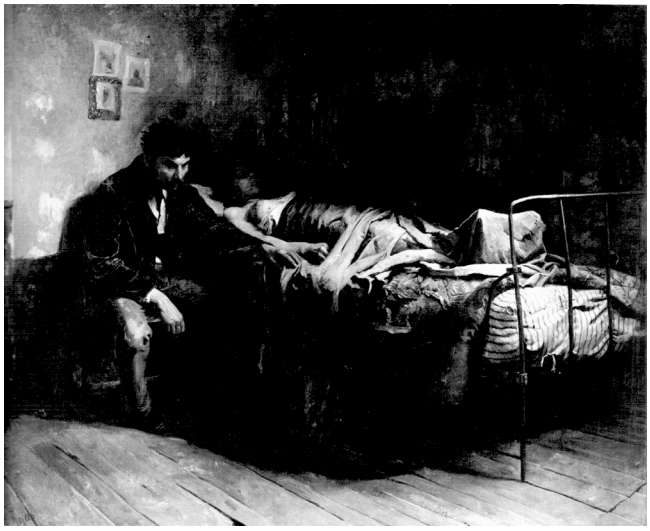

Figure 2. La Miseria by Cristóbal Rojas (1886). The author, suffering from tuberculosis, depicts the social aspect of the disease, and its relation with living conditions at the close of the 19th century.

UNDERSTANDING THE NATURE OF THE BEAST

A series of major scientific discoveries in the 1800s finally established both the infectious nature and the precise etiology of TB. Into the nineteenth century, TB was commonly perceived as a hereditary disease, particularly in northern Europe, because of the frequency by which it afflicted multiple members of the same family.[6] Englishman Benjamin Marten offered one of the earliest theories that TB may actually be caused by a microorganism. In his book *A New Theory of Consumptions*, published in 1720, Marten – building off of Anton van Leeuwenhoek's discovery of bacteria in 1676 – suggested that the cause of TB "may possibly be some certain species of *Animalcula* or wonderful minute living Creatures, that, by their peculiar Shape, or disagreeable Parts, are inimicable [sic] to our Nature."[34]

It was French physician René Théophile Hyacinthe Laennec who set the stage for our modern understanding of the pathophysiology of TB. In *Traité de médecine sur les maladies de la poitrine* (Treatise on Diseases of the Chest), translated in 1821, Laennec provided detailed descriptions of the pathologic changes seen in TB-infected lungs and established that similar lesions found in other parts of the body were due to TB as well. "These secondary eruptions of tubercles are not confined to the lungs," Laennec wrote. "They may make their appearance in many other organs."[35] Laennec himself died of TB at age 45.[29]

Another Frenchman, the surgeon Jean-Antoine Villemin, established the infectious nature of TB. In his study *Etudes sur la Tuberculosis* (Studies on Tuberculosis), published in 1868, Villemin described the transmission of TB between various species such cows, rabbits and humans. Villemin's experiments included injecting healthy rabbits with pulmonary tissue from deceased TB patients and, weeks later, observing TB lesions within the inoculated animals when he autopsied them.[28] "[A]t the end of three months and fourteen days," Villemin wrote,

> There was no appreciable change in the health of the animal, [and so] we sacrificed it and noted the following: The lungs are full of large tubercular masses, formed, in an obvious manner, from the agglomeration of numerous granulations . . . The other rabbit, who had shared the same conditions of life with the inoculated rabbit . . . did not show a single tubercle.[36]

While Laennec and Villemin made great strides in the characterization of TB, it was German physician Robert Koch who finally identified the causative agent of TB, presenting his findings to a scientific meeting in Berlin in 1882.[37,38] (Figure 3.[39]) Koch called the bacteria *Bacillus tuberculosis*, later to be renamed *Mycobacterium tuberculosis*. In implicating *M. tuberculosis* as the causative agent of TB, Koch used an approach – developed from ideas of his mentor, Jakob Henle[40] – that is still considered the standard for the experimental demonstration of the pathogenicity of a microorganism: (1) The suspected causal organism must be constantly associated with the disease; (2) the suspected causal organism must be isolated from an infected host and grown in pure culture; and (3) when a healthy susceptible host is inoculated with the pathogen from pure culture, symptoms of the original disease must develop.[38,41]

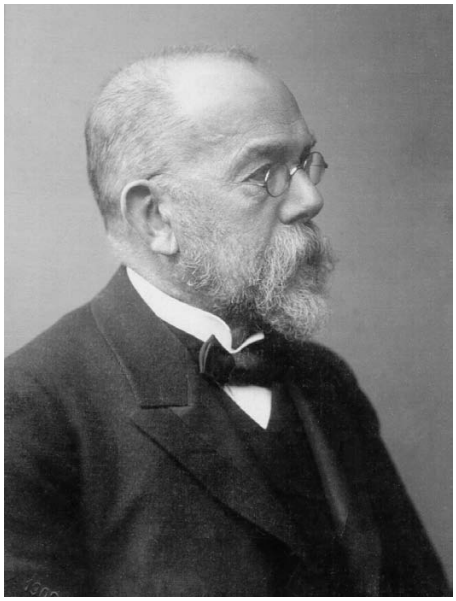

Figure 3. Robert Koch, discoverer of *Mycobacterium tuberculosis*.

It is difficult to overstate the significance of Koch's contributions to the modern understanding of TB, let alone the field of medical microbiology in general. In addition identifying *M. tuberculosis* as the cause of TB and introducing his postulates for implicating specific microorganisms with disease, Koch also identified the causative agents of anthrax, wound fever and cholera.[41] Koch developed culture media for growing bacteria, invented new techniques for staining them, refined animal models of infectious disease, improved methods for microphotography, and mentored many other medical microbiologists who would make their own major contributions to medicine and bacteriology.[41] In 1905 Koch was awarded the Nobel Prize in Medicine or Physiology for his work in identifying the cause of TB.

It was in fact a failure of Koch to develop a treatment for TB that led to a major advance in the diagnosis of TB. In 1890 Koch claimed to have isolated a substance that could

"render harmless the pathogenic bacteria that are found in a living body and do this without disadvantage to the body."[1,42] The substance, which would become known as tuberculin, was made from supernatants of *M. tuberculosis* cultured in broth.[1,37] Although Koch's claim was rapidly debunked, in developing tuberculin, Koch had demonstrated that the cell-mediated immune response was a key factor in determining the manifestation of TB disease.[37] It was almost in passing that Koch noted the potential diagnostic value that this discovery entailed.

However, Clemens Freiherr von Pirquet, a Viennese pediatrician, seized upon the discovery and developed an intracutaneous tuberculin test, which he used initially to detect latent TB among the children he treated.[43] Further refinement of the tuberculin test by Charles Mantoux and Florence Siebert led to the purified protein derivative (PPD) that is still used today – virtually unchanged – for the tuberculin skin test (TST).[1,44]

The arsenal for TB diagnosis expanded further when, in 1895, five years after Koch's overstated presentation on tuberculin, German physicist Conrad Wilhelm Röntgen discovered x-rays.[45] Soon after, American inventor Thomas Edison developed the first practical fluoroscope, and by 1897 fluoroscopic examinations of lungs for TB and other diseases were already being performed.[6] Although useful for detecting abnormalities of the lung, including ones that can be caused by TB, chest radiography is no longer considered a reliable method for diagnosing or monitoring TB patients.[46]

TUBERCULOSIS CARE BEFORE CHEMOTHERAPY

From early times into the nineteenth century, treatment for TB changed little from the fresh air, rest and healthy diet that Greek physician Galen recommended patients over two millennia ago. Sanatoriums institutionalized this approach, segregating patients from the demands of normal living and providing a place of rest and comfort – and time for meditation on life and society. As Thomas Mann wrote in his famous novel, *The Magic Mountain*, which was set in a sanatorium:

> And life? Life itself? Was it perhaps only an infection, a sickening of matter? Was that which one might call the original procreation of matter only a disease, a growth produced by morbid stimulation of the immaterial? The first step toward evil, toward desire and death, was taken precisely then, when there took place that first increase in the density of the spiritual, that pathologically luxuriant morbid growth, produced by the irritant of some unknown infiltration; this, in part pleasurable, in part a motion of self-defense, was the primeval stage of matter, the transition from the insubstantial to the substance. This was the Fall.[47]

The Channing Street Home for the Sick and Destitute, established in Boston in 1857 by Harriet Ryan, was perhaps the first institution established specifically as a refuge for TB patients.[29] Two years later, the German physician Herman Brehmer started the first sanatorium for the treatment of TB in the Silesian Mountains of southwestern Germany. Brehmer's regiment of rest, rich diet and supervised exercise was rapidly imitated throughout much of Europe and North America.[29] (Figure 4.[48]) While it is unclear that sanatoriums provided clinical benefit to the patients, they undoubtedly reduced transmission.

Figure 4. Patients at the Ninette Tuberculosis Sanitorium, Manitoba, Canada.

Twenty years after Louis Pasteur first developed the means for attenuating the virulence of microbes for the production of effective vaccines, Albert Calmette and Camille Guérin, physician and veterinarian respectively, harnessed these new techniques to produce strains of *M. tuberculosis* that could not progress to TB disease but nevertheless provided some resistance to reinfection.[1] The vaccine was named "Bacille Bilié Calmette et Guérin" because in developing it, the Frenchmen exploited the discovery that growing *M. tuberculosis* in ox bile reduced the bacteria's virulence. The name was quickly shortened to Bacille Calmette Guérin, and then to simply BCG.[1] Estimates of the level of protection provided by BCG have varied wildly, and a number of clinical trials have suggested that the vaccine is largely ineffective.[49] It is therefore difficult to determine the impact BCG has had on TB control. Nevertheless, BCG – which is currently developed using *M. tuberculosis*'s close relative, *M. bovis* – has become one of the most widely used vaccines in the world.

Prior to the discovery of effective chemotherapy agents for the treatment and eradication of TB infection, a variety of surgical interventions were attempted. Collapse of diseased pulmonary cavities was invariably the goal of such surgeries, the idea being that "resting" the lung tissue would give it a better chance of recovery. The outside surface of the lung is held against the inside of the thoracic cavity by the surface tension of naturally occurring liquid called pleural fluid. When air is introduced into this pleural space, it disrupts the bond between the two surfaces and the lung collapses. This introduction of air – called pneumothorax – typically only occurs due to trauma, such as when a broken rib punctures the pleural space. However, pneumothoraces intentionally induced by surgically opening a hole in the chest cavity became a widely used treatment for TB in the nineteenth century.[29]

Other surgical approaches to TB treatment included thoracoplasty, which involved rib removal in order to collapse the chest wall and associated portion of the lung; phrenicecomy, which involved crushing of the phrenic nerve in order to paralyze the diaphragm; and pneumoperitoneum, which involved introduction of air into the abdominal space in order to put pressure on the thoracic cavity.[29,50] In some cases an entire diseased lung was removed.[50] While some patients clearly benefited from these interventions, effectiveness was highly variable and there were many potential complications including infections and highly compromised respiration.[29,50]

ANTITUBERCULOSIS CHEMOTHERAPY

With its thick, lipid-laden cell wall, *M. tuberculosis* is naturally protected from many of the body's antibacterial defenses as well as numerous foreign chemicals.[28] The discovery of the first antibacterial chemotherapeutic agent, Prontosil, in 1932 signaled the beginning of a new era in medicine and would quickly lead to a revolution in the treatment of TB.[51] By 1945, the first chemotherapeutic agents with effect on *M. tuberculosis*, para-amino salicylic acid (PAS) and thiosemicarbazone, had been discovered respectively by the chemists Jorgen Lehmann and Gerhard Domagk (the latter of whom had originally discovered Prontosil).[1,50,52,53] However, these drugs were only able to slow but not reverse infection – in other words, they were *bacteriostatic* but not *bacteriocidal* drugs.

In 1943, the American soil microbiologist Selman Waksman, working with Elizabeth Bugie and Albert Schatz, was able to isolate the antibiotic streptomycin, a chemical produced by the soil actinomycete *Streptomyces griseus*.[54,55] (Figure 5.[56]) In less than a year, physicians Corwin Hinshaw and William Feldman had used the chemical to successfully treat a woman deeply ill with pulmonary TB, resulting in a remarkable recovery and long-term cure.[54,57] Although streptomycin was found to have significant limitations – including that it had toxicity to the auditory nerve[58] and that bacteria could quickly develop resistance[59] – it was also soon observed that these problems could be significantly mitigated through a combined therapy of streptomycin and PAS.[60]

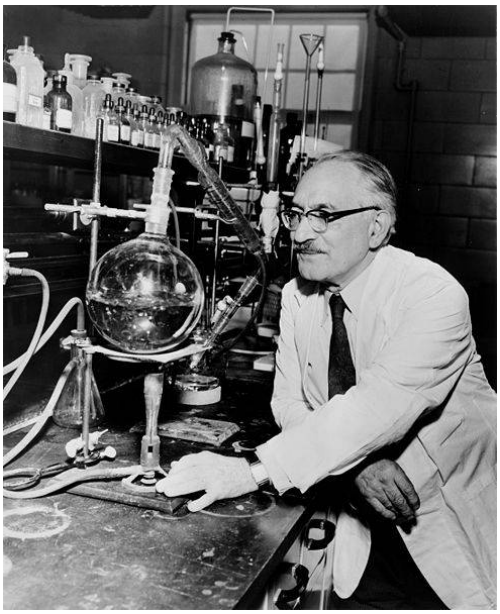

Figure 5. Selman Waksman was awarded the 1952 Nobel Prize for the discovery of streptomycin. He also coined the term "antibiotic" and was involved with the discovery of many other antibiotics such as neomycin, clavacin, antinomycin and candidin.

At the beginning of the 1950s, pharmaceutical companies discovered isoniazid,[61-63] the first TB drug that was cheap and effective and had low toxicity.[28] Isoniazid rapidly became part of new combination therapies along with PAS and streptomycin that for the

first time could reliably and safely cure many TB patients within 18 to 24 months of treatment.[64] In the mid-1960s, rifamycins were discovered[65,66] and this was followed by the development of several other effective anti-TB drugs[67,68] as well as the rehabilitation of an older compound, pyrazinamide.[69-71]

With this expanded battery of medications, there was new interest in examining shorter courses of anti-TB therapy.[67] Through the support of the British Medical Research Council, a multicenter randomized controlled trial run in Kenya between 1972 and 1974 demonstrated that the period of TB treatment could be shortened to 6 months by the inclusion of rifampicin and pyrazinamide in the regimen.[72]

From the advent of effective chemotherapy for TB, the hospital-based aspect of treatment represented a major obstacle to access for most patients worldwide. A British study led by the physician Wallace Fox in Madras, India was designed to compare the outcomes of patients treated at home versus in a hospital.[72] The results were striking: not only was treatment success similar in both groups, the household contacts of home-based patients appeared to face little risk of contracting TB once the patient was undergoing treatment.[68] Home-based TB care held the promise of vastly wider treatment access, although Fox and his colleagues realized that the regularity of drug-taking had to be better ensured.[73] "Irregularity," he noted, "[had] been a problem throughout the course of treatments."[74]

Perceiving the fundamental importance of this challenge, Fox followed up his initial research with investigations in Madras examining the feasibility of supervised therapy. Despite the poverty of his patient population, the distance that they often lived from health clinics, and the lack of adequate transportation, Fox concluded that "Long term daily supervised administration can be organized under special circumstances, even in developing countries."[74,75] His work would lay the conceptual foundation for what would be called directly observed therapy, short course (DOTS). This approach, which has become a foundation of modern of TB care, involves observation by a health care provider or community health worker of each dose of chemotherapy a patient receives.[74] Modern short-course TB therapy, when administered correctly, is curative about 98 percent of the time and is considered one of the most cost-effective treatments available for any chronic disease.[76] In the developed world at least, these therapies dramatically changed the landscape of TB disease.

SECTION 1, PART 2

TUBERCULOSIS TODAY

Tuberculosis is thus two things at once: a completely curable disease and the leading cause of young adult deaths in much of the world.

- Paul Farmer, "Consumption of the Poor," *Ethnography*, 2000.

