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NOVEL DIAGNOSTIC APPROACH FOR TUBERCULOSIS DIAGNOSIS

by

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Declaration

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Abstract

There is a clear need for a rapid, inexpensive point-of-care diagnostic test for tuberculosis (TB). The aim of this study was to analyze the performance of a novel rapid diagnostic test for TB, GeneXpert MTB/RIF, in symptomatic adults and to compare it to other commercially available nucleic acid amplification assays and to standard microbiological smear and culture. The GeneXpert system performs real time, nested PCR from sputum and provides a result within two hours of sampling. The result includes a semi-quantitative assessment of bacillary load in the sample and simultaneously detects rifampicin resistance.

This study was part of a cross-sectional, multi-centre clinical trial. The Cape Town component of this study was conducted at three sites, one hospital based and other two community clinics, all with high TB/HIV coinfection rate.

Among 43.2% of patients diagnosed with TB during the evaluation study, GeneXpert detected TB in 95.5% of all culture positive cases. In smear positive patients, sensitivity was 99.0% and in smear-negative, culture positive patients, 86.1%. Specificity in patients who were culture negative and clinically diagnosed as non-TB after follow up was 98.4%. Sensitivity and specificity of GeneXpert in detecting rifampicin resistance was 100% comparing to phenotypically detected drug resistance.

GeneXpert is a highly promising novel tool for the rapid diagnosis of adult TB. Future studies are needed to establish the performance and impact of GeneXpert when performed at the level of the microscopy centre.

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Acknowledgements

This evaluation study of the FIND/Cepheid GeneXpert MTB/RIF assay for the detection of pulmonary tuberculosis in sputum of symptomatic adults was carried out at the Institute of Infectious disease and Molecular Medicine (IIDMM) and the National Health Laboratory Services (NHLS) laboratory, a part of Groote Schuur Hospital (GSH), with Foundation for Innovative New Diagnostics (FIND) as study sponsor. Patients were recruited at GF Jooste Hospital - Manenberg, Khayelitsha Site B Community Health Clinic - Khayelitsha and Hannan Crusaid Treatment Centre - Gugulethu, Cape Town.

The study principal investigator was Dr M. Nicol, who provided logistical support and guidance throughout the study, with collaborators Dr G. Hussey (IIDMM) and Dr C. Boehme (FIND), who provided study protocol. Clinical personnel were recruiting patients at selected locations, with medical doctors T. Oni and Dr S. Lawn and study nurses, L. Bashe, Y. Hlombe and R. Tsekela and a driver who transported samples from recruiting sites to NHLS, M. Baliwe.

Traditional smear, culture, speciation and drug susceptibility testing for MTB was performed at NHLS thanks to the laboratory personnel: Dr A. Whitelaw, head of NHLS microbiology laboratories at Groote Schuur, K. Mentoor, head of the TB laboratory and R. Jacobson, who did preliminary sample analysis, sample randomisation, decontamination and culture cultivation. Y. Ghebrekristos and C. Visser performed speciation testing and additional molecular tests on drug resistant samples, and helped with establishing molecular tests in the IIDMM molecular laboratory.

Researcher F. Liesengang and I performed molecular tests on all samples including Xpert and Cobas Amplicor, with support from R. Thomas form Roche Products (Pty) Ltd - Diagnostics Division, in establishing assay in the IIDMM molecular and TB laboratory.

Results were entered into a case report form and data were captured and sent to the FIND general database thanks to J. Workman and S. Adams.

We had monitoring visits from our sponsor, FIND, every three months and monitor Dr P. Nabeta helped us with maintaining the study flow and with problems we encountered on a daily basis.

A clinical review committee comprised of Dr M. Mendelson, Dr G. Meintjies, T. Oni, local TB clinicians and Dr M. Nicol reviewed discrepant cases requiring follow-up considering smear,

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culture and culture-based DST results (not GeneXpert MTB/RIF and alternative NAAT results) as well as clinical records and radiographic information.

I extend my gratitude to my supervisor, Professor Dr M. Nicol, who took me into the medical microbiology laboratory and guided me through the project.

I would like to thank all the volunteers participating in the study and I hope that once Xpert MTB assay has passed the demonstration study and becomes available to the public, it will significantly complement current diagnostic tools. In addition, I have great expectations for GeneXpert MTB/RIF system as diagnostic tool in helping to fulfil the goals of WHO to halt and to begin to reverse the incidence of TB by 2015.

My role in the study was as a researcher, establishing and performing molecular tests GeneXpert MTB/RIF assay, Cobas Amplicor and Hain MTBDRplus and a clinical operation manager, helping the principal investigator coordinate the research team, site organisation and data capturing and statistical analysis. I accept responsibility for the conduct of the study and support all presented data. I have performed this analysis and write up independently.

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Abbreviations

AFB	acid fast bacilli
AIDS	acquired immunodeficiency syndrome
APC	antigen presenting cells
ART	antiretroviral therapy
BCG	bacille Calmette-Guérin
BSL	biosafety level
CCD	charge coupled device
CDC	Centers for Disease Control and Prevention
CFP-10	culture filtrate protein 10
CFU	colony forming units
CR3	complement receptor-3
CSF	cerebrospinal fluid
Ct	threshold cycle
CTAB	cationic detergent cetyltrimethylammoniumbromide
CXR	chest X-ray
DC	dendritic cell
DNA	deoxyribonucleic acid
DOR	diagnostic odds ratio
dsDNA	double-stranded deoxyribonucleic acid
DST	drug susceptibility test
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
EMB	ethambutol
EPTB	extrapulmonary tuberculosis
ESAT-6	early secretory antigen target 6
FDA	Food and Drug Administration
FIND	Foundation for Innovative New Diagnostics
FU	follow-up
GC	guanine and cytosine
GFJ	GF Jooste Hospital

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GSH	Groote Schuur Hospital
GU	growth units
HIV	human immunodeficiency virus
IC	internal control
IFN- γ	Interferon γ
IGRA	Interferon-γ release assay
IIDMM	Institute of Infectious disease and Molecular Medicine
IL-12	interleukin 12
INH	isoniazid
LAM	lipoarabinomannan
LAMP	loop-mediated isothermal amplification
LCx	ligase chain reaction
LJ	Löwenstein-Jensen
LPA	line probe assays
LTBI	latent tuberculosis infection
MDR TB	multidrug-resistant tuberculosis
MGIT	Mycobacteria Growth Indicator Tube
MHC	major histocompatibillity complex
MIC	minimal inhibitory concentration
MODS	microscopic observation drug susceptibility
MSF	Medecins Sans Frontieres
MTB	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
NAAT	nucleic acid amplification test
NALC	N-Acetyl-L-cysteine
NaOH	sodium hydroxide
NHLS	National Health Laboratory Services
NPV	negative predictive value
NRA	nitrate reductase assay
NTM	non-tuberculous mycobacteria
PANTA	polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PIM	phosphatidylinositol mannoside

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PPD	purified protein derivative
PPV	positive predictive value
PZA	pyrazinamide
RD	regions of difference
RD1	region of difference-1
RIF	rifampicin
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SDA	strand displacement amplification
SDS	sodium dodecyl sulfate
SIRE	streptomycin, isoniazid, rifampicin, ethambutol
SM	streptomycin
SNP	single nucleotide polymorphisms
ssDNA	single-stranded deoxyribonucleic acid
STAG	Strategic and Technical Advisory Group
TB	tuberculosis
TLR	toll-like receptors
Tm	melting temperature
TMA	transcription-mediated amplification
TNF-α	tumor necrosis factor α
TST	tuberculin skin test
UDG	uracil DNA glycosylase
WCP	Western Cape Province
WHO	World Health Organization
XDR TB	extensively drug resistant tuberculosis

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I Literature review

This literature review will briefly review the epidemiology, pathology, clinical features and treatment of tuberculosis (TB), followed by a more detailed discussion of TB diagnostics with particular focus on the molecular diagnostic approach and commercial tests currently available.

1 Epidemiology

1.1 Global tuberculosis epidemiology

Tuberculosis is the seventh leading cause of death worldwide, second only to human immunodeficiency virus (HIV) acquired immunodeficiency syndrome (AIDS) among infectious diseases (Keeler et al., 2006). There are 2 billion people today infected with *Mycobacterium tuberculosis* (MTB) and 1.7 million deaths worldwide are attributed to TB (WHO, Acute Respiratory Infections, 2009). In 1990 there were 6.6 million TB cases, while in 2007 there were an estimated 9.27 million cases per year (WHO report, 2009).

Most of the estimated TB cases (92%) in 2007 were in developing countries (WHO report, 2009). Also, 98% of TB-related deaths occur in the developing world (Charles et al., 2006). TB is generally referred to as a poverty-related disease. The TB epidemic has been contained in developed countries by introducing health measures such as screening, surveillance and follow-up and a general improvement in living conditions (Modi et al., 2009). However, international travel and countries caught in war and conflict have contributed to a TB increase worldwide (Frothingham et al., 2005, Modi et al., 2009).

It was estimated that 15% of TB cases were HIV-positive and 79% of these HIV-positive cases were in the WHO African region (WHO report, 2009). The number of AIDS-related deaths has declined by over 10% over the past five years (WHO AIDS epidemic update, 2009) and about half a million deaths occurred among HIV-positive patients diagnosed with TB in 2007- which means that one in four TB deaths was HIV-related (WHO report, 2009). The paradoxal effect of antiretroviral therapy (ART) on HIV/TB coinfected patients provokes the immune system to act against TB antigens and further complicates the patient's condition (WHO, Acute Respiratory Infections, 2009).

HIV has distorted demographic statistics in the TB epidemic. TB is no longer disease of older men; HIV has created a population of immune-depressed hosts, perfect for TB to thrive. So now, in a setting where HIV prevalence exceeds 1%, patients with TB/HIV coinfection are younger and tend to be more frequently female (Lawn et al., 2006, WHO report, 2009).

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Children under the age of 14 contributed 10% to the total number of new cases in Africa and 2% in developed countries in 2004 (Dye, 2006), but this figure is influenced by difficulties in diagnosing TB in children. Unfortunately, children suffer from severe TB morbidity and mortality, especially in endemic areas (Marais et al., 2007).

Even with rising awareness of TB presence worldwide and global projects aimed at reducing rates of TB incidence, Africa and Europe will still not be able to reach the WHO goal of halving the prevalence of TB disease from 300/100 000 to 150/100 000 and deaths from 30/100 000 to 15/100 000 by 2015 (WHO report, 2009).

1.2 Tuberculosis in South Africa

South Africa's contribution to the worldwide incidence in 2007 was estimated to be 0.46 million total cases per year (WHO report, 2009). The incidence of all TB types in South Africa rose sharply from 169/100 000 in 1998 to 739.6/100 000 in 2007. In 2007 the incidence of all TB cases in the Western Cape Province was 1005.7/100 000 (Health Systems Trust, Tuberculosis).

As in other developing countries with underdeveloped health care infrastructure, estimates were higher than reported cases and in South Africa there was a difference of 150 000 unreported cases of TB (WHO report, 2009).

Antenatal HIV prevalence has risen in South Africa from 1.7% in 1991 to 28% in 2007 (Health Systems Trust, HIV). It is estimated that HIV positive incident TB cases represented 73% of all TB cases in 2007, which is by far the worse incidence among TB cases in the world (WHO report, 2009).

1.3 Multidrug resistant tuberculosis

MTB has affected humankind since 3000- 5000 BC. It reached its climax during the seventeenth and eighteenth century when 1 in 5 deaths were attributed to TB (Iseman, 1994). The first remedy for TB, streptomycin (SM), was discovered in 1943 and initial reports were highly promising (Feldman et al., 1948). However, it was soon discovered that different strains of the same species vary greatly in sensitivity and that some gain resistance (Waksman et al., 1945, Iseman, 1994). Changes were introduced into the drug regimen for TB, while MTB adaptation to administered drugs followed.

In the 1960s rifampicin (RIF) was introduced as a drug used in combination therapy, and it lead to a decline in the number of TB cases worldwide (Johnson et al., 2006). Later outbreaks of TB

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resistant to drugs used in combinational therapy occurred in the 1980s, in association with the HIV epidemic (Small et al., 1993).

At present, combinational therapy of first-line drugs: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) or streptomycin (SM) for 6 months is recommended (Treatment of tuberculosis Guidelines, 2009). If TB is caused by an MTB strain resistant to the two first-line drugs INH and RIF, it is recognised as multidrug resistant-TB (MDR TB) (Zhang et al., 2009).

Worldwide, 5% of all TB cases are MDR TB, based on data from more than 100 countries collected during the last decade (WHO, Tuberculosis facts, 2008). There were an estimated 0.5 million cases of MDR TB in 2007 and more than half were among new TB cases (WHO, Tuberculosis facts, 2008).

INH mono-resistance is common, with 6.7% of new TB cases INH resistant in surveyed countries (Wright et al., 2009). However, RIF mono-resistance is rare. Thus, RIF resistance is used as a surrogate marker for MDR-TB as nearly 90% of RIF resistant strains are also INH resistant (Johnson et al., 2006).

South Africa, with almost 16 000 MDR cases annually, is ranked fourth among the leading countries representing 0.5 million MDR cases in 2007 (WHO report, 2009). In South Africa, 1.8% of all new TB cases are MDR and 6.7% of previously treated TB cases are MDR (WHO report, 2009).

Out of all MDR cases worldwide in 2007, 6.6% were found to be infected with strains resistant to first-line drugs (RIF and INH) and two classes of second-line drugs, a fluoroquinolone (such as ofloxacin) and an injectable drug (amikacin, kanamycin or capreomycin) and were therefore defined as extensively drug resistant or XDR TB (Schaaf et al., 2009, Wells et al., 2007). XDR TB had been identified in 57 countries in all regions of the world by 2009 (WHO report, 2009). Mortality in high HIV prevalence settings was 40% among patients with MDR TB and 51% among patients with XDR TB, within the first 30 days after sputum collection (Gandhi et al., 2010). In a recent outbreak in a rural area of KwaZulu-Natal in South Africa, 52 out of 53 people infected with XDR strains died on average within three weeks from diagnosis (Gandhi et al., 2006).

The collision of two epidemics, TB and HIV, has created extraordinary rates of mortality, the majority occurring before a diagnosis is made (Gandhi et al., 2010). A rapid diagnostic test that

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is sensitive in HIV infected patients and can be used at any rural health centre level is urgently needed.

So far, attempts to challenge the MDR TB epidemic with available diagnostics, drugs, and vaccines have not had the desired success. Fortunately, rapid diagnostic tests are in late stages of clinical evaluation, drug candidates with new mechanisms of action have recently shown positive results in trials, and six vaccines are in human trials (Borgdorff et al., 2009).

2 Mycobacterium tuberculosis

2.1 *M. tuberculosis* structure and morphology

Mycobacterium tuberculosis complex (MTBC) is a group of closely related species and subspecies that include MTB, namely *Mycobacterium africanum, Mycobacterium bovis, Mycobacterium caprae, Mycobacterium microti*, and *Mycobacterium pinnipedii* (Gordon et al., 2009). MTBC is clustered with Gram-positive bacteria, since it has a peptidoglycan layer. However, MTB peptidoglycan has some distinctive characteristics as its outer layer consists of glycolipids – major cell wall component, lipoarabinomannan (LAM), polysaccharides and unique lipids with a hallmark molecule of *Mycobacteria*, mycolic acid (Cole et al., 1998, Chatterjee, 1997). In addition, such membranes form dye complexes that are not decolourised following exposure to acidic ethanol or mineral acids. This property is recognised as acid fastness (Barksdale et al., 1977).

Almost 60% of the bacteria's dry weight consists of lipids, which makes it highly hydrophobic and as a consequence, impermeable for drugs (Cole et al., 1998). *In vivo* fatty acids are a major nutrient source and besides their role as nutrients, fatty acids, primarily in the form of mycolic acids, are a source of pathogenicity (Ehrt et al., 2007). Some differences in the host's immune response to different strains are related to the lipid composition in MTB's cell envelope (Ehrt et al., 2007).

Mycobacteria are obligate aerobes, non-spore forming and non-motile (Dinnes et al., 2007, Barksdale et al., 1977). Microscopically, mycobacteria appear as spherical cells although, in cultures, mycobacteria show an inhibition of post-divisional cell separation forming short filaments, which can be branched (Barksdale et al., 1977). Tubercle bacilli from clinical specimens are usually rod-like cells. Individual bacilli are 0.5 to 1.0 μm in diameter and 1.5 and 10 μm long (Figure 1) (Friedman, 2000).

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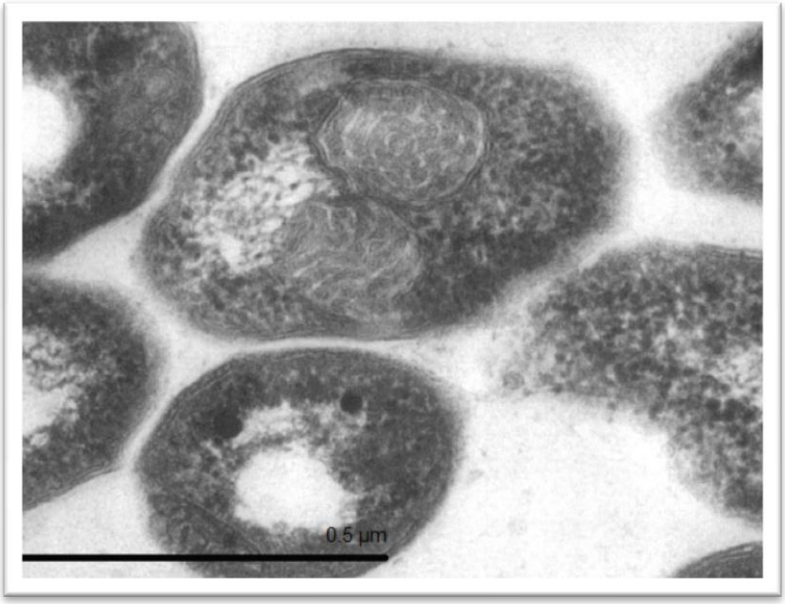

Figure 1 Actively growing MTB H37Rv 102, (adapted from Barksdale et al., 1977)

2.2 Nutritional factors and growth

In vitro MTB can grow in simple synthetic media with glycerol and ammonium salts with nutritional preference for lipids like free fatty acids found in fresh eggs (Löwenstein-Jensen medium) or purified oleic acid in Middlebrook's medium (Bannister et al., 1996). MTB can persist in a medium with fatty acids as its sole carbon source; moreover it depends on fatty acid metabolism to resist being killed by the immune system (Ehrt et al., 2007). A high concentration of long chain fatty acids has an inhibitory effect on MTB growth so albumin is added to the medium to buffer the concentration of fatty acid (Bannister et al., 1996). Although MTB utilises oxygen metabolism it has low reproductive rates and long generation times.

If MTB is grown on a solid culture it forms rough, dry, and cauliflower-like colonies (Gordon et al., 2009). In solution, MTB forms surface pellicles, (hence the name mycobacterium or mould-like) and even if non-ionic detergents are used to grow it in a more dispersed form, it seldom grows as a single cell. Shaking is a preferred method of cultivation, at a carbon dioxide concentration of 6-8% (Barksdale et al., 1977). If other bacteria are presented in an inoculum they will rapidly overgrow MTB, so samples used to inoculate media require decontamination.

In vivo, MTB is usually ingested by the lungs' macrophages, and within hours of infection bacteria are found in phagolysosomes, whereas some escape and are found in the cytoplasm (McDonough et al., 1993). Virulent MTB strains (see chapter 1, section 3.5), can persist and proliferate in a macrophage, by inhibiting fusion with lysosomes. Conversion into dormant form

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is proposed to be influenced by oxygen limitation since it changes the metabolic state of bacteria (Rustad et al., 2009, Boshoff et al., 2005). While in dormant state, MTB cannot be detected using culture, but PCR has detected MTB in murine models (De Wit et al., 1995).

2.3 *M. tuberculosis* genome and evolution

MTB has a large genome. MTB strain H37Rv has 411 529 bp, and is remarkably guanine and cytosine (GC) rich; 65.6% of the whole genome comprises GC pairs (Cole et al., 1998). In 1998, when the first MTB genome was sequenced, it was the second largest bacterial genome available (the largest was *Escherichia coli*).

Since the publication of the H37Rv genome there have been nearly 20 MTBC strains with complete or partially complete genomes sequenced (Gordon et al., 2009). Identifying the differences between genomes of virulent and non-virulent mycobacteria leads toward understanding phenotypic diversity and is a base for new vaccine, drug and diagnostic approaches.

Differences in MTB strains' genomes are analysed within larger polymorphic regions, greater than 0.5 kb, named regions of difference (RD) loci. The most widely used vaccine containing viable attenuated BCG strain has portions of RD1 region missing, while the recombinant BCG:RD1 has at most partially restored it's virulence (Pym et al., 2002).

From an evolutionary perspective it was believed that MTB emerged as a human disease 10 000 -15 000 years ago as a consequence of a bovine to human transmission, at the time cattle were domesticated (Cole et al., 1998). However, recent discoveries imply co-evolution of bovine and human TB strains, since human bone samples dated from the Pleistocene era have TB-like lesions (Donoghue et al., 2004); while both cattle and humans have the same ancestral origin in environmental mycobacteria (Stinear et al., 2008). It appears that the direct ancestor of present-day MTB emerged in East Africa, where co-evolution between the bacterium and man-shaped tuberculosis formed a lifelong asymptomatic symbiosis with this pulmonary disease (Gutierrez et al., 2005, Gordon et al., 2009).

MTBC shows an extraordinary genetic homogeneity (with 0.01–0.03% variations between genomes within MTBC), and does not allow any lateral exchange of genetic material, leading to clonal evolution (Gordon et al., 2009). Even with exclusive homogeneity, several distinctive strains and strain lineages emerged from this clonal evolutional pattern, mostly by silent single nucleotide polymorphisms (SNP) as well as deletion, duplication and insertion deletions (Ernst et al., 2007). Based on the absence of a specific RD locus named TbD1, MTB strains can be

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divided into ancestral and modern strains. Modern strains are characterised by the absence of the TbD1 region (Brosch et al., 2002). Lineages like W-Beijing, Haarlem, CAS and MTB H37Rv, belong to the modern families. Ancestral strains like the EAI family have an intact TbD1 region and are prevalent in India, Bangladesh, and South East Asia (Gordon et al., 2009, Brosch et al., 2002). Since ancestral strains are mostly found in one part of the world, it may indicate that they have preference for a specific host and this fact is of critical when developing new vaccines, and new drugs.

3 Pathology

TB has been a disease of humans for thousands of years (Donoghue et al., 2004), but it was only in 1882 that MTB was found to be the cause of disease, when Robert Koch discovered that tubercle bacillus is the etiologic agent responsible for TB (Koch 1882, Gordon et al., 2009).

As mentioned above, one third of the world's population is infected with MTB but only 5% to 10% of this population has a lifetime risk of developing active tuberculosis (Van Crevel et al., 2002). MTB is either eradicated by the immune system or remains dormant for years. Factors influencing the outcome of infection could be either pathogen or host related (McDonough et al., 1993, Brosch et al., 2002, Caws et al., 2008).

3.1 Infection on a cellular level

MTB is an airborne pathogen and infection is caused by inhalation of droplet nuclei no bigger than 5 µm in diameter, from a cough or sneeze carrying MTB. Droplets remain in the air for several hours and if during that time enough droplets containing bacilli are inhaled MTB will cause infection in two thirds of immunocompetent individuals. (Beggs et al., 2003)

Once inhaled by a human host and deposited in alveoli MTB initiates infection (Quast et al., 2006). At the same time host immunologic defence starts. Alveolar macrophages or dendritic cells (DC) first encounter MTB in the lungs. Three types of receptors have been identified for phagocyosis of MTB by macrophages and DCs: complement receptors (responsible for uptake of opsonized MTB), and manose and scavenger receptors for uptake of nonopsonized MTB (Van Crevel et al., 2002). Phagocytosis initiates a cascade of events engaging innate immunity while initiating the intracellular life-stage of MTB in a phagosome (Korbel et al., 2008). At this point MTB can take the course of a disease or it can be cleared by the immune system. The outcome depends on the microbicidal capacity of host phagocytes and virulence factors of the ingested mycobacteria (Van Crevel et al., 2002).