WHERE WE ARE NOW

"It is a sad reflection on society's incompetence that, more than 30 years after the methods for cure and prevention were evolved and before the advent of the HIV pandemic, there were already more patients with active tuberculosis in the world than there had been in the 1950s" wrote the renown TB researcher Sir John Crofton in 1994.[77] Despite the revolution in TB treatment in the 1950s and 1970s, TB remains today a leading cause of morbidity and mortality worldwide.[78]

In fact, with an estimated 8.8 million new TB cases worldwide in 2010 alone, there are more people falling ill with TB in recent years than ever before.[78] One-in-three people on Earth is latently infected with TB, each with the potential to progress to active, infectious disease.[79] Each year, 1.7 million people die from TB[80], making it responsible for more deaths worldwide than any other single infectious microbe.[81] And the burden of disease falls disproportionately on lower resource areas of the world. About 85 percent of all TB cases occur among the poor of Asia and Africa.[78]

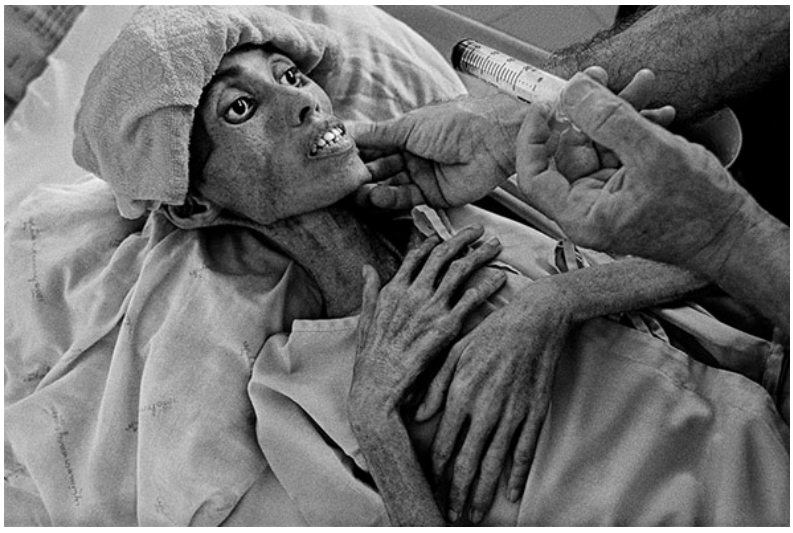

Figure 6. A volunteer in the AIDS hospice at the Buddhist temple in Wat Phrabat Nampu, Lop Buri, Thailand, gives water to a patient coinfected with AIDS and TB.

Since HIV/AIDS was first identified 30 years ago, it has contributed heavily to the current TB crisis.[82] Through its destruction of the body's cell-mediated immunity, HIV co-infection dramatically increases the risk of developing active TB disease[83,84] and facing a premature death.[85-87] (Figure 6.[88]) Indeed, TB is the number one killer of people infected with HIV, contributing to 350,000 deaths – a full quarter of all TB-related deaths each year.[78] The lethal combination of TB and HIV has been particularly devastating on

the African continent, where 82 percent of the world's HIV co-infected TB patients live.[78] (Figure 7.[78])

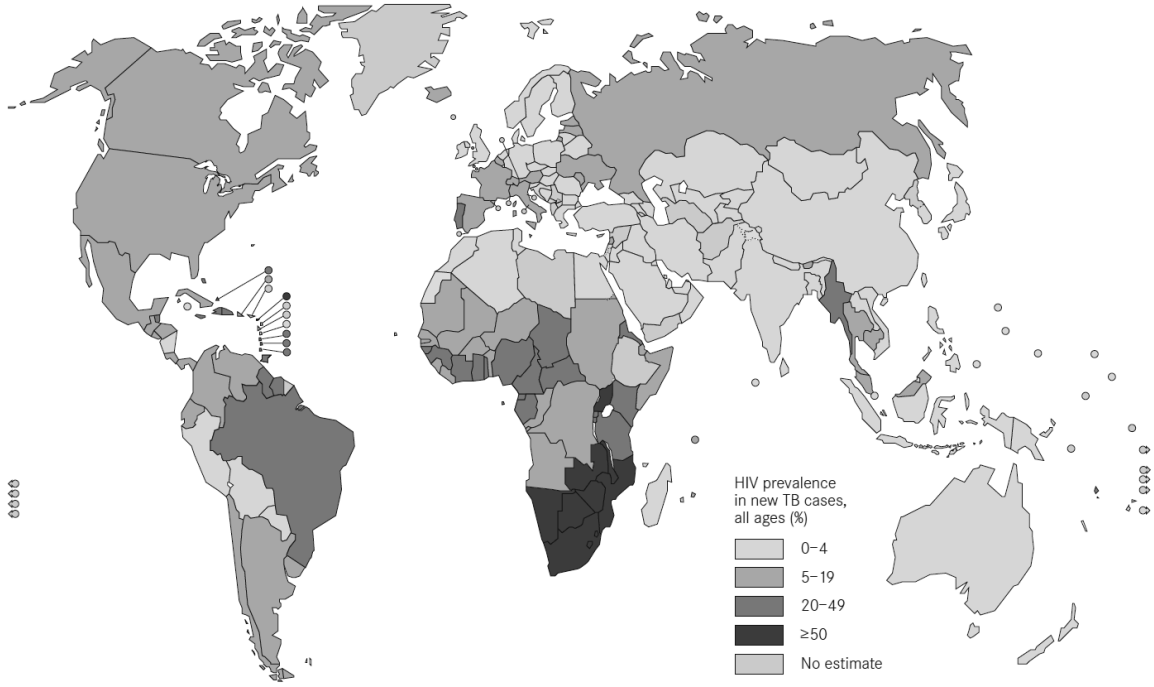

Figure 7. Estimated HIV prevalence in new tuberculosis cases in 2010.

In addition to HIV, over the past two decades multidrug-resistant (MDR) TB has arisen as a worldwide challenge to TB control.[89] Responsible now for 4 percent of all TB cases[89] and 150,000 deaths a year[90], MDRTB is difficult to diagnose in developing countries due to the lack of capable laboratories,[89] leading to delayed diagnosis or misdiagnosis.[91,92] This in turn raises the risk of transmission, morbidity and mortality. Drug treatment for MDRTB patients can cost 200 fold the cost treatment of patients with more drug-susceptible TB[90], and even when appropriate drugs are available, treatment success is lower.[93] Moreover, in recent years, extensively drug-resistant (XDR) TB[94] and even strains resistant to all known anti-TB drugs have been identified.[95]

The decline of TB disease in wealthy countries bred complacency towards the ongoing TB problem both at home and abroad. From the 1950s until the 1990s, interest in TB control severely waned, particularly in Europe and the U.S., which led to lost clinical expertise and a decline of the TB control infrastructure.[77] Several TB research journals were discontinued and organizations like the American Lung Association – formerly called The National Association for the Study and Prevention of Tuberculosis – chose to refocus on other issues.[96] Important targeted funding for TB provided to low-resource regions by the U.S. Agency for International Development shrank to zero.[97]

When the WHO took the unprecedented step in 1993 of declaring TB a global public health emergency,[98] the international community was woefully ill equipped for the challenge ahead. A study published by the WHO two years before found that the majority

of countries had no built-in mechanisms at all for monitoring treatment outcomes for TB patients. And in most of these countries, less than half of TB patients were even covered by treatment services.[99] As the Commission on Research for Development noted in 1990, ‘‘The magnitude of the tuberculosis problem is matched only by its relative neglect by the international community.”[100]

MYCOBACTERIUM TUBERCULOSIS AND DISEASE

To understand potential strategies for improved TB control, it is important to briefly discuss the responsible bacteria. (Figure 8.[101])

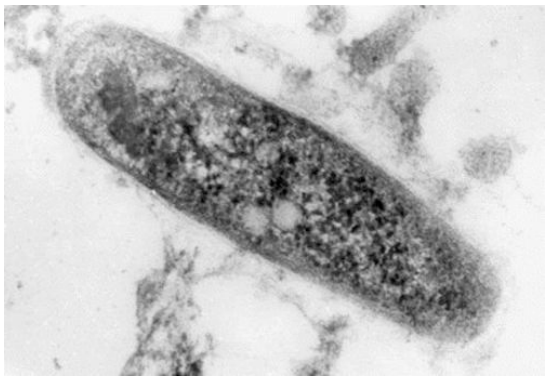

Figure 8. Thin section transmission electron micrograph of *Mycobacterium tuberculosis*.

Development of TB disease requires infection by bacteria belonging to the *Mycobacterium tuberculosis complex* (MTBC). This usually occurs when the potential host is exposed to airborne droplets containing MTBC bacilli. These infectious droplets are produced in large quantities when someone with an active pulmonary TB infection coughs and can remain in the air for several hours.[102,103] Upon inhalation by the potential host, the bacilli can establish infection within the lungs. Theoretically a single bacterium is sufficient for the establishment of a TB infection,[104] although prolonged exposure to multiple inocula of infective aerosols is usually required.[81] It is estimated that one patient with active pulmonary TB disease infects on average 10-15 additional people every year that they go untreated.[105]

Exposure to infectious droplets usually does not lead to TB infection, thanks to multiple host defenses that protect exposed individuals. Initially the bacteria must be able to avoid being trapped by the ciliated epithelium and respiratory mucosa.[106] And bacteria that reach the distal portions of the airways normally face a vigorous response from alveolar macrophages and other immune cells.

Macrophages and dendritic cells attempt to engulf (phagocytose) bacteria with which they come into contact. However, *M. tuberculosis* is able to survive within the phagosomal compartment inside these antigen-presenting cells (APCs) by preventing fusion of the phagosome with lysosomes (*i.e.*, phagosomal maturation), the acidic contents of which would destroy the mycobacteria.[107] The APCs, however, are still able to process bacterial peptides and present these antigens on specialized cell-surface receptors called major histocompatibility complexes (MHCs), of which there are two types, class I and class II. These complexes, when mounted with an antigen, function as means of activating other immune cells against the infection.[81] (Figure 9.[108])

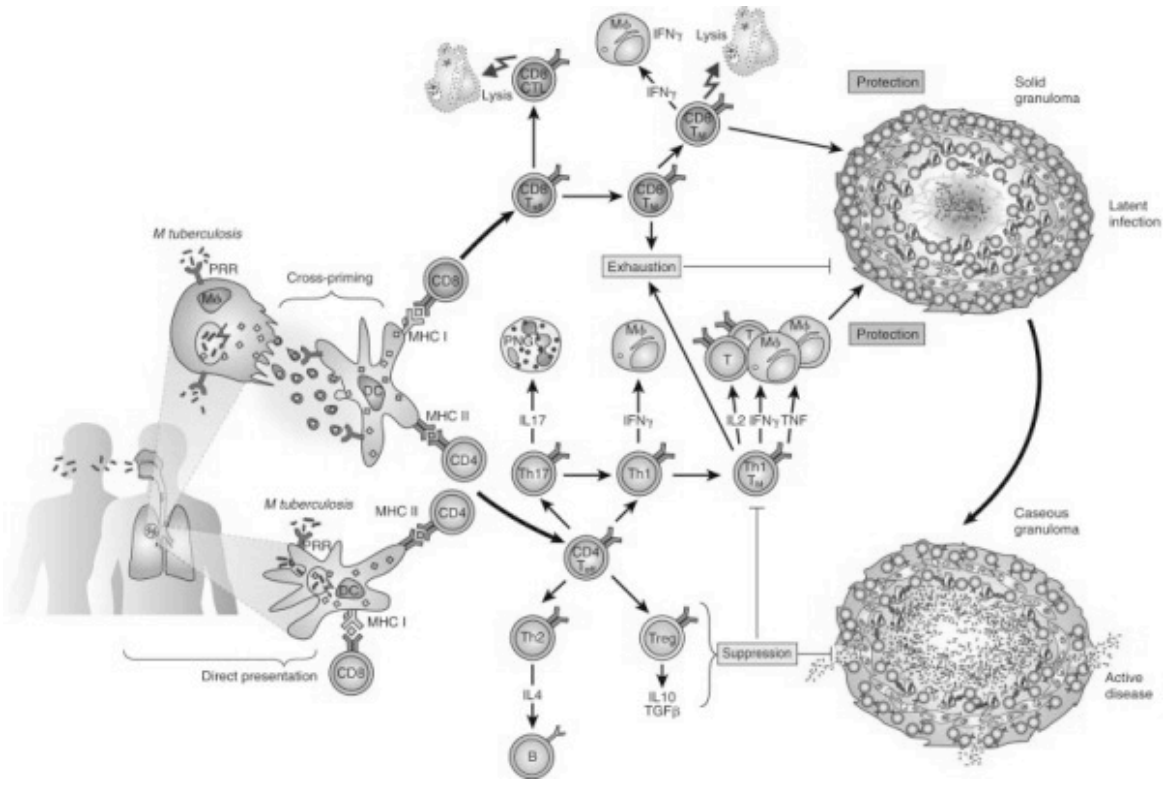

Figure 9. Overview of the immune response in tuberculosis.

T-lymphocytes encounter and are transformed by these antigen-presenting MHC molecules. T_1 lymphocytes that encounter the MHC class I on infected APCs are activated and release cytokines that recruit and activate macrophages and other immune cells against the infection.[109-111] Meanwhile, T_2 lymphocytes are activated by MHC class II molecules, leading to the release of other cytokines and activation of cytotoxic T lymphocytes.[109-111] In a process that is not well understood, activated macrophages are able to overcome the defenses of *M. tuberculosis* against phagosomal maturation and can eliminate their phagocytized mycobacteria.[6] Some macrophages even merge and become what are called multinucleated giant cells. In addition, activated cytotoxic T lymphocytes seek out and lyse infected macrophages and directly attack the mycobacteria that they contain.[6]

The convergence of activated macrophages and T-lymphocytes around the site of infection leads to the formation of an inflammatory nodule called a granuloma such that, even though usually a small number of mycobacteria survive in a dormant state and remain viable, they are sealed off from the rest of the body.[112-114] Inflammatory cytokine release precipitates the development of local fibrosis around the exterior zone of the granuloma, and eventually necrosis often develops within – usually described as "caseous" because of its crumbly, cheese-like consistency.[6,106]

Even if the bacteria are able to take hold, the vast majority of hosts are able to contain the infection and avoid any outward signs of disease. The infection is considered latent in these individuals. Latently infected individuals are noncontagious and even without any

treatment, 90-95 percent will never develop an active infection and experience TB disease.[108] (Figure 10.[115])

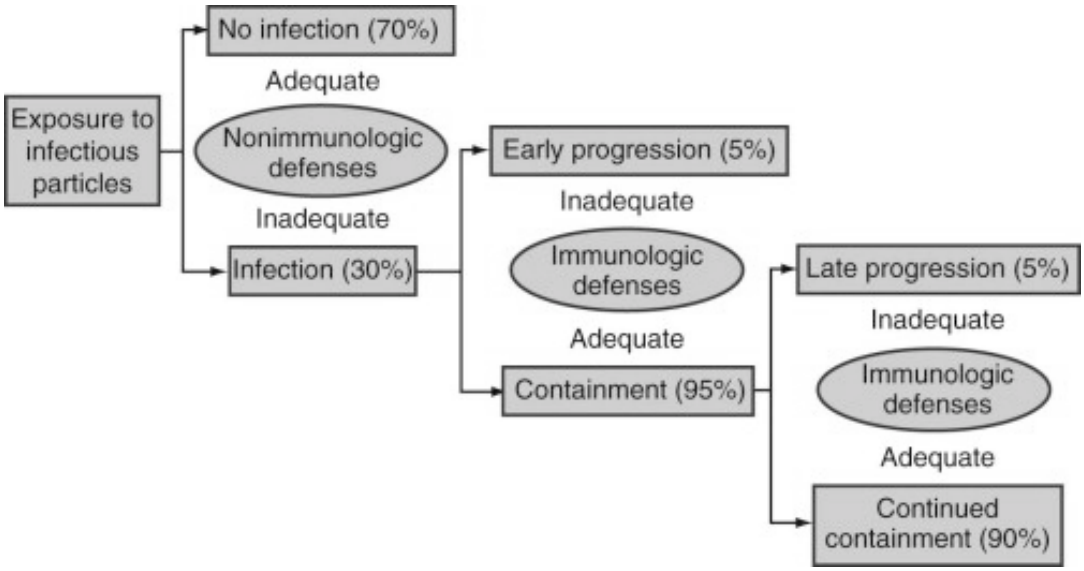

Figure 10. Consequences of exposure to an infectious source of tuberculosis.

In some cases, however, the infection is not contained. Those most at risk for developing disease are children and adults who are elderly, malnourished, co-infected with HIV, or otherwise immuno-compromised. Prolonged inflammatory activity leads to destruction of bronchial walls, creating cavitations in the lungs and allowing bacterial spread into the airways.[6] From there they can be coughed up and transmitted through the aerosol expectorations.[116] Bacteria can also spread within the respiratory tract and cause TB bronchopneumonia, pleuritis, empyema, and pulmonary effusions.[6] Moreover, coughed-up bacteria can be easily swallowed, placing patients at risk of intestinal TB infection.[6]

Inflammation can also disrupt pulmonary lymphatic and blood vessels, allowing TB bacteria to enter pulmonary and systemic circulation. This can lead to disseminated infection both throughout the lungs and potentially single or multiple other organ systems, such as the liver, bone marrow, spleen, adrenal glands, CNS, urogenital organs, gastrointestinal tract, skin and lymph nodes.[6,106] Widely disseminated infection is called *miliary* TB – its name drawn from the distinctive pattern observed on x-ray of many tiny spots – not unlike small millet seeds – that represent sites of infection.[6,106] (Figure 11.[117])

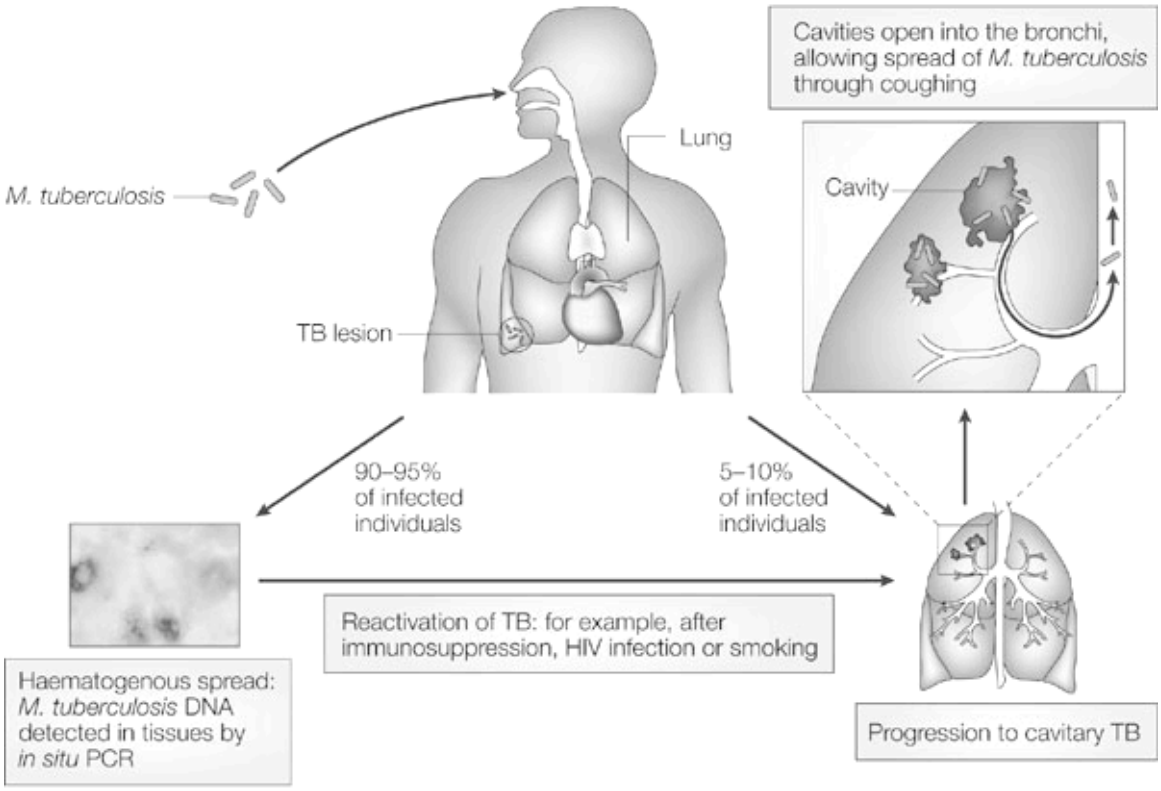

Figure 11. The natural history and spectrum of tuberculosis.

Given *M. tuberculosis's* predilection for pulmonary infection, cough is the most identifiable symptom of TB. Initially dry, the cough becomes increasingly productive as the disease progresses and more respiratory tissues become involved.[118] Patients cough up sputum, which is mucus from the lower airways, along with bacteria and inflammatory cells. Eventually blood begins to also streak the sputum as airway vasculature is damaged. Characteristic x-ray findings include indications of infiltrates in the lung apices that over time coalesce to form cavities as the lung tissue is progressively destroyed.[118]

In addition to cough, active TB infections are associated with chest pain, shortness of breath, fever, night sweats, fatigue, appetite loss, and unintentional weight loss.[6] Left untreated, pulmonary TB is fatal more than 50 percent of the time, typically following a progressive deterioration over 2 to 5 years. In patients with AIDS or other T-cell deficiency conditions the disease course can be much more rapid.[118]

Active TB infections in organ systems other than the lungs are less common than pulmonary disease but a significant problem among certain patient subgroups, particularly children and individuals with compromised immune systems. More than half of all individuals co-infected with both AIDS and TB experience extrapulmonary disease.[119] TB can infect nearly any part of the body, although the most common sites of infection outside the lungs are the lymphatics, pleura, bone and joints, genitourinary,

meningeal, and peritoneal.[120] (Figure 12.[115]) As such, TB can masquerade as numerous other types of disease depending on the organ systems affected, making correct diagnosis challenging. Potential symptoms and signs range from ascites, chronic cervical lymphadenopathy and lymphatic pleocytosis of the cerebral spinal fluid to monoarticular joint inflammation, pericardial effusion, and vertebral osteomyelitis.[119]

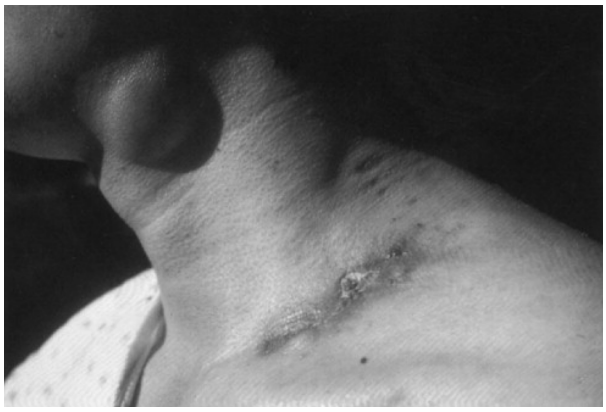

Figure 12. Tuberculous lymphadenitis. There is swelling of an anterior cervical node and a chronic sinus tract with cutaneous scarring in the supraclavicular area.

POVERTY AND TUBERCULOSIS CONTROL

Poverty, both relative and absolute, is a constant burden on human society and has not necessarily improved in modern times. At the beginning of the twentieth century the richest 20 percent of the world's population was nine times wealthier than the poorest 20 percent; that ratio had grown to 140 times as of 2009.[121] Between 1975 and 1995 the number of people living in extreme poverty in the world more than doubled. Currently, a quarter of humanity lives on less than $1 a day.[122] Moreover, poverty refers not only to low income but also the lack of material well-being, lack of adequate services, and lack of social and political empowerment.[6]

The burden of TB falls disproportionately on the most vulnerable populations – the poor and socially marginalized. Differences in TB burden strongly align with socioeconomic differences between countries, within countries and within communities.[123] Developing countries account for 95 percent of TB cases and 98 percent of deaths due to TB.[124] It is estimated that more than half of new TB patients live on less than $2 a day.[125] More than 15 percent of those who die of TB are in the economically productive age group of 15-49 years.[126] And just as poverty leads to poor health, poor health exacerbates poverty.[127,128] - It is estimated that in India alone the direct and indirect economic costs of TB total at least $3 billion per year.[129]

And even wealthy countries have areas of poverty, which beget health disparities. African-Americans and other non-whites in San Francisco, for example, have incidence rates of TB comparable to some high TB-burden countries of sub-Saharan Africa.[125] Study after study in both poor and wealthy countries has demonstrated the association between social marginalization and TB risk, whether examining prisoners, the homeless or certain ethnic minorities.[130-135]

Although the causal linkages between poverty and TB are not fully understood, poverty entails increased exposure to many risk factors for TB infection and development of disease.[123] These factors include exposure to people with active TB disease[136-138], HIV

infection[139], overcrowded living conditions[138], malnutrition[140], alcohol abuse[141,142], smoking[143,144], indoor air pollution exposure[145], and depression.[146,147] (Figure 13.[148])

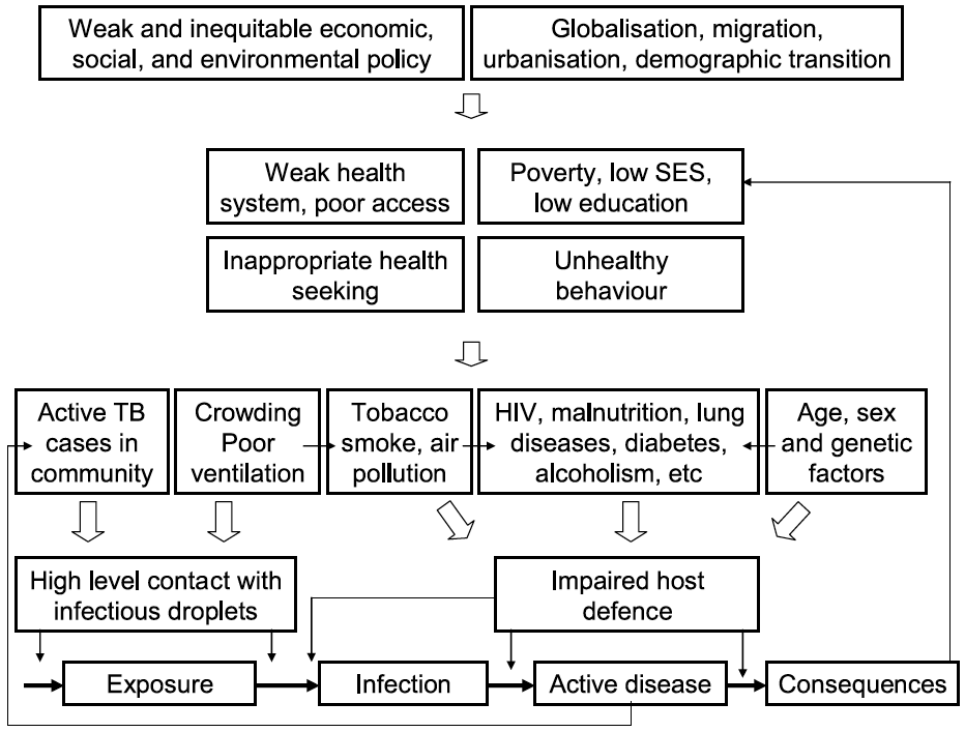

Figure 13. Risk factors and upstream determinants of TB.

The diagnostic process is costly and inconvenient for many patients, especially the poor, for whom their health concerns must complete with many other vital priorities.[126] Numerous potential barriers obstruct these patients from accessing diagnostic and other health services.[149]

Charges for health services[150-152]; the costs of transportation, accommodations and subsistence when seeking healthcare[153-157]; as well as the costs of lost income, lost productivity and lost time[127,128] can impose difficult, if not insurmountable economic hardships on poor patients. Many of the poor live in rural areas or other locations geographically remote from services for TB diagnosis and treatment[153-156], which is a particular challenge given that multiple trips to multiple providers are commonly necessary prior to and following diagnosis.[153,156,158,159]

Often sociocultural barriers also impede access to services – especially for women – including the threat of social stigma and the lack of knowledge about both TB and what TB services are available.[153,155,160] Furthermore, the health system itself can present significant barriers, such as a lack of responsiveness to patients, lack of culturally appropriate services, and poor coordination of services.[153,157,158,161,162]

DEVELOPMENT AND TUBERCULOSIS

High prevalence of TB in much of the developing world makes TB control a priority there, but the disease perversely erodes the kind of socioeconomic development that would serve indirectly to control it. Tuberculosis, above all else, is a disease of poverty, and the two combine in a vicious cycle. Overcrowded housing facilitates transmission, and factors such as malnutrition, drug addiction, poor hygiene and co-infection with other diseases contribute to reducing host resistance to TB infection.[125] And illness with TB undercuts one's ability to earn an income to maintain an adequate diet and have appropriate clothing and shelter.

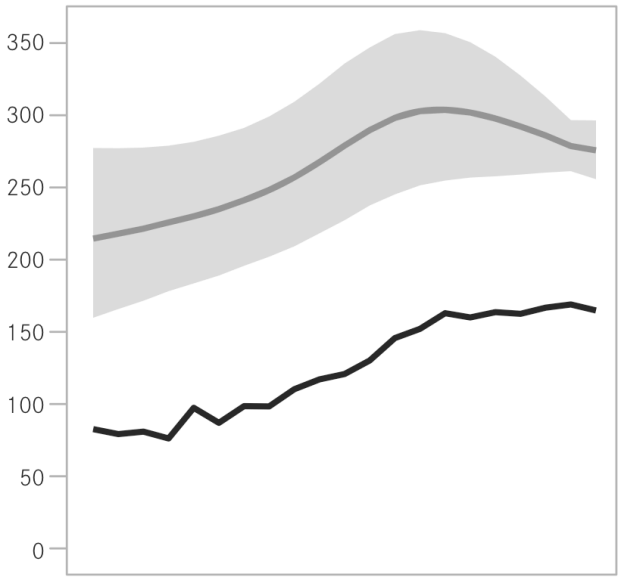

Figure 14. Case notification and estimated TB incidence rates per 100 000 pop in WHO Africa region, 1990–2010. Trends in case notification rates (new and relapse cases, all forms) (lower line) and estimated TB incidence rate (upper line). Shaded areas represent uncertainty bands.

In recognition of the close marriage between TB and poverty, the UN General Assembly included TB control targets among the indicators for measuring progress towards achieving the Millennium Development Goals for the reduction of world poverty.[163] Reflected in the goals also set by the World Health Assembly and the Stop TB Partnership of the WHO, these targets aim to halve TB prevalence and death rates by 2015, compared with 1990 levels, and eliminate TB as a public health problem by 2050.[164] Process targets identified in order to attain these goals include detection of at least 70 percent of new smear-positive pulmonary TB cases and successful treatment of at least 85 percent of these cases. In 2010 the WHO reported that new case detection had reached a rate of 63 percent and that treatment success was 86 percent.[164]

Although case detection has significantly improved in many parts of the world, efforts have stalled in important regions of high TB burden. In 2010, for example, the estimated case detection rate in the WHO Africa Region was 60 percent.[78] (Figure 14.[78]) Moreover, TB incidence is falling at a rate of less than 1 percent per year. At this point, in order to reach the goal of eliminating TB by the year 2050, incidence would have to fall by an average of 16 percent annually over the next 40 years.[123]

TESTS FOR DIAGNOSING TUBERCULOSIS

A number of technologies are available for screening for or diagnosing TB: microscopy, mycobacterial growth detection, nucleic acid amplification testing, the tuberculin skin test, and interferon-γ release assay. Microscopic detection of microbes in clinical specimens is the oldest laboratory-based diagnostic strategy for infectious diseases and remains the most widely used method of diagnosing TB.[6] Since most TB disease involves pulmonary infection and productive cough, microscopy typically involves examination of a sample of sputum that has been smeared and fixed onto a slide.

Although *M. tuberculosis* is technically a Gram-positive bacterium based on its cell wall structure, in practice it is very difficult to detect under a microscope using Gram stain.[125,165] Identification is therefore done using stains that take advantage of the mycobacterium's resistance to decoloration by acid – thus the term "acid-fast" bacilli, or AFB – which is due to the high lipid content of the mycobacterial cell wall.[125] In Ziehl-Neelsen (ZN) staining, the specimen is exposed first to carbol fuchsin and then to an alcohol-acid mix. Carbol fuchsin is normally washed away by the alcohol and acid, but mycobacteria hold onto it. When the specimen is finally counter-stained with methylene blue, mycobacteria appear pink against a bluish background.[166] (Figure 15.[115]) In Auramine-O staining, auramine instead of carbol fuchsin enters the bacteria and binds to DNA.[167] Auramine is visualized through the use of a fluorescence microscope while ZN-stained slides are examined with a standard light microscope.[6] Ziehl-Neelson-stained smears must be viewed under high-power magnification while auramine-stained smears can be viewed at lower magnifications because the fluorescence is easier to detect.[6]

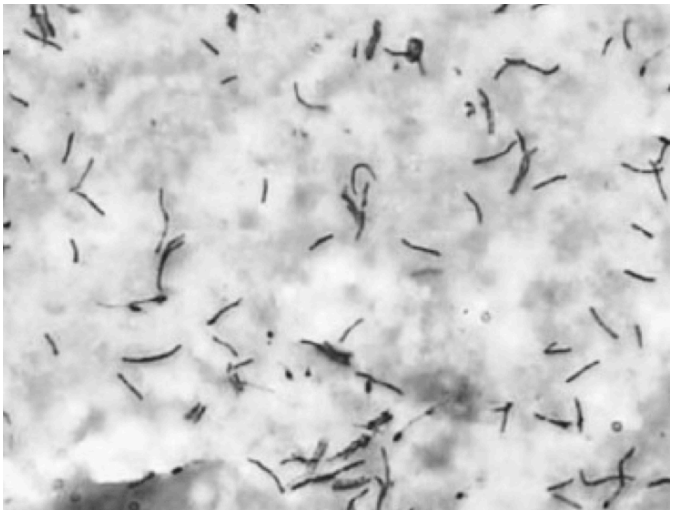

Figure 15. Mycobacterium tuberculosis as seen via sputum smear microscopy with Ziehl-Neelsen staining.

Specimen culture is the most sensitive manner to detect the presence of mycobacteria and is still considered a gold standard among TB diagnostics. However, it is not without very important disadvantages. *M. tuberculosis* is very slow growing, requiring up to 6-8 weeks for a determination to be made using conventional solid culture media.[125]This is far too long to wait to initiate treatment. Liquid culture with a growth indicator can greatly reduce culture time, improve sensitivity and be paired with drug-susceptibility testing (DST).[168-170] However, culture and the necessary specimen processing constitute a complex process requiring well-trained personnel and a well-equipped laboratory, which

makes this diagnostic approach expensive and difficult-to-implement in most high-burden countries.[171] (Figure 16.[172])

An alternative to microscopy and culture is the molecular detection of *M. tuberculosis* genes through the use of polymerase chain reaction (PCR) and other amplification techniques – which as a group are referred to as nucleic acid amplification testing (NAAT). These assays for TB have demonstrated excellent sensitivity, specificity and speed.[173-175] In addition, they are considered more effective than culture at detecting TB in specimen from smear-negative patients.[176]

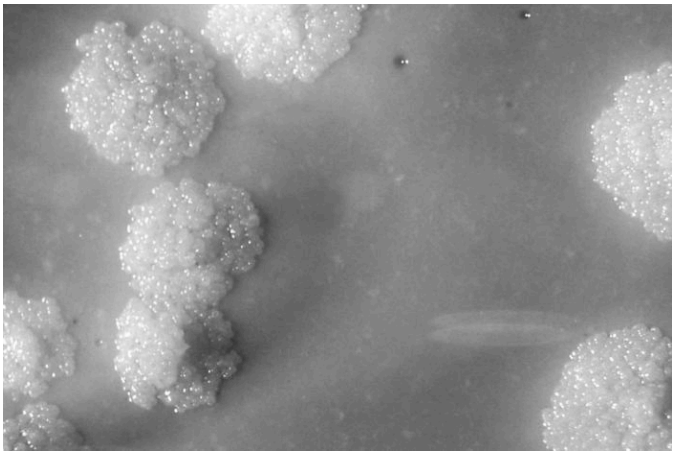

Figure 16. This close-up of a *Mycobacterium tuberculosis* culture reveals the organism's colonial morphology.