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MTB can survive phagocytosis, escape phagolysosomal fusion and proliferate within macrophages since it has a wide spectrum of survival strategies (Martino, 2008). MTB uses passive phagocytosis to enter macrophages, since it binds to: complement receptors (CR3) (opsonized phagocytosis), mannose-binding receptors, and scavenger receptors (non-opsonized phagocytosis) (Korbel et al., 2008, Van Crevel et al., 2002). MTB uses its cell wall components, mannose-containing cell wall glycolipids, like LAM and phosphatidylinositol mannoside (PIM) to bind with phagocyte surface receptors (Korbel et al., 2008). This silent phagocytosis enables MTB to enter phagocytes without triggering a reactive oxygen respiratory burst or other cytocidal mechanisms (McDonough et al., 1993, Korbel et al., 2008). Thus, MTB can persist and proliferate within macrophages and to some extent within DC, and this will lead to disruption of the macrophage (Korbel et al., 2008, Van Crevel et al., 2002). When this happens, blood monocytes and other inflammatory cells are attracted to the lungs, beginning the second stage of the disease.

Disruption of macrophages attracts blood monocytes into the lungs and they differentiate into macrophages that will also ingest MTB without being harmed, creating an event loop enabling MTB to proliferate and thrive within the macrophage (Van Crevel et al., 2002). This stage lasts for 2-4 weeks until T-cell acquired immunity develops (Quast 2006, Van Crevel et al., 2002).

3.2 Immune response to *M. tuberculosis*

The innate immune system, and subsequently acquired immune system, can clear MTB infection or sustain disease development. Once MTB gets into the lungs, factors at epithelial surfaces like collectins, defensins and natural antibodies may interfere with further MTB infection (Korbel et al., 2008).

Then, MTB is recognised as a pathogen by a macrophage (Figure 2) using evolutionary conserved recognition patterns – MTB's LAM resembles Gram-negative bacterial lipopolysaccharide (LPS) engaging toll-like receptors (TLR) (Korbel et al., 2008, Martino, 2008). Sensory functions of TLRs promote signalling cascades of inflammation while engaging MyD88, responsible for releasing chemokines and pro-inflammatory cytokines (Reiling et al., 2008, Van Crevel et al., 2002). The downstream signalling cascade upon activation of TLRs and MyD88 leads to the transcription of genes involved in the activation of the innate host defence, particularly cytokines such as tumour necrosis factor α (TNF-α), and interleukin 12 (IL-12) and chemokines (Jo, 2008).

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Cytokine production is a vital step in response to MTB infection. TNF-α is a proinflammatory cytokine, produced by macrophages while initiating autoinduction of microbicidal activity. IL-12 is produced by macrophages upon MTB phagocytosis, and is responsible for activation of Interferon γ (IFN- γ) production and initiation of adaptive immunity (Van Crevel et al., 2002, Jo, 2008). IL-1 is also a proinflammatory cytokine, and IL-15 and IL-18 work in conjunction with IL-12.

Figure 2 M. tuberculosis (orange) binds to the TLR-2 receptor on the surface of a macrophage (green), (adapted from Kaufmann, 2008)

Only after 9-14 days of extensive growth of the MTB population in alveoli, are the bacteria first detected in the draining lymph node. Upon IL-12 stimulation, DC migrates to the nearest lymph node and presents MTB antigens to naïve T-cells (Cooper et al., 2007). As the naïve T-cells become activated, they proliferate and begin to accumulate in the lungs. Migration of activated T-cells is also delayed and takes place 15-18 days post infection (Cooper, 2009). Activated CD4+ T-cells undergo clonal expansion and migrate to the lungs where they start producing cytokines of the Th1 milieu and represent the main source of IFN- γ. IFN- γ activates a bactericidal mechanism in macrophages, which in collaboration with TNF-α, activates synthesis of oxidative stress proteins. These enzymes are able to synthesize reactive nitric and oxygen intermediates, with microbicidal effect.

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3.3 Tuberculosis disease

Once acquired immunity is activated, 2-3 weeks after infection, T-cells have migrated to the site of infection and activated macrophages kill intracellular bacteria. By doing so, a necrotic centre is created, also known as a caseous centre (from the Latin *caseus*, meaning cheese), with necrotic tissue centrally and granulation tissue containing viable phagocytes and T-cells peripherally. Until this point MTB grows logarithmically with a 28h doubling time, while formation of the caseous centre inhibits growth of mycobacteria to some extent (Cooper, 2009, Van Crevel et al., 2002). Only at this point does the infected patient become tuberculin positive (see chapter 1, section 7.1), and this is usually 1-2 months after exposure to MTB (Quast et al., 2006).

MTB infection contained within the caseous lesion can heal; it may become latent or dormant; it may disseminate MTB into the bloodstream or lymph drains; it may liquefy and form a cavity (Quast et al., 2006, Van Crevel et al., 2002). Cavity formation provides the best conditions for extracellular growth of MTB. The cavity can enlarge and may cause rupture of bronchi, allowing MTB to enter airways and the outside environment, spreading the disease (Van Crevel et al., 2002).

Figure 3 shows possible outcomes following infection with MTB with number of cases worldwide.

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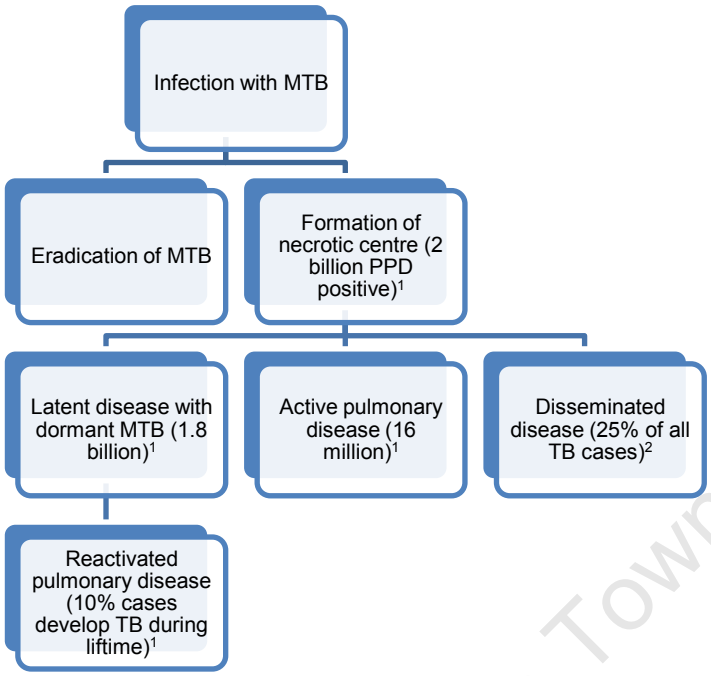

Figure 3 Chronological events following MTB infection, adapted from Van Crevel et al., 2002, ((1)-Rustad et al., 2009, (2)-Harries et al., 2004)

Latent tuberculosis infection (LTBI) is characterised by MTB persistence within a cavity (Van Crevel et al., 2002, Rustad et al., 2009). A spectrum of MTB dormancy is characterised as a latent disease: cavitary lesions can be calcified with few viable bacteria; caseous lesions can contain low number of bacteria; or evolving lesions can contain replicating bacteria but they are actively destroyed by the immune system (Rustad et al., 2009). The risk of reactivation or progression from LTBI to active disease is greatest within the first 2 years after infection and subsequently declines over time but does not disappear (Keeler et al., 2006).

Active TB can arise as a rapidly progressive primary disease or from reactivation of latent infection. Primary disease occurs in only 3% to 10% of those exposed, while most of the infected individuals are able to keep the disease latent or heal (Quast et al., 2006). The clinical outcome of primary disease can be tuberculous pneumonia, hyperinflation and collapse/consolidation or pleural effusion (Harries et al., 2004).

Reactivating latent tuberculosis or exogenous reinfection is referred to as postprimary TB (Quast et al., 2006). Postprimary TB usually affects the lungs but can involve any other part of the body. The clinical picture of postprimary TB consists of upper lobe involvement with extensive lung destruction. Patients with these lesions are the main transmitters of infection in the community (Harries et al., 2004).

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TB can affect any part of the body (nervous system, pleura, lymph nodes, bones and joints, pericardia, skin, larynx, gastrointestinal and genitourinary systems, eyes, endocrine system and ears) after MTB enters the blood stream and lymphatic system and this condition is generally referred to as extrapulmonary tuberculosis (EPTB) (Drobniewski 2005). Up to 25% of TB cases may present with EPTB (Harries et al., 2004). EPTB ranges from localised extrapulmonary infection to disseminated disease and most common forms of EPTB are: disseminated disease causing miliary TB or TB meningitis, pleural effusion, lymphadenopathy, pericardial disease. EPTB can also have coexistent pulmonary TB (Harries et al., 2004, Jacob et al., 2008).

Vaccination against TB with *Mycobacterium bovis* BCG affects acquired T-cell immunity, but is more effective against disseminated infection than against pulmonary disease (Colditz et al., 1994).

3.4 Risk factors

If the host immune system is uncompromised it typically clears TB infection and only 10% of infected immunocompetent individuals develop disease (Corbett et al., 2003). This number is much higher if the immune system is compromised. HIV infection is the most important factor in altering the course of a TB epidemic. In settings where TB and HIV infection overlap, TB is the main cause of morbidity and mortality in HIV infected population (Harries et al., 2004). TB/HIV coinfected patients have an estimated 10% risk of developing active TB each year (Corbett et al., 2003).

In Africa the major consequence of TB/HIV coinfection is the high risk of reactivation of LTBI in HIV infected persons and the high risk of progressive TB by reinfection owing to the high TB transmission rate in the community (Lienhardt et al., 2005). HIV positive patients may be at 10 times greater risk of developing MDR TB (Dinnes et al., 2007). TB in HIV positive patients has unusual clinical features: atypical pulmonary manifestations or false-negative microbiological results, which can cause diagnostic difficulties (Dinnes et al., 2007).

Other factors affecting immune response include genetic defects in IFN-γ production, therapeutic interference in TNF-α activity (treating rheumatoid arthritis), nutritional factors (vitamin D deficiency), and ageing and alcohol or drug abuse, all of which may negatively alter the outcome of infection with MTB (Schwenk et al., 2000).

Malnutrition and poverty were the most important TB risk factors before the HIV era. Low protein intake and a consequently depressed immune system were the main reason for TB epidemics through history (Schwenk et al., 2000).

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Age is an important factor in disease progression, and in settings where transmission of MTB has been stable or increasing for many years, the incidence rate is highest among young adults, and most cases are recently infected. As transmission falls, patients are likely to be older adults, and a higher proportion of cases are due to the reactivation of latent infection (Dye et al., 2006).

Environmental factors associated with TB are mainly products of poor living conditions: a high number of adults in the household, risk of TB is higher in singles than in married individuals, smoking (with a dose–effect relationship), a history of asthma, and a family history of previous TB (Lienhardt 2005).

3.5 *M. tuberculosis* strains and virulence

Virulence of the strain and tropism for specific tissues may determine the course of the disease (Diagnostic Standards, 2000). Also, MTB's tendency to acquire drug resistance as well as transmissibility between human hosts may be influenced by the genetics of MTB strain types (Wirth et al., 2008).

The cell envelope is responsible for most immunological characteristics of MTB. Members of the W-Beijing family of MTB strains have been associated with extensive morbidity and mortality worldwide (Bifani et al., 2002). Moreover, infection with W-Beijing accelerates time to death in mice, even though growth of these strains in murine models is not significantly different from several non-WBeijing isolates. The outcome of infections with different strains correlates with their ability to induce a strong Th1 response and those differences are likely to be attributable to the lipid composition of the cell envelope (Ehrt et al., 2007).

In vivo generation time may be concurrent with strain virulence: virulent laboratory strain H37Rv has a generation time of 31h, while a strain isolated during a TB outbreak has shown to grow more rapidly with a cellular generation time of 25h (Valway et al., 1998).

Using comparative genomics and molecular epidemiological tools it is demonstrated that the global population structure of MTB is defined by six phylogeographical lineages and that these are adapted to particular human populations. Those results agree with the observation that geographic subdivision exists for MTB and suggest that human migrations leading to human population bottlenecks might be responsible for this area-specific distribution of the pathogen (Arora et al., 2009).

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4 Clinical manifestations of tuberculosis

Pulmonary TB can present with a wide variety of non-specific symptoms like cough, with or without mucus production. Symptoms like weight loss, malaise, fever, night sweats, as products of TNF-α activity, are also common. The patient usually presents to the health professional after ≥2 weeks of persistent productive cough. Laboratory studies usually reflect a non-specific reaction to the infection: anaemia, leukocytosis, and an elevated sedimentation rate are common in TB (Quast et al., 2006).

Children have different manifestations of the disease, highly dependent on their age and immunological status: newborns usually present with miliary TB and TB meningitis, and older children infected with TB can develop primary pulmonary tuberculosis (Cruz, et al., 2007). Childhood tuberculosis is manifested with strong inflammatory response to a relatively low burden of organisms, making it difficult to diagnose TB in culture (Cruz, et al., 2007).

4.1 Chest X-ray

Chest radiography is often used in the diagnosis of patients with active pulmonary TB. Pulmonary TB can present with any radiographic abnormality; or it can appear completely normal. In patients with reactivated pulmonary TB, lesions are found in upper lobes while in primary TB, the radiograph often shows abnormalities in lower or middle lobes predominantly with infiltrate without cavitation and associated hilar adenopathy. TB in the lower lobes takes a longer time to diagnose than upper lobe TB, and is associated with a high mortality rate (Quast et al., 2006).

HIV positive patients often have an atypical chest X-ray for TB. The degree of immunosupression reflects on a radiograph such that in mild case of immunosupression, the appearance is typical for TB (with cavitation and upper lobe infiltrates). In severe immunocompromise, the appearance is often atypical (with no cavitations) (Harries et al., 2004). Some 36% of HIV infected patients have a primary pattern and 28% have a post-primary MTB pattern. In HIV infected patients with less than 200 $CD4^+$ T-cells/μl a normal chest X-ray was seen in 21% of all cases (Tuberculosis. Clinical diagnosis and management, 2006).

5 Treatment of tuberculosis

The goal of TB treatment is to eliminate all TB bacilli from the infected individual and avoid emergence of TB resistance. The general approach is to administer multiple drugs to which the organism is susceptible, and to add at least two new drugs if treatment failure is suspected, to ensure the safest and most effective therapy in the shortest period (McPhee, 2008).

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Treatment of TB requires combination therapy with isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) or streptomycin (SM). Patients with previously untreated TB can be effectively treated with a six to nine month regimen. The initial phase consists of daily INH, RIF, PZA and EMB for two months. The second phase consists of RIF and INH for a minimum of four months or at least three months beyond conversion of sputum and culture to negative for MTB (Centers for Disease Control and Prevention (CDC), TB in persons not infected with HIV). Treatment of HIV positive patients can be longer, depending on the $CD4^+$-T-cell count and rifabutin can be used instead of rifampin which can interact with certain antiretroviral agents (CDC, TB Treatment of Drug-Susceptible Tuberculosis Disease in HIV-infected persons, 2003).

The treatment is long and can last up to nine months (CDC TB Treatment of Drug-Susceptible Tuberculosis Disease in Persons Not Infected with HIV, 2003) Patient adherence to treatment is problematic and rates of treatment completion are often low. Failure to adhere to the full treatment leads to the emergence of drug resistant strains due to inadequate dosing, making MTB growth conditions less bactericidal (Lipstich et al., 1998). The second reason for drug resistance is based on compartmentalisation of infection, since MTB grows inside and outside of macrophages, at different pH conditions within lung cavities. This may lead to suboptimal drug concentration, leading to resistance (Gillespie 2002, Lipstich et al., 1998). Also, the size of the initial bacterial population is associated with the possibility of resistance, based on mathematical models (Gillespie 2002).

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5.1 Isoniazid

INH or isonicotinic acid hydrazide is a nicotinamide analog and has been used for TB treatment since 1951 (Johnson et al., 2006, Slayden et al., 2000). It enters the cell as a prodrug and gets activated by the catalase peroxidase encoded by *katG.* Activated INH affects mycolic acid biosynthesis while binding to ACP (enoyl-acyl carrier) reductase encoded by the *inhA* locus. The InhA-NADH complex becomes locked and aborts the reduction step in mycolic acid synthesis. This eventually results in loss of cell wall integrity and the bacteria die (Johnson et al., 2006).

INH is used in the early stage of TB therapy. It is active mainly against MTB growing aerobically in pulmonary cavities and it rapidly reduces the number of MTB cells in sputum (Gillespie 2002).

Mutation in the catalase gene *katG* results in resistance to INH. Also, several point mutations have been identified, associated with the structural *inhA* gene that result in decreased binding affinity to NADH. *InhA* promoter mutations are abundant and they result in overexpression of

InhA leading to low level INH resistance. Today 70% - 80% of INH resistance is due to *katG* and *inhA* mutations (Johnson et al., 2006). Another gene associated with INH resistance is the *ahpC* gene coding alkyl hydroperoxide reductase, responsible for a detoxifying effect on organic peroxides (Johnson et al., 2006). INH drug resistance based solely on *katG* mutation was detected in 63% of cases and on *inhA* mutation in only 2.5% of cases, while *katG*/*inhA* and *katG*/*ahpC* double mutations were detected in 21% and 13% respectively, of examined cases in a study performed in South Africa (Kiepiela et al., 2000).

5.2 Rifampicin

RIF has been used for TB treatment since 1972, and in combination with PZA allowed shortening the TB treatment from one year to six months (Johnson et al., 2006). RIF is a semi-synthetic derivative originating from rifamycin B, produced by *Streptomyces mediterranei* (Floss et al., 2005).

RIF binds to the RNA polymerase β subunit encoded by *rpoB*, preventing RNA chain elongation. RIF sterically blocks the extension of the nascent RNA chain in the initial stage, but once RNA synthesis has progressed beyond this point it is no longer sensitive to RIF (Floss et al., 2005). The inactivated RNA polymerase remains bound to the promoter, blocking initiation by uninhibited enzymes and eventually killing the organism (Floss et al., 2005, Johnson et al., 2006).

RIF is effective against semi-dormant MTB that are metabolizing slowly, killing persistent MTB and sterilising sputum (Gillespie 2002, Lipsitch, et al., 1998).

There have been several nucleotide changes, insertions, deletions and multiple nucleotide changes detected in the *rpoB* all leading to RIF resistance. More than 95% of all mutations are located in the 81bp core region (also known as RIF-resistance determining region RRDR) (Somoskovi et al., 2001).

5.3 Pyrazinamide

PZA, a nicotinamide analogue, inactivates the enzyme involved in fatty-acid synthesis. PZA is a prodrug, and it is activated by MTB enzyme pyrazinamidase (Wade et al., 2005). PZA is activated at lower pH, as in caseous necrotic foci, where other drugs are inactivated (Johson et al., 2006, Gillespie 2002). PZA is specific for MTB and it is responsible for killing persistent, nonreplicating bacilli with low metabolic activity (Johnson et al., 2006, Wade et al., 2005).

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Resistance to PZA emerges from mutation in the pyrazinamidase gene *pncA* accounting for 97% of resistant strains. Mutations in the *pncA* gene are highly diverse and scattered along the gene (Wade et al., 2005).

5.4 Ethambutol

EMB inhibits synthesis of arabinogalactan, a cell wall component of mycobacteria and is only active against growing MTB. EMB inhibits the polymerization of arabinan by blocking arabinosyl transferase (*embB*) involved in cell wall biosynthesis (Wade et al., 2005, Johnson et al., 2006).

Resistance mutations are found in the *emb*ABC operon, while routine phenotypic analysis fails to identify EMB resistance in most cases (Wade et al., 2005).

5.5 Streptomycin

Streptomycin was the first antibiotic used in the treatment of TB and today is an alternative first-line drug. SM is aminocyclitol glycoside and it interacts with ribosomal 16S rRNA and ribosomal protein, causing an mRNA misfit and consecutive misreading and inhibition of protein synthesis (Johnson et al., 2006). SM kills actively growing MTB (Wade et al., 2005).

Mutations in *rrs*, coding 16S rRNA and *rps*L coding ribosomal protein 12S can lead to drug resistance in 20% and 50% of cases respectively (Wade et al., 2005).

5.6 Second-Line drugs

Patients with MDR TB can be cured with appropriate management based on second-line drugs. The following drugs can be classified as second-line drugs:

- first-line oral agents: PZA, EMB, rifabutin
- injectable agents: aminoglycosides (kanamycin and amikacin) polypeptides (capreomycin)
- fluoroquinolones: ofloxacin, ciprofloxacin, levofloxacin, moxifloxacin
- oral bacteriostatic second-line agents: cycloserine, terizidone, ethionamide, protionamide
- agents with unclear role in treatment of drug resistant-TB: linezolid, amoxicillin/clavulanate, thioacetazone, imipenem/cilastatin, high-dose isoniazid, clarithromycin (WHO, Treatment of tuberculosis: guidelines, 2010).

MDR TB treatment can be standardised or individualised, but in any case must be more closely supervised by the clinician. Since MDR MTB isolates are resistant to RIF and INH it is necessary to include at least four second-line drugs to which the organism is susceptible (WHO,

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Treatment of tuberculosis: guidelines, 2010). Unfortunately, second-line drugs are more toxic and less effective than first-line drugs, while treatment is prolonged and significantly more expensive (Johnson et al., 2006). If a patient is INH mono-resistant, he can be successfully treated with a combination of RIF, PZA and EMB or SM for up to 12 months (McPhee, 2008).

XDR TB is defined as MDR TB that is resistant to any one of the fluoroquinolones as well as to at least one of three injectable second-line drugs (amikacin, capreomycin or kanamycin). Among MDR and XDR TB cases, capreomycin resistance is associated with worst prognosis and death outcomes (Migliori et al., 2008).

Agents with an unclear role in the treatment of drug resistant TB can be used in patients with XDR TB (WHO, Treatment of tuberculosis: guidelines, 2010).

6 Traditional tests for diagnosis

6.1 Clinical diagnosis

The diagnosis of TB is based on a combination of symptoms, clinical signs and investigations. Clinical diagnosis of pulmonary TB is based on passive case-finding in patients presenting with a persistent cough (Keeler et al., 2006). Diagnosis cannot be made from a single piece of evidence and investigation including diagnostic tests should be interpreted according to the clinical scenario and sensitivity and specificity of each test (Knottnerus et al., 2002). A history of a recent TB contact, recent travel to, or residence in endemic areas, can influence the diagnosis (CDC Guidelines for the Investigation of Contacts of Persons with Infectious Tuberculosis, 2005).

Common clinical signs of pulmonary TB are: loss of appetite, weight loss, fever, night sweats, chest pain, haemoptysis, and breathlessness (Siddiqi et al., 2003). TB has a gradual onset of symptoms over a period of time, which could be neglected by the patient, resulting in a delay of weeks or months before seeking medical attention (Schoch et al., 2007).

6.2 Clinical specimens

Mycobacterial disease may occur in almost any site in the body, so a variety of clinical materials may be submitted to the laboratory for examination: sputum (natural or induced), bronchial washings, gastric aspirate, blood, urine, cerebrospinal fluid, pleural fluid, material from abscesses, endometrial scrapings, bone marrow, and other biopsy specimens or resected tissue (Bannister et al., 1996).

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The majority of clinical specimens sent to the mycobacterial laboratory are contaminated by rapidly growing normal physiological flora. To maximise the yield, contaminated specimens require treatment with a digestion and decontamination procedure. The most widely used agent for digestion is N-Acetyl-L-cysteine (NALC). Sodium citrate is added to NALC to bind heavy metal ions, which would otherwise inactivate NALC (Kubica et al, 1963). Sodium hydroxide (NaOH) is used for digestion and decontamination at a final concentration of 1% - 1.25%. In developing countries the final NaOH concentration is often increased from 1.25% to 1.5% to reduce contamination in cultures, but small reduction in contamination rates makes this method toxic to MTB, while reducing the culture positivity rate from 21% to 11% (Peres et al., 2009).

The sample has to be subsequently vortexed to enhance mucolytic activity, centrifuged and concentrated (Kubica et al, 1963). Keeping in mind the infectious nature of the sample, these are hazardous steps and have to be performed in BSL 3 laboratory.

There are decontamination kits on the market, with ready to use reagents (Mycoprosafe, Salubris, Inc., USA), but they still require vortexing and concentration.

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6.3 Traditional tests for detecting tuberculosis

Traditional tests used for detecting TB are based on:

- microscopy
- culture
- tuberculin skin test

6.3.1 Microscopy

Microscopy is a 100 year-old technique, used to examine clinical specimens or culture isolates for the presence of the acid-fast property of MTB. A variety of different stains is available, but most commonly used are carbolfuchsin-based methods (Ziehl–Neelsen, and Kinyoun) and fluorescence based auramine–rhodamine fluorochrome methods.

Sputum must contain 5,000 – 10,000 bacilli/ml for AFB to be detectable by microscopy (Diagnostic Standards, 2000). The number of bacilli is determined by the type of tuberculosis lesion. Thus, a cavity of about 2cm in diameter opening into a bronchus may contain 100 million bacilli, whereas a non-cavitary lesion of the same size may contain only 100-1000 bacilli. Since the latter scenario is likely to be observed in early disease, moderately advanced non-cavitary disease as well as HIV-positive patients and children, the sensitivity of the test is greatly reduced in those groups (Toman et al., 2004).