Nucleic acid amplification assays have historically involved a cost and complexity that have prevented implementation in low-resource countries. The recently developed and WHO-endorsed Xpert MTB/RIF may change this trend. Xpert MTB/RIF is a sensitive and specific automated NAAT assay that uses molecular probes and real-time PCR to detect both *M. tuberculosis* and rifampicin resistance. No sputum preparation is required, minimum laboratory expertise is necessary, test results can be available in less than two hours and the test performs well in populations where both TB and HIV are endemic.[177-179] The device offers to make drug resistance testing more available and to bolster TB case finding. However, limitations remain: the expense of the device and diagnostic cartridges, the relatively short shelf-life of the cartridges, the operating temperature and humidity requirements, the need for constant electrical supply, the device's unknown durability, and its need for yearly servicing.[180]

For more than 100 years, the tuberculin skin test (TST) has been the only screening tool available for evaluating latent infection with TB. A standard TST involves the intracutaneous injection of 0.1 mL of purified protein derivative (PPD) on the forearm – although any accessible area is adequate – that is conventionally read 48 to 72 hours later. Under most circumstances, the observation of an induration 10 mm or more in diameter is considered indicative of infection with *M. tuberculosis*.[115] Interpretation of the TST results, however, can be confounded by patients who have been exposed to nontuberculous mycobacteria, vaccinated with BCG, or who have any number of conditions that can interfere with cell-mediated immunity, from HIV and corticosteroid use to malnutrition and old age.[115]

In an effort to surmount some of the limitations posed by the TST, a new approach to testing for immune system exposure to *M. tuberculosis* has been developed over the past 10 years – the interferon-γ release assay (IGRA). This assay measures interferon-γ release by peripheral blood mononuclear cells after in-vitro exposure to TB-specific antigens.[181] The major advantage of IGRA tests over the TST is that they are significantly more specific and are unaffected by BCG vaccination. Moreover, their sensitivity is comparable or better than the TST.[181] There are currently two commercially available IGRA tests, T-SPOT and QuantiFERON-TB Gold, and they have become the gold standard in wealthy countries for identifying people previously exposed to *M. tuberculosis*. (Figure 17.[182])

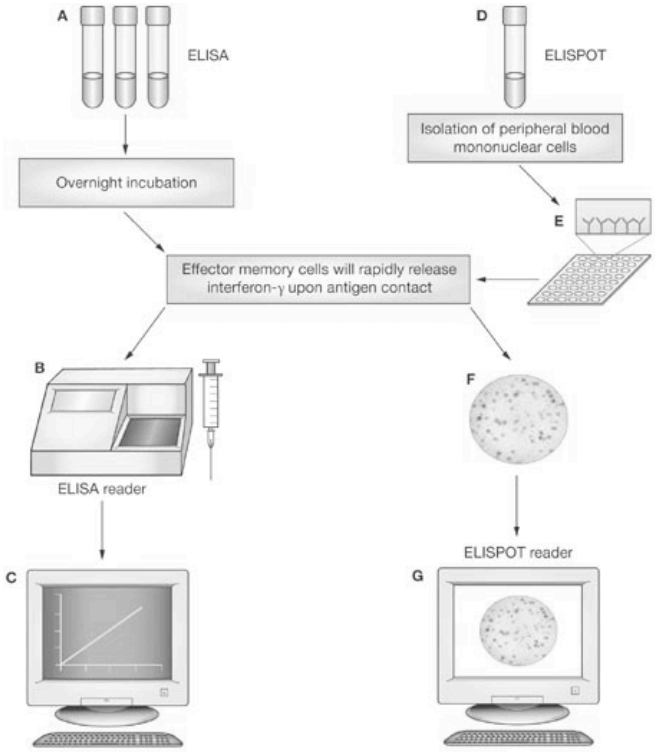

Figure 17. T-cell interferon-γ release assays for the diagnosis of *M. tuberculosis* infection.

Although offering advantages over the TST, IGRA tests have variable sensitivity and, like the TST, are less reliable among patient populations with higher rates of immunosuppressive conditions.[181] This is problematic given that individuals with TB in high-burden countries are also at higher risk for advanced disease, malnutrition, and co-infections such as HIV.[183] Moreover, immune-based tests such as IGRA tests and the TST measure a cellular immune response to exposure to *M. tuberculosis* and do not directly detect the bacteria or allow distinction between recent or distant exposure.[181] Therefore, results of these tests cannot be used alone to diagnosis active TB disease, nor can they conclusively rule out disease in a patient with other indications of possible infection.[181,184,185]

ONGOING CHALLENGES TO MICROSCOPY ACCESS

Despite the development of improved diagnostics and an estimated $1 billion spent worldwide annually on testing for TB[186], there has been little impact in endemic regions. TB patients can face delays of many weeks[156,159,187-189], and dropout from the diagnostic or treatment process is common.[190-194] Dropout rates as high as 13 percent have been observed among suspected TB cases who failed to return after providing an initial sputum sample – including over 5 percent of patients who were found to have smear-positive TB.[190] And even when TB-infected patients succeed in receiving a diagnosis, many never actually begin treatment.[195]

Weaknesses of the microscopy process contribute significantly to suboptimal case detection. The sensitivity of sputum-smear microscopy is probably in the range of 60–70 percent for HIV-negative adult populations. Considerable variation exists, however, with studies suggesting sensitivities ranging from 30 percent to over 95 percent[196] – with fluorescence more sensitive than conventional microscopy.[196] Numerous factors can undermine sensitivity including duration of slide examination,[154] the experience of the microscopist,[197,198] the concentration of bacteria in the sample used,[46] the preparation of the sputum smear, [199] and the number of specimens examined from each patient.[200] Microscopy is also significantly less sensitive among patient populations with a high HIV burden,[201] and does not provide a means for differentiation between mycobacterial species or for the evaluation of drug-susceptibility.

Poor infrastructure, limited available trained staff, and shortages of reagents, microscopes and other important materials and equipment often hobble microscopy services. Microscopy labs at peripheral health centers are often comprised of no more than a few people in a single room with badly maintained microscopes, unreliable electricity and scarce clean water.[202,203] (Figure 18.[204]) Lab workers are too few in number to typically handle the high-volume workloads.[202] In addition to impeding work, scarce supplies such as gloves and masks place lab employees at elevated risk from infectious exposures.

Figure 18. Typical one-room microscopy lab in rural Uganda.

Microscopy labs frequently receive limited support from the government. The majority of high-burden countries spend less than $0.50 per capita on their national TB control programs.[78] Typically, national TB programs provide few education or training programs, inadequate logistical support, insufficient monitoring of microscopy test quality, and outdated or nonexistent national standards for laboratory testing.[202]

Often, microscopy and other lab-based diagnostic services are simply not available. Of the 36 countries in 2010 that had the highest TB or MDR-TB burden, 20 failed to meet the recommended minimum capacity of one laboratory for culture and drug-susceptibility testing per 5 million population.[78] And among the 22 high TB-burden countries that

account for 80 percent of the world's TB, more than one-third fail to meet the minimum concentration of microscopy centers needed to meet population needs, 1 per 100,000 individuals.[78] (Figure 19.[78]) Experts estimate that at least a total 13,000 more microscopy centers would need to be built in high burden countries to meet demand.[205]

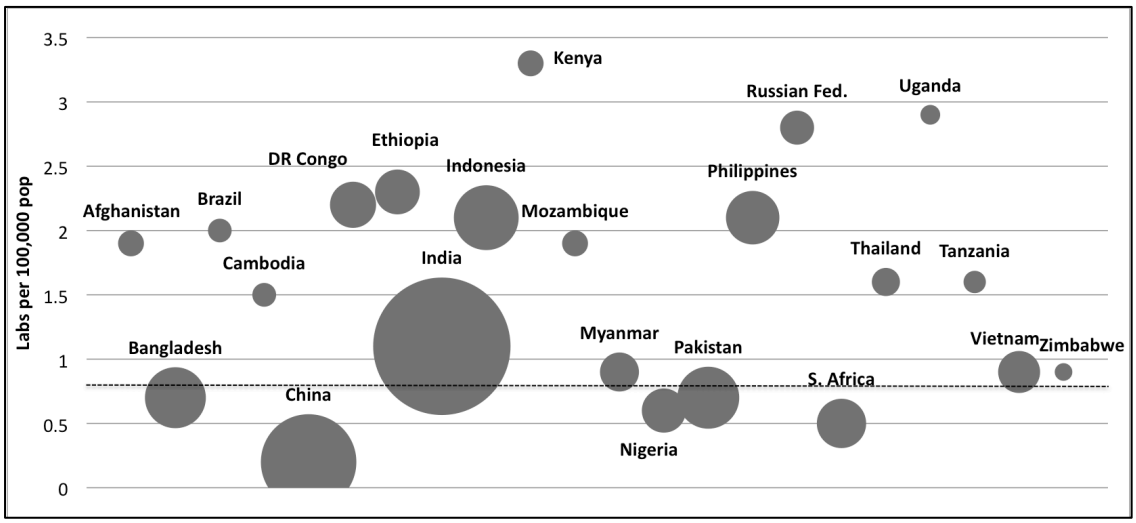

Figure 19. Density of microscopy labs in highest TB-burden countries. Circle area represents relative estimated total tuberculosis cases.

Geographic maldistribution further exacerbates problems of access to microscopy services. Like with all lab-based services, microscopy centers are typically overwhelmingly concentrated in more affluent, urban areas, leaving large swathes of the country with far more limited access to these services. Numerous studies have found that rural residence is a risk factor for late TB diagnosis.[153,188,206] Moreover, even within urban areas, inequitable distribution of resources can leave some neighborhoods well-serviced while higher-risk urban slums have far more limited access.[149]

OPPORTUNITIES FOR IMPROVED MICROSCOPY

Given that for at least the next 5 to 10 years a new diagnostic test is unlikely to be widely available to TB control programs in developing countries[207], it is a research imperative to identify ways in which, in the meantime, the quality of existing services can be improved in the regions with the greatest need and the least resources. The WHO and the TB research community have given greater attention in recent years to strategies for optimizing microscopy services. A number of important strategies have been identified for enhancing microscopy, including physical or chemical sputum processing, fluorescence microscopy (FM) instead of conventional microscopy, and reduced serial sputum specimen collection.

In conventional preparation of a sputum smear slide, a small portion of sputum is smeared directly onto the glass microscopy slide without any intermediary processing. This is called direct (or unconcentrated) microscopy. A number of physical and chemical

methods of processing sputum prior to creation of the smear have been investigated for potential value in improving the sensitivity of microscopy. These methods include centrifugation, sedimentation, and the application of bleach. Centrifugation concentrates the cellular components of the sputum, facilitating identification of any AFB potentially present when the smear is examined. The mechanism of chemicals such as bleach is unclear but they may serve to concentrate the sputum or they may improve sensitivity by digesting debris and hereby providing for a clearer field of view.[208]

Although previous systematic reviews have suggested that sputum processing may provide improved sensitivity[199], more recent research has cast doubt on many of these techniques[209], particularly their value at the peripheral health service level.[210]

The advent of FM has provided new potential for improved sensitivity and other enhancements compared to conventional light microscopy (LM). A large systematic review found that the sensitivity for detection of AFB is on average 10 percent higher with FM as compared to conventional microscopy and that the specificity of the two techniques is comparable (at nearly 100 percent).[196] (Figure 20.[211]) FM may also perform better with specimens from HIV-positive patients, a population in which TB is currently often missed.[212]

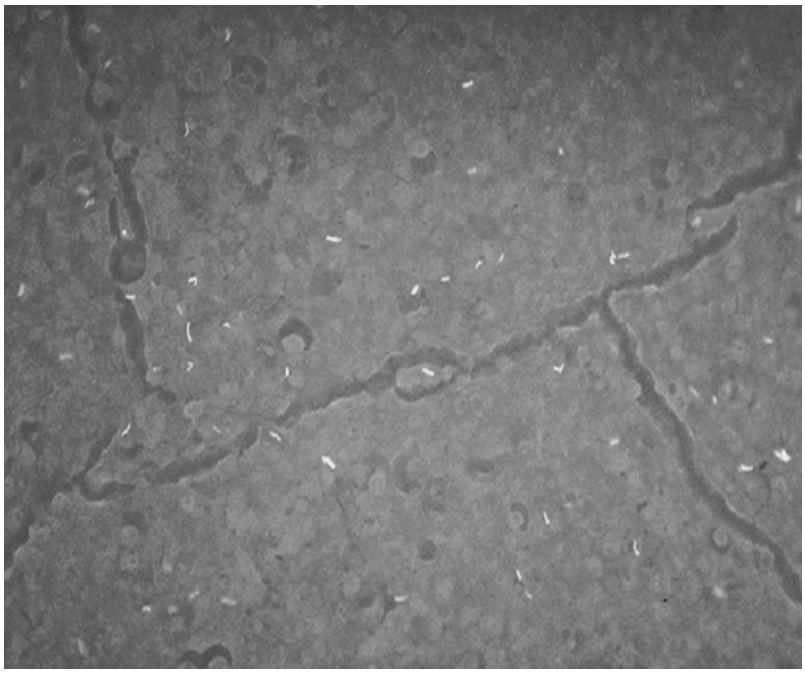

Figure 20. Acid-fast bacteria as seen by fluorescence microscopy.

Moreover, because smears can be examined at lower magnification – meaning the field of view is larger – FM provides the advantage of faster slide reading compared to conventional microscopy using ZN staining.[213] One recent study found a 4-fold difference in examination time between conventional microscopy and FM. Such reductions could have a substantial impact on the management of workload at laboratories in low-income countries.

Beyond addressing the variable sensitivity of smear microscopy, efforts have been made to identify strategies for alleviating the high rates of patient dropout during the diagnostic process. Prior to 2007, international TB guidelines advised the microscopic examination of three serial sputum specimens for evaluation of individuals suspected of having pulmonary TB. According to this convention, the first sputum sample was collected from the patient when s/he initially presented, the second the next morning, and a third the

following day (the so-called 'spot-morning-spot' system). In addition, a case was considered positive after at least two smear examination results were positive for AFB.

In 2007, following a series of studies and systematic reviews that demonstrated both the negligible value-added of the third specimen[200,214-217] and the improved sensitivity gained when one positive smear instead of two was the threshold for identifying a case as positive,[217] the WHO endorsed a new two-specimen ('spot-morning') case-finding strategy as well as a lower threshold for positive case identification.[218,219] It is worth noting that this two specimen-based approach held valid among patient populations with high HIV prevalence.[220]

More recent research found that examination of two sputum samples collected consecutively on the same day provides comparable performance to the two-day, two-sample approach[221-223] and allows the patient to avoid multiple trips to the laboratory prior to completion of the evaluation. Following a systematic review and meta-analysis of published and unpublished data from seven diagnostic studies, in 2009 the WHO changed its case-finding policy yet again and recommended that countries who have effectively implemented the two-day case-finding approach should move towards adoption of a strategy involving the collection of two sputum specimen collected consecutively on the same day.[224]

Further simplification of the case-finding strategy appears possible. A recent study from Uganda found that in a setting with a high burden of TB and HIV infection, microscopic examination of two smears prepared from the same sputum specimen was equally sensitive to the now-standard two specimen-based approach.[225]

FUTURE DIRECTIONS

Mobile phone technology and imaging analysis algorithms offer some of the most promising opportunities for improving access to microscopy-based diagnostic services in low-resource, rural areas where TB is endemic.

In the past decade, mobile telephony has increased at three-times the rate of other modes of telecommunications such as landline telephones and the internet.[226] Over 76 percent of the world's population is estimated to have a mobile phone subscription, and even in the developing world, penetration is estimated at over 67 percent.[226] Moreover, subscriptions in developing countries represent well over the majority of all subscriptions.[226] Although mobile phone coverage does not necessarily mirror multimedia messaging service (MMS) availability, recent studies suggest widespread availability, for example, throughout most of Africa.[227] (Figure 21.[227])

Numerous efforts have arisen in recent years to leverage the wide availability of mobile phones and MMS services to increase access to microscopy-based diagnostic services. Recent studies have demonstrated the feasibility of using mobile phones to capture images of various pathologies or microscope specimen and transmit the compressed images by MMS to remote sites for evaluation by specialists.[227-229] Other researchers have focused on transforming the mobile phone into a mobile microscope. Mobile phones have been successfully modified into lens-less microscopes with resolution adequate for imaging various micro-particles, platelets, red and white blood cells, and the waterborne parasite *Giardia lamblia*.[230,231] Mobile phone-mounted light microscopes have also been developed that are capable of imaging *P. falciparum*-infected and sickle red blood cells through LM and *M. tuberculosis*-infected sputum samples in fluorescence with LED excitation.[232]

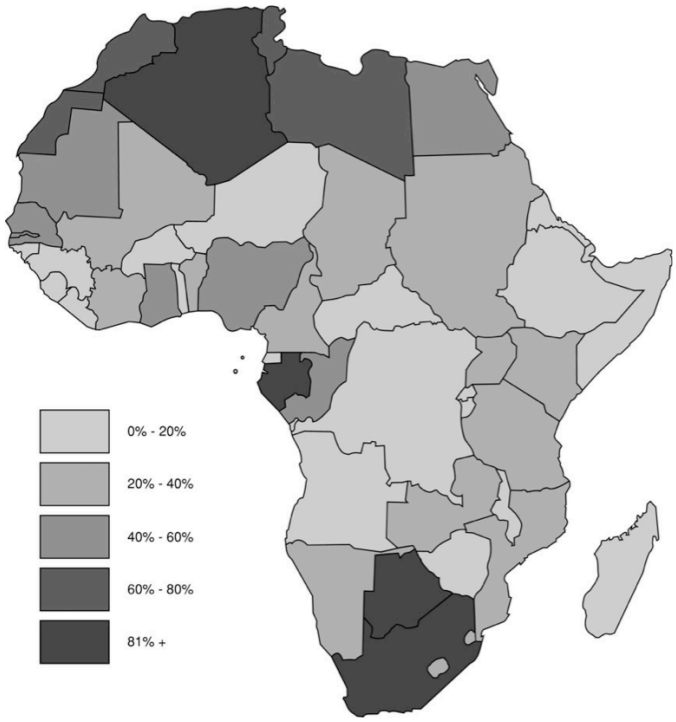

Figure 21. Mobile phone subscriptions with declared availability of MMS, per 100 pop.

In addition to telemedicine, the utilization of mobile phones and other digital imaging devices for microscopy is providing an opportunity for the use of computer algorithm-based image analysis in order to screen imaged specimens for pathogens. Several groups, for example, have explored automated detection of *M. tuberculosis* in images from FM[233-236] as well as LM.[237-239] Computer-driven image analysis could eventually replace specimen examination by human microscopists and allow point-of-care microscopy-based diagnostic assessments in places where such services are currently limited or unavailable.

SECTION 2:

EVALUATION OF A NOVEL, MOBILE DIGITAL FLUORESCENCE IMAGING DEVICE FOR MICROSCOPIC IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS*

Asa Tapley[1,✦], Neil Switz[2,✦], Clay Reber[2], J. Lucian Davis[3,4,5], Cecily Miller[3], Daniel Fletcher[2], Adithya Cattamanchi[3,4,5]

[1] UC Berkeley-UCSF Joint Medical Program, Berkeley, CA, USA
[2] Bioengineering, University of California, Berkeley, Berkeley, CA, USA
[3] Division of Pulmonary & Critical Care Medicine and
[4] Francis J. Curry International Tuberculosis Center, Department of Medicine, San Francisco General Hospital, University of California San Francisco, San Francisco, California, USA;
[5] Makerere University-University of California San Francisco Research Collaboration, Kampala, Uganda
✦ These authors contributed equally to this work.

Address for correspondence:
Adithya Cattamanchi, M.D., M.A.S., San Francisco General Hospital, Room 5K1 – Pulmonary Division, 1001 Potrero Avenue, San Francisco, CA 94110. E-mail: acattamanchi@ medsfgh.ucsf.edu

AUTHOR CONTRIBUTIONS

AT helped design the study, participated in data collection and analysis, and wrote the first draft of the manuscript. NS developed CellScope hardware and software, and helped train CellScope readers. CR participated in data collection. JLD helped design the study and revise the manuscript. CM analyzed the data. DF helped conceive of the study and coordinated development of CellScope hardware and software. AC helped conceive of the study, coordinated data analysis, and helped revise the manuscript.

INTRODUCTION

Tuberculosis (TB) continues to be a worldwide scourge, responsible for more deaths than any other infectious disease besides HIV/AIDS and overwhelmingly affecting the poor.[171] As part of the Millennium Development Goals established by the United Nations in 2000, the international community set new case detection targets based on epidemiologic models that estimated TB elimination would require at least 70% detection of smear-positive patients.[2] Now, as then, sputum-smear microscopy is the most widely available method of diagnosing TB.[78] However, despite enormous investments in TB control over the subsequent decade, in 2010 alone an estimated 8.8 million people became newly infected with TB, one-third of whom went undiagnosed.[78] This failure is largely due to smear microscopy-based detection rates of 60% or less in high TB burden regions such as Africa and Southeast Asia.[78] Although recent advances in molecular detection methods suggest promising future alternatives to microscopy[177], to date these tests rely on expensive equipment that is poorly suited for peripheral, low-resource areas.[180] Consequently, improved access to high-quality smear microscopy is a global priority.[164]

Rapid, relatively simple and highly specific in regions with a high prevalence of TB[196], smear microscopy is undermined by several factors. Limited availability of microscopy services plagues many regions[78], and the sensitivity of microscopy can vary significantly, depending in part on the diligence with which specimens are collected, slides prepared, and smears examined.[202] In low-resource settings, poor quality assurance as well as shortages of reagents, functional microscopes, and skilled microscopists further reduce the effectiveness of services.[202,240]

Beyond the introduction of fluorescent staining, which has proven to increase sensitivity[196], the methods of smear microscopy has changed little over the past century and still consist of direct visualization of a stained smear under a microscope by a trained expert. By contrast, pathology in high-income countries increasingly involves the use of digital images viewed on high-resolution monitors, an approach that has maintained or improved diagnostic accuracy.[241,242] Whereas the type of expensive, bulky technology employed in digital pathology is impractical for the low-resource settings where TB is endemic, advances in opto-electronics have brought the promise of improved TB diagnosis through microscopy-based digital imaging devices that are both portable and low cost.

Recent studies have described a number of novel portable digital microscopy devices[232,243-245], which exploit new technologies such as tiny, long-lasting light-emitting diodes (LEDs)[246] and complementary metal-oxide semi-conductor (CMOS) image sensors with high sensitivity and fine pixel size.[247] However, these studies have been largely proof-of-concept in nature. In this study we describe the first time such a digital microscopy device has been evaluated for diagnostic accuracy compared to conventional methods.

We report here on the performance of CellScope, a portable digital imaging device using LED illumination and capable of both light microscopy (LM) and FM. Our primary objective was to determine whether the diagnostic accuracy of LED FM with CellScope was non-inferior to conventional methods of LED FM involving commercial fluorescence microscopes.

MATERIALS & METHODS

DEVICE DESCRIPTION

The CellScope is a small (20 x 20 x 10 cm), light (3 kg) portable battery powered digital fluorescence microscope (Figure 1). The system is built around a 20× 0.4 numerical aperture (NA) microscope objective providing 0.76 μm resolution and a 0.64 x 0.49 mm sample-referenced field of view. Fluorescence excitation is provided by a 1-watt, 460nm LED via a 0.65 NA condenser. Typical fluorescence exposure times for the 8-bit CMOS image sensor (quantum efficiency 45%) are 100-500 ms, depending on staining, but with negligible dark noise in all cases. Images are samples at above Nyquist frequency, allowing images to be blown up with proper interpolation for viewing at apparent magnification of 100-200×.

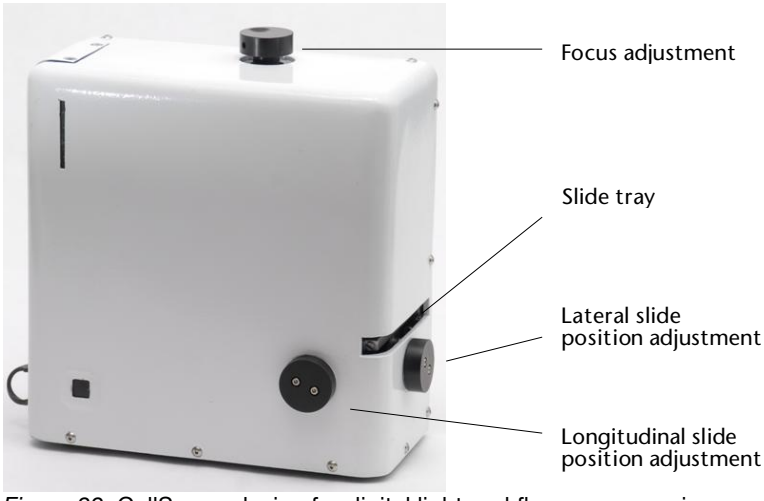

Figure 22. CellScope device for digital light and fluorescence microscopy.

Focus and slide position were adjusted manually by knobs. All other operations are performed via password-protected customized software installed on a low-cost E10IL2 Notebook laptop computer (EliteGroup, Taipei, Taiwan; 1.6 GHz processor, 1 GB of RAM, and 26.6-cm transistor liquid crystal display with 1024x600 pixel resolution) running Microsoft Windows operating system (Microsoft Corp, Redmond, WA) and connected to the device via a standard universal serial bus (USB) 2.0 cable.

SAMPLE SELECTION

This study included smear microscopy slides prepared from sputum specimens submitted by 585 consecutive adults (age ≤ 18 yr) with cough between 2 and 24 weeks' duration

enrolled in the International HIV-Associated Opportunistic Pneumonias (IHOP) study at Mulago Hospital (Kampala, Uganda) between September 2007 and January 2008. We excluded slides only if corresponding culture results were unavailable.

Details of patient enrollment and evaluation for the IHOP study have been published previously.[212,225] Briefly, patients submitted two sputum samples to the Uganda National Tuberculosis Reference Laboratory (NTRL) for all mycobacterial studies. Experienced NTRL technicians prepared smears on glass slides, stained them using auramine-O, and interpreted results using LED FM (Lumin; LW Scientific, Lawrenceville, GA; magnification × 400) in accordance with standard algorithms.[225,248,249] In addition, the technicians cultured sputum sediment on Löwenstein-Jensen media.[225,249] After initial smear examination, all slides were stored in opaque, sealed slide boxes and transported to the second study site, a temperature- and light-controlled laboratory space at UC Berkeley where all further aspects of the study were completed.

DIGITAL FLUORESCENCE MICROSCOPY

Two pre-doctoral students (AT and CR) blinded to the original FM and culture results re-examined smears using two identical CellScope devices. Both students had no prior experience with microscopy, but were provided training on operation of the device and AFB identification, including instruction by a CellScope engineer (NS), review of WHO and IUATLD smear microscopy guidelines, and instruction on staining and slide reading protocols by expert microscopists at the San Francisco Department of Public Health.

The students re-stained slides in batches[250] (≤ 20 slides) using the 3-minute F.A.S.T. auramine-O stain kit (QBC Diagnostics, Port Matilda, PA),[251] and read slides within 24 hours of staining. A wax pencil was used to circumscribe each smear to provide a highly visible boundary line during reading.

Slide reading and the imaging with the CellScope device was done according to a standardized protocol (see Online Supplement for details). Reading time was collected for all slides. 535 consecutive slides were divided equally between the two students, read once, and scored dichotomously as positive or negative based on the detection of AFB within one smear length. The remaining 50 slides were read twice by each student and scored semi-quantitatively using an adaptation of the IUATLD/WHO semi-quantitative grading system for LED FM.[249] An over-labeling system was implemented by a study coordinator not involved in slide reading for purposes of blinding (Figure 2).

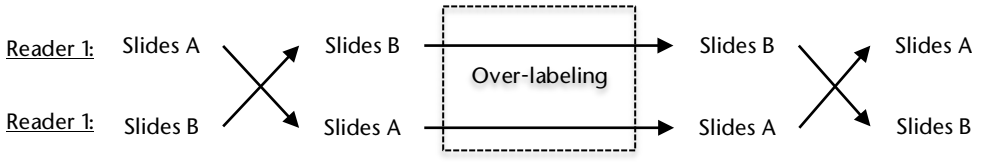

Figure 23. Reading strategy for subset of 50 semi-quantitatively scored slides. The subset was divided into two sets of 25 slides each (denoted here as slides A and slides B).

STATISTICAL ANALYSIS

Our primary objective was to demonstrate that the sensitivity and specificity of CellScope-based LED FM performed by inexperienced readers was non-inferior to conventional LED FM performed by experienced technicians. The sample size for the study was fixed by the number of slides available for the analysis and supported a non-inferiority margin of 15% with 80% power and a 5% significance level based on a one-sided equivalence test of correlated proportions (Power Analysis and Sample Size; NCSS, Kaysville, UT). In calculating this, we assumed that the sensitivity of conventional LED FM would be 60% using mycobacterial culture as the gold standard[252] and that the difference in sensitivity and specificity between LED FM with CellScope and conventional methods would be 0%.

We calculated the sensitivity and specificity of the smear microscopy techniques in reference to culture results. We compared the sensitivity and specificity of the techniques using McNemar's paired test of proportions and reported the exact binomial 95% confidence interval (CI) for sensitivity and specificity differences. We evaluated correlation between the techniques as well as intra- and inter-reader reliability using weighted kappa statistics based on the IUATLD External Quality Assessment guidelines.[253] We performed all data analysis using STATA 10.0 software (Statacorp LP, College Station, TX) and Microsoft Excel 2008 (Microsoft Corp, Redmond, WA).

ETHICS

The study protocol was approved by institutional review boards at Makerere University, Mulago Hospital, the Uganda National Council for Science and Technology, and the University of California, San Francisco. University of California, Berkeley determined that the proposed study was not human subject research based on the use of de-identified microscopy smears.

RESULTS

STUDY POPULATION

Of the total 585 slides read, 60 slides (10%) lacked associated culture results or other data and were excluded from analysis. Table 1 shows the characteristics of the patients corresponding to the remaining 525 slides. Two hundred forty-six (47%) were women and 380 (72%) were HIV-infected. The median age of the patient population was 32 years (interquartile range [IQR] 27-39) and the median $CD4^+$ T-lymphocyte count among HIV-infected patients was 55 cells/μl (IQR 19-175). *M. tuberculosis* was cultured from 227 (39%) specimens.

Seven of the 60 slides excluded from analysis were among the 50 slides semi-quantitatively scored, leaving 43 from this subset for analysis. Sex and age characteristics were no different between patients represented by this subset compared to the remaining 482 (P = 0.32 and 0.89 respectively). HIV infection, however, was less prevalent (53 vs.

72%, difference 21%, 95% CI 7-34%), as was culture positivity (23 vs. 41%, difference 18%, 95% CI 2-33%).

TABLE 1. DEMOGRAPHICS AND CLINICAL CHARACTERISTICS

Characteristic, N (%)	Study cohort (N = 525)
Male	246 (47%)
Age in years, median (IQR)	32 (27 to 39)
HIV seropositive	380 (72%)
CD4 T-lymphocyte count, median (IQR)	55 (19 to 175)
Culture positive	207 (39%)

Definition of abbreviation: HIV = human immunodeficiency virus; IQR = Interquartile range

LED FM WITH CELLSCOPE VERSUS CONVENTIONAL METHODS

Using culture as a reference standard, conventional LED FM was more sensitive (70 vs. 63%, difference 7%, 95% CI 1-13%) than LED FM with CellScope (Table 2). We also found that conventional LED FM was more specific (93 vs. 85%; difference 7%, 95% CI, 3-12%) than with CellScope. However, these differences, including their confidence intervals, were within the preselected 15% margin of non-inferiority. Sensitivity was similar between both readers ($P = 0.45$), whereas specificity was slightly different (90 vs. 80%, difference 10%, 95% CI 2.2-18%).

TABLE 2. DIAGNOSTIC ACCURACY OF LIGHT-EMITTING DIODE FLUORESENCE MICROSCOPY WITH CELLSCOPE VERSUS WITH CONVENTIONAL METHODS

	CellScope LED FM	Conventional LED FM	Difference (95% CI)	P value
Overall (N = 525)				
% Sensitivity	63	70	7 (1 to 13)	0.029
% Specificity	85	92	7 (3 to 12)	< 0.01

Definition of abbreviations: CI = confidence interval; LED = light-emitting diode; FM = fluorescence microscopy

CellScope and conventional LED FM were positive in a similar proportion of patients (34 vs. 32%, $P = 0.38$), with moderate correlation between techniques (agreement 84%; unweighted kappa, 0.64). Among 83 discordant pairs of results, a greater proportion were positive with CellScope and negative with conventional LED FM (55 vs. 45%) but the difference was nonsignificant ($P = 0.38$).

EXAMINATION TIME

Median slide examination time for LED FM with CellScope was 252 seconds (IQR, 137-358). Examination time varied between slides scored as positive versus those scored as negative. Median examination time was 80 seconds (IQR, 32-194) for positive slides versus 297 seconds (IQR, 225-385) for negative slides.