The AFB smear provides an index of the degree of infectiousness and provides valuable information for decision makers regarding infection control risk, although smear-negative patients have been shown to be infectious (Behr et al., 1999). Patients with smear negative, culture positive TB appear to be responsible for 17% of TB transmissions (Dinnes et al., 2007).

Sensitivity of the microscopy ranges from 34% - 80% and is highest in patients with advanced cavitary disease, and lowest in those with a weak cough (Davies et al., 2008). In MDR and XDR TB patients, smear positivity was 60% (Gandhi et al., 2010). Rates of smear positivity in HIV-infected patients vary from 30% to 80%, depending on immune status: when $CD4^+$ T-cell count is higher than 200 cells/μl patients present with pulmonary cavities and sputum smear is positive in 70% of patients, and as the number of $CD4^+$ T-cell decreases so does the sensitivity of the test (Charles et al., 2006).

It has been recently recommended by the WHO to take two sputum specimens, on the day of the patient's visit, which is a reduction from the traditional three samples, but it is as effective and reduces laboratory work and cost and patient compliance (Harries et al., 2000, WHO, Reduction of number of smears for the diagnosis of pulmonary TB).

Microscopy is inexpensive, quick and specific for high prevalence settings and detects the most infectious subset of patients. Downfalls of AFB are, unfortunately numerous:

- sensitivity varies between laboratories and is only 40% - 60% under field conditions and in the HIV infected population sensitivity may be as low as 20% (FIND Diagnostics for tuberculosis, 2006)
- it is difficult to maintain equipment in field settings
- it is highly operator dependent
- even it is considered fast, in practise it often still takes one to seven days for the final result to be made available to the clinician
- there is usually a need for sample duplicates (sensitivity increases from 22% - 43% to 50% - 70% if more than one sample is inspected) (Toman et al., 2004)
- sample volume influences the result and 5ml or more is desirable (sensitivity was increased from 72% to 92% when only samples of 5ml were processed) (Royal College of Physicians of London, Tuberculosis, 2006)
- bacillary load can vary and depends on time of the sampling (morning versus spot), method of sampling (with or without induction), severity of the disease and existence of cavitary lesions

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- specificity is impaired if specimens are obtained from individuals who are heavily colonised with nontuberculous mycobacteria (NTM) or have chronic disease due to NTM (Cole et al., 2005)
- drug resistance and viability of the organism cannot be established
- some food particle precipitates, inorganic materials and artefacts may give false positive results (Toman et al., 2004)
- only one third of microbiologists are senior microbiologists who can interpret a Gram stain the identify a pathogen at an early stage (Baron, 2006)

Even with all the shortcomings, AFB direct smear remains "the gold standard" (since it demonstrates existence of mycobacteria in sputum) in developing countries, especially in peripheral health facilities, since there is no infrastructure for further testing. However, even peripheral health facilities often lack trained microscopists, properly functioning microscopes and adequate reagents (Keeler et al., 2006).

Several attempts have been made in order to improve the performance of smear microscopy. Concentrating the sample by adding sodium-based reagent and centrifugation increases sensitivity, but this still requires BSL 2 capacities. At point-of-care, especially in rural settings, there is no infrastructure for introducing laboratory equipment like centrifuges or biosafety cabinets (Pai et al., 2008). Use of household bleach or ammonium sulphate and overnight or short-term sedimentation is more sensitive than direct smear, but still higher sensitivity is obtained if centrifugation follows bleach treatment (Steingart et al., 2006, Van Deun et al., 2000).

A great advantage in TB diagnosis would be to develop a new technique or improve the existing one in order to improve smear performance and at the same time have simple instruments easy to implement at the point-of-care level. The use of fluorescence method represents a significant advance in that context. It has been shown that the auramine and auramine/rhodamine method has an advantage over ZN staining, because it is easier to detect a fluorescent rod against a darker background. The fluorescence method allows the technician to scan the slide at a lower magnification and thus observe a larger area than with ZN smears. These factors reduce the time spent per slide and lead to greater sensitivity (Somoskövi et al., 2001).

However, the fluorescence method is more expensive and is not widely available in the developing world, considering the cost of the fluorescent microscope as well as reagents. More

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recently much cheaper, light emitting diode (LED) microscopy is likely to make fluorescent microscopy more widely available (Pai et al., 2008).

There is a clear need for a novel diagnostic test that can be introduced at the same level of health facility as smear microscopy, and with the same or higher specificity but with significantly improved sensitivity.

6.3.2 Culture

A definitive diagnosis of tuberculosis is still dependent on the isolation of MTB by cultivation. The goal is to detect viable mycobacteria in clinical samples. As few as 10 to 100 organisms are needed for a positive culture (Toman et al., 2004). A significant advantage of culture is that species identification as well as drug susceptibility testing is made possible. Culture is the most sensitive test available: sensitivity of culture ranges from 80% to 93% (sensitivity rates up to 98% are reported) while specificity is 98% (Davies et al., 2008, Dinnes et al., 2007). Culture can be used to detect TB from biopsy material and needle aspirates, meaning it is also applicable for extrapulmonary TB.

The biggest downfall of culture analysis is unfortunately that it time consuming. With a median time of more than four weeks for a conclusive result and four to six weeks more for DST it does not bring valuable information to the clinician at the moment of establishing the diagnosis and commencing treatment. Culture also requires more technical expertise and extensive laboratory infrastructure than microscopy and it is restricted to referral centres (Davies, et al., 2008, FIND Diagnostics for tuberculosis, 2006).

Culture is considered the “gold standard” for TB detection. But in practise, there are still cases when bacillus is not successfully cultured. As a result a significant proportion of patients are started on empiric TB treatment if the clinical suspicion is strong enough (Davies, et al., 2008).

6.3.2.1 Solid culture

Traditional cultivation involves solid media, such as that of egg-based Löwenstein-Jensen (LJ), or agar-based Middlebrook 7H9, 7H10, 7H11. Both can be made into selective media by adding a cocktail of antibiotics allowing only MTB to grow. MTB is detected by the specific growth morphology: colonies are rough, dry, flat, and beige-coloured or granular and with time they grow into a cauliflower shape (Dinnes et al., 2007). The method is cost effective in comparison with liquid automated methods (FIND Diagnostics for tuberculosis, 2006). It is valuable to examine culture morphology and detect mixed cultures, and the solid medium allows this (Diagnostic Standards, 2000).

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The biggest drawbacks of the method is that it is time-consuming, taking up to four weeks for a positive result, but incubation is necessary for six to eight weeks in order to be classified as negative (Davies et al., 2008). Each culture has to be examined weekly (Moore et al., 2005). MTB grows better on LJ medium, with higher sensitivity, but more rapidly on the agar medium (Diagnostic Standards, 2000).

Several approaches have been made to improve time to result using solid culture. Blood agar slants, made from sheep blood, may be a good substitute for LJ as it saves about one third of the time for rapid detection of MTB, and it is suitable for resource-limited settings (Mathur et al., 2009). The thin layered agar method is as sensitive as LJ and results are available within two weeks (Martin et al., 2009).

A recent improvement is the commercially available solid culture-based colorimetric method, TK Medium (Salubris, Inc., USA). Metabolic activity of growing MTB changes the colour of the culture media, at the same time enabling positive identification of MTB. Time to detection is two weeks, and further advantages of the test are: low-cost, simplicity, and possibility for DST (Pai et al., 2006). However this method appears to have been abandoned by developer.

6.3.2.2 Liquid culture

The use of a liquid medium for cultivating MTB systems has led to a considerable shortening of the time (one to four weeks) required for the detection of mycobacteria (FIND Diagnostics for tuberculosis, 2006). Sensitivity of liquid culture methods is generally higher than of solid culture; specificity is also higher for liquid media, especially since contamination rates with non-AFB are higher in solid culture (Cruciani et al., 2004). Several automated and semi-automated methods and detection approaches are used: radiometric, oxygen quenching, and redox-reaction.

Firstly, the radiometric approach was used as a semi-automated system. The BACTEC 460 radiometric method uses 7H12 liquid medium containing ^{14}C-lebeled palmitic acid and detects radioactive $^{14}CO_2$ as a catabolic product (Ramachandran et al., 2003). The result is expressed as an arbitrary growth rate, enabling reproducibility. It has enhanced sensitivity and the time to positive result is shortened. The same culture can be used for DST, once it is reported positive for microbial growth and the result is obtained after 10 days. The radiometric system's main limitations are the high cost of disposal of the radioactive waste and the need for specific laboratory infrastructure and instrumentation (Ganeswrie et al, 2004).

In order to resolve issues around radioactive waste and simplify the procedure, a fluorescence measuring system was developed. The principle is based on measuring the metabolic activity of

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growing bacteria. The Mycobacteria Growth Indicator Tube (MGIT) contains modified Middlebrook 7H9 broth with an oxygen quenching-based fluorescent sensor. Oxygen is initially dissolved in the media and it quenches fluorescence. Consumption of the dissolved oxygen by the growing MTB results in a fluorescence from the sensor at the bottom of the MGIT, when illuminated with an ultraviolet lamp (Piersimoni et al., 1998, Palomino et al., 2008). The fluorescence system is usually preferable because of the increased convenience of the technology.

The automatic BACTEC MGIT960 system (Becton, Dickinson and Company, USA) performs incubation of an MGIT tube at 37^0C and reads the tubes continuously inside the machine, analysing the fluorescence signal for mycobacterial growth, and interpreting each MGIT as positive or negative for MTB (Palomino et al., 2008).

MGIT is currently considered as the "gold standard" (FIND report, 2008) but the biggest drawback for this technology is that it is not available to the wide population; many problems have been encountered while implementing the system in laboratories, in developing countries, generally managing a medium load of samples, while the biggest issue is high cost (Palomino et al., 2008).

A new pricing agreement between low income countries and BD for the BACTEC MGIT960 system should make MGIT available to additional high burden countries (FIND press release, 2007).

There are several new liquid systems on the market having similar performance characteristics to the MGIT 960:

- BacT/Alert 3D system (bioMerieux Inc., France), measures carbon dioxide production
- Versa TREK system (Trek Diagnostic systems Inc., USA), measures changes in gas pressure
- MB redox (Heipha diagnostika, Germany) detects colour change (red particles become visible) upon redox reaction between CO_2 and tetrazolium salt-containing tube (Dinnes et al., 2007, Palomino et al., 2008, Grandjean et al., 2008, Cambau et al., 1999).

6.4 Diagnosis of tuberculosis in smear negative cases

In low resource/ high prevalence settings TB diagnosis is relying on sputum smear since it is highly specific (Keeler et al 2006). However, smear has impaired sensitivity in people living with HIV and in children.

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People living with HIV are 20 to 30 times more likely to develop TB than HIV negative people. Moreover, as HIV increases the risk of developing active TB, so does TB accelerate the progression of HIV into AIDS. Without adequate TB treatment, approximately 90% of HIV positive people die of TB within months of infection (WHO, TB/HIV facts 2009).

In a recent study two groups of HIV positive and HIV negative TB suspects were recruited and examined for TB culture and smear: direct smear was positive for 47% and 83.1% of all culture positive but HIV negative and HIV positive patients, respectively (Eyangoh et al., 2008).

Over 250 000 children develop TB and 100 000 children will die each year from TB, since TB has been largely misdiagnosed in children under 10 years old. Diagnosis of TB in children is influenced by the children inability to cough up enough sputum to be analyzed for smear or culture and CXR of children are difficult to interpret as the typical shadow is rarely seen. Hence the diagnosis is based on the clinical features: cough, weight loss, history of contact with a TB patient (WHO TB and Children, 2006).

In order to improve diagnosis in HIV positive patients and in children, existing methods of diagnosing TB should be evaluated in those groups and new approaches made. A number of studies aiming at those groups are ongoing and they should make an impact on changing clinical practice in those patients.

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7 Immunological diagnosis

Demonstration of mycobacteria is often not possible, due to the paucibacillary nature of the illness in some cases, for example in children. Immunodiagnostic methods are used as an alternative option in order to increase detection rate in such cases, since they are considered cost effective and rapid (Palomino et al., 2007).

Assays available today use various immunological approaches in response to TB antigens: they detect antibodies in blood (complement fixation, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA)), or T-cell based assays (detect up-regulation of IFN-γ, and other Th1 cytokines, or delayed-type hypersensitivity (DTH) reaction).

7.1 Tuberculin skin test

Tuberculin skin test (TST) is a century-old test based on intradermal introduction of tuberculin, a concentrated protein mixture known as purified protein derivative (PPD) derived from MTB. The method was described by Mantoux who noticed that results of DTH reaction to tuberculin are

reproducible; if one measures the size of the induration produced after injection of a standardised dose (Palomino et al., 2007).

The skin test is simple, inexpensive, and easy to administer and read. It can be done on site, but it requires two visits by the patient one for antigen administration and the second after 48h-72h for interpretation of the result based on the skin reaction. There are several practical difficulties in performing the test: the return rate for the test reading can be very low, the injection sometimes causes painful skin inflammation and scarring (Dinnes et al., 2007). The test is used for surveillance for TB in a selected settings or, previously, to select subjects for BCG revaccination (Dinnes et al., 2007, Palomino et al., 2007).

The dose of antigen, method of application and interpretation of results vary between settings. More than 10mm-15mm for the skin reaction is considered a positive result, depending on whether there is a history of BCG vaccination and more than 5mm for HIV infected patients.

The test has 70% sensitivity in active TB patients, and in immuncompromised is as low as 30% (Palomino et al., 2007). The reason for the low sensitivity is a generally low immune response, especially in HIV-infected patients. In addition, antigen recognition in TB is very variable from individual to individual and may be specific to a stage of disease (Gennaro, 2000). TST can give a false positive result: PPD shares antigens with other environmental mycobacteria; a positive test may result from prior BCG vaccination; repeated TST may have a booster effect (Dinnes et al., 2007). It also cannot distinguish between active and latent infection.

7.2 Serological tests

Serological tests are based on detecting humoral immune response to TB antigens. Several serological tests for detecting active TB and LTBI have been developed over the years. They are simple, rapid and potentially useful in settings where culture is not routinely available.

Serological tests available at present perform poorly for the diagnosis of active TB and in their ability to distinguish between active and latent TB infection. Their use cannot therefore be supported (Steingart et al., 2007). Responses to TB antigens vary individually however sensitivities are generally poor, ranging from 16% - 57% whilst specificities of 62% - 100% have been reported for commercial assays (Pottumarthy et al., 2000). Serological tests are not recommended for paediatric TB and development of new diagnostics is recommended by the WHO (WHO, A research agenda for childhood tuberculosis, 2007).

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Serological test accuracy is influenced by the demographic and environmental determinants; exposure to atypical mycobacteria, BCG vaccination and HIV prevalence can influence the result (Dinnes et al., 2007, Lalvani et al., 2007). New approaches and new antigens are necessary for serological assays in order to improve their performance. They should be able to distinguish between active and latent infection, to increase specificity in order to discriminate between BCG vaccination and NTM infection, and monitor response to therapy. They should be simple, safe and rapid (less than 30 minutes) and cost-effective (Talbot et al., 2004). Performance should be evaluated in children and HIV-infected patients (Steingart et al., 2007).

Commercial serology assays use single or compound TB antigens such as: antigen-60, LAM, 38 kDa protein, or 16 kDa protein, while typically using ELISA as the detection technique. A recently developed ICT TB Test (ICT Diagnostics, Australia) is an immunochromatographic test, using strip technology, and is simple to use, especially at point-of-care, but with variable sensitivity, ranging from 20% - 73%, and specificity rates between 80% - 100% (Ongut et al., 2006). Detection of active and latent TB and uninfected individuals should be improved by using multiantigen cocktails (Gennaro, 2000).

7.3 Interferon-γ release assays

Interferon-γ release assays (IGRA) have been designed to replace the TST. Mycobacterial antigens specific for MTB are used for in vitro stimulation of peripheral blood mononuclear cells (PBMC) or whole blood. IGRA has several advantages over TST: a single patient visit is required, there is reduced bias in interpretation of the result, a booster effect does not occur, and there is greater sensitivity in detecting LTBI and active TB in HIV-infected people (Dinnes et al., 2007)

Antigens included in IGRA are RD1-specific antigens: early secretory antigen target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) and TB10.4 protein in QuantiFERON-TB. The RD1 region is missing in BCG strains so RD1-antigens will not stimulate a BCG-related cellular response (Lavani et al., 2001).

These tests need to be performed in an equipped tissue culture laboratory where blood cell separation and cultivation is followed by interferon-gamma ELISA or enzyme-linked immunospot ELISPOT assay. IGRA that are now commercially available are: the ELISPOT T SPOT-TB assay (Oxford Immunotec, UK), and ELISA based QuantiFERON-TB, and its enhanced version QuantiFERON-TB Gold In-Tube assay (Cellestis International, Australia).

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Interpretation still depends on the clinical situation. As for TST, IGRAs cannot distinguish latent from active disease. Only if prevalence of TB is low and the clinical situation highly suggestive of TB, then a positive IGRA result can be used as an indication for active TB. In a high prevalence setting it can mean either latent or active disease. It is not uncommon to have false negative results in active TB (Dinnes et al., 2007).

8 Molecular tests

Molecular tests include a wide group of methods that utilise different nucleic acid moieties. The unique sequences of nucleic acid specific to each species make it distinguishable from different species which provides a sounds basis for developing a microbial diagnostic test. Hence, molecular tests have developed in the past few decades into essential tools in microbiology. In terms of sensitivity, specificity and most of all speed, molecular tests offer excellent performance.

Molecular identification may be applied in three scenarios: identification of an organism isolated in pure culture, rapid identification of organisms in clinical specimens and identification of uncultivable organisms present in specimens (Speers, 2006).

8.1 Molecular approaches to detecting tuberculosis

Deciphering the MTB genome of strain H37Rv in 1998 was a benchmark in MTB research (Cole et al, 1998). This achievement made a breakthrough in functional and comparative genomics for this organism. Those events initiated development of the more sophisticated molecular diagnostic tools. New molecular tools for detecting TB emerged from the demand for rapid and reliable diagnosis, as well as a means for differentiation of MTB from NTM and for rapid DST.

8.2 Nucleic acid amplification tests

Nucleic acid amplification-based pathogen diagnostic tests are able to detect small amounts of genetic material, by amplifying a DNA or RNA sequence, from micro-organisms. The most important feature of molecular testing is that the result can be obtained within hours.

There are several amplification methods and the most common NAAT is based on polymerase chain reaction (PCR). PCR has evolved from end-point reaction to real-time PCR detection where detection is done while the reaction is ongoing. NAAT uses different enzymes and strategies, such as amplification of different targets within DNA or RNA. Most NAAT-based tests involve three steps: DNA/RNA isolation, amplification and detection.

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8.2.1 Polymerase chain reaction

Since the first publication of the polymerase chain reaction (PCR) amplification technique in 1985 (Saiki, et al., 1985) considerable progress has been made in pathogen detection and identification. The first semi-automated amplification system for pathogen detection, Cobas Amplicor was on the market in 1995 (DiDomenico et al., 1996, Thon et al., 2001). Since then, nucleic acid amplification techniques have been refined and different approaches have been deployed in order to make it simple, cost effective and applicable for clinical use.

PCR is the most widely used amplification technique for detection and characterisation of pathogens. It can give a simple answer whether a pathogen is present in the sample or not. PCR amplifies DNA, but in conjunction with reverse transcriptase it can amplify a sequence originating from the RNA. Reverse transcription polymerase chain reaction (RT-PCR) can assess viability of an organism in question if short-lived RNA is targeted.

The reaction is performed by consecutive temperature alterations. High temperature melts the double stranded DNA (dsDNA), then the temperature is reduced to allow primers to anneal to the template and finally the temperature is set at an optimal temperature for polymerase enzymatic activity of incorporating deoxyribonucleotide triphosphates (dNTPs) into a new strand (Kubista et al., 2006). PCR targeted at a specific sequence within a genome requires optimal conditions for amplification:

- template DNA
- dNTPs, the four nucleotide triphosphates
- primers, flanking the sequence to be amplified
- heat-stable DNA polymerase
- Mg^{2+} ions, stabilising double stranded dsDNA
- buffer
- temperature cycling

At the beginning of PCR, the temperature should be high enough to separate the DNA template strands, since if dsDNA is not fully separated when temperature is lowered, strands will reanneal before primers have the same opportunity (Kubista et al., 2006).

Primers should have a 40% - 60% GC content, be relatively short and similar in size (18-25 bases), be free of repetitive motifs, and long stretches of polypurines or polypyrimidines (Roux, 1995). Annealing temperature should be few degrees below primer melting temperature.

Mg^{2+} ions stabilise dsDNA and raise the temperature of DNA annealing or (melting temperature) Tm; thereby a shift in Mg^{2+} ion concentration determines the reaction specificity: low Mg^{2+}

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concentration requires more stringent base pairing in the annealing step, but it can also result in a low yield of PCR product, whereas high Mg^{2+} concentration enables primer binding to a non-specific template and may result in amplification of unwanted product (Roux, 1995).

Hot-start polymerases require a heat activation step prior to PCR, which is a practical advantage, since it can be added to the master mix prior to template without a possibility of elongation before the fist melting-annealing step. Elongating temperature is usually set to be the optimal temperature for polymerase, and it is 72^0C for polymerase isolated from *Thermus aquaticus* or Taq polymerase (Kubista et al., 2006).

PCR specificity is enhanced by nested PCR, involving a second round of PCR with an internal set of primers. This procedure involves more manipulation and extra cost, but more importantly it increases the chance of sample contamination, since it involves handling already amplified DNA product. One solution is to have PCR and nested PCR done in the same tube (Wilson et al., 1993). By doing this, both sensitivity and specificity rises and the possibility of cross-contamination at this stage is less likely.

PCR in microbial detection has been used in diagnostic laboratories in most of the cases as an "in-house" method. Assays are referred to as ''in-house'', if they are based on a protocol developed in a non-commercial laboratory. In addition many of the PCR based technologies have already been developed into commercially available diagnostic kits and assays for use in clinical microbiology.

PCR is a widely used method for MTB detection, and in 80% of all "in-house" NAAT protocols PCR is the amplification method of choice (Flores et al., 2005). Time to diagnosis is usually two days and it is cost effective in comparison with culture (Muhumuza et al., 2006).

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8.2.2 Real-time PCR

A major advance in amplification has been the development of systems that allow monitoring amplification in real-time. The first application of real-time PCR used ethidium bromide and a CCD camera to monitor amplification of PCR (Muhumuza et al., 2006). The accumulation of fluorescence during thermocycling is directly related to the starting number of DNA copies (Higuchi et al., 1993). The fewer cycles necessary to produce a detectable fluorescence, the greater the number of target sequences. In this way DNA can be amplified and quantitated at the same time. Besides quantitation there are several practical advantages of real-time PCR over conventional PCR: the reaction is performed in a closed tube and there is no need for

subsequent post-PCR detection and amplicon handling, thereby reducing the possibility of amplicon contamination (Higuchi et al., 1992).

There are two general approaches to quantifying real-time PCR product: standard curve or absolute quantitation and relative quantitation (Arya et al., 2005). The standard curve is made up using known concentrations of the gene in question and determining a threshold cycle (Ct) for each sample. Ct is the number of PCR cycles at which the detecting signal is greater than the threshold. The Ct number is lower if the concentration of sample is higher. Ct is specific for each experiment and it should not be used as the nominal value; rather internal control (IC) should be included in each run. The threshold is an arbitrary value of fluorescence, usually selected by the instrument or thermal cycler, but it can be adjusted for each experiment (Arya et al., 2005). Relative quantitation requires IC amplified at the same time as the template sample. IC has a defined numbers of copies and it is usually very similar to the target gene. According to the proportional relationship between the IC's Ct and sample's Ct it is possible to estimate the number of gene copies in the original sample. Ct is defined as number of cycles necessary to reach the signal threshold line, so that signal can be distinguished from the background (Heid et al., 1996).

Real time PCR detection is made possible by complex reactions of emitting or quenching fluorescence. The first attempt at emitting fluorescence was done using fluorescence dyes intercalating into DNA. As mentioned above, the first real-time PCR detection used ethidium bromide, which becomes fluorescent upon intercalating into DNA. Asymmetric cyanine intercalator dyes with two aromatic rings are used nowadays; they have no fluorescence in solution, but when they bind to a DNA minor groove, they become brightly fluorescent when exposed to the appropriate wavelength. Intercalating dyes widely used today are: SYBR Green I, BEBO, LC Green, and SYTO9. This is the simplest and most cost-effective detection system. The downside is that some dyes can inhibit PCR at certain concentrations (Monis et al., 2005)

The second approach is to use labelled primers and probes. Probes can be covalently bound to one or two dyes. Probes based on a single dye are actually fluorophores that change their fluorescence properties upon binding to the nucleic acid. In case of two dyes, fluorophores are covalently bound to the probe or primer which is structurally designed to bring one fluorophore in contact with another fluorophore or a quencher (Monis et al., 2005). Summarised mechanisms or reporters used in real-time PCR are as follows:

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Taqman probe or hydrolysis probe: The probe has a fluorophore on the 5' end and a quencher on the 3' end. Once the probe is bound to the ssDNA, Taq polymerase hydrolyses the probe near the 5' end with its exonuclease activity and releases fluorophore into the solution, thereby separating it from the quencher.