INTER- AND INTRA-READER RELIABILITY ANALYSES

Tables 3, 4a and 4b show results of a sub-analysis of the 43 slides with complete data that were semi-quantitatively scored twice by both readers. Based on these results, inter-reader reliability was moderate (agreement, 90%; weighted kappa, 0.65) (Table 3) and intra-reader reliability varied by reader (agreement, 85 vs. 71%; weighted kappa, 0.48 vs. 0.11) (Table 4a and 4b).

TABLE 3. INTER-READER COMPARISON OF SEMI-QUANTITATIVE SCORES FOR LIGHT-EMITTING DIODE FLUORESENCE MICROSCOPY WITH CELLSCOPE

		Reader 1 (N = 43)					
		Negative	Scanty	1+	2+	3+	Total
Reader 2 (N = 43)	Negative	29	5				34
	Scanty	2					2
	1+		1	2			3
	2+	1			1		2
	3+					2	2
	Total	32	6	2	1	2	43

TABLE 4a. INTRA-READER COMPARISON OF SEMI-QUANTITATIVE SCORES FOR LIGHT-EMITTING DIODE FLUORESENCE MICROSCOPY WITH CELLSCOPE: READER 1

		1st Read 1 (N = 43)					
		Negative	Scanty	1+	2+	3+	Total
2nd Read (N = 43)	Negative	25	5				30
	Scanty	7	1		1		9
	1+			1			1
	2+			1			1
	3+					2	2
	Total	32	6	2	1	2	43

TABLE 4b. INTRA-READER COMPARISON OF SEMI-QUANTITATIVE SCORES FOR LIGHT-EMITTING DIODE FLUORESENCE MICROSCOPY WITH CELLSCOPE: READER 2

		1st Read 1 (N = 43)					
		Negative	Scanty	1+	2+	3+	Total
2nd Read (N = 43)	Negative	24	1	2	1	2	30
	Scanty	6					6
	1+	1	1	1			3
	2+	1					1
	3+	2			1		3
	Total	34	2	3	2	2	43

DISCUSSION

Increasing access to high-quality smear microscopy is crucial to global TB control. In this study, the first to evaluate the diagnostic accuracy of a portable digital microscopy device compared to conventional methods, we found that LED FM by CellScope was non-inferior to conventional LED FM involving commercial laboratory fluorescence microscopes. These findings are particularly significant given a substantial training and experience advantage enjoyed by technicians performing the conventional method. Our results suggest that portable digital microscopy could make an important contribution to expanding access to microscopy services in low-income, high TB burden settings where laboratory infrastructure and trained medical personnel are scarce.

Prior approaches to integrated, portable digital microscopy have, like CellScope, capitalized on advances in LEDs and image sensors, although system designs have varied. Optofluidic microscopy devices have recently been able to accommodate fluorescence, but they continue to require assumptions about the specimen such as flow speed and optical working distance.[254] Lensless holographic microscopy devices have succeeded in achieving high-resolution imaging, although they require that specimen to be immobilized at a fixed working distance.[255] Recent prototypes of array microscopy platforms allow imaging of separate fields of view without need for scanning, but improvements in image quality are needed.[244] None of these portable digital microscopy devices have been subjected to rigorous evaluation of diagnostic accuracy compared to conventional methods, as we present here.

CellScope has a number of design features that optimize it for use in limited-resource, peripheral settings. Its LED bulbs are long-lasting, have low energy requirements, and cost little to replace.[246] The inexpensive CMOS image sensor used in CellScope is no different than that found in a typical commercial mobile camera phone. The device is capable of LM as well as FM and, unlike most fluorescence microscopes, can be used in direct sunlight. CellScope's battery life of over 5 hours is a valuable asset in areas with unreliable electricity. And the estimated manufacturing cost of CellScope is less than a low-cost commercial LED fluorescence microscope. Although the current design is not standalone and requires connection to a computer, the use of inexpensive netbook laptops

contains costs and minimally affects portability. In future designs the computing tasks of CellScope could be easily handled by a smartphone.

Although in this study we evaluated CellScope as an alternative method for a microscopist to visualize smears, the device's digital images along with computing power and the opportunity for connection to mobile phone networks open exciting avenues for new applications of the device. Mobile phone coverage in most low-income countries is extensive.[226] Previous studies have demonstrated the feasibility of using cellular networks to transmit images to remote experts for consultation and quality assurance purposes.[227,256] The same technology can also be harnessed through geographic information systems (GIS) to generate detailed maps, spatial distributions of disease incidence and performance indictors of disease control.[257] Moreover, a number of recent studies have indicated significant progress in the development of reliable algorithms for the detection of AFB in images of smear microscopy slides[233-235], suggesting that computer algorithm-based analysis of CellScope's digital images could soon be used to assist or even replace human readers.

Our study has several potential limitations. First, the moderate sample size, which was fixed by the availability of slides, limited our statistical power and therefore the narrowness of the non-inferiority margin. However, given that the technicians who performed the conventional LED FM had a significant training and experience advantage over the CellScope technicians, we believe that the selected non-inferiority margin was justified. Second, the intra-reader variability raises concerns about reliability, although we believe that this may be a reflection of learning taking place between the first and second readings. Further evaluation is needed. Third, because the CellScope technicians were raised and educated in the US, our findings should be validated with technicians from low-resource regions who have less familiarity with personal computers and electronics. Such evaluation could identify important opportunities for improvements in the user interface. Finally, our study population consisted of hospitalized patients, many of whom had advanced HIV infection. While the specific values that our study determined for sensitivity and specificity of the techniques may not be generalizable to an ambulatory or low HIV-infected population, we would expect that differences between techniques would be conserved.

In summary, our findings suggest that LED FM by the digital imaging device CellScope is non-inferior to LED FM by conventional laboratory-based methods involving standard fluorescence microscopes. This study is the first to evaluate the diagnostic accuracy of a portable digital microscopy device compared to conventional methods. The device's portability as well as the opportunities it offers for telemicroscopy and automated AFB detection makes CellScope an attractive alternative microscopy platform for TB control. Additional implementation studies should be conducted in more diverse patient populations and comparing LED FM by CellScope and conventional methods with both techniques performed by similarly trained technicians from low-resource areas.

REFERENCES

1 Daniel TM. The history of tuberculosis. *Respiratory Medicine* 2006; 100: 1862–1870.

2 Ernst JD, Trevejo-Nuñez G, Banaiee N. Genomics and the evolution, pathogenesis, and diagnosis of tuberculosis. *J Clin Invest* 2007; 117: 1738–1745.

3 Gutierrez MC, Brisse S, Brosch R, *et al.* Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. *PLoS Pathog* 2005; 1: e5.

4 Gutacker MM, Smoot JC, Migliaccio CAL, *et al.* Genome-wide analysis of synonymous single nucleotide polymorphisms in Mycobacterium tuberculosis complex organisms: resolution of genetic relationships among closely related microbial strains. *Genetics* 2002; 162: 1533–1543.

5 Sreevatsan S, Pan X, Stockbauer KE, *et al.* Restricted structural gene polymorphism in the Mycobacterium tuberculosis complex indicates evolutionarily recent global dissemination. *Proc Natl Acad Sci USA* 1997; 94: 9869–9874.

6 Schaaf H, Zumla A. Tuberculosis: A Comprehensive Clinical Reference. Philadelphia, Saunders/Elsevier, 2009.

7 Ziskind B, Halioua B. [Tuberculosis in ancient Egypt]. *Rev Mal Respir* 2007; 24: 1277–1283.

8 Cave AJE, Demonstrator A. The evidence for the incidence of tuberculosis in ancient Egypt. *British Journal of Tuberculosis*; 33: 142–152.

9 Morse D, Brothwell DR, Ucko PJ. Tuberculosis in ancient Egypt. *Am Rev Respir Dis* 1964; 90: 524.

10 Roberts CA, Buikstra JE. The bioarchaeology of tuberculosis: a global perspective on a re-emerging disease., University Press of Florida, 2003.

11 Zink A, Haas CJ, Reischl U, Szeimies U, Nerlich AG. Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. *J Med Microbiol* 2001; 50: 355–366.

12 Zink AR, Grabner W, Nerlich AG. Molecular identification of human tuberculosis in recent and historic bone tissue samples: The role of molecular techniques for the study of historic tuberculosis. *Am J Phys Anthropol* 2005; 126: 32–47.

13 Donogue H, Spigelman M, Greenblatt C, *et al.* Tuberculosis: From Prehistory to Robert Koch, as Revealed by Ancient DNA. *Lancet Infect Dis* 2004; 4: 584–592.

14 Donoghue HD. Insights gained from palaeomicrobiology into ancient and modern

tuberculosis. *Clin Microbiol Infect* 2011; 17: 821–829.

15 Nerlich AG, Haas CJ, Zink A, Szeimies U, Hagedorn HG. Molecular evidence for tuberculosis in an ancient Egyptian mummy. Lancet. 1997; 350: 1404 EP –.

16 Ke Y. African Origin of Modern Humans in East Asia: A Tale of 12,000 Y Chromosomes. *Science* 2001; 292: 1151–1153.

17 Gibbons A. Human anthropology. Modern men trace ancestry to African migrants. *Science* 2001; 292: 1051–1052.

18 Relethford JH. Genetic evidence and the modern human origins debate. *Heredity* 2008; 100: 555–563.

19 Goebel T, Waters MR, O'Rourke DH. The Late Pleistocene Dispersal of Modern Humans in the Americas. *Science* 2008; 319: 1497–1502.

20 Daniel TM. The origins and precolonial epidemiology of tuberculosis in the Americas: can we figure them out? *Int J Tuberc Lung Dis* 2000; 4: 395–400.

21 Merriam-Webster, Inc. Merriam-Webster's Medical Dictionary. Dictionary.com. 1995.http://dictionary.reference.com/browse/phthisis (accessed 18 Jul.2011).

22 Finer KR. Tuberculosis. Philadelphia, Chlesea House Publishers, 2003.

23 Hippocrates. Aphorisms. St Peter Port, Dodo Press, 2009.

24 Mays S, Fysh E, Taylor G. Investigation of the link between visceral surface rib lesions and tuberculosis in a medieval skeletal series from England using ancient DNA. *Am J Phys Anthropol* 2002; 119: 27–36.

25 Mays S, Taylor GM, Legge A, Young D, Turner Walker G. Paleopathological and biomolecular study of tuberculosis in a medieval skeletal collection from England. *Am J Phys Anthropol* 2001; 114: 298–311.

26 Krause AK, Library of Congress YPC. Tuberculosis and public health. sn (Baltimore), 1928.

27 Grigg E. The arcana of tuberculosis; with a brief epidemiologic history of the disease in the USA III. *Amer Rev Tuberc* 1958; 78: 426.

28 Murray JF. A Century of Tuberculosis. *Am J Respir Crit Care Med* 2004; 169: 1181–1186.

29 Daniel TM. Captain of death: the story of tuberculosis. University of Rochester Press, 1999.

30 Dickens C. The life and adventures of Nicholas Nickleby. New York, Harper, 1873.

31 Quiki. History of tuberculosis. History of Tuberculosis. http://www.qwiki.com/q/?_escaped_fragment_=/History_of_tuberculosis (accessed 20 Sep.2011).

32 Davies RP, Tocque K, Bellis MA, Rimmington T, Davies PD. Historical declines in tuberculosis in England and Wales: improving social conditions or natural selection? *Int J Tuberc Lung Dis* 1999; 3: 1051–1054.

33 Eshun-Wilson I, Havers F, Nachega JB, *et al.* Evaluation of Paradoxical TB-Associated IRIS With the Use of Standardized Case Definitions For Resource-Limited Settings. *Journal of the International Association of Physicians in AIDS Care (JIAPAC)* 2010; 9: 104–108.

34 Doetsch RN. Benjamin Marten and his "New Theory of Consumptions"., 1978.

35 Laennec R. A treatise on the disease of the chest. Forbes J, trans. New York: Library of the New York Academy of Medicine. 1962.

36 Villemin JA. Études sur la tuberculose. Paris, JB Baillière et Fils, 1868.

37 Schluger NW. The Pathogenesis of Tuberculosis: The First One Hundred (and Twenty-Three) Years. *Am J Respir Cell Mol Biol* 2005; 32: 251–256.

38 Koch R. Die aetiologie der tuberculose. Berl. *Klin Wochenschr* 1882; 19: 221–230.

39 Harries A. Robert Koch and the discovery of the tubercle bacillus: the challenge of HIV and tuberculosis 125 years later. *Int J Tuberc Lung Dis* 2008; 12: 241–249.

40 Evans AS. Causation and disease: the Henle-Koch postulates revisited. *Yale J Biol Med* 1976; 49: 175–195.

41 Kaufmann SHE. Future Vaccination Strategies against Tuberculosis: Thinking outside the Box. *Immunity* 2010; 33: 567–577.

42 Koch R. A Further Communication on a Remedy for Tuberculosis. *Br Med J* 1891; 1: 125–127.

43 Pirquet von C. Frequency of tuberculosis in childhood. *Journal of the American Medical Association* 1909; 52: 675.

44 Daniel TM. Florence Barbara Seibert and purified protein derivative., 2009.

45 Underwood EA. Wilhelm Conrad Röntgen (1845-1923) and the Early Development of Radiology. *Proceedings of the Royal Society of Medicine* 1945; 38: 697.

46 Toman K, Frieden T, Health Organization W. Toman's tuberculosis: case detection, treatment, and monitoring. Geneva, World Health Organization, 2004.

47 Mann T. The Magic Mountain. New York, Alfred A. Knopf, 1951.

48 Carr I. Hippocrates on the Red. http://www.umanitoba.ca/libraries/units/health/resources/hipredninette.html.

49 Andersen P. TB vaccines: progress and problems. *Trends Immunol* 2001; 22: 160–168.

50 Rosenblatt M. Pulmonary tuberculosis: evolution of modern therapy. *Bulletin of the New York Academy of Medicine* 1973; 49: 163.

51 Schaaf HS, Marais BJ. Management of multidrug-resistant tuberculosis in children: a survival guide for paediatricians. *Paediatric Respiratory Reviews* 2011; 12: 31–38.

52 Sweany HC, Turner GC. A preliminary report on the use of para-amino salicylic acid in the treatment of pulmonary tuberculosis. *Dis Chest* 1949; 16: 633–60, illust.

53 Domagk G. Investigations on the Antitubereulous Activity of the Thiosemicarbazones in Vitro and in Vivo. *American Review of Tuberculosis and Pulmonary Diseases* 1950; 61: 8–19.

54 Schatz A, Bugle E, Waksman SA. Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram-Positive and Gram-Negative Bacteria. *Proceedings of the Society for Experimental Biology and Medicine* 1944; 55: 66–69.

55 Sakula A. Selman Waksman (1888-1973), discoverer of streptomycin: A centenary review. *British Journal of Diseases of the Chest*; 82 IS -: 23–31.

56 Science History of August 16. http://chemistry.about.com/od/augustinscience/tp/august16history.htm.

57 Pfuetze KH, Glover RP, White EF, Feldman WH, Hinshaw HC. Clinical Use of Streptomycin in the Treatment of Tuberculosis. *Chest* 1946; 12: 515–519.

58 Heilman DH, Heilman FR, Hinshaw HC, Nichols DR, Herrell WE. Streptomycin: absorption, diffusion, excretion and toxicity. *The American Journal of the Medical Sciences* 1945; 210: 576.

59 Finland M, Murray R. Development of streptomycin resistance during treatment. *Journal of the American Medical Association* 1946; 132: 16–21.

60 Medical Research Council. Treatment of Pulmonary Tuberculosis with Para-aminosalicylic Acid and Streptomycin. *Br Med J* 1949; 2: 1521.

61 Domagk G, Offe HA, Siefken W. [Additional investigations in experimental chemotherapy of tuberculosis]. *Dtsch Med Wochenschr* 1952; 77.

62 Benson CA, Williams PL, Cohn DL, *et al.* Clarithromycin or rifabutin alone or in combination for primary prophylaxis of Mycobacterium avium complex disease in patients with AIDS: A randomized, double-blind, placebo-controlled trial. The AIDS Clinical Trials Group 196/Terry Beirn Community Programs for Clinical Research on AIDS 009 Protocol Team. *J Infect Dis* 2000; 181: 1289–1297.

63 Bernstein J, Lott WA, Steinberg BA, Yale HL. Chemotherapy of experimental tuberculosis. V. Isonicotinic acid hydrazide (nydrazid) and related compounds. *Am Rev Tuberc* 1952; 65: 357–364.

64 Crofton J. Chemotherapy of pulmonary tuberculosis. *Br Med J* 1959; 1: 1610.

65 Sensi P. History of the development of rifampin. *Rev Infect Dis* 1983; 5 Suppl 3: S402–6.

66 Sensi P, Margalith P, Timbal MT. Rifomycin, a new antibiotic; preliminary report. *Farmaco Sci* 1959; 14: 146–147.

67 Saltini C. Chemotherapy and diagnosis of tuberculosis. *Respiratory Medicine* 2006; 100: 2085–2097.

68 Mitchison DA. Antimicrobial therapy of tuberculosis: justification for currently recommended treatment regimens. *Semin Respir Crit Care Med* 2004; 25: 307–315.

69 Yeager R, Munroe W, Dessau FI. Pyrazinamide (aldinamide) in the treatment of pulmonary tuberculosis. *Amer Rev Tuberc* 1952; 65: 523.

70 McCune RM Jr, Tompsett R. The fate of Mycobacterium tuberculosis in mouse tissues as determined by the microbial enumeration technique: I. The persistence of drug-susceptible tubercle bacilli in the tissues despite prolonged antimicrobial therapy. *J Exp Med* 1956; 104: 737.

71 McCune RM Jr, McDermott W, Tompsett R. The fate of Mycobacterium tuberculosis in mouse tissues as determined by the microbial enumeration technique. II. The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. *J Exp Med* 1956; 104: 763.

72 Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. *Int J Tuberc Lung Dis* 1999; 3: S231–S279.

73 Fox W. The problem of self-administration of drugs; with particular reference to pulmonary tuberculosis. *Tubercle*; 39: 269–274.

74 Bayer R, Wilkinson D. Directly observed therapy for tuberculosis: history of an idea. *Lancet* 1995; 345: 1545–1548.

75 Fox W. Self-administration of medicaments. A review of published work and a study of the problems. *Bulletin of the International Union Against Tuberculosis* 1962; 32: 307.

76 Murray CJ, Styblo K, Rouillon A. Tuberculosis in developing countries: burden, intervention and cost. *Bull Int Union Tuberc Lung Dis* 1990; 65: 6–24.

77 Zumla A, Mwaba P, Huggett J, Kapata N, Chanda D, Grange J. Reflections on the white plague. *Lancet Infect Dis* 2009; 9: 197–202.

78 World Health Organization. Global tuberculosis control: WHO report 2011. Geneva, Switzerland, World Health Organization, 2011.

79 Lönnroth K, Castro KG, Chakaya JM, *et al.* Tuberculosis control and elimination 2010-50: cure, care, and social development. *Lancet* 2010; 375: 1814–1829.

80 Health Organization W. Global Tuberculosis Control 2010. 2010; : 1–218.

81 Mandell GL, Bennett JE, Dolin R, Kelvin G. Mandell, Douglas and Bennett's principles and practice of infectious diseases., 7th ed. Philadelphia, Elsevier Churchill Livingstone, 2009.

82 Harries AD, Zachariah R, Corbett EL, *et al.* The HIV-associated tuberculosis epidemic--when will we act? *Lancet* 2010; 375: 1906–1919.

83 Nunn P, Williams B, Floyd K, Dye C, Elzinga G, Raviglione M. Tuberculosis control in the era of HIV. *Nat Rev Immunol* 2005; 5: 819–826.

84 Corbett EL, Marston B, Churchyard GJ, De Cock KM. Tuberculosis in sub-Saharan Africa: opportunities, challenges, and change in the era of antiretroviral treatment. *Lancet* 2006; 367: 926–937.

85 Kyeyune R, Boon den S, Cattamanchi A, *et al.* Causes of early mortality in HIV-infected TB suspects in an East African referral hospital. *J Acquir Immune Defic Syndr* 2010; 55: 446–450.

86 Lawn SD, Myer L, Bekker L-G, Wood R. Burden of tuberculosis in an antiretroviral treatment programme in sub-Saharan Africa: impact on treatment outcomes and implications for tuberculosis control. *AIDS* 2006; 20: 1605–1612.

87 Lawn SD, Harries AD, Anglaret X, Myer L, Wood R. Early mortality among adults accessing antiretroviral treatment programmes in sub-Saharan Africa. *AIDS* 2008; 22: 1897–1908.

88 XDRTB.org. http://xdrtb.org/photographs.php.

89 Gandhi NR, Nunn P, Dheda K, *et al.* Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. *Lancet* 2010; 375:

1830–1843.

90 World Health Organization. Multidrug and extensively drug-resistant TB (M/XDR-TB). Geneva, Switzerland, World Health Organization, 2010.

91 Hoek KGP, Van Rie A, van Helden PD, Warren RM, Victor TC. Detecting Drug-Resistant Tuberculosis: The Importance of Rapid Testing. *Molecular Diagnosis &# 38; Therapy* 2011; 15: 189–194.

92 Singla R, Sarin R, Khalid UK, *et al.* Seven-year DOTS-Plus pilot experience in India: results, constraints and issues. *Int J Tuberc Lung Dis* 2009; 13: 976–981.

93 Kwon YS, Kim YH, Suh GY, *et al.* Treatment outcomes for HIV-uninfected patients with multidrug-resistant and extensively drug-resistant tuberculosis. *Clin Infect Dis* 2008; 47: 496–502.

94 Shah NS, Wright A, Bai G-H, *et al.* Worldwide emergence of extensively drug-resistant tuberculosis. *Emerging Infect Dis* 2007; 13: 380–387.

95 Velayati AA, Masjedi MR, Farnia P, *et al.* Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran. *Chest* 2009; 136: 420–425.

96 Tuberculosis (TB): Age of Optimism. National Institute of Allergy and Infectious Diseasehttp://www.niaid.nih.gov/topics/tuberculosis/Understanding/history/Pages/historical_optimism.aspx (accessed 11 Aug.2011).

97 Hopewell PC. Tuberculosis control: how the world has changed since 1990. *Bull World Health Organ* 2002; 80: 427–427.

98 Ducati RG, Ruffino-Netto A, Basso LA, Santos DS. The resumption of consumption -- a review on tuberculosis. *Mem Inst Oswaldo Cruz* 2006; 101: 697–714.

99 Arata K. The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle*; 72: 1–6.

100 Commission on Health Research for Development. Health research: essential link to equity in development. New York, Oxford University Press, 1990.

101 Introduction to Drug Discovery. http://www.interactive-consultancies.co.uk.

102 Cole EC, Cook CE. Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies. *Am J Infect Control* 1998; 26: 453–464.

103 Nardell EA. Catching droplet nuclei: toward a better understanding of tuberculosis transmission. *Am J Respir Crit Care Med* 2004; 169: 553–554.

104 Nicas M, Nazaroff WW, Hubbard A. Toward understanding the risk of secondary airborne infection: emission of respirable pathogens. *J Occup Environ Hyg* 2005; 2: 143–154.

105 World Health Organization. World Health Organization fact sheet. Tuberculosis, No. 104. Geneva, Switzerland, World Health Organization, 2010.

106 Raviglione MC, O'Brien RJ. Tuberculosis. In: Longo DL, Fauci AS, Kasper DL, Hauser SL, Jameson JL, Loscalzo J, eds. Harrison's Principles of Internal Medicine, 18th ed. New York, McGraw-Hill, 2012.

107 Clemens DL, Horwitz MA. Characterization of the Mycobacterium tuberculosis phagosome and evidence that phagosomal maturation is inhibited. *J Exp Med* 1995; 181: 257–270.

108 Kaufmann SHE, Hussey G, Lambert P-H. New vaccines for tuberculosis. *Lancet* 2010; 375: 2110–2119.

109 Cooper AM. Cell-mediated immune responses in tuberculosis. *Annu Rev Immunol* 2009; 27: 393–422.

110 Huynh KK, Joshi SA, Brown EJ. A delicate dance: host response to mycobacteria. *Curr Opin Immunol* 2011; 23: 464–472.

111 Schluger NW, Rom WN. The host immune response to tuberculosis. *Am J Respir Crit Care Med* 1998; 157: 679–691.

112 Zumla A, James DG. Granulomatous infections: etiology and classification. *Clin Infect Dis* 1996; 23: 146–158.

113 Paige C, Bishai WR. Penitentiary or penthouse condo: the tuberculous granuloma from the microbe's point of view. *Cell Microbiol* 2010; 12: 301–309.

114 Russell DG. Who puts the tubercle in tuberculosis? *Nat Rev Microbiol* 2007; 5: 39–47.

115 Mason RJ. Murray and Nadel's textbook of respiratory medicine. Elsevier Saunders, 2005.

116 Russell DG, Barry CE, Flynn JL. Tuberculosis: what we don't know can, and does, hurt us. *Science* 2010; 328: 852–856.

117 Rook GAW, Dheda K, Zumla A. Immune responses to tuberculosis in developing countries: implications for new vaccines. *Nat Rev Immunol* 2005; 5: 661–667.

118 Ray C, Ryan K. Mycobacteria. In: Ray C, Ryan K, eds. *Sherris Medical Microbiology*, 5th ed. New York, McGraw-Hill.http://www.accessmedicine.com/content.aspx?aID=6943397.

119 Golden MP, Vikram HR. Extrapulmonary tuberculosis: an overview. *Am Fam Physician* 2005; 72: 1761–1768.

120 Peto HM, Pratt RH, Harrington TA, LoBue PA, Armstrong LR. Epidemiology of extrapulmonary tuberculosis in the United States, 1993-2006. *Clin Infect Dis* 2009; 49: 1350–1357.

121 DeGraw D, editor. Amped status report Part I., Amped Status, New York, NY, USAhttp://ampedstatus.com/the-eco- nomic-elite-vs-the-people-of-the-united-states-of-america- part-i (accessed 18 Sep.2011).

122 Shah A, editor. Poverty around the world., Global Issueshttp://www.globalissues. org/article/4/poverty-around-the-world (accessed 18 Sep.2011).

123 Lönnroth K, Jaramillo E, Williams BG, Dye C, Raviglione M. Drivers of tuberculosis epidemics: the role of risk factors and social determinants. *Soc Sci Med* 2009; 68: 2240–2246.

124 WorldMapper. A to Z index of maps. WorldMapperhttp://www.worldmapper. org (accessed 18 Sep.2011).

125 Davies PDO, Barnes PF, Gordon SB. Clinical Tuberculosis. Hodder Arnold, 2010.

126 Benatar SR, Upshur R. Tuberculosis and poverty: what could (and should) be done? *Int J Tuberc Lung Dis* 2010; 14: 1215–1221.

127 McIntyre D, Thiede M, Dahlgren G, Whitehead M. What are the economic consequences for households of illness and of paying for health care in low- and middle-income country contexts? *Soc Sci Med* 2006; 62: 858–865.

128 Russell S. The economic burden of illness for households in developing countries: a review of studies focusing on malaria, tuberculosis, and human immunodeficiency virus/acquired immunodeficiency syndrome. *Am J Trop Med Hyg* 2004; 71: 147–155.

129 Frieden T, Mulling J. Research for Action-Understanding and Controlling Tuberculosis in India. *WHO Regional Office for south-East Asia, New Delhi* 2000.

130 Baussano I, Williams BG, Nunn P, Beggiato M, Fedeli U, Scano F. Tuberculosis incidence in prisons: a systematic review. *PLoS Med* 2010; 7: e1000381.

131 Burki T. Tackling tuberculosis in London's homeless population. Lancet. 2010; 376: 2055–2056.

132 Haddad MB, Wilson TW, Ijaz K, Marks SM, Moore M. Tuberculosis and homelessness in the United States, 1994-2003. *JAMA* 2005; 293: 2762–2766.

133 Centers for Disease Control and Prevention (CDC). Trends in tuberculosis--United

States, 2010. *MMWR Morb Mortal Wkly Rep* 2011; 60: 333–337.

134 Serpa JA, Teeter LD, Musser JM, Graviss EA. Tuberculosis disparity between US-born blacks and whites, Houston, Texas, USA. *Emerging infectious diseases* 2009; 15: 899–904.

135 Manangan L, Elmore K, Lewis B, *et al.* Disparities in tuberculosis between Asian/Pacific Islanders and non-Hispanic Whites, United States, 1993-2006. *Int J Tuberc Lung Dis* 2009; 13: 1077–1085.

136 Morán-Mendoza O, Marion SA, Elwood K, Patrick D, FitzGerald JM. Risk factors for developing tuberculosis: a 12-year follow-up of contacts of tuberculosis cases. *Int J Tuberc Lung Dis* 2010; 14: 1112–1119.

137 Sia IG, Orillaza RB, St Sauver JL, *et al.* Tuberculosis attributed to household contacts in the Philippines. *Int J Tuberc Lung Dis* 2010; 14: 122–125.

138 Baker M, Das D, Venugopal K, Howden-Chapman P. Tuberculosis associated with household crowding in a developed country. *J Epidemiol Community Health* 2008; 62: 715–721.

139 Corbett EL, Watt CJ, Walker N, *et al.* The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. *Arch Intern Med* 2003; 163: 1009–1021.

140 Cegielski JP, McMurray DN. The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. *Int J Tuberc Lung Dis* 2004; 8: 286–298.

141 Lönnroth K, Williams BG, Stadlin S, Jaramillo E, Dye C. Alcohol use as a risk factor for tuberculosis - a systematic review. *BMC Public Health* 2008; 8: 289.

142 Rehm J, Samokhvalov AV, Neuman MG, *et al.* The association between alcohol use, alcohol use disorders and tuberculosis (TB). A systematic review. *BMC Public Health* 2009; 9: 450.

143 Slama K. Global perspective on tobacco control. Part I. The global state of the tobacco epidemic. *Int J Tuberc Lung Dis* 2008; 12: 3–7.

144 Slama K, Chiang C-Y, Enarson DA, *et al.* Tobacco and tuberculosis: a qualitative systematic review and meta-analysis. *Int J Tuberc Lung Dis* 2007; 11: 1049–1061.

145 Lin H-H, Ezzati M, Murray M. Tobacco smoke, indoor air pollution and tuberculosis: a systematic review and meta-analysis. *PLoS Med* 2007; 4: e20.

146 Deribew A, Tesfaye M, Hailmichael Y, *et al.* Common mental disorders in TB/HIV co-infected patients in Ethiopia. *BMC Infectious Diseases* 2010; 10: 201.

147 Aghanwa HS, Erhabor GE. Demographic/socioeconomic factors in mental disorders associated with tuberculosis in southwest Nigeria. *J Psychosom Res* 1998; 45: 353–360.

148 Ramsay A. Making the Most of Poor Diagnostics: increasing access to tuberculosis treatment through optimized smear microscopy services. *Dissertation: Universiteit van Amsterdam* 2010; : 1–150.

149 World Health Organization. Addressing poverty in TB control. World Health Organization, 2005http://whqlibdoc.who.int/hq/2005/WHO_HTM_TB_2005.352.pdf.

150 Long Q, Smith H, Zhang T, Tang S, Garner P. Patient medical costs for tuberculosis treatment and impact on adherence in China: a systematic review. *BMC Public Health* 2011; 11: 393.

151 Mauch V, Woods N, Kirubi B, Kipruto H, Sitienei J, Klinkenberg E. Assessing access barriers to tuberculosis care with the tool to Estimate Patients' Costs: pilot results from two districts in Kenya. *BMC Public Health* 2011; 11: 43.

152 Zhang T, Tang S, Jun G, Whitehead M. Persistent problems of access to appropriate, affordable TB services in rural China: experiences of different socio-economic groups. *BMC Public Health* 2007; 7: 19.

153 Storla DG, Yimer S, Bjune GA. A systematic review of delay in the diagnosis and treatment of tuberculosis. *BMC Public Health* 2008; 8: 15.

154 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; 11: 40–45.

155 Mesfin MM, Newell JN, Walley JD, Gessessew A, Madeley RJ. Delayed consultation among pulmonary tuberculosis patients: a cross sectional study of 10 DOTS districts of Ethiopia. *BMC Public Health* 2009; 9: 53.

156 Huong NT, Vree M, Duong BD, *et al.* Delays in the diagnosis and treatment of tuberculosis patients in Vietnam: a cross-sectional study. *BMC Public Health* 2007; 7: 110.

157 Finnie RKC, Khoza LB, van den Borne B, Mabunda T, Abotchie P, Mullen PD. Factors associated with patient and health care system delay in diagnosis and treatment for TB in sub-Saharan African countries with high burdens of TB and HIV. *Trop Med Int Health* 2011; 16: 394–411.

158 Long Q, Li Y, Wang Y, *et al.* Barriers to accessing TB diagnosis for rural-to-urban migrants with chronic cough in Chongqing, China: a mixed methods study. *BMC Health Serv Res* 2008; 8: 202.

159 Yimer S, Bjune G, Alene G. Diagnostic and treatment delay among pulmonary tuberculosis patients in Ethiopia: a cross sectional study. *BMC Infectious Diseases* 2005; 5: 112.

160 Cramm JM, Finkenflügel HJM, Møller V, Nieboer AP. TB treatment initiation and adherence in a South African community influenced more by perceptions than by knowledge of tuberculosis. *BMC Public Health* 2010; 10: 72.

161 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; 3: 1088–1095.

162 Kiwuwa MS, Charles K, Harriet MK. Patient and health service delay in pulmonary tuberculosis patients attending a referral hospital: a cross-sectional study. *BMC Public Health* 2005; 5: 122.

163 United Nations. Millennium Development Goals Indicators. Millennium Development Goals Indicators. 2006.

164 Partnership Stop TB. The global plan to stop TB: 2011-2015. *World Health Organization* 2010.

165 Ratledge C, Dale J. Mycobacteria: Molecular biology and virulence. Wiley-Blackwell, 1999.

166 Gilpin C, Kim SJ, Lumb R, Rieder HL, Van Deun A, Working Group on Sputum Smear Microscopy. Critical appraisal of current recommendations and practices for tuberculosis sputum smear microscopy. In: The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease. 2007: 946–952.

167 Fluorescence microscopy for tuberculosis diagnosis. 7: 236–237.

168 Moore DAJ, Evans CAW, Gilman RH, *et al.* Microscopic-observation drug-susceptibility assay for the diagnosis of TB. *N Engl J Med* 2006; 355: 1539–1550.