Molecular beacon probe: Molecular beacons are self-complementary single-stranded nucleotides that form a hairpin loop. On one side of the loop structure there is a fluorophore and on the other a quencher. When a probe binds to the target sequence fluorescence is emitted since the fluorophore and quencher are further apart.

FRET probe: Fluorescence resonance energy transfer probes are two separate fluorescently labelled oligonucleotides designed to bind to a DNA sequence bringing them in close proximity, thereby enabling energy transfer from one fluorophore to another and emitting fluorescence of a different wavelength.

Scorpion primers: Scorpion primers perform a dual function of probe and a primer. In structure it is a molecular beacon. The link between the beacon and the primer has a PCR blocker to prevent replication of the beacon sequence. The loop is complementary to the sequence synthesised immediately after the primer. Once the sequence after the primer has been synthesised, the loop unwinds exposing the fluorophore and emitting fluorescence (Coleman et al., 2006, Monis et al., 2005, Kubista et al., 2006).

Instruments used for real-time PCR are commercially available and the main differences between them are the excitation and emission wavelengths, single or multiple wavelengths, speed, and the number of reactions that can be run in parallel. Instruments may hold microtiter plates from 96 to 384 wells. Instruments with independent sample tubes, such as the Cepheid SmartCycler (Cepheid, USA), recently appeared on the market (Kubista et al., 2006). Such instruments have independent modules and each can perform operator instructed real-time PCR under individual heating and optical protocols, so that different tests can be done at the same time. This is, most probably, the way forward for non-commercial, "in-house" diagnostic assays (see chapter 1, section 8.5)

Real-time PCR is the greatest advance since PCR found its place among diagnostic tests. Real-time PCR is automated, is adjustable to any protocol when primers are designed, and minimises the risk of contamination. Specificity is enhanced if nested set of primers are used (Wilson et al., 1993)

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8.2.3 Ligase chain reaction

Ligase chain reaction (LCx) uses thermostable DNA ligase and four primers: two pairs of forward primers and their complements. Forward primers are made in a way that once they attach to ssDNA a gap of 1-3 bases between them is formed. The next step takes place at the temperature optimal for DNA polymerase which fills the gap between primers. Thermostable DNA ligase acts to covalently connect extended primers. Product is detected using a capture system (see in detail below) (Coleman et al., 2006, Ausina et al., 1997, Monis et al., 2005).

8.2.4 Strand displacement amplification

Strand displacement amplification (SDA) is an isothermal reaction that uses a combination of exonuclease deficient DNA polymerase (Klenow fragment is omitted), restriction endonuclease and two sets of primers. The reaction mixture contains also dTTP, dCTP, dGTP and modified hemi-phosphorothioate dATPs. The reaction course is performed at 370C. The amplification process can be divided in two phases: target generation and amplification.

Target generation uses a first set of primers with a specific restriction site. These primers anneal to the ssDNA, after short heating, close to the target sequence. This initiates DNA polymerase binding to the primers and their elongation toward the target sequence. Since DNA polymerase lacks endonuclease activity, the primer is not degraded. A second set of primers, bumper primers, bind within the target sequence and their extension displaces the newly synthesised strand containing the restriction enzyme site. This strand now becomes a template for further amplification since it is a target for reverse first primer as well as bumper primer.

The restriction enzyme makes a nick only in the original double stranded molecule including the first set of primers. If the first set of primer is abundant, then each time they anneal to the template end elongate, endonuclease will recognise the restriction site and make a nick, and this is a starting point for DNA polymerase to start synthesising new strands and displacing the downstream strand (Walker et al., 1992, Walker, 1993).

8.2.5 Transcription-mediated amplification

Transcription-mediated amplification (TMA) targets RNA molecules, abundantly present in each cell, and transforms them into cDNA, thereby starting with an about 10 000 transcriptional active DNA template instead of 1-2 copies of genomic DNA template.

Amplification is an isothermal, two step process: rRNA is copied into cDNA using reverse trancriptase and then RNA polymerase is used to make amplicons of the target RNA using cDNA as a template.

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To begin the reaction, a primer that is complementary to target rRNA is added to initiate the synthesis of cDNA using reverse transcriptase. RNase H degrades the primer upon cDNA synthesis, leaving single stranded cDNA. Then, a DNA-specific primer initiates DNA polymerase binding and dsDNA is produced. The final product is dsDNA matching the target RNA region with an RNA polymerase binding region. RNA polymerase binds to the dsDNA and generates RNA via transcription. Finally, the labelled probe complementary to the DNA amplicon is used to detect the amplified sequence (Coleman et al., 2006).

8.2.6 Loop-Mediated Isothermal Amplification

Loop-Mediated Isothermal Amplification (LAMP) is isothermal amplification based on strand displacement reaction, carried out at a higher incubation temperature at 65^0 C. Reaction mixture includes Bst DNA polymerase, with strand displacement activity, and a set of four primers, that together recognise six distinct sequences on the target DNA. LAMP reaction is based on a combination of cDNA synthesis, strand displacement DNA synthesis and formation of stem-loop DNA, by self-primed DNA synthesis. The product is a mixture of stem-loop DNAs with various lengths and cauliflower-like structures with multiple loops. Detection of DNA amplification is measured as turbidity or precipitate forming. Pyrophosphate, which is produced as a by-product, yields a white precipitate of magnesium pyrophosphate that can be detected visually. Furthermore, the increase in the turbidity of the reaction mixture correlates with the amount of DNA synthesised, allowing real-time monitoring of the LAMP reaction by real-time measurement of the turbidity (Palomino, 2009, Boehme et al., 2007, Notomi et al., 2000).

8.3 Clinical use of nucleic acid amplification tests

Performance analysis of both “in-house” and commercial NAAT for the diagnosis of TB demonstrates a wide range of performance. Overall, performance of commercial NAAT for both smear positive and negative samples 85% (ranging from 36% -100%) and 97% (ranging from 54% -100%) for sensitivity and specificity, respectively (Ling et al., 2008).

Most studies report NAAT detection of MTB being 5 to 100 cells, when using dilutions of broth-grown organisms. However, sensitivity is much lower when used on actual samples due to the inhibitory substances in a specimen which interfere with PCR or other amplification methods; also it could be that MTB isolated from humans is more rigid and resistant to lysis or difficult to concentrate by centrifugation (Cole et al., 2005). Further performance of molecular tests is likely to be affected by the low concentration of bacilli present in smear negative sputum (Ling et al., 2008).

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Real-time PCR systems developed previously for MTB diagnosis showed good levels of sensitivity, 90% - 100% only on AFB smear-positive samples (Dalovisio et al., 1996). The use of more specific primers and multi primers led to increase in sensitivity in smear negative samples (Broccolo et al., 2003). So far real-time PCR has not been applicable in the field at point-of-care, and that would be a significant break-through in diagnostic.

Current NAAT are typically utilised in conjunction with, not instead of culture. The implication is that if the calculated sensitivity of a NAAT is 80% this does not mean that two out of every 10 specimens containing MTB would be missed, but rather that 8 out of 10 people are actually diagnosed the same day (Meltzer, 1998).

The prevalence of disease in the setting should be included when analysing the performance of NAAT. The actual clinical utility of the NAAT depends on how common TB is in the affected population (see chapter 3, section 12.2). Low prevalence of disease gives rise to high false positive rates and whilst the probability of having disease while having a positive test result rises with increasing prevalence. In high burden countries a test with a high negative predictive value, would be of particular value, since this could be used to rule out TB in patients presenting with related conditions causing symptoms indistinguishable from those associated with TB (Cole et al., 2005).

One potential drawback of NAAT is the possibility of false positive results from culture negative patients, who were on TB treatment at the time of testing. Nucleic acid can be detected in respiratory specimens that yield no organisms on culture since dead cells might be detected and give a false positive result.

The Food and Drug Administration (FDA) initially approved two commercial tests for use only with AFB positive respiratory specimens:

- Amplified Mycobacterium Tuberculosis Direct Test (MTD) (Gen-Probe®, US) approved in 1995
- Amplicor® Mycobacterium Tuberculosis Test (Amplicor MTB) (Roche® Diagnostic Systems, Inc., US), approved in 1996

AMTD was improved recently and as AMTD2 it is cleared by the FDA for testing both smear positive and negative samples, as well as non-respiratory specimens.

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8.4 When to use nucleic acid amplification testing

CDC guidelines regarding NAAT for TB diagnosis were first established in 1996, and updated in 2000 and 2009 (CDC Guidelines, 1996; CDC Guidelines, 2000; CDC Guidelines, 2009). CDC recommendations regarding NAAT for TB diagnosis are that NAAT should be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB. Also, culture should remain the gold standard for laboratory confirmation of TB and it is still required for isolating bacteria for drug-susceptibility testing and genotyping. Nonetheless, NAAT should become standard practice for patients suspected to have TB, and all clinicians and public health TB programmes should have access to NAAT for TB diagnosis to shorten the time needed to detect TB from one to two weeks to one to two days. However, currently available NAATs should not be ordered routinely when the clinical suspicion of TB is low, because the positive predictive value of the NAAT is <50% for such cases (CDC Guidelines, 2009).

The CDC states that rapid laboratory confirmation of TB also can help reduce inappropriate use of fluoroquinolones as empiric monotherapy for pneumonias. That practise is suspected to lead to development of fluoroquinolone-resistant MTB and it delays the initiation of appropriate anti-TB therapy.

The costs of adding NAAT to the routine testing of respiratory specimens from patients suspected to have TB might be considerable (operating costs exceed $100 per AMTD test); however, NAAT has the potential to provide overall cost savings to the treatment centre and TB control programme through reduced costs for isolation, reduced costs of contact investigations of persons who do not have TB, and increased opportunities to prevent transmission. For procedural and economic reasons, NAAT might be impractical in laboratories with a small volume of testing. Referral of samples to high-volume laboratories might be preferable to improve cost-efficiency, proficiency, and turnaround times.

Overall, clinicians should interpret all laboratory results on the basis of the clinical situation. A single negative NAAT result should not be used as a definitive result to exclude TB, especially when the clinical suspicion of TB is moderate to high; rather, to expedite testing for an alternative diagnosis, or to prevent unnecessary TB treatment (CDC Guidelines, 2009).

8.5 “In-house” nucleic acid amplification test

Investigators design their own protocols for “in-house” assays. They are commonly used in developing countries where commercial kits may not be affordable. The accuracy of NAAT for TB has been extensively studied since the early 1990s (Flores et al., 2005). A major conclusion

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in evaluating such assays is great variation in sensitivity and specificity. Sensitivity estimates have been lower in smear negative pulmonary TB and extra-pulmonary TB and higher in smear positive pulmonary TB. The most common cause of impaired sensitivity, besides low bacillary load, is the presence of inhibitors of enzymatic amplification and the lack of specificity is mainly due to sample handling and carryover of target DNA from samples containing heavy MTB load (Flores et al., 2005).

Assay characteristics vary among laboratories as do laboratory techniques, but some general characterisation of all assays used can be made; all assays include sample preparation, PCR and analysis of the product.

8.5.1 Sample preparation

Samples used are mainly sputum samples, digested and decontaminated. Samples other than sputa used in different studies were: cerebrospinal fluid (CSF), joint fluid, abdominal fluid, pleural fluid and urine, and these are usually concentrated prior to DNA extraction (Almeda et al., 2000).

Sample preparation involves:

- disrupting the thick MTB cell wall
- removing PCR inhibitors
- DNA precipitation and extraction

The sample is usually decontaminated and concentration is performed prior to DNA isolation. Typically, the first step in DNA extraction and preparation for PCR involves protease digestion (proteinase K), extraction of the solvent and nucleic acid precipitation. This is a time-consuming and complicated protocol. Another approach is to use a chaotropic agent (guanidinium thiocyanate) for cell lysis and capturing DNA onto silica. This is generally referred to as the chemical approach (Almeda et al., 2000).

Samples may contain inhibitors of PCR. Examples of common PCR inhibitors are heme, heparin, IgG, and lipids (Akane et al., 1994, Kubista et al., 2006). Some detergents like sodium dodecil sulphate (SDS) and cationic detergent cetyltrimethylammoniumbromide (CTAB) are known to form complexes with polysaccharides and proteins, thereby enhancing purity of the extract (Rafi et al., 2007).

Cells can be disrupted mechanically (using sonicator, vortex); or mechanical and chemical methods can be combined (Wilson et al., 1993). The easiest way is to use the sample as a

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heat-killed suspension, concentrated by centrifugation. This crude mixture contains a significant proportion of PCR inhibitors as well as debris that also disables amplification.

Examples of older isolation procedures involve:

- washing bacteria in a buffer, incubating with lysozyme with vigorous shaking, then increasing temperature (up to 65^0C) to break the MTB membrane and adding SDS/proteinase K/CTAB and isolating DNA by extracting with chloroform/isoamyl alcohol, precipitating with isopropanol and washing with ethanol
- lysis by boiling with Triton and glass beads followed by phenol/chloroform extraction and ethanol precipitation
- consecutive cycles of boiling and freezing in Triton (Hermans et al., 1990, Portillo et al., 1991, Suffys et al., 2000)

Those protocols illustrate a decade-old approach to DNA isolation: time-consuming, cumbersome, and not practical. About 20 samples would keep a laboratory technician occupied the whole day, just for the DNA isolation. So far, approaches to DNA isolation have been impractical, since each requires BSL 3 or BSL 2 facilities and they are difficult for scaling up and automation.

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8.5.2 Amplification

PCR is performed using a defined set of primers and DNA polymerase. Cycling conditions are adjusted according to the enzyme and sequence.

8.5.3 Choice of sequence

The majority of tests target DNA, since it is more stable and always present; not dependent on environmental or metabolic conditions. On the other hand, mRNA is a marker for organism viability and it is present in more copies; but generally molecular diagnosis is less concerned with the viability status but rather presence in a patient's specimens (e.g. detection of MTB DNA in CSF of a patient with suspected meningitis).

Most "in-house" PCR assays amplify a 250 - 520bp long fragment of IS*6110*. However, the number of IS*6110* elements differs between different MTB strains, varying between 1-25 copies (Arora et al., 2009). *M. tuberculosis* strains with low copy numbers of IS*6110* have been more frequently isolated from Asian patients than from patients of European origin, and some previous studies reported strains with no IS*6110* segments (Ali et al., 2007). In addition, there is

recent evidence that the IS*6110* RFLP banding pattern changes during transmission, especially in high incidence settings (Hanekom et al., 2008).

Other amplification targets are: 16S rRNA, 23S rRNA, genes coding 32-kDa and 38-kDa proteins, *dnaJ*, *hsp65*, IS*986*, internal transcribed spacer region between 16S rRNA and 23S rRNA, region 650-900, rpoB and phospholipase C gene fragments (*plcA* and *mpt40*) (Greco, et al., 2009; Suffys et al., 2000, Portillo et al.,1991).

Some genes are predominantly used for nested PCR. The gene coding for Mpb-64, secreted immunogenic protein, has been used as a marker for MTB, using a nested PCR with an inner segment 200bp long (Rafi et al., 2007). A gene coding for 65-kDa protein was also used as a MTB marker for nested PCR with the inner sequence 220bp long.

Some protocols targeted two genes: IS*6110* plus either genes coding 38-kDa protein, or MPB624 (Greco et al., 2009).

8.5.4 Detection

Agarose gel electrophoresis is a simple technique, but it is not suitable for a large number of samples or automation. Still it is the most frequently used detection technique for “in-house” assays.

Hybridization on microplates can be used if oligonucleotide capture probes are synthesised and immobilised to the wells on the microtiter plate. Amplified DNA and labelled DNA is bound to the immobilised probes and detected using any colorimetric method. However, it makes no significant impact on the accuracy of the test if the hybridization method is used instead of agarose gel electrophoresis. Colorimetric detection has been described using DNA-binding proteins, biotin and the digoxigenin technique (Wilson et al., 1993).

Since real-time PCR enables PCR and detection at the same time, there is no need for an additional instrument and separated post-PCR area. Turnaround time is shorter and the time after sample preparation is basically hands-off time.

8.5.5 “In-house” nucleic acid amplification tests performance review

Performance of in-house NAATs is highly variable. In a meta-analysis sensitivity estimates ranged from 9.4% to 100% and specificity ranged from 5.6% to 100% (Flores et al., 2005). Pooled sensitivity in a different meta-analysis was 96% and pooled specificity was lower and variable 81% ranging from 78% to 84%. Only one study had substantially low sensitivity, 20%

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and this may be due to the amplification region in question being IS*6110,* appearing in a low copy number in MTB isolates from India (Greco et al., 2009).

The general conclusion is that “in-house” NAATs are a broad group of diagnostic tests with significant heterogeneity in performance and diagnostic accuracy. Some variables within the different tests seem to increase the accuracy: use of specific amplification targets and the use of nested PCR (Flores et al., 2005).

8.6 Commercial nucleic acid amplification tests

An industry survey undertaken in the late 1990s identified more than 50 companies engaged in TB diagnostic research and development. Also recent technical advances in diagnostics and mycobacteriology lead to the development of a number of commercial TB diagnostic tests (Perkins et al., 2006). Each NAAT uses a different method to amplify specific nucleic acid regions in the Mycobacterium tuberculosis complex. Some of the commercially available tests for TB are:

- Roche Amplicor MTB test
- Cobas Amplicor
- GenProbe Amplified M. tuberculosis Direct test (AMTD)
- Abbott LCx test, discontinued
- BD-ProbeTec (SDA)

8.6.1 Amplicor MTB test

The AMPLICOR *M. tuberculosis* assay is produced by Roche Molecular System, USA

8.6.1.1 Description of the test

Amplicor MTB assay is a PCR-based qualitative test based on detecting the 584-bp segment of the 16S rRNA MTB gene. The test consists of three steps: specimen preparation, amplification of target sequence by PCR in presence of biotinylated primers; hybridization of the amplified products to oligonucleotide probes specific to MTB and detection of probe-bound amplification product by colour formation (Piersimoni et al., 2003).

- DNA isolation

The procedure starts with 100µl concentrated sputum sample. Aliquot of the sediment sample is mixed with wash solution and centrifuged. After this step supernatant is removed and lysis reagent is added to the pellet. This, rather simple step, is of most importance for TB detection: since the pellet contains DNA, disturbance of the pellet can affect the yield of DNA and

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performance of the test. The suspension is heated to complete lysis of the mycobacteria. The lysed material is then neutralised.

- Amplification

For amplification, a neutralised specimen is added to the master mix reagent. The latter is prepared by the addition of uracil *N*-glycosylase enzyme (AmpErase; Roche Molecular Systems, Inc.) to an amplification mixture containing excess nucleotides (dATP, dGTP, dCTP and dUTP in place of dTTP), biotinylated primers, and thermostable *Taq* polymerase just prior to the amplification process. The primers KY18 and KY75 are used to amplify a 584-bp sequence located in a highly conserved region of the 16S rRNA gene of *Mycobacterium* spp. The PCR procedure is carried out by using a thermocycler (Meltzer, 1998).

Carry-over contamination from previous PCR can be a significant problem, due to the abundance of PCR products. Carry-over contamination can be controlled by incorporating dUTP in all PCR products (by substituting dUTP for dTTP, or by incorporating uracil during synthesis of the oligodeoxyribonucleotide primers) and treating all subsequent, fully preassembled starting reactions with uracil DNA glycosylase (UDG), followed by thermal inactivation of UDG. UDG cleaves the uracil base from the phosphodiester backbone of uracil-containing DNA, from previous amplification but has no effect on natural (thymine-containing) DNA ready to be amplified. The resulting apyrimidinic sites block replication by DNA polymerases, and are very labile to acid/base hydrolysis. Because UDG does not react with dUTP, and is also inactivated by heat denaturation at 55^{o} C, prior to the actual PCR, carry-over contamination of PCR amplicons can be controlled effectively if the contaminants contain uracils in place of thymines (Longo et al., 1990).

Each PCR reaction is comprised of three steps: denaturation, annealing, where biotinylated primers hybridize to the denaturated target, and extension, where dNTPs are added to the biotinylated primers and incorporated into the new DNA strand by *Taq* polymerase.

A profile is made up by 37 cycles of PCR resulting in a theoretical billion-fold amplification within the 2h (Meltzer, 1998).

- Detection

After amplification, double strand products are denaturated by adding denaturation solution, followed by room temperature incubation.

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Denatured nucleotide sequences are added to a microwell plate coated with a DNA probe specific for the MTB complex. If the product hybridizes to the probe it is subsequently detected by an avidin-horseradish peroxidase conjugate-tetramethylbenzidine substrate system. The reaction is stopped by the addition of hydrosulfuric acid, and the absorbance is read at a wavelength of 450nm. Specimens giving an absorbance value of 0.350 are considered positive (Schrim et al., 1995). Positive and negative amplification controls are included in each run. The result is ready after 6.5 h.

8.6.1.2 Test performance review

The biggest strength of the Amplicor MTB assay is good accuracy 96% in smear positive cases (Table 2). This is why it is an FDA approved NAAT for TB detection in smear positive samples only.

Sensitivities in respiratory specimens (compared with culture and clinical diagnosis) are considerably lower in smear negative specimens, 61%. Also there is a correlation between test sensitivity and the number of tested specimens for each patient (the performance of NAAT is affected by the mycobacterial burden and its distribution in the sample) (Piersimoni et al., 2003). The test performs poorly in extrapulmonary specimens especially pleural fluids, gastric aspirates, lymph nodes and CSF with sensitivity ranging form 27.3% - 85%. There are no significant differences in performance between manual Amplicor MTB and Cobas Amplicor (Table 2).

Manual Amplicor MTB does not have internal control, so the presence of PCR inhibitors cannot be detected. Most PCR inhibitors are found in extrapulmonary specimens (Piersimoni et al., 2003).

Sample preparation requires a BSL3 laboratory, since the sample has to be decontaminated and concentrated and DNA is isolated in the BSL3 cabinet, also designated pre-PCR. Separate PCR and post-PCR areas are required. Analysis of 20 samples takes 1.5 days.

8.6.2 Cobas Amplicor

The trend in molecular diagnostics is to put all processes on one platform and avoid operator involvement as much as possible. Roche and Cobas (Hoffmann-La Roche Ltd, Germany) made a system in order to perform PCR and detection of products in a way that minimises the operator's hands-on time and the risk of cross-contamination during sample manipulation.

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8.6.2.1 Description of the test

The COBAS® AMPLICOR Analyzer (automated test Cobas Amplicor will be referred to as Amplicor for the reminder of the thesis) is a benchtop system designed to fully automate the amplification and detection steps, as it combines five instruments into one: thermal cycler, automatic pipettor, incubator, washer and reader. This was the first automated system for diagnosing infectious disease using PCR (DiDomenico et al., 1995).

The Cobas Amplicor Analyzer instrument is used for amplification of the MTB gene target and uses the same PCR strategy as the Roche Amplicor MTB manual system. The same 584bp sequence within a highly conserved region of the 16S rRNA gene of MTB is targeted.

Important features introduced in Amplicor detection system are:

- A species-specific oligonucleotide capture probe is coated with magnetic particles to capture an amplicon specific for the MTB complex (not hybridized in a plate with probes as in Roche Amplicor MTB)
- An internal control is used; the primer pair allows simultaneous amplification of the MTB target and an internal control (IC) (Piersimoni et al., 2003)

The internal control is a DNA plasmid with a length and base composition similar to that of MTB and a primer binding region sequence identical to the target sequence of MTB. The internal control (IC) is present in each reaction mixture (20 copies of IC plasmid per 50uL) and is co-amplified with the clinical sample and detected separately with an oligonucleotide probe specific for the resulting IC amplicon. Only 20 copies of IC are introduced in each test sample to generate a positive result at the limit of test sensitivity (Reischl, et al., 1998)

8.6.2.2 Test performance review

Test has similar performance as manual test with sensitivity in smear positive samples 96% and smear negative 64%. Specificity is 99% in smear positive samples.

The instrument is reliable and does not need high maintenance. A run takes about 6 hours and maximises the operator's walk-away time. It can accommodate 20 samples plus two positive and two negative controls. After amplification, new sets of samples can be loaded while amplified samples are moved on the detection rack. The system is barcode enabled. It can be used for tests other than MTB: HIV-1, hepatitis C, hepatitis B, Cytomegalovirus, *Chlamydia trachomatis, Neisseria gonorrhoea, Mycobacterium avium,* and *Mycobacterium intercellulare.*

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The instrument has simple software in charge of the process. The system is robust and can only be deployed in a laboratory environment. It cannot detect drug resistance. Samples could be prepared in advance, except for the positive and negative control, which should be prepared just before the run. Some reagents are protein-based and thermo sensitive. The substrate is light sensitive and is also prepared just before the run. Proper handling of reagents is of the utmost importance.

8.6.3 Gen-Probe AMPLIFIED M. tuberculosis direct test

The AMTD assay is produced by Gen-Probe, Inc., USA

8.6.3.1 Description of the test

The AMTD test utilises Transcription-Mediated Amplification (TMA) and hybridization to qualitatively detect MTB rRNA. Theoretically the assay should have enhanced sensitivity in a sample with a small bacterial load, since it detects rRNA which is presented at approximately 2000 copies per cell. A result is obtained within 2.5 to 3.5 hours. The AMTD test cannot ascertain drug susceptibility. The AMTD test consists of two parts: isolation of DNA and an amplification-detection step, which takes place in a single tube (Palomino et al., 2007).

- RNA isolation

The sputum sample is pre-treated with NaOH/NALC. 450µl of the sample is treated with detergent and afterward nucleic acids are released from mycobacterial cells by sonication. Heat is used to denature the nucleic acids and disrupt the secondary structure of the rRNA.

- Amplification

The Gen-Probe TMA method, using a constant 42°C temperature, then amplifies a specific mycobacterial rRNA target by transcription of DNA intermediates, resulting in multiple copies of mycobacterial RNA amplicon. MTB sequences are then detected in the RNA amplicon using the hybridization method. The MTB hybridization reagent contains a single-stranded DNA probe with a chemiluminescent label. This probe is complementary to MTB complex-specific sequences.

- Detection

When stable RNA:DNA hybrids are formed between the probe and the specific sequences, the hybridized probe is selected and measured in a luminometer (El-Sayed Zaki et al., 2008, Piersimoni et al., 1998).

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8.6.3.2 Test performance review

The first version of the test (AMTD1) as well as the second one (AMTD2) are approved by the FDA. The new version incorporates three main changes:

- an increase in the initial amount of pre-treated specimen from 45 to 450µl
- a reduction in the incubation time of the amplification reaction (60 to 30 minutes)
- elimination of the termination reaction (Gamboa et al., 1998)

AMTD's performance is similar to Amplicor in smear positive samples with sensitivity 97% and 95% - 97% for AMTD and Amplicor respectively (Table 2), but AMTD is better in smear negative samples, with sensitivities of 73% - 83% and 43% - 74% for AMTD and Amplicor respectively (Forbes et al., 1997). Sensitivities in smear negative samples from 65% - 92% have been reported in per patient analysis (Bergmann et al., 1999). Hence, it has been approved by the FDA for use in smear negative samples as well as extrapulmonary samples. The FDA excluded patients on TB treatment for more than seven days within the previous 12 months from testing on any NAAT, since it may give false-positive results.

On the other hand AMTD does not have an internal control, and that is one of the test's biggest drawbacks. So, a negative result could actually be the result of PCR inhibition. If a sample is suspected to be inhibited (AFB smear positive), a second reaction should be run, with the same sample spiked with the known target nucleic acid and then amplified. This increases the cost of the test, and decreased its usefulness.

8.6.4 Abbott LCx Assay

The LCx MTB assay, ABBOTT LCx probe system was produced by Abbott Laboratories, Abbott Park, USA, but production is discontinued.

8.6.4.1 Description of the test

LCx Mycobacterium Tuberculosis Assay (LCx) is based on the ligase chain reaction. The assay uses a semi-automated system and allows direct detection in clinical specimens. The amplified sequence is specific for all MTB complex strains (Ausina et al., 1997, Garrino, et al., 1999).

The target sequence is the gene-encoding a 38kDa protein, Antigen b. Four primers in pairs recognise and hybridize to the complementary single-strand MTB target sequence. The target sequence is annealed and exposed using high temperature. Primers are designed so that once they hybridize to a target DNA a gap of few nucleotides remains between them. Polymerase fills the gap between them and, once the gap is filled, ligase covalently joins the pair of probes,

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hence an amplification product is formed which is complementary to the original target gene. In that way product is amplified and analysed on the Abbott LCx Analyzer.

- DNA isolation

A volume of 500µl concentrated sample, treated for potential inhibitors using SDS/NaOH, is placed in an LCx specimen tube. After two washings and concentration steps the sample is exposed to heat at 95^0 for 20 minutes and subsequently to mechanical lysis in the LCx Lysor for 10 min.

- Amplification

After DNA isolation, the sample is added to the master mix, containing thermostable DNA ligase, DNA polymerase, dTTP and four oligonucleotide probes labelled with haptens. Amplification is completed after 37 cycles on the LCx Thermal Cycler.

- Detection

The amplified product is transferred to the LCx Analyzer for detection. Detection is enabled by capture and detection hapten on each paired probes, thus the amplification product has capture hapten on one end and detection hapten on the other. The reaction mixture is incubated with microparticles coated with anti-capture hapten, after which the mixture is exposed to the glass fiber matrix to which microparticle complexes bind irreversibly. Following the washing step, bound microparticles are incubated with antihapten-alkaline phosphatase conjugate which binds to the detection hapten. After adding the substrate, product is detected by fluorescence measurement (Ausina et al., 1997).

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8.6.4.2 Test performance review

Lack of sensitivity is its main shortcoming: 78% and 37% in smear positive and smear negative groups respectively (Garrino et al., 1999). It has been modified in the field in terms of increasing the concentration of target DNA, numbers of amplification cycles, lowering cut-off values, and increasing the number of washing steps to remove inhibitors. Since, even with all those improvements sensitivity was still unsatisfying; the test was removed from the market in 2002.

The price varied between $30 per sample, if test is run at full capacities (20 samples and 4 controls), and $200 if only one sample is analysed. The test is therefore affordable if performed on a batch basis, which counteracts its usefulness, since the main benefit from the test lies in obtaining results the same day (Garrino et al., 1999).

8.6.5 BDProbeTec ET system

The BD ProbeTec ET Direct TB System (DTB) is produced by Becton Dickinson Biosciences Microbiology Products, USA.

8.6.5.1 Description of the test

The BDProbeTec system uses a strand displacement amplification (SDA) technique for rapid detection of MTB. The assay uses a thermophilic version of SDA to enzymatically replicate target sequence. Recently a new system was developed, called BDProbeTec ET, which couples SDA to fluorescent energy transfer (ET) detection (Berrett et al., 2002, Bergmann et al., 2000).

- DNA isolation

Specimens are decontaminated using NaOH-NALC and 500µl aliquots are used for further analysis. DNA isolation involves one washing step, concentrating, and pellet heat lysis at 105^0 for 30 minutes. The next step is resuspending of the pellet in the lysis buffer, neutralisation and sonication for 45 minutes.

- Amplification

The amplified region is a 95bp long fragment from IS*6110.* Nucleic acid amplification is isothermal and based on homogenous strand displacement amplification. An internal amplification control is run with each sample to confirm validity of amplification and detect potential inhibitory factors.

The prepared sample is transferred into a priming plate and incubated for 20 minutes at room temperature and then heated for 10 minutes. After that, samples are transferred to an amplification plate containing 96 wells and placed in the amplification/reader instrument.

- Detection

Fluorescence detection is simultaneous with amplification.

8.6.5.2 Test performance review

BDProbeTec ET system's biggest strength is high sensitivity and specificity 98% and 97% respectively (Table 2); higher than other currently available commercial tests. It has internal control in the same PCR reaction mix, so the presence of inhibitors can be detected. Cross-over post-PCR contamination is minimised since amplification and detection happen in the same sealed well and there is no need for a post- PCR designated area. All reagents are stable at room temperature, which is another advantage.

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DNA isolation is similar to the Amplicor, and needs additional instruments like: centrifuge, water bath, and sonicator. DNA isolation is labour-intensive, and a crucial step is removing the supernatant from the pelleted sample.

8.6.6 Commercial nucleic acid amplification test performance review

Two methods (Amplicor and BDProbe ET) are fully automated, but they still require cumbersome sample preparation and DNA extraction in BSL 3 facilities. This step extends time to result, usually for one more day. None of the methods gives any quantitative value regarding the microbial load. Only two methods (Amplicor and BDProbe ET) include an internal control, to detect inhibitors in the sample. Amplicor has a method for carry-over prevention. Cost per sample depends on the number of samples processed at the time, since the price is lower if the test is run at full capacities. Price per sample also depends on where the test is performed. Only two tests (Amplicor and AMTD) have FDA approval.

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Commercial nucleic acid amplification tests characteristics					
Characteristic	**Amplicor MTB**	**Amplicor**	**AMTD**	**LCx**	**BDProbe ET**
Automated	No	Yes	No	Semi	Yes
Sequence	16S rRNA	16S rRNA	rRNA	gene coding 38kDa protein	IS*6110*
NAAT method	PCR	PCR	TMA	LCR	SDA
Detection method	Hybridization/ colorimetric	Hybridization/ colorimetric	Chemiluminiscence	Fluorescence	Fluorescence
DNA quantitation	No	No	No	No	No
Number of samples per run	Depends on thermocycler's capacity	24	Depends on luminometer capacity	24	96
Internal control	No	Yes	No	No	Yes
Positive and negative controls per run	2	4	2	4	4
Carryover prevention	dUTP-UDG	dUTP-UDG	No	No	No
PCR inhibitors	Not detected	Detected	Not detected	Removed	Detected
Time	8h	8h	2.5 – 3.5h	6h	3h
Hands-off time	2h	6.5h	2h	2.5h	2h
Sample preparation time	1h 30 min	1h 30 min	30min	2h	1h
Sample volume µl	100	100	450	500	500
Cell lysis and DNA isolation	Reagent/heat	Reagent/heat	Sonication/heat	Reagent/heat/ mechanical	Reagent
Sample decontamination	Yes	Yes	Yes	Yes	Yes
Labour cost ($20/hour)	$160	$160	$50	$120	$60
FDA approval	Yes	Yes	Yes	No	No

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Table 1 Technical characteristics of NAAT, TMA (transcription mediated amplification), LCR (ligase chain reaction), SDA (strand displacement amplification) (Piersimoni et al., 2003, Neonakis et al., 2008, Palomino 2005)

In general, performance of commercial NAATs is similar, however, AMTD appears to perform best in smear negative samples, and BDProbe has the highest sensitivity in smear positive sample. The diagnostic odds ratio (DOR) is the ratio of the odds of positivity in patients with disease to the odds of positivity in disease-free patients; it may be used as a useful summary of test performance. A DOR value of less than one indicates that a test more frequently positive on control subjects, whilst increasing values above one are associated with improved performance.

Table 2 summarizes the performance data of commercial NAATs. AMTD and BDProbe have the highest DOR values.

Commercial nucleic acid amplification tests performance					
Performance	**Amplicor MTB**	**Amplicor**	**AMTD**	**LCx**	**BDprobe**
Sensitivity S+ % (range)	96 (94-97)	96 (95-97)	97 (95-98)	96 (94-98)	98 (96-99)
Sensitivity S- % (range)	61 (57-65)	64 (59-69)	76 (70-80)	57 (50-64)	71 (66-76)
Specificity S+ % (range)	97 (96.8-97.4)	99.3 (99.2-99.4)	97 (96.6-97.4)	98 (97.8-98.5)	97 (96.4-97.4)
Specificity S- % (range)	83 (80-86)	74 (68-80)	96 (93-97)	71 (64-78)	89 (84-93)
DOR S+	117 (56-246)	99 (56-173)	314 (99-995)	42 (12-142)	181 (39-834)
DOR S-	77 (51-115)	220 (144-335)	157 (48-510)	71 (38-132)	96 (53-175)

Table 2 Performance of NAATs S+ (smear positive sample), S- (smear negative sample), DOR (diagnostic odds ratio), (Greco, et al., 2006)

Performance of Amplicor			
Type of specimen	**Sensitivity**	**Specificity**	**Study**
Culture positive	91%	100%	Kim et al., 2008
Culture positive smear negative	59%	100%	Takakura et al., 2005
Culture positive smear positive	95%	100%	Takakura et al., 2005
Culture positive	81%	100%	Takakura et al., 2005
Culture positive	73%	100%	Ozkutuk et al., 2006
Culture positive smear negative	50%	NA	Goessens et al., 2005
Culture positive smear positive	95%	NA	Goessens et al., 2005
Culture positive	78%	98%	Goessens et al., 2005
Culture positive	73%	100%	Alfaresi et al., 2006
Culture positive	92%	100%	Abu-Amero et al., 2004

Table 3 Performance of Amplicor for diagnosis of TB in adult patients

Amplicor is widely used automated NAAT for detection of MTB. It has been used since 1997 for diagnosis of TB in smear negative and smear positive sputum samples. It was chosen as a comparison test in this study, since it has high sensitivity and specificity in pulmonary samples and it is very well described and evaluated automated MTB detection system (Table 3).

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9 Phenotypic Drug Susceptibility Testing

A recent WHO recommendation is to perform DST for INH and RIF on initial isolates from all patients in order to identify drug resistance. Furthermore, DST should be repeated if the patient continues to produce culture positive sputum after three months of treatment or develops positive cultures after a period of negative cultures (WHO, Use of Liquid TB Culture and Drug Susceptibility Testing, 2007).

Phenotypic determination of MDR TB using first-line drugs in inoculated culture is the benchmark for DST, but it can only be applied in a well equipped laboratory and may take many weeks for the final result. The WHO and Stop TB Partnership have called for the development of a more rapid and feasible DST method that could be deployed in low resource settings. It appears that the molecular approach is the way forward and the candidate test is based on the ability to detect mutated MTB genes that are responsible for drug resistance, particularly to INH and RIF.

9.1 Traditional phenotypic methods

The traditionally accepted methods include:

- proportion method
- absolute concentration method
- resistance ratio method

The most commonly used is the proportion method on LJ medium or Middlebrook agar and it compares the number of colonies on two plates with and without the drug and requires a minimum of three to four weeks to produce results (Palomino et al, 2008). The absolute concentration method involves inoculating a commercially available plate with serial concentrations of the drug, allowing determination of the minimal inhibitory concentration (MIC). The resistance ratio method compares the MIC of the isolate to the MIC of a strain with a drug susceptible phenotype.

These provide valuable information for deciding on treatment, but the result is usually available only after six to eight weeks after diagnosis (Palomino et al, 2008).

9.2 Newer phenotypic methods

9.2 1 Liquid culture

The radiometric Bactec 460 system greatly decreased the turnaround time for DST. The test can be performed in the same tube as culture, just by adding one antibiotic. Still a major

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inconvenience is the use of radioactive materials especially for low-resource countries (Palomino et al., 2008).

Bactec MGIT (MGIT) has been successfully used for DST. It has been evaluated for use for a range of antibiotics namely: RIF, INH, EMB, SM, PZA, amikacin, capreomycin and ethionamid; and it shows high accuracy, especially for RIF and INH. Antibiotic is added to one MGIT tube which is then inoculated with the culture. A second tube is inoculated with the same culture but without antibiotic. Comparing growth in tube with antibiotic and control tube allows determination of susceptibility or resistance. MGIT has also been evaluated for direct DST from patient samples (rather than from cultures). This considerably shortens the time to result to about six days whilst maintaining a high degree of result accuracy. It may, however, be associated with increased cost, since if it were routinely performed on all samples for TB testing, a high proportion would typically be culture-negative (El-Sayed Zaki et al., 2007).

Bactec MGIT has a manual version as well as an automated format, and each has its advantages: manual for laboratory in a low-resource and low sample turnover and automated for laboratories testing larger number of samples. In the manual system, a drug-containing tube and a control tube are inoculated with the prepared sample and incubated. From the third day both tubes are analysed for fluorescence. The presence of an orange fluorescence until the 14th day of incubation, in the antibiotic containing tube at the same time as in the control tube, is interpreted as resistance to the drug; otherwise, the strain is considered to be susceptible (Palomino et al., 2007).

Bactec MGIT has 95% - 98% and 99% - 100% sensitivity for detection of resistance of INH and RIF respectively when compared to radiometric Bactec 460, as stated in a meta-analysis of MGIT comparative studies (Piersimoni et al., 2006) However, liquid systems are prone to contamination and therefore require stringent quality assurance. In addition, they are more expensive and in the case of the automatic system, require investment in equipment (Pai et al., 2009).

Two other commercial and automated methods for DST are the MB/BacT system (Organon Technika) and the ESP culture system II (Accumed International, Chicago, IL, USA). Both systems rely on expensive equipment and have also been evaluated in several studies.

9.2.2 Solid culture

Several solid culture systems for DST are available. The commercial E-test system (AB BIODISK, Sweden), based on strips with impregnated gradients of antibiotics for the

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determination of drug susceptibility, allows the reading of MIC minimal inhibitory concentrations directly on agar plates. Sensitivity in comparison with the traditional proportion method is 90% (Palomino et al., 2005).

The thin layered agar method (see chapter 1, section 6.3.2.1) used for detection of MTB, can be used for DST. It has a similar time to result as MGIT, 11 days and sensitivity and specificity of 100% for both RIF and INH compared with the proportion method in agar. It has contamination rate of 4.4% (Robledo et al., 2008).

9.2.3 The microscopic observation drug susceptibility assay

Recently, new rapid culture-based tests have been described. These include the microscopic observation drug susceptibility (MODS) assay, where direct specimen is cultured with and without antibiotic in a 24-well plate with liquid selective media where MTB growth can be visually observed for characteristic cord formation using an inverted microscope (Reddy et al., 2010, Migliori et at., 2008). The test is rapid (mean time to result 7 days) and may be less costly than MGIT testing. Sensitivity is 97% and 100% for detection of resistance to INH and RIF respectively and 97% for detection of MDR TB. The MODS is significantly cheaper then automated culture system, per tube, but labour cost might be higher (Moore et al., 2006).

But unfortunately, logistics relating to this method are complicated since a skilled technician is required, plates must be checked daily until a point of observed growth, methods are labour intensive, and biosafety protocols are the same as in the reference laboratory, since live MTB is dealt with. In addition, MODS assay requires an inverted microscope for observation of MTB growth. Considering the price of the microscope, and the fact that BSL 3 facilities are required, this may make MODS difficult to implement in rural environments or developing regions where these tests were actually intended to be applied.

9.2.4 Colorimetric methods

Several colorimetric methods have been developed for the MTB DST. They are based on reduction of colour indicator added to the culture in a microplate to analyse growth after MTB has been exposed to antibiotics (Palomino et al., 2007). Resistance is detected by a change in colour of the indicator, which is directly proportional to the number of viable MTB in the medium (Palomino et al., 2008).

Time to result is between 7-14 days. In addition this method does not need sophisticated equipment and is therefore less expensive to perform than some other liquid culture techniques for DST. But since the microplate needs to be opened, the test requires BSL 3 capacities. It has

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sensitivity and specificity for detecting RIF resistance ranging from 89% - 100% and 90% - 100% respectively. Sensitivity and specificity for INH resistance was from 90% - 100% and 88% - 100% respectively (Martin et al., 2007).

9.2.5 Phage-based methods

Phages are highly specific and can infect a single species. Phage-based tests use the ability of an MTB phage to grow inside MTB. If a reporter gene, like the firefly luciferase gene, is incorporated into the genome of the MTB-specific phage, MTB can be detected by gene product activity, in this case emitted light (Palomino et al., 2007).

A commercially available phage assay, FASTPlaque-TB-MDRi® (Biotec Laboratories Ltd, UK) has been developed for RIF DST. This is a phage amplification-based test, and it is intended for direct use on sputum specimens. Drug resistance is diagnosed when MTB is detected in samples that contain antibiotic. A sensitivity of 97% - 100% and specificity of 84% - 100% was reported for detection of RIF resistance (Palomino et al., 2008).

9.2.6 Nitrate reductase assay

The nitrate reductase assay (NRA) is based on MTB's reductive capacity, and if MTB growth occurs, MTB reduces nitrate to nitrite, which is detected by adding a chemical reagent to the culture medium. LJ medium is used for the test. The advantage of this method is that a result is obtained more rapidly (before growth can be readily observed). The result is measured after 10 days. An evaluation of this method for three first line drugs: INH, RIF and EMB demonstrated accuracy ranging from 96% - 98% (Palomino et al., 2007).

NRA is less expensive than liquid culture techniques for DST. NRA does not need sophisticated equipment, and it can be performed in BSL 2 laboratories in low resource settings (WHO, New laboratory diagnostic tools, 2008).

10 Molecular assays for detection of multidrug resistant tuberculosis

Conventional methods for DST are slow and expensive. In routine analysis in developing regions, definite diagnosis regarding DST using traditional culture based methods can be established only after two months (Van Deun et al., 2009). Also, conventional methods are not applicable at point-of-care. Therefore, research is heading toward fast and simple options, like molecular assays.

Molecular methods for detecting MDR TB are based on NAAT in conjunction with electrophoresis, sequencing or hybridization. The main advantage of molecular testing is time,

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since there is no need to grow the organism and subculture for DST. So far, several methods have been deployed for MDR TB testing: direct sequencing has proved expensive and time consuming (Garcia de Viedma, et al., 2003), real-time PCR has proven highly sensitive and specific although, it is complicated for routine use, expensive and less applicable in low resource settings (Palomino et al., 2009), and hybridization-based line probe assays (LPA) were, until recently, the only cost-effective tool for MDR TB testing, with good sensitivity/specificity performance (Rossau et al., 1997, Palomino 2005).

10.1 Line probe assays

10.1.1 INNO-LiPARif.TB

INNO-LiPA is an MDR TB detection kit produced by Innogenetics, Belgium. It is based on a reverse hybridization assay, referred to as a line probe assay.

In this assay concentrated specimen is used. The target sequence is within the *rpoB* gene. Nested PCR is used to amplify the sequence and during amplification, product is biotinylated. Post-PCR analysis involves hybridization between the product and a set of 10 probes immobilised as parallel lines on a strip. According to the hybridization pattern, visible after colorimetric reaction, the presence and absence of the mutations can be assessed.

Each strip contains conjugate control and an MTB control probe, to determine if hybridization was effective and if the genome originates from MTB, respectively. The MTB control is also part of the *rpoB*, and it is present in all MTB complex strains. There are five overlapping probes for wild-type *rpo*B and four probes for the most frequently observed mutations. If a mutation is in fact present in one of the target regions, a mismatch created will prevent the amplicon from hybridization to the probe under stringent conditions. There are also four probes on a strip that hybridize with the amplicons carrying specific mutations.

Interpretation of results is based on a specific scheme of probe appearance. If all probes for wt are present, the sample is considered RIF sensitive. If one is missing, even without the mutated probe visualisation, the sample is considered RIF resistant (Rossau et al., 1997).

10.1.2 Hain MTBDRplus

Hain MTBDRplus (assay will be referred to as Hain for the reminder of thesis) is manufactured by Hain Lifescience GmbH, Germany. The assay is the most widely used molecular assay for DST. It detects most common mutations related to RIF and INH (low and high level) resistance. It is also based on the reverse hybridization assay.

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The technique may be applied either directly to the concentrated sample or on cultured isolates and performance is still excellent for RIF (Pai et al., 2009). A simple procedure is used for DNA extraction involving sonication and heating. The sample is amplified using any commercially available DNA polymerase kit, with primers and amplification control provided by the manufacturer. PCR is performed in the thermocycler according to a protocol for the specific DNA polymerase used and the Hain, combined.

Hybridization is either automated or manual, using strips with immobilised probes. Visualization is obtained after a final colorimetric reaction.

Each strip has amplification and hybridization control. There is also an MTB control, which detects all MTB complex strains. There are three locus controls for *rpoB*, *katG* and *inhA* loci. There are eight overlapping *rpoB* wild type probes, followed by four mutation probes, with most common mutations within the selected gene. There is one *katG* wt probe and two mutation probes; and also two *inhA* wt probes and four mutation probes for the corresponding wt segment.

The result gives evidence of a mutation in any one of three genes, hence RIF and INH resistance can be established (Lacoma et al., 2008, Barnard et al., 2008).

10.1.3 Line probe assays detecting of multidrug resistant tuberculosis performance review

Molecular assays for rapid detection of RIF resistance could significantly reduce the need for conventional laboratory infrastructure, though still costly and inadequate for most high-burden countries and developing countries. Rapid MDR TB screening tests would impact morbidity, mortality and transmission of MDR strains if assays are introduced in screening (WHO, Molecular line probe assay, 2008).

So far two commercial molecular assays, Hain and INNO-LiPA, have been widely used and characterised. Performance in detecting RIF resistance had a pooled sensitivity of 97% and specificity of 99% for INNO-LiPA and sensitivity of 98% and specificity of 99% for Hain. Pooled sensitivity for INH resistance detection was 85% and the pooled specificity 99% (WHO, Molecular line probe assay, 2008).

Both tests have been used widely in resource rich setting since 1999. In the resource limited setting both assays have been under evaluation until recently and they have become part of the

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national TB programme in some countries, like South Africa, since 2006 (WHO, Molecular line probe assay, 2008).

The result of the test is easy to interpret and it is informative for epidemiological study as well, since it gives a broad overview of the local mutations involved in drug resistance. Another important feature of line probe assays that they are useful for identification of MTB.

Limitations of the test related to laboratory capabilities. Line probe assays are not suitable for point-of-care diagnostic evaluation. They require highly trained staff, BSL3 facilities and designated PCR processing areas. The commercial line probe assay is intended for cultured isolates and smear positive samples. It is not sufficiently evaluated for smear negative and extrapulmonary samples.

In case of mixed infection, the analysed sample might hinder the actual result, since the test will detect a wild type pattern, and a mutated pattern, and it depends on the intensity of wt and a mutated probe. It is then up to the operator's subjective opinion to decide if the sample has a drug resistant strain or not.

Also, resistance can exist even if a wild type pattern is observed. Some of the possible scenarios leading to this result are:

- sample containing more than one MTB strains can be composed in a way that one of the strains contains wt and the mutation is not covered by the mutation probes
- if amplification was not efficient, because the starting concentration of bacilli was low, especially in paediatric patients or extrapulmonary TB, or in case DNA extraction was not effective, or PCR inhibitors were present, hybridization might show pale bands, and confirmation or resistance is not reliable

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Commercial line probe assays characteristics		
Characteristic	**Hain**	**INNO-Lipa**
Drug resistance	Rifampicin/Isoniazid (high and low)	Rifampicin
Automated	Manual and automated	Manual and automated
No. of samples per run	12 (manual) 48 (automated)	48 (automated)
Region amplified	*rpoB, inhA, katG*	*rpoB*
Isolation method	Sonication/heat	Detergent/heat
NAAT method	PCR	Nested PCR
Detection method	Hybridization/strip blot/colorimetric	Hybridization/strip blot/colorimetric
Amplification control	Yes	No
Conjugate control	Yes	Yes
MTB specific probe	Yes	Yes
Carryover prevention	No	No
Time	7 h + sample preparation	12 h + sample preparation
Sample volume	500	500
Price per test $	$57-120	$45-116

Table 4 Technical characteristics of Hain and Inno LIPA (Lacoma et al., 2008, Barnard et al., 2008)

LPAs are recommended only for smear positive samples, but not approved for smear negative samples. In a South African evaluation, when a small number of smear negative samples were analysed on Hain, 80% gave interpretable results for RIF resistance and 74% gave interpretable results for INH resistance. Furthermore, 100% sensitivity for resistance detection was observed in interpretable samples for both RIF and INH resistance. In the same study performance of Hain in detecting resistance in smear-positive samples was excellent, with sensitivity and specificity for RIF both 99%, for INH 94% and 100% respectively and MDR 99% and 100% respectively.

Commercial line probe assays characteristics		
Performance in smear positive samples	**Hain**	**INNO-LiPA**
RIF sensitivity% (range)	98.1 (95.9-99.1)	97 (95-98)
RIF specificity% (range)	98.7 (97.3-99.4)	99 (98-100)
INH sensitivity% (range)	84.3 (77-90)	
INH specificity% (range)	99.5 (98-100)	

Table 5 Performance of Hain and INNO LiPA (WHO, Molecular line probe assay, 2008)

As part of its global laboratory initiative the WHO Strategic and Technical Advisory Group for Tuberculosis (STAG) in 2008 recommended the use of LPA for direct detection of resistance to RIF and INH in settings where the test can be reliably performed.

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Performance of Hain					
Specimen	**RIF sens**	**RIF spec**	**INH sens**	**INH spec**	**Study**
Direct smear positive	99%	99%	94%	100%	Barnard et al., 2008
Culture isolate	98%	98%	90%	100%	Hillemann et al., 2007
Clinical strains	92%	NA	73%	NA	Lacoma et al., 2008
Culture isolate	95%	100%	82%	100%	Huang et al., 2009

Table 6 Performance of Hain in RIF and INH resistance detection, RIF sens (RIF sensitivity), RIF spec (RIF specificity), INH sens (INH sensitivity), INH spec (INH specificity)

Hain assay is the used in most reference laboratories as DST on direct samples and culture isolates. It was chosen as a comparison test in order to evaluate performance of GeneXpert MTB/RIF in detecting RIF resistance, since it detects the same gene and mutations leading to resistance to RIF.

11 Commercial assays for culture speciation

Until recently, species identification was based on phenotypic characteristics, such as growth rate, pigmentation, and colonial morphology. Even though this method is straightforward it is very often incorrect and cumbersome. Hence, line probe assays are made into commercial assays used to detect species, like GenoType Mycobacterium AS and GenoType Mycobacterium CM (Hain Lifescience GmbH, Germany) and INNO-LiPA MYCOBACTERIA. In 2008 a very simple new test was introduced for confirming MTB grown in culture, named the Capilia TB test, developed by FIND and Tauns Co. Japan.

The test is based on lateral flow technology and allows confirmation of MTBC in 15 minutes. By detecting MPB64, with a specific monoclonal antibody using an immunochromatographic assay, MTB is readily detected in the culture.

The assay is based on a double antibody sandwich technique in which an antibody labelled by colloidal gold reacts with target antigens to form an antigen-antibody complex. This complex migrates across a chromatographic carrier such as filter paper. The complex is captured by a second antibody fixed in the middle of a chromatographic carrier. If the target antigens are present in the specimen, a colour reaction is caused by the labelled colloidal particles at the site where the second antibody is fixed and the specimen is regarded as positive.

The test procedure is very simple and since TB antigen MPB64 is extracellular protein, there is no need for specific sample preparation, if the test is performed from a specimen obtained from

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liquid culture. Sensitivity and specificity are 99.2% and 100% respectively (Wang et al., 2007, Hillemann et al., 2005).

12 Introducing new diagnostic tests

Many aspects of TB management need improvement: vaccine, treatment regimens, and diagnostic tools for tuberculosis. If those aspects of TB control are improved they may lead to reduction of global incidence of TB by 71%. But if there is no improvement in TB control 101.7 million new TB cases and 17.9 million TB-related deaths are expected between 2015 and 2050 (Abu-Raddad et al., 2009).By introducing a fast, sensitive, diagnostic test at point-of-care would directly impact duration of infectiousness and indirectly reduce incidence in TB. It has been calculated that a new NAAT would therefore prevent 24.4 million active TB cases and 4.2 million deaths between 2015 and 2050 (Abu-Raddad et al., 2009).

In order to meet expectations the new diagnostic should assist in many areas of TB care and control such as:

- screening tests for use in health clinics to distinguish active tuberculosis from all other conditions that may cause the same symptoms
- monitoring treatment response, to determine whether there is bacterial resistance to specific drugs
- detection of latent infection in people at greatest risk for progression to active TB following exposure

In terms of global market for TB diagnostics requirements, the new development should produce a tool that responds to all the current shortcomings and have an affordable price. At the moment the world spends an estimated $1 billion per year on diagnostics for TB (FIND Diagnostics for tuberculosis, 2006)

The performance of a new desired diagnostic test should be compared to available tests. New test should be able to detect TB with at least the same sensitivity as culture and it should be as simple as direct smear microscopy and applied at point-of-care.

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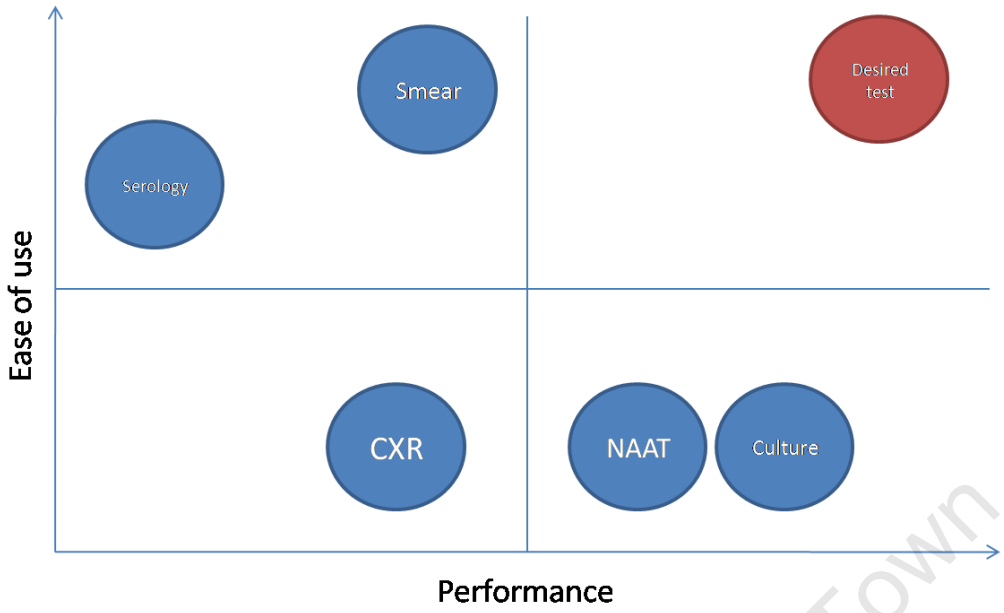

Figure 4 New desired diagnostic test and current diagnostics (Adapted from FIND Diagnostics for tuberculosis, 2006)

A number of commercial diagnostic tests are in the evaluation phase or have been introduced.

Tests under evaluation include:

- Breathalyzer screening tests (Rapid Biosensor Systems Ltd and others); could be used for screening at point-of-care where patient would cough into a disposable device, which is then analyzed for volatile organic compounds specific for MTB
- TB Patch Test; is an improvement of TST since patch delivers MPT64 an MTB-specific antigen, causing irritation in active TB patients after three to four days after application. It does not cross-react with BCG vaccination or NTM, and it can be used at health post
- Vital fluorescent staining of sputum smears; fluorescent dye is used to analyze smears and visualizes only living bacteria. It would be potentially be useful to assess response to therapy (WHO, New laboratory diagnostic tools, 2008)
- Loop-mediated isothermal amplification (LAMP) assay (Eiken Chemical Co.); based on molecular amplification, but does not require PCR or BSL 3 laboratory, cost effective and simple. Isolation does not require centrifugation and amplification but only a simple heating block. It is intended for use in low resource laboratory settings (Boehme et al., 2007)

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Tests already in use include a test for lipoarabinomannan (LAM) detection in urine. LAM was found in urine of TB patients, and this allows a different approach to specimen handling, since urine is not infectious. ELISA and dipstick methods have been developed. The test shows poor sensitivity in HIV-negative patients and variable (but but generally poor) sensitivity in HIV-positive patients. Patients with advanced Immunosuppression are more likely to have a positive test (Lawn et al, 2009). The test was previously marketed by Chemogen and currently by Inverness Medical Innovations, Inc (Mandelson et al., 2007, Boehme et al., 2005)

13 GeneXpert MTB/RIF

Cepheid and FIND have developed a fully automated molecular test for TB and MDR TB case detection for TB endemic countries. The performance of the test is evaluated in this thesis.

The GeneXpert platform (referred to as Xpert in the reminder of thesis) has integrated direct sputum sample preparation, NAAT and detection in a disposable cartridge. The system is completely automated and closed.

Result is obtained after 1 hour and 45 min. The only manual step is the addition of a bactericidal buffer to sputum before transferring it to the cartridge.

NAAT is based on hemi-nested real time PCR to amplify an MTB sequence involved in RIF resistance. Each cartridge contains probes specific to the *rpoB* gene regions where RIF mutations occur. Each probe is labelled with molecular beacons.

The test is intended for use at point-of-care facilities in high burden countries, since it requires only an electricity supply. This is the first molecular assay for TB intended for use at this level.

In comparison to other molecular and traditional assays, advantages of Xpert are:

- detection of TB and MDR TB at the same time
- result is obtained in < 2h
- may be used in point-of-care facilities (microscopy sites)
- closed system, inactivated MTB used for analysis
- limited sample pre-treatment
- no need for BSL 3 facilities
- no need for separate PCR preparation, amplification and detection areas
- no need for technicians skilled in TB detection or molecular biology

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13.1 Description of the test

The test requires an Xpert platform, and a disposable cartridge. Platforms are available with one, four or 16 independent modules, with each module accommodating one cartridge at a time. Each module performs a test independently of other modules on the instrument; hence, it is not necessary batch samples. This allows the technician to run the test immediately after sputum is obtained and a result is ready after 1h and 30min, while the patient is still waiting.

Sample preparation involves adding a sodium hydroxide based reagent or sample reagent. After 15min of exposure to the reagent, MTB is inactivated and can be transferred to the cartridge. The sample reagent liquefies sputum samples so that they could be tested within the cartridge and to decontaminate each sample to reduce possible biohazards. The goal was to achieve a minimum 6-log kill of MTB present in the sputum to comply with international decontamination standards (Helb et al., 2010). This is the end of hands-on time.

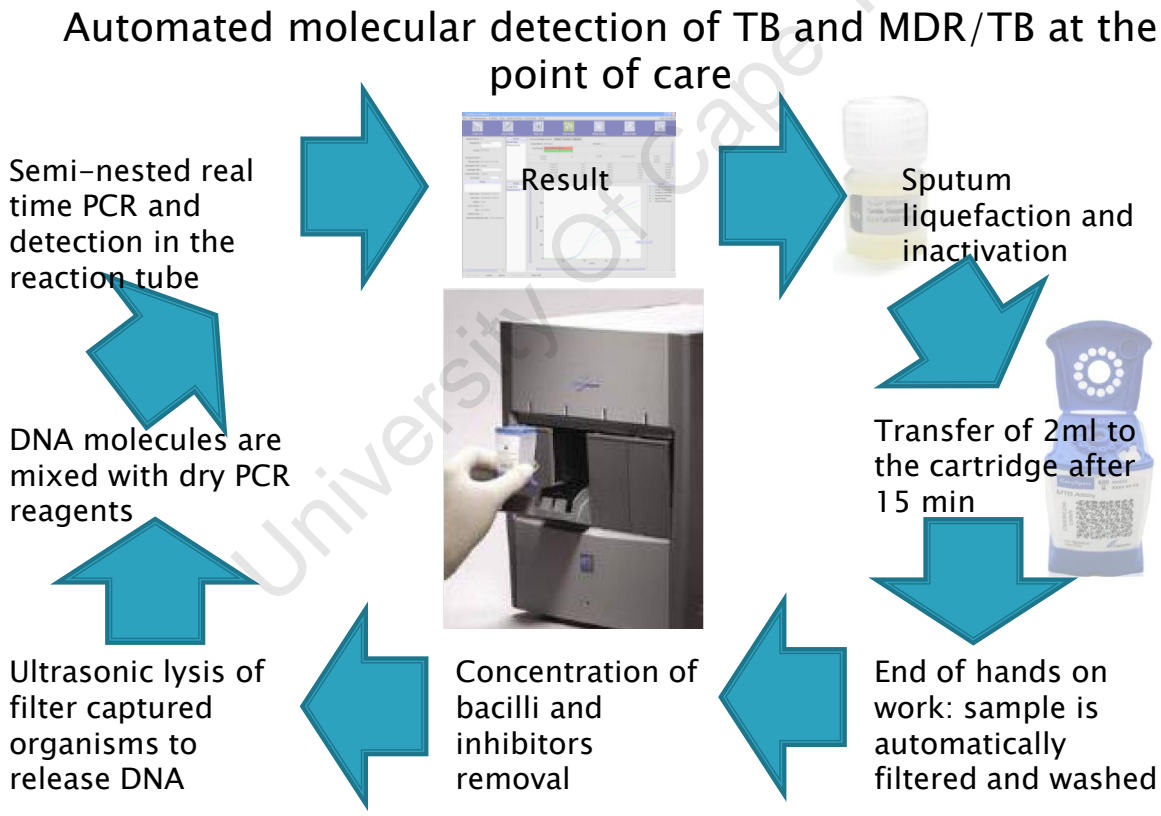

Figure 5 Xpert assay procedure (adapted from Boehme et al., unpublished work)

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13.2 GeneXpert MTB/RIF Cartridge

The cartridge is a disposable unit with necessary reagents for real time PCR including lyophilized enzymes, and six labelled probes. Real time PCR is performed on the instrument. During the test, the following steps are performed on the cartridge:

- passes the sample through the filter to discard larger particles, afterwards DNA is extracted using sonication
- moves the sample and reagents into different chambers in the cartridge for sample preparation
- hydrates the reagent beads
- performs probe checks to ensure that the sample preparation is successful
- moves the sample and reagent mixture into the reaction tube
- starts the PCR cycles and real-time detection

The cartridge holds the samples and reagents needed for the test. Each cartridge consists of the following components:

- processing chambers (hold samples, reagents, processed sample, and waste solutions)
- one chamber is designated as an air chamber to equilibrate pressures within the cartridge
- valve body (rotates and allows fluid to move to different cartridge chambers and to the reaction tube)
- reaction tube (enables rapid thermal cycling and optical excitation and detection of the tube contents)

Cartridge is a closed-system vessel. Inside the cartridge, the specimen is isolated, PCR inhibitors are removed, and specimens are ultrasonically lysed. After the sample is processed, it is mixed with PCR reagents and moved into the integrated reaction tube. The reaction tube is automatically inserted into the I-CORE module when the cartridge is loaded into the instrument.

13.3 GeneXpert MTB/RIF module

Each instrument has 1-16 modules (there is 124 module system being developed), including a heating and cooling system, connected to a PC. The same system can be used for different tests, using different cartridge, heating/cooling cycles and result analysis: vancomycin resistant *Enterococcus*, *Clostridium difficile,* methicillin resistant *Staphylococcus aureus,* enteroviral meningitis, group B *Streptococcus*, FII G20210A and FV Leiden, influenza infection and identification of new variant H1N1 influenza (Rossney et al., 2008, Seme et al., 2008)

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Modules can be replaced in case of troubleshooting without affecting the rest of modules and instrument. In addition, the system is designed to perform a self-test before each test starts to verify that the system is functioning properly.

Each instrument module contains the following components that enable automated sample processing in the cartridge and filling of the tube with the sample-reagent mixture for PCR:

- valve drive (rotates the cartridge valve body to address the different cartridge chambers)
- syringe pump drive (dispenses fluids into the different cartridge chambers)
- ultrasonic horn (lyses the sample)
- I-CORE module (performs PCR amplification and detection)

13.4 I-CORE Module

The I-CORE (Intelligent Cooling/Heating Optical Reaction) module is the hardware component within each instrument module that performs PCR amplification and fluorescence detection. When the cartridge is inserted into the module a reaction tube fits into a tiny slot inside I-CORE. As the reaction inside the cartridge is running, the sample and reagent mixture are pushed from the cartridge into the reaction tube. During the amplification process, the I-CORE heater heats up and the fan cools down the reaction tube contents, while optical blocks excite the dye molecules and detect the fluorescence emitted. There are six optical blocks calibrated for reported dyes.

13.4.1 Heating and Cooling Mechanisms

Within the I-CORE, the heater consists of two ceramic plates with high thermal conductivity in order to assure equal and fast heat transfer.

Thermistor is attached directly to each plate to monitor its temperature. A high efficiency fan cools the reaction tube during thermocycling, while the instrument firmware controls the temperature inside the instrument module. The firmware enables a stringent control system to ensure rapid heating of the plates.

13.4.2 Optical System

Within the I-CORE, the optical system consists of two blocks:

- six-colour excitor module (contains high intensity light-emitting diodes (LEDs) to excite the reporter dye molecules)
- six-colour detector module (contains silicon photodetectors and filters to detect the six spectral bands)

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The optical blocks are positioned within the I-CORE in a way that they are in very close proximity to the reaction tube, thereby enabling excitation and emission of reaction mixture. By using probes labelled with different fluorescent reporter dyes, up to six targets can be detected simultaneously in a single reaction tube (Table 7). The emission spectra of fluorescent dyes can overlap, and a particular dye could produce signal in more than one channel. To compensate for the spectral overlap, the system uses appropriate calibration and data analysis algorithms to determine the concentrations of each reporter dye.

Reporter dyes and their excitation wavelengths		
Reporter dye	**Excitation nm**	**Emission nm**
CF1	375-405	420-480
FAM	450-495	510-535
Alexa Fluor 532	500-550	565-590
Texas Red	555-590	606-650
Alexa Fluor 647	630-650	665-685
CF6	630-650	>700

Table 7 Reporter dyes used for Xpert

13.5 Results

Results are obtained after 1h and 30 minutes. They do not need to be interpreted by the technician, since the system incorporates an algorithm to automatically determine the presence of TB as well as RIF resistance. If none of the reporter dyes (named A, B, C, D, E) are detected but internal control (named SPC), the test is negative (Figure 6). If all dyes are detected, test is MTB positive (Figure 7). If less than five but more than two dyes are detected, test is RIF resistant (Figure 8), since probes complementary to wt did not hybridize properly to the *rpoB* amplicon due to the mutation. This was defined by the presence of greater than a 3.5-cycle difference in the cycle threshold values between the earliest and latest *rpoB* probe signals or if one to three *rpoB* probes did not produce any measurable signal (Helb et al, 2010). Figure 8 shows RIF resistant case where one of the probes (probe E) was not detected.

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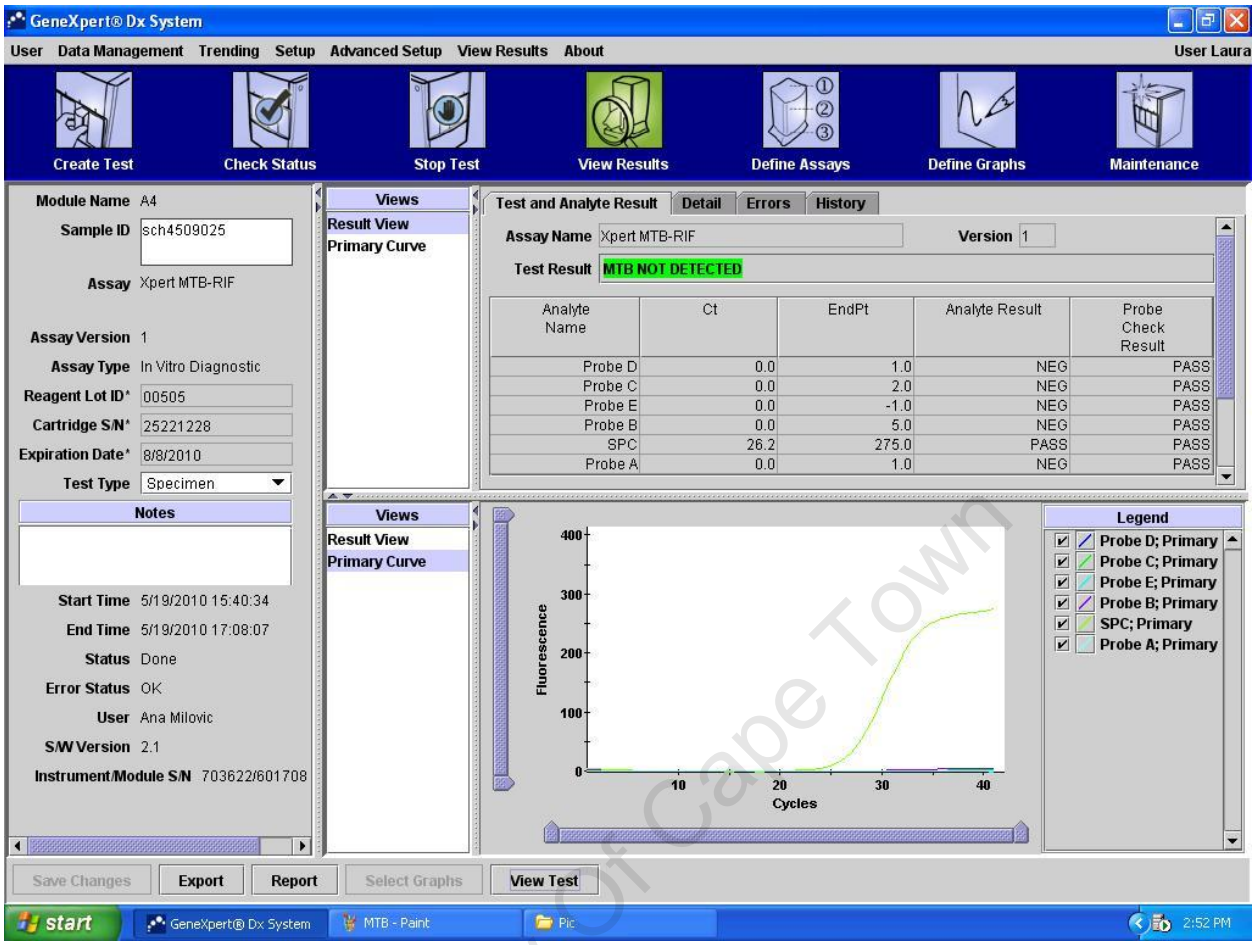

Figure 6 Xpert negative result

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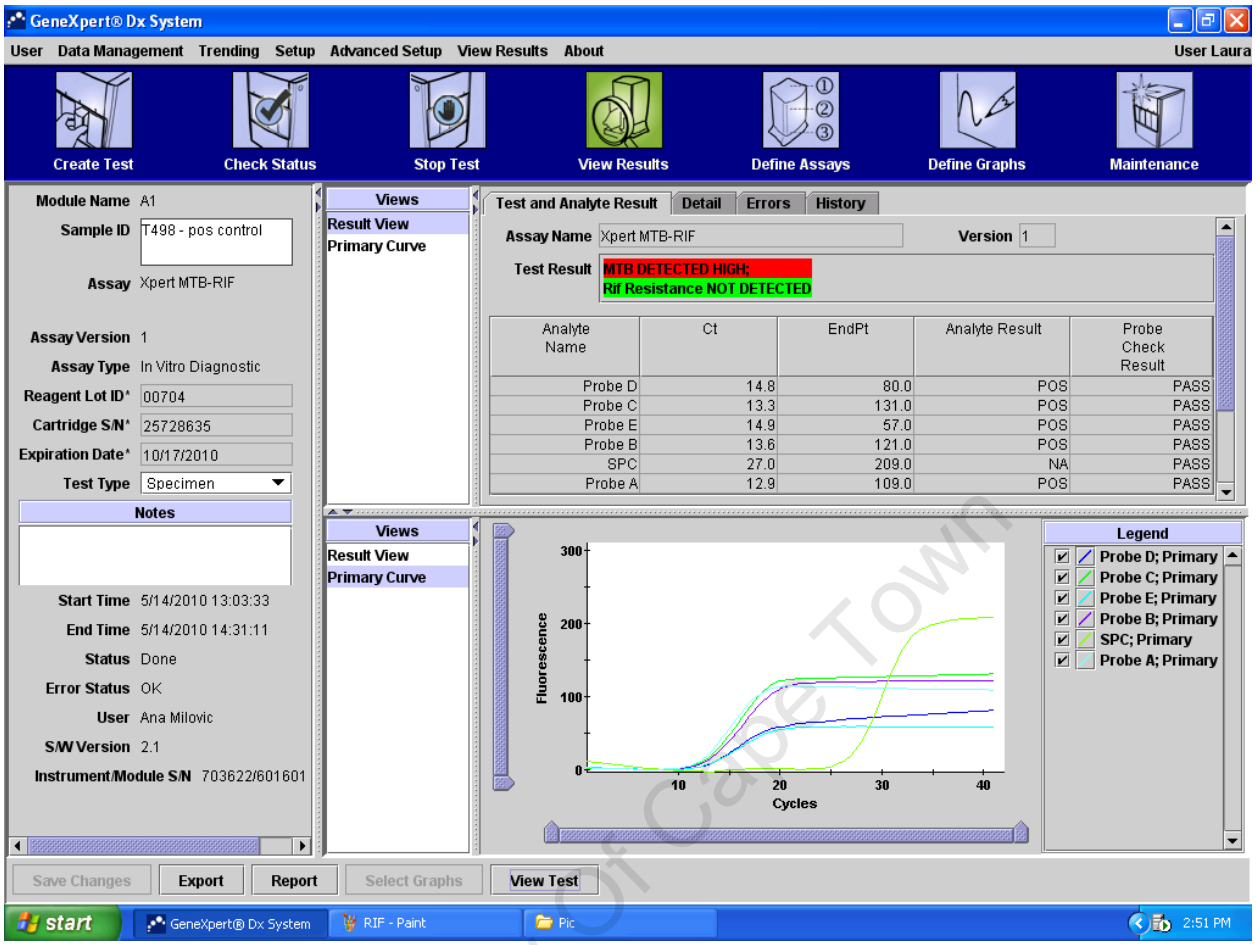

Figure 7 Xpert MTB positive result, RIF resistance not detected

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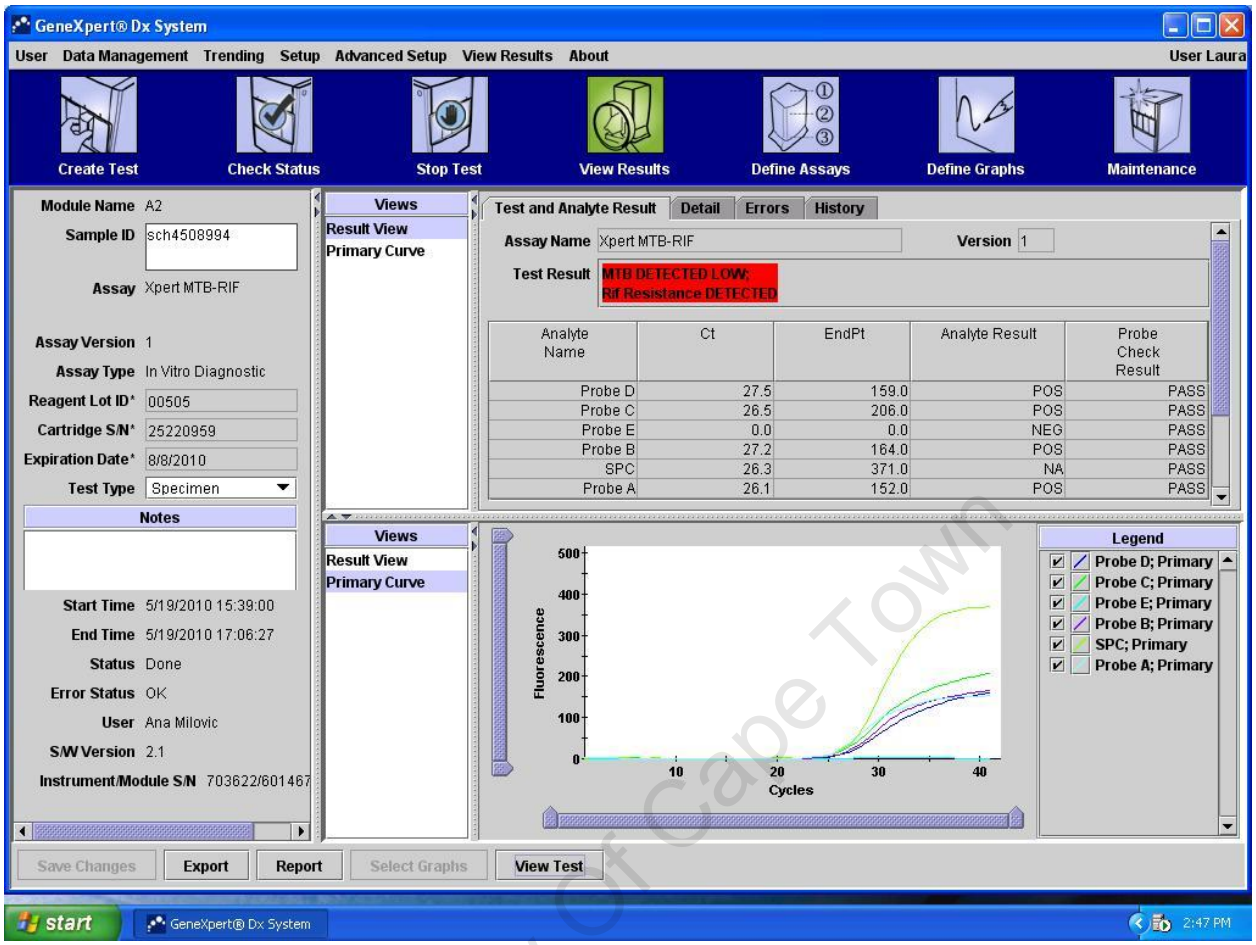

Figure 8 Xpert MTB positive result, RIF resistance detected

14 Summary

There is an urgent need for a rapid, sensitive diagnostic test for TB which can be used at or close to the point-of-care. Such a new test could potentially reduce TB incidence by 13% - 42%.

With the improvement of NAAT techniques in TB detection, sensitivity of tests for TB detection has been rising. However, none of the commercially available assays is in a format which could be implemented close to point-of-care, but are rather intended for use in centralized, well-equipped laboratories with highly trained staff. Moreover, the performance of existing commercial assays for smear-negative TB is suboptimal.

The Xpert system, which incorporates integrated sample preparation, DNA extraction and detection, is a format which could potentially be applied close to point-of-care. This study aims to assess the performance of Xpert for diagnosis of TB in a population with a high prevalence of HIV infection (and hence smear-negative TB).

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II Aims

The aim of the study is to evaluate the performance and operability of GeneXpert MTB/RIF when performed under controlled laboratory conditions for the diagnosis of TB in patients with suspected TB.

Further, the study aims to compare the performance of Xpert in detecting TB and RIF resistance when compared with MGIT and LJ culture result as the “gold standard” and when compared with two commercially available NAATs, Amplicor and Hain.

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III Materials and methods

1 Recruitment and study flow

Study clinicians and recruitment staff screened the patients to identify TB suspects (see chapter 3, section 1.2). Patients were asked to participate in the study and informed consent was obtained. Thereafter, patients' medical history was taken and clinical examination was performed.

HIV testing was advised for all patients with unknown HIV status, but left to the discretion of the attending clinician. Chest radiography was performed on all study participants and a CXR digital image was stored along with the source documents.

Patients were instructed how to produce the first spot sputum, and asked to return the next day with a morning sputum sample. The second spot sputum was provided on the second visit.

Sample analysis took place at the NHLS TB laboratory at Groote Schuur Hospital, Cape Town and TB laboratory at the IIDMM, Cape Town. Smear and culture analysis was performed at NHLS laboratory and molecular tests at IIDMM.

Sample processing was performed according to study protocol only if all three samples arrived at the NHLS laboratory. If there was not a full set of samples, or samples were not of sufficient volume (less than 1.5mL), samples were processed for smear and culture according to the NHLS protocol but excluded from the study.

Any remaining pellet was stored at -20^{0}C for the duration of the study; at least one positive Xpert cartridge was stored at +4^{0}C; culture isolates from all positive cultures were stored at -80^{0}C and MGIT and LJ tubes with positive cultures were stored at room temperature. This was necessary for further analysis of discrepant cases.

Drug susceptibility testing was carried out for the first positive culture of each specimen. First-line drug testing (INH and RIF) was done, and then if proven RIF resistant, second-line drug testing followed for ethambutol, ethionamide, ofloxacin and amikacin.

1.1 Recruitment sites

The trial site in Cape Town was chosen for its high HIV prevalence among an MTB infected population: 70% in the Western Cape and 2.6% MDR cases of all TB cases (MSF report 2009, Health Systems Trust. HIV).

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Recruitment took place at three sites:

- GF Jooste Hospital - Manenberg, Cape Town
- Khayelitsha (Site B) Community Health Clinic – Khayelitsha, Cape Town
- Hannan Crusaid Treatment Centre – Gugulethu, Cape Town

GF Jooste hospital (GFJ) is located in Manenberg, Cape Town, in the Western Cape Province (WCP) of South Africa. GFJ provides secondary treatment and represents a referral facility for the antiretroviral clinics located within areas of North Khayelitsha, Nyanga, Gugulethu, Mitchell's Plain, Heideveld, Crossroads, Manenberg, Strandfontein and Philippi.

These areas are populated with the most socially and economically disadvantaged communities in the WCP (Kevany, 2005). GFJ hospital serves a population of approximately 1.2 million people and HIV prevalence in 2004 in this population was 10% (Meintjes, unpublished data).

Since GFJ is a referral hospital for many primary health facilities, we expected cases with more advanced TB to be recruited at this site.

Khayelitsha Site B community clinic is situated in the largest informal settlement in the WCP. The township is home to at least 500,000 people, over half of whom are unemployed. Khayelitsha has one of the highest burdens of both HIV infection and TB in the country and globally. In 2007, antenatal HIV prevalence was 30% and the case notification rate for TB was 1,500 per 100,000 people per year. About a fifth of TB patients die while waiting for a diagnosis or after treatment begins. One in six patients who start TB treatment drop out during the course of treatment. Treatment success in this environment is as little as 30% while the vast majority of drug resistant cases have a poor treatment outcome (MSF report, 2009).

The Hannan Crusaid Treatment Centre is situated at the Gugulethu Community Health Centre in the Nyanga district of Cape Town. Gugulethu has predominantly informal housing, with high levels of overcrowding and poverty. In 2005, the Gugulethu/Nyanga district HIV prevalence rate was 29.1%, making it the second highest prevalence district in the province (Desmond Tutu HIV Foundation, 2010).

1.2 Inclusion criteria

Patients were recruited into one of two groups: the *case detection group* and the *MDR risk group*.

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Case detection group:

- Clinically suspected to have TB
- Persistent, productive cough for ≥ 2 weeks
- Volunteers to provide three sputum specimens of at least 1.5ml over the course of two days
- Age 18 and above
- No TB treatment for the past 60 days and not started on TB treatment for >48 hours

MDR risk group:

- Re-treatment cases
- Non-converting pulmonary TB cases
- Contact of MDR case
- Clinically suspected or confirmed to (still) have TB
- Volunteers to provide three sputum specimens of at least 1.5ml over the course of two days
- Age 18 and above

For the *case detection group*, TB suspects were eligible for recruitment if they had not started TB treatment >48 hours prior to recruitment, and had not been on any TB treatment <60 days before recruitment. However, for the *MDR risk group*, pulmonary TB cases were eligible if they were suspected to be treatment failures. If that was the case, duration of treatment did not matter even if the treatment was ongoing.

Patients on TB treatment in the *MDR risk group* with negative culture results were excluded from the study regardless of their smear microscopy result. Since both smear microscopy and Xpert assay can detect dead cells, and DNA from dead cells, respectively, inclusion would negatively impact assessment of Xpert specificity.

1.3 Sputum sample collection

Samples were collected in sterile, screw-capped, leak-proof, wide-mouthed containers. Two basic types of sputum specimens were collected:

- Spot specimens
- Morning specimens

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Spot specimens were collected at the health facility. If the initial volume of the sample was too low, the patient was asked to collect sputum within an hour in the same container. Two spot specimens were taken over the period of two days.

Morning specimens were collected early in the morning of the second visit. Morning samples are more likely to contain secretions from the lower airways and hence be of better quality than the spot samples.

Sample collecting procedure:

- Patient was sitting or standing
- Patient was instructed to rinse the mouth with water before expressing the sputum
- Patient was instructed to inhale deeply, cough vigorously, and expectorate the sputum into container
- If the patient did not cough spontaneously, he/she was instructed to take several breaths and hold the breath momentarily, thereby inducing the cough
- If a patient could not produce a sample spontaneously, induced sputum was collected using a nebuliser containing saline solution
- Patient was instructed to hold the sterile container close to the mouth and release the specimen without spilling or touching the inside of the container or the lid
- The lid was firmly screwed on the container
- Expectoration was performed in an isolated outdoor area

Labelling the container was done before it was used. Information on the container included two labels: one containing patient ID, initials, date of collection and if induction was used; and the other one containing study number and method of sampling (spot or morning sample).

At the NHLS TB laboratory, the sample was visibly examined for:

- Food particles
- Blood staining
- Viscosity

At the health facility and also before processing at the NHLS laboratory, the sample was held at 4^0 - 8^0C. All samples analyzed for smear and Xpert were not stored for longer period of time than overnight, or for no longer than 48h if sample was obtained at the end of working week.

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Decontaminated and concentrated pellets form sputum were stored at -20^{0}C according to manufacturer's instructions.

Samples were carried in the safety transport box the same day. Safety measures were taken during sample transport to NHLS and IIDMM.

1.4 TB diagnosis

Diagnosis of TB was based on a combination of rapid identification of bacilli using direct microscopy combined with culture with species identification and antibiotic sensitivity. The following tests were performed on each patient at the initial visit (day1 and day2):

- Smear microscopy; two specimens of concentrated sputum and one direct smear per patient
- LJ culture; two concentrated specimens per patient
- MGIT culture; two concentrated specimens per patient
- Capilia; speciation of positive cultures
- Smear microscopy; identification of acid fast bacilli in positive cultures
- MGIT SIRE (streptomycin, isoniazid, rifampicin and ethambutol) method; DST for first-line and second-line drugs on all culture positive specimens
- NAAT Amplicor; one concentrated sputum per patient
- NAAT Hain; one concentrated sputum per patient

DST was performed for first-line drugs (INH, RIF, and EMB). If the patient was resolved as MDR after first-line DST, the sample was further tested for second-line drugs (PZA, amikacin and ofloxacin). In case of discrepant RIF DST results, the test was repeated and an additional molecular Hain test was performed on culture isolates. Culture isolates were also sequenced.

The TB treatment decision was made by the clinic staff, not influenced by results of Xpert, Amplicor or Hain. Samples sent for Xpert were two decontaminated and concentrated sputum samples, as leftover samples after smear and culture was performed. One direct sputum sample was sent for Xpert after direct smear was performed. Samples sent for Amplicor and Hain were decontaminated and concentrated samples.

After two months a number of "cases" and "control" patients were followed up. At two months follow up randomly selected smear, culture, Amplicor and Xpert negative patients were also invited for the follow up as a comparison group, in the remainder of the thesis those patients are

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referred to as “control” group. Followed up “cases” were Amplicor and/or Xpert positive but culture and smear negative cases.

At follow up smear, culture and Xpert was repeated (see chapter 3, section 1.6). The patient was diagnosed as a TB case if TB was culture confirmed by MGIT and/or LJ (“culture confirmed cases” or C+) or if TB was diagnosed by panel of TB clinicians (“clinical TB”) blinded to Xpert results and independent from the study. The independent panel of clinicians revised cases and CXRs after 2 months of therapy and assigned them to a diagnostic category, either “non-TB” or “clinical TB”. This clinical review did not include a systematic scoring of CXRs, but rather relied on the overall impression of the panel. In case of dissenting opinions, the diagnosis made by 2/3 committee members was considered the final diagnosis. For the remainder of this report, patients who were diagnosed as TB patients according to panel decision are referred to as “clinical TB” patients.

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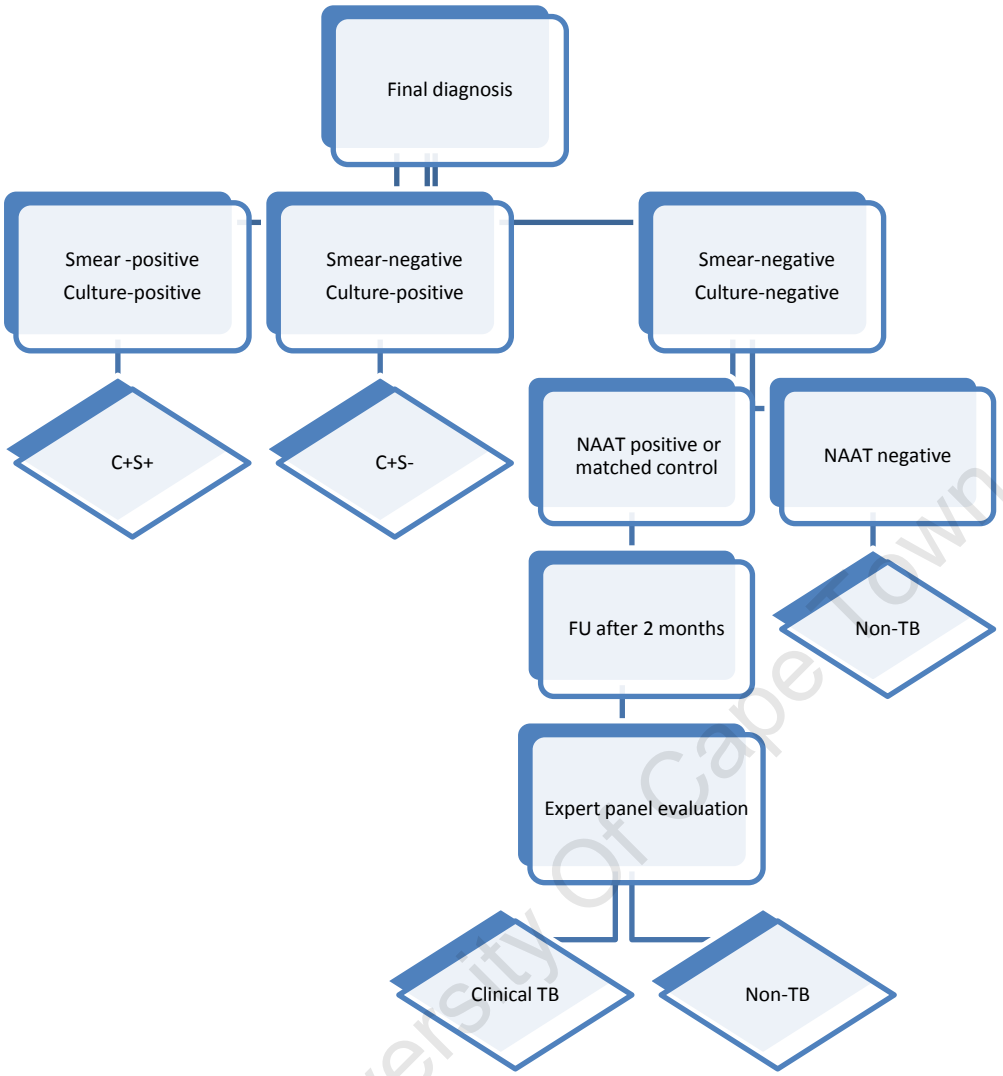

Figure 9 TB diagnostic algorithm

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1.5 Sample analysis workflow

Three samples were obtained from each patient. Each one was labelled and randomised but not before all three samples arrived in the laboratory. Thereafter, two randomly selected samples (labelled S1 and S2), were decontaminated and concentrated prior to further testing whilst the third sample was analysed as direct sputum (labelled S3).

Sample S1 was decontaminated and concentrated, analysed for smear, culture (solid and liquid), speciation, Amplicor and Xpert. If the sample was culture positive, DST was carried out. Sample S2 was also decontaminated and concentrated, analysed for smear, culture (solid and liquid), speciation, Hain and Xpert. If the sample was culture positive, DST was carried out.

Sample S3 was analysed by direct smear and direct Xpert (no sample concentration). The two decontaminated and concentrated samples are referred to as “concentrated samples” and the third sample as “direct sample” for the remainder of this report.

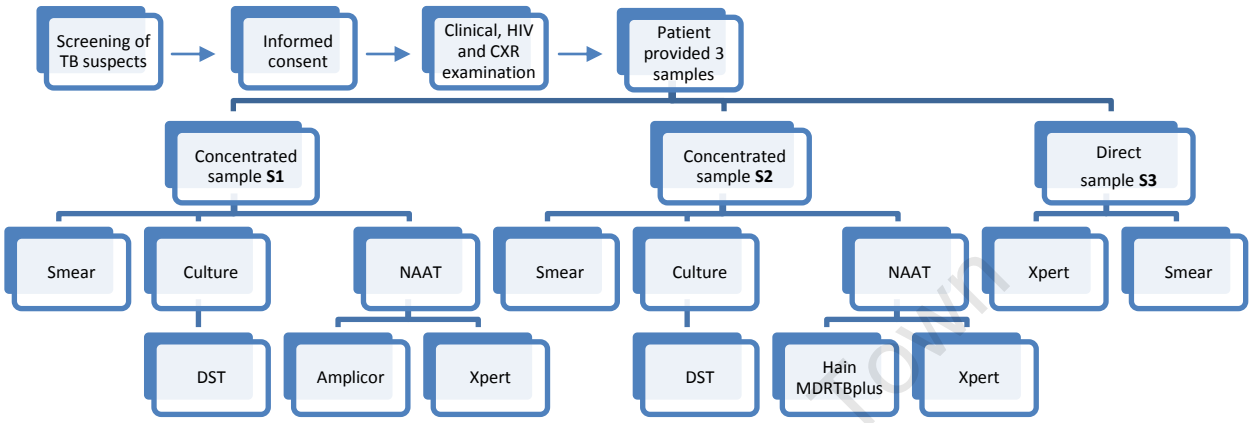

Figure 10 Sample analysis workflow

1.6 Follow-up procedure

Follow-up (FU) clinical and laboratory evaluation was performed on all smear and culture negative, but Xpert or Amplicor positive patients referred to as “cases”. FU was conducted two months after the initial visit. The same number of randomly selected smear, culture, Amplicor and Xpert negative patients were also invited for the follow-up as a comparison group, in the remainder of the thesis those patients are referred to as “control” group.

Patients were not followed up for clinical response to treatment.

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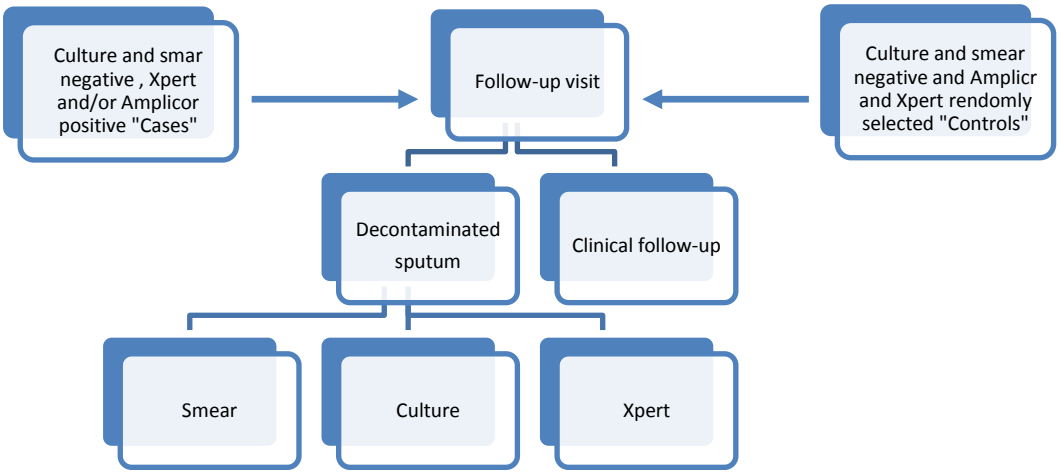

Figure 11 Follow-up workflow

At the follow-up visit the patient was re-examined and provided a fourth sputum for further laboratory analysis, to determine any remaining suspicion of TB. The fourth sample, labelled S4, was decontaminated and concentrated; and then analysed for smear, Xpert, MGIT culture and if culture positive, for DST. At the follow-up, a second CXR was taken and a general clinical examination was performed.

1.7 Ethical considerations

Patients participating in the study benefited from the currently best possible diagnostic analysis funded by study sponsors.

Patients were informed about the study by recruiting personnel and all patients signed informed consent in their own native language. Informed consent was provided in Xhosa and English. Patients were compensated for travel and time. Patients gave consent for HIV testing or consent to have their HIV status revealed to the study clinicians from their medical records.

Since this was an evaluation trial, no medical decisions were made based on the Xpert result.

The patient provided only three specimens during the baseline study and a fourth one if follow-up was required. No sample bank was created.

Only authorised medical personnel had access to patients' personal data. Samples were labelled with barcodes and the same barcodes were used throughout the workflow and connected to the patient's source document and medical files.

Formal ethical approval for the study was obtained from Research Ethics Committee, with reference number REC REF: 012/2007.

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2 HIV testing

Patients with unknown HIV status were offered to confirm their HIV status using Determine - HIV 1/2 test (Inverness Medical Innovations, Inc.). The test detects antibodies to HIV-1 and HIV-2 in infected individuals. It is based on immune-chromatographic testing of whole blood. The result is visually observed after 15 minutes, up to an hour. Positive tests were confirmed by ELISA.

If the patient had been previously tested for HIV and the result was noted in clinical records, this information was used as the HIV result in the study.

3 Sputum decontamination for culture and microscopy

Sputum was decontaminated and concentrated using the Kubica method (Kubica et al, 1963). NaOH/NALC/Citrate solution is added directly to the sample container in a 1:1 v/v ratio, making a final concentration of 1.5% NaOH. The sample was vortexed for 30s and shaken at room temperature for 20 minutes to enhance mucolytic activity. The sample was transferred to a sterile centrifuge tube and phosphate buffer, and pH 6.8 was added with a final volume of 45ml. The sample was centrifuged at 3500g at 10^0C for 15 minutes. Supernatant was discarded according to the laboratory safety regulations and the remaining pellet was resuspended with phosphate buffer to 2ml. One drop of the concentrated sample was used for smear microscopy. MGIT vials (7ml) and LJ-medium slopes were inoculated with 0.5ml and 0.2ml of the sample respectively.

Sample S1 was used for NAAT Amplicor as well as for Xpert assay. The volume of the sample required for Amplicor analysis was 100µl. The volume used for Xpert was at least 0.5ml and up to 1ml. The rest of the pellet was stored in case repetition was needed.

Sample S2 was analysed using Hain and Xpert. Both assays require a minimum 0.5ml pellet. The rest of the pellet was stored in case repetition was needed.

Work was done in a biosafety level (BSL) 3 laboratory. A biosafety cabinet class 2 was used for the entire procedure. All containers containing specimens were labelled according to the patient study ID number and sample number (1, 2 and 3 for baseline and 4 for follow-up samples). Necessary measures were taken to avoid cross-contamination of the samples including: processing one sample at a time and recapping each sample after adding buffer, using disposable Pasteur pipettes and using fresh buffer each time. Buffer is used within 24 hours after all components are combined. Buffer also loses its mucolytic activity on standing.

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4 Semi-quantitative mycobacterial smear

The presence of mycobacteria in the sample was determined using auramine staining. The sample was fixed to the glass plate and air dried. The sample area was covered with auramine phenol mixture for 15min. The slide was washed with distilled water and drained, then decolourised with 0.5% acid-alcohol for 3 minutes and rinsed with water. Counterstaining was performed using 0.5% potassium permanganate for 30s - 60s. Longer exposure to the counterstaining agent leads to fluorescence quenching. The slide was rinsed with water and then dried.

The slide was microscopically examined using a fluorescence microscope and an x40 noncover-glass objective lens. At least 100 fields were read in a sweeping patter, avoiding repeated examination of the same area.

The smear was made directly from sputum (sample 3) as well as from concentrated sputum (samples 1 and 2). In case of direct sputum, areas most likely to contain mycobacteria, i.e., necrotic areas and areas that are tinged with blood were selected form sample.

Semi-quantitative smear interpretation	
Number of AFB	**Result**
0/100 fields	negative
1-9/100 fields	number of AFB recorded
10-99/100 fields	+
1-10/1 field	++
>10/1 field	+++

Table 8 Semi-quantitative mycobacterial smear

5 MGIT culture

Commercial MGIT tubes (BD Microbiology Systems, Cockeysville, MD, USA) were used for the rapid cultivation of MTB. Each tube was enriched with 0.8ml growth supplement, containing oleic acid, albumin, dextrose, and catalase, and an antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) (Huang et al., 2001). PANTA was initially dissolved in 15ml MGIT Growth Supplement. To maintain the concentration of CO_2 in the tube, and lower the risk of contamination, each tube was opened at the time for as short a time as possible. Tubes were inoculated with 0.5ml of sample, then recapped and inverted several times to mix. Tubes and caps were wiped with mycobactericidal disinfectant. Inoculated tubes were left for 30 minutes at room temperature before putting them in the MGIT 960 instrument. Each tube was scanned and placed into the MGIT 960 drawer into the slot identified

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by the instrument. MGIT tubes were incubated until the instrument flagged them positive or negative. All MGIT tubes were incubated for six weeks before they were considered negative for MTB.

Each MGIT tube was visually inspected for mycbacterial growth on removal from the instrument. Typically MTB growth appears granular with slight turbidity and the growth settles on the bottom of the tube. Species identification for positive tubes was carried out using Tauns Capilia (see, chapter 3, section 7).

6 LJ culture

LJ egg slants were inoculated with 0.2ml of resuspended processed sputum sample. Slants were laid with medium facing up for 30 minutes to allow bacteria to adhere to the surface. LJ tubes were incubated at 37^0C for up to eight weeks and examined for growth weekly.

Culture was continuously examined until:

- colonies were large enough to count
- contamination was apparent
- no growth was visible after eight weeks

In case of visible growth, MTB was confirmed by an AFB smear. Cultures completely overgrown by bacterial or fungal contamination within three weeks were discarded and the result recorded as contaminated. If contamination occurred during three to eight weeks of incubation, or in case of mixed LJ culture, MTB colonies were recultured. Species identification for positive tubes was carried out using Auramine smear and Tauns Capilia.

Reports were reported semi-quantitatively. The number of colonies on the slope were counted and reported.

Semi-quantitative LJ culture interpretation	
Number of AFB colonies	**Result**
0	negative
0-19	number of AFB colonies recorded
20-100	+
101-200	++
201-500	+++
>500	++++

Table 9 Semi-quantitative LJ culture

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7 Species identification

Species identification was carried out on the first positive tube for each specimen, either MGIT or LJ using Tauns Capilia (Capilia TB, Tauns, Numazu, Japan) (Hillemann et al., 2005).

No sample preparation was required for AFB positive MGIT tubes. Species identification from LJ slope was carried out from a bacterial colony picked with a 1mm diameter micro loop. The colony was dissolved in 0.2ml Tauns extraction buffer in Eppendorf tube and vortexed.

The LJ colony suspension or AFB positive MGIT media was applied to the sample slot on the Capilia TB specimen placing area. The reading area was observed after 5 minutes for presence of red or deep purple bands for control and the test bands. For specimens with negative or invalid test after 5 min, the test was read again after 1 hour.

8 Drug susceptibility testing

DST was performed in MGIT tubes. Drug-containing tubes were prepared for each culture positive specimen according to the following scheme:

Drug susceptibility testing	
Result	**Procedure**
Positive, non-contaminated MGIT and positive LJ	DST performed from MGIT
Positive, non-contaminated MGIT and negative LJ	DST performed from MGIT
Negative or contaminated MGIT and positive LJ	DST performed from LJ
Positive, contaminated MGIT and negative LJ	MGIT decontaminated, if successful DST
Negative non-contaminated MGIT and negative LJ	No DST
Negative or contaminated MGIT and negative LJ	No DST

Table 10 DST inoculum source and relevant procedure

Each specimen was tested for first-line drugs: INH and RIF. Drug susceptibility was determined by comparing growth in subcultured MGIT tube with and without drug.

Growth control tubes were prepared by adding 0.8ml of four primary drugs, SM, INH, RIF EMB, a combination known as SIRE supplement, to the drug-containing tube and growth control tube. Drug-containing tubes were prepared by adding 0.1ml of the appropriate reconstituted drug solution into each of the MGIT tubes. Standard drug concentrations used for MGIT are as follows:

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Drugs used for susceptibility testing	
Drug used for analysis	**Standard Drug Concentration (µg/ml)**
STR	1.0
INH	0.1
RIF	1.0
EMB	5.0
PZA	100.0

Table 11 Drug concentration used for DST

8.1 Preparation of inoculum from MGIT Tube

The day that the MGIT tube was flagged for positive culture was considered Day 0. The tube was kept in the incubator for at least one more day or up to five days before DST. If a tube had been left in the incubator for more than five days, it was subcultured in a fresh MGIT tube until it turned positive again.

If processed tube was of Day 1 or Day 2, the tube was vortexed and left undisturbed for five to ten minutes for large clumps to settle. Undiluted supernatant was used for inoculation of the drug-containing tube and 1:100 dilution was made for growth control tube by adding 0.1ml of MGIT supernatant to 10ml sterile saline. The growth control tube and drug-containing tube were inoculated with 0.5ml of corresponding diluted and concentrated bacterial suspension.

If the processed tube was of Day 3-5, the tube was mixed well and left to settle and then diluted 1:4, by adding 1ml of broth to 4ml of sterile saline. This inoculum corresponds to undiluted inoculum of Day1-2 and from this step onward it was processed in the same way as previously described.

8.2 Preparation of inoculum from LJ media

Colonies from LJ media were used if they were no more than 15 days incubated after the first appearance of positive growth. A sterile loop was used to scrape as many colonies as possible without removing solid medium and avoiding very young colonies because among them growth rate for sensitive and resistant colonies might be different. Growth was transferred into a sterile tube containing 4ml of sterile saline and 3-4ml of 3mm glass beads. The tube was vortexed for 2-3 minutes to break large clumps. After that the tube was left to settle for 20 min. Supernatant was transferred to another sterile tube and allowed to settle for another 15 min. Supernatant was transferred once again to the new tube and at this stage turbidity should have been greater than McFarland 0.5 standard. Turbidity was adjusted to McFarland 0.5 standard by adding sterile saline. This suspension was diluted 1:5 by adding 1ml of suspension to 4ml of sterile

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saline. The growth control tube was inoculated with 0.5 ml of 1:100 dilution of suspension, making a final 1:500 diluted culture inoculum. Drug-containing tubes were inoculated with 0.5ml of original 1:5 diluted suspension.

8.3 Incubation and interpretation of results

Tubes were placed into a BACTEC instrument and incubated for 4-21 days, until the growth control reached 400 growth units (GU). At this point GU in the drug containing vial was evaluated. If the GU in the RIF tube was 100 or more the result was interpreted as resistant. If growth in the drug-containing tube was 100 GU or less than in the control tube reaching 400 GU, containing 100 times more diluted inoculum, the specimen was considered sensitive.

9 Hain MTBDR*plus* assay

Hain test was performed as additional DST. The test is based on the DNA STRIP technology and permits molecular genetic identification of the MTB complex and its resistance to RIF and/or INH from pulmonary smear positive direct patient material. Identification of rifampicin resistance is enabled by the detection of the mutations in the 81bp core region of the *rpoB* gene. More than 96% of the RIF's resistant strains have a mutation within the 81bp core region of the *rpoB* gene that encodes the β subunit of DNA-dependent RNA polymerase (Bártfai et al., 2001, Garcia et al., 2001).

For testing high level isoniazid resistance *katG* gene (coding catalase peroxidase) is examined and for testing of low level isoniazid resistance the promoter region of *inhA* gene (coding NADH enoyl ACP reductase) is examined.

Procedure is divided into three steps:

- DNA isolation
- multiplex amplification with biotinylated primers
- reverse hybridization (chemical denaturation of the amplified products, hybridization of the single-stranded, biotin labelled amplicons to membrane-bound probes, stringent washing, addition of strepavidin/alkaline phosphatase (AP) conjugate and AP mediated staining reaction

The test procedure was performed in a unilateral direction: the master mix was combined in a DNA-free area, before any sample manipulation. Sample labelled 2 was used for this test. Samples were processed at level 3 BSC and concentrated after which they were added to the

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master mix in a designated pre PCR area. The actual test was performed in a designated post PCR area.

9.1 DNA isolation

Isolation was performed in a BSL3 laboratory. The concentrated sample S2 was used for this test. The sample volume of 0.5 mL was incubated for 20 minutes at 95^0C to destroy viable MTB. The sample was then incubated for 15 minutes in an ultrasonic bath and centrifugated at maximum speed (Eppendorf 5417C, maximum speed 25000g; 16400rpm) for five minutes. Supernatant was used for the test.

9.2 Amplification

The master mix was made using Hot Star Taq Polymerase and primers (PNM) designed by Hain Lifescience. Master mix was prepared in a DNA-free cabinet. Volume was calculated according to the number of samples needed plus one.

Amplification mix	
Reagent	**Volume µL**
Buffer 10x	5
PNM	35
Mg	1
Q 5x	4
Taq	0.2
Total Volume	45.2

Table 12 Master mix preparation protocol

The PCR mixture was combined with the sample in a BSC and 5µL of processed sample was added to each PCR tube. PCR was performed on Applied Biosystems Thermal Cycler 2720 according to the amplification profile.

Amplification profile		
Time	**Temperature ^{0}C**	**Number of cycles**
15 min	95	1
30 sec	95	10
2 min	58	
25 sec	95	40
40 sec	53	
40 sec	70	
8 min	70	1
∞	4	∞

Table 13 Hain amplification profile

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The first step in amplification enables Hot Star Taq Polymerase heat activation. Since polymerase is inactivated until this step, it is possible to add polymerase to the master mix and at the last step add DNA.

Two amplification steps are introduced to ensure denaturisation and hybridisation of GC-rich DNA. The first amplification step is intended for correct primer binding and the second for actual amplification.

9.3 Hybridization and detection

Hybridization was performed with the GT Blot 48 (Hain Lifescience), which is an automated hybridization machine (Protocol available from manufacturer on request, http://www.hain-lifescience.de/en/). After hybridization and washing, strips were removed and allowed to air dry. Strips were analysed according to the manufacturer's instructions:

If conjugate control (CC) was not detected the test was repeated using PCR product. If amplification control (AC) was not detected PCR was repeated using the stored remainder of the sample. If MTB complex control (TUB) was not detected and AC and CC were detected, the sample was considered TB negative. If the strip was indicated as TB positive but either locus control zones for *rpoB, inhA* or *katG* was absent or wt and mut probe bands were less intense than AC sample, it was considered indeterminate for drug susceptibility but MTB positive.

TB positive, with at least one *rpoB* wild type band missing and/or a mutated gene present was considered RIF resistant. A TB positive result with at least one *katG* and/or *inhA* wt band missing and/or mut gene probe present was considered INH resistant.

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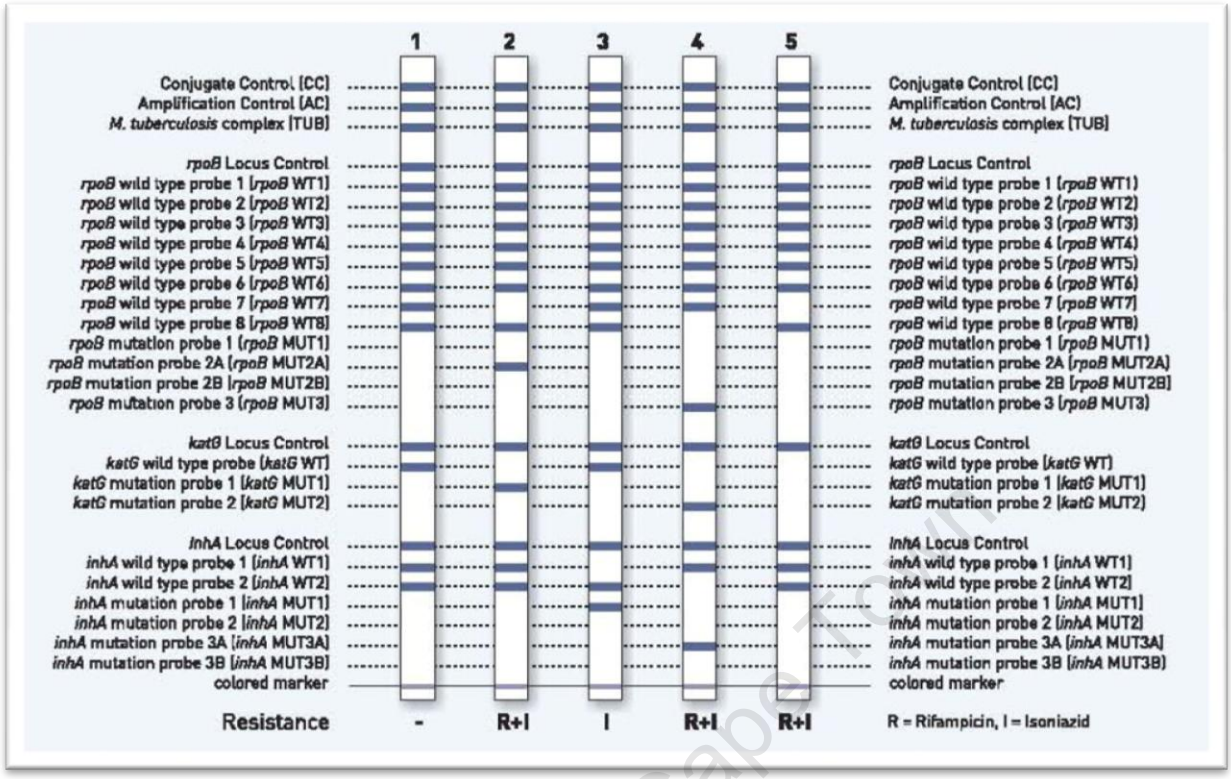

Figure 12 Hain strips reading scheme (adapted from Hain MTBDRplus procedure manual)

10 Cobas Amplicor test

Amplicor is an automated MTB test designed to detect MTB in a concentrated sample. Sample decontamination and isolation are manual steps and PCR and product detection is automated and performed on an Amplicor instrument. The sample labelled S1 was processed using Amplicor.

10.1 DNA isolation and reagent preparation

Workflow in the laboratory was performed in a unidirectional manner. Master mix reagents, designed by Roche as part of the Amplicor MTB test kit, were combined according to the manufacturer's instructions. The master mix was loaded on to PCR tubes at the beginning of the procedure. The master mix contained internal control in order to eliminate false negative results due to PCR inhibition.

The sample load per run was 20 samples plus two positive and negative controls. A concentrated sputum sample was used for the test and the volume was 0.1 mL. Each sample was washed, pelleted using centrifugation, lysed using reagent and incubated at 60^0C in a dry heat block for 45 minutes.

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Master mix (0.05ml) was added to a set of 12 tubes loaded on a ring and subsequently 0.05ml sample template was added and loaded on the instrument.

10.2 PCR and detection

PCR reagents were loaded on to the instrument before the run. Each reagent has a different expiry date after first usage, so a close monitoring of reagent usage was done as part of the routine instrument maintenance.

Results were expressed as an absolute absorbance value at 660nm and detected as positive or negative after comparison with the value of internal control. The run was considered valid if a correct result was detected for positive (absorbance >2.0) and negative control (absorbance <0.25) of the run. In the case of incorrect absorbance for either positive or negative control, the run was considered invalid and it was repeated.

If the result was negative and the internal control result was negative, the test was considered indeterminate and it was repeated if there was more than 0.1mL of concentrated sample left. If the result was positive and the internal control was either positive or negative, the sample was considered positive.

11 GeneXpert MTB/RIF

11.1 Sample preparation procedure for sputum 1 and 2 (concentrated samples)

Samples S1 and S2 were concentrated samples analysed on Xpert. No more samples than the number of available Xpert modules (up to eight) were processed at a time. Sample volume of minimum 0.5mL up to 1 mL was used for Xpert analysis, and the rest of the sample was stored at -20^{0}C. If samples were not processed on Xpert immediately after collection they were stored at 4^{0}C for maximum of two days.

Sample preparation for Xpert took place in a BSL 3 laboratory during the evaluation study, even though the proposed use of Xpert is intended as a bench-top procedure.

Sample reagent was added to the sample container to make up a final volume of 2ml, mixed vigorously and placed in the cabinet at room temperature for 15 minutes. During the incubation the sample was shaken once again. At the end of 15 minutes, the sample was considered non-infectious and it was transferred to a cartridge using a disposable Pasteur pipette. Cartridges were then loaded onto the Xpert instrument and a result was obtained after 1h30min.

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11.2 Sample preparation procedure for sputum 3 (direct sputum sample)

Sample S3 was an untreated sputum sample. Sputum volume was estimated in the leak-proof sputum collection cup by comparing with a set of sputum container samples with known volumes and sample reagent was added directly to the sputum contained in a 1:2 up to 1:4 v:v ratio. The sample was shaken vigorously and left for 15 minutes in the BSC. During incubation it was shaken once again. After incubation the sample was transferred to a cartridge and loaded onto the Xpert instrument.

11.3 Amplification

After loading the cartridge on to the instrument, all further steps were automatic and reactions were contained within a closed cartridge system. Firstly, the sample was pressed through a filter to remove any impurities or clumps. Then an ultrasonic horn was beaked down the cell and DNA released. DNA solution was then transferred between compartments within the cartridge and mixed with lyophilized constituents of the master mix. Amplification mix was then exposed to the heating/cooling plates and amplification was monitored real-time using a six-colour detector module. After the first amplification process, the amplification mix was removed from the PCR/detection micro tube and mixed with a nested set of primers. Nested PCR was then subsequently performed in the micro tube, and detected in real-time.

11.4 GeneXpert MTB/RIF results interpretation and evaluation

All Xpert tests, for which the result was “invalid”, “indeterminate”, “error” or “no result” were repeated if there was enough sample (with or without sample reagent) volume left. If result message was “invalid” or “error” with respect to MTB detection it meant that run was faulty and internal control was not detected. Borderline RIF resistance was described as result message “indeterminate” or “no result”. Results after repetition were considered final results. Results MTB negative or MTB positive, with either RIF resistance detected or RIF resistance not detected, were considered as final Xpert results. The result was as described as semi-quantitative since the MTB load was characterised as very low, low, medium and high.

12 Data analysis

12.1 Per patient and per sample analysis

Data analysis based on test results was performed on per patient and per sample.

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Per sample analysis criteria	
Resolution	**Description**
Smear positive specimen	Positive smear (scanty, 1+,2+,3+)
Exclusion (smear)	Smear positive specimen with both negative or contaminated cultures
Culture positive specimen	LJ and/or MGIT positive culture confirmed for MTB complex
Exclusion (cross