169 Srisuwanvilai L-O, Srisuwanvilai L-O, Srisuwanvilai L-O, *et al.*

170 Sorlozano A, Soria I, Roman J, *et al.* Comparative evaluation of three culture methods for the isolation of mycobacteria from clinical samples. *J Microbiol Biotechnol* 2009; 19: 1259–1264.

171 Lawn SD, Zumla AI. Tuberculosis. *Lancet* 2011; 378: 57–72.

172 Kubica G. Mycobacterium tuberculosis culture. Atlanta, Public Health Image Library.

173 Greco S, Girardi E, Navarra A, Saltini C. Current evidence on diagnostic accuracy

of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax* 2006; 61: 783–790.

174 Greco S, Rulli M, Girardi E, Piersimoni C, Saltini C. Diagnostic accuracy of in-house PCR for pulmonary tuberculosis in smear-positive patients: meta-analysis and metaregression. *J Clin Microbiol* 2009; 47: 569–576.

175 Flores LL, Pai M, Colford JM, Riley LW. In-house nucleic acid amplification tests for the detection of Mycobacterium tuberculosis in sputum specimens: meta-analysis and meta-regression. *BMC Microbiol* 2005; 5: 55.

176 Sarmiento OL, Weigle KA, Alexander J, Weber DJ, Miller WC. Assessment by meta-analysis of PCR for diagnosis of smear-negative pulmonary tuberculosis. *J Clin Microbiol* 2003; 41: 3233–3240.

177 Boehme CC, Nabeta P, Hillemann D, *et al.* Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med* 2010; 363: 1005–1015.

178 Bowles EC, Freyée B, van Ingen J, Mulder B, Boeree MJ, Van Soolingen D. Xpert MTB/RIF®, a novel automated polymerase chain reaction–based tool for the diagnosis of tuberculosis [Technical note]. *Int J Tuberc Lung Dis* 2011; 15: 988–989.

179 Scott LE, McCarthy K, Gous N, *et al.* Comparison of Xpert MTB/RIF with Other Nucleic Acid Technologies for Diagnosing Pulmonary Tuberculosis in a High HIV Prevalence Setting: A Prospective Study. *PLoS Med* 2011; 8: e1001061.

180 Evans CA. GeneXpert--a game-changer for tuberculosis control? *PLoS Med* 2011; 8: e1001064.

181 Pai M, Zwerling A, Menzies D. Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Ann Intern Med* 2008; 149: 177–184.

182 Lange C, Hellmich B, Ernst M, Ehlers S. Rapid immunodiagnosis of tuberculosis in a woman receiving anti-TNF therapy. *Nat Clin Pract Rheumatol* 2007; 3: 528–534.

183 Lange C, MORI T. Advances in the diagnosis of tuberculosis. *Respirology* 2010; 15: 220–240.

184 Mazurek GH, Weis SE, Moonan PK, *et al.* Prospective comparison of the tuberculin skin test and 2 whole-blood interferon-gamma release assays in persons with suspected tuberculosis. *Clin Infect Dis* 2007; 45: 837–845.

185 Losi M, Bossink A, Codecasa L, *et al.* Use of a T-cell interferon-gamma release assay for the diagnosis of tuberculous pleurisy. *Eur Respir J* 2007; 30: 1173–1179.

186 Research SPF, Diseases TIT, Foundation for Innovative New Diagnostics. Diagnostics for tuberculosis: global demand and market potential. World Health Organization, 2006.

187 Sreeramareddy CT, Panduru KV, Menten J, den Ende Van J. Time delays in diagnosis of pulmonary tuberculosis: a systematic review of literature. *BMC Infectious Diseases* 2009; 9: 91.

188 Cambanis A, Yassin MA, Ramsay A, Bertel Squire S, Arbide I, Cuevas LE. Rural poverty and delayed presentation to tuberculosis services in Ethiopia. *Trop Med Int Health* 2005; 10: 330–335.

189 Sendagire I, Schim Van der Loeff M, Mubiru M, Konde-Lule J, Cobelens F. Long delays and missed opportunities in diagnosing smear-positive pulmonary tuberculosis in Kampala, Uganda: a cross-sectional study. *PLoS ONE* 2010; 5: e14459.

190 Khan MS, Khan S, Godfrey-Faussett P. Default during TB diagnosis: quantifying the problem. *Trop Med Int Health* 2009; 14: 1437–1441.

191 Rao NA, Anwer T, Saleem M. Magnitude of initial default in pulmonary tuberculosis. *J Pak Med Assoc* 2009; 59: 223–224.

192 Quy HT, Lan NTN, Lonnroth K, Buu TN, Dieu TTN, Hai LT. Public-private mix for improved TB control in Ho Chi Minh City, Vietnam: an assessment of its impact on case detection. *Int J Tuberc Lung Dis* 2003; 7: 464–471.

193 Creek TL, Lockman S, Kenyon TA, *et al.* Completeness and timeliness of treatment initiation after laboratory diagnosis of tuberculosis in Gaborone, Botswana. *Int J Tuberc Lung Dis* 2000; 4: 956–961.

194 Sai Babu B, Satyanarayana AVV, Venkateshwaralu G, *et al.* Initial default among diagnosed sputum smear-positive pulmonary tuberculosis patients in Andhra Pradesh, India. *Int J Tuberc Lung Dis* 2008; 12: 1055–1058.

195 Belaye AK, Squire SB, Salaniponi FML, Mundy CJF, Theobald S, Kemp J. 'Lost' smear-positive pulmonary tuberculosis cases: where are they and why did we lose them? *Int J Tuberc Lung Dis* 2004; : 25–31.

196 Steingart KR, Henry M, Ng V, *et al.* Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis* 2007; 6: 570–581.

197 Nguyen TN, Wells CD, Binkin NJ, Pham DL, Nguyen VC. The importance of quality control of sputum smear microscopy: the effect of reading errors on treatment decisions and outcomes. *Int J Tuberc Lung Dis* 1999; 3: 483–487.

198 Endo S, Trono M, Fujiki A, Macalalad N. Operational conditions influencing the

proficiency of AFB microscopy services in the Philippines. *Int J Tuberc Lung Dis* 2007; 11: 293–299.

199 Steingart KR, Ng V, Henry M, *et al.* Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. *Lancet Infect Dis* 2006; 6: 664–674.

200 Mase SR, Ramsay A, Ng V, *et al.* Yield of serial sputum specimen examinations in the diagnosis of pulmonary tuberculosis: a systematic review. *Int J Tuberc Lung Dis* 2007; 11: 485–495.

201 Steingart KR, Ramsay A, Pai M. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis. *Expert Rev Anti Infect Ther* 2007; 5: 327–331.

202 Parsons LM, Somoskövi A, Gutierrez C, *et al.* Laboratory Diagnosis of Tuberculosis in Resource-Poor Countries: Challenges and Opportunities. *Clinical Microbiology Reviews* 2011; 24: 314–350.

203 Petti CA, Polage CR, Quinn TC, Ronald AR, Sande MA. Laboratory medicine in Africa: a barrier to effective health care. *Clin Infect Dis* 2006; 42: 377–382.

204 Tapley A. Typical one-room microscopy lab in Uganda. Jinja, Uganda, Personal photo collection.

205 Paramasivan CN, Lee E, Kao K, *et al.* Experience establishing tuberculosis laboratory capacity in a developing country setting. *Int J Tuberc Lung Dis* 2010; 14: 59–64.

206 Lienhardt C, Rowley J, Manneh K, *et al.* Factors affecting time delay to treatment in a tuberculosis control programme in a sub-Saharan African country: the experience of The Gambia. *Int J Tuberc Lung Dis* 2001; 5: 233–239.

207 Guillerm M, Usdin M, Arkinstall J. Tuberculosis diagnosis and drug sensitivity testing: An overview of current diagnostic pipeline. 2007.

208 Srikanth P, Kamesh S, Daley P. Bleach Optimization of Sputum Smear Microscopy for Pulmonary Tuberculosis. *Indian J Tuberc* 2009; 56: 174–184.

209 Cattamanchi A, Davis JL, Pai M, Huang L, Hopewell PC, Steingart KR. Does bleach processing increase the accuracy of sputum smear microscopy for diagnosing pulmonary tuberculosis? *J Clin Microbiol* 2010; 48: 2433–2439.

210 Bonnet M, Gagnidze L, Guerin PJ, *et al.* Evaluation of Combined LED-Fluorescence Microscopy and Bleach Sedimentation for Diagnosis of Tuberculosis at Peripheral Health Service Level. *PLoS ONE* 2011; 6: e20175.

211 Klatt EC. Tuberculosis tutorial.

http://library.med.utah.edu/WebPath/TUTORIAL/MTB/MTB.html.

212 Cattamanchi A, Davis JL, Worodria W, *et al.* Sensitivity and specificity of fluorescence microscopy for diagnosing pulmonary tuberculosis in a high HIV prevalence setting. *Int J Tuberc Lung Dis* 2009; 13: 1130–1136.

213 Van Hung N, Sy DN, Anthony RM, Cobelens FG, van Soolingen D. Fluorescence microscopy for tuberculosis diagnosis. *Lancet Infect Dis*; 7: 238–239.

214 Yassin MA, Cuevas LE. How many sputum smears are necessary for case finding in pulmonary tuberculosis? *Trop Med Int Health* 2003; 8: 927–932.

215 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; 8: 824–828.

216 Rieder HL, Chiang C-Y, Rusen ID. A method to determine the utility of the third diagnostic and the second follow-up sputum smear examinations to diagnose tuberculosis cases and failures. *Int J Tuberc Lung Dis* 2005; 9: 384–391.

217 Bonnet M, Ramsay A, Gagnidze L, Githui W, Guerin PJ, Varaine F. Reducing the number of sputum samples examined and thresholds for positivity: an opportunity to optimise smear microscopy. *Int J Tuberc Lung Dis* 2007; 11: 953–958.

218 World Health Organization. Proposal for a revision of the case definition of "Sputum Smear-Positive Tuberculosis.." Geneva, 2007.

219 World Health Organization. Proposed reduction of number of smears for the diagnosis of pulmonary TB: background document. Geneva, 2007.

220 Noeske J, Dopico E, Torrea G, Wang H, Van Deun A. Two vs. three sputum samples for microscopic detection of tuberculosis in a high HIV prevalence population. *Int J Tuberc Lung Dis* 2009; 13: 842–847.

221 Cambanis A, Yassin MA, Ramsay A, Squire SB, Arbide I, Cuevas LE. A one-day method for the diagnosis of pulmonary tuberculosis in rural Ethiopia. *Int J Tuberc Lung Dis* 2006; 10: 230–232.

222 Rawat J, Biswas D, Sindhwani G, Masih V. An alternative 1-day smear microscopy protocol for the diagnosis of pulmonary tuberculosis. *Respirology* 2010; 15: 1127–1130.

223 Al-Aghbari N, Al-Sonboli N, Yassin MA, *et al.* Multiple sampling in one day to optimize smear microscopy in children with tuberculosis in Yemen. *PLoS ONE* 2009; 4: e5140.

224 World Health Organization. Same-day diagnosis of tuberculosis by microscopy: policy statement. Geneva,

2007http://whqlibdoc.who.int/publications/2011/9789241501606_eng.pdf.

225 Cattamanchi A, Huang L, Worodria W, *et al.* Integrated strategies to optimize sputum smear microscopy: a prospective observational study. *Am J Respir Crit Care Med* 2011; 183: 547–551.

226 Teltscher S, Magpantay E, Gray V, Olaya D, Vallejo I. Measuring the Information Society: The ICT Development Index. Geneva, Switzerland, Telecommunication Development Bureau, International Telecommunications Union, 2009.

227 Bellina L, Missoni E. Mobile cell-phones (M-phones) in telemicroscopy: increasing connectivity of isolated laboratories. *Diagn Pathol* 2009; 4: 19.

228 Zimic M, Coronel J, Gilman RH, Luna CG, Curioso WH, Moore DAJ. Can the power of mobile phones be used to improve tuberculosis diagnosis in developing countries? *Trans R Soc Trop Med Hyg* 2009; 103: 638–640.

229 Frean J. Microscopic images transmitted by mobile cameraphone. *Trans R Soc Trop Med Hyg* 2007; 101: 1053.

230 Mudanyali O, Tseng D, Oh C, *et al.* Compact, light-weight and cost-effective microscope based on lensless incoherent holography for telemedicine applications. *Lab Chip* 2010; 10: 1417–1428.

231 Tseng D, Mudanyali O, Oztoprak C, *et al.* Lensfree microscopy on a cellphone. *Lab Chip* 2010; 10: 1787–1792.

232 Breslauer DN, Maamari RN, Switz NA, Lam WA, Fletcher DA. Mobile phone based clinical microscopy for global health applications. *PLoS ONE* 2009; 4: e6320.

233 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; 21: 277–282.

234 Veropoulos K. Machine learning approaches to medical decision making. University of Bristol, 2001.

235 Forero MG, Cristóbal G, Desco M. Automatic identification of Mycobacterium tuberculosis by Gaussian mixture models. *J Microsc* 2006; 223: 120–132.

236 Manuel G Forero, Filip Sroubek, Gabriel Cristóbal. Identification of tuberculosis bacteria based on shape and color. *Real-Time Imaging* 2004; 10: 251–262.

237 Costa MGF, Costa Filho CFF, Sena JF, Salem J, de Lima MO. Automatic identification of mycobacterium tuberculosis with conventional light microscopy. *Conf Proc IEEE Eng Med Biol Soc* 2008; 2008: 382–385.

238 Khutlang R, Krishnan S, Dendere R, *et al.* Classification of Mycobacterium tuberculosis in images of ZN-stained sputum smears. *IEEE Trans Inf Technol Biomed* 2010; 14: 949–957.

239 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; 12: 579–582.

240 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; 2: 756–765.

241 Ozluk Y, Blanco PL, Mengel M, Solez K, Halloran PF, Sis B. Superiority of virtual microscopy versus light microscopy in transplantation pathology. *Clinical Transplantation* 2011; : no–no.

242 Rocha R, Vassallo J, Soares F, Miller K, Gobbi H. Digital slides Present status of a tool for consultation, teaching, and quality control in pathology. *Pathology Research and Practice* 2009; 205: 735–741.

243 Miller AR, Davis GL, Oden ZM, *et al.* Portable, Battery-Operated, Low-Cost, Bright Field and Fluorescence Microscope. *PLoS ONE* 2010; 5: e11890.

244 McCall B, Pierce M, Graviss EA, Richards-Kortum R, Tkaczyk T. Toward a low-cost compact array microscopy platform for detection of tuberculosis. *Tuberculosis* 2011; 91: S54–S60.

245 Ghosh KK, Burns LD, Cocker ED, *et al.* Miniaturized integration of a fluorescence microscope. *Nat Meth* 2011; 8: 871–878.

246 Hänscheid T. The future looks bright: low-cost fluorescent microscopes for detection of Mycobacterium tuberculosis and Coccidiae. *Trans R Soc Trop Med Hyg* 2008; 102: 520–521.

247 Keller P. Recent Innovations in CMOS Image Sensors. 2011; : 1–5.

248 Kent P, Kubica G. Public health mycobacteriology - a guide for the level III laboratory. Atlanta, Georgia, USA, U.S. Centers for Disease Control and Prevention, 1985.

249 World Health Organization. Laboratory services in tuberculosis control. Part II: Microscopy. Geneva, Switzerland, World Health Organization, 1998.

250 Kam KM, Yip C-W, Tang HS, Van Deun A. Bulk acid-fast staining of sputum smears: time to end a taboo. *Int J Tuberc Lung Dis* 2009; 13: 1119–1123.

251 Hendry C, Dionne K, Hedgepeth A, Carroll K, Parrish N. Evaluation of a Rapid Fluorescent Staining Method for Detection of Mycobacteria in Clinical Specimens.

J Clin Microbiol 2009; 47: 1206–1208.

252 Cattamanchi A, Dowdy DW, Davis JL, *et al.* Sensitivity of direct versus concentrated sputum smear microscopy in HIV-infected patients suspected of having pulmonary tuberculosis. *BMC Infectious Diseases* 2009; 9: 53.

253 World Health Organization, Association of Public Health Laboratories, CDC, IUATLD, KNCV Tuberculosis Foundation,, Research Institute of Tuberculosis. External Quality Assessment for AFB Smear Microscopy. Geneva, Switzerland, World Health Organization, 2002.

254 Pang S, Han C, Lee LM, Yang C. Fluorescence microscopy imaging with a Fresnel zone plate array based optofluidic microscope. *Lab Chip* 2011; 11: 3698–3702.

255 Zhu H, Yaglidere O, Su T-W, Tseng D, Ozcan A. Cost-effective and compact wide-field fluorescent imaging on a cell-phone. *Lab Chip* 2011; 11: 315–322.

256 Tuijn CJ, Hoefman BJ, van Beijma H, Oskam L, Chevrollier N. Data and Image Transfer Using Mobile Phones to Strengthen Microscopy-Based Diagnostic Services in Low and Middle Income Country Laboratories. *PLoS ONE* 2011; 6: e28348.

257 Nadol P, Stinson KW, Coggin W, *et al.* Electronic tuberculosis surveillance systems: a tool for managing today's TB programs. *Int J Tuberc Lung Dis* 2008; 12: 8–16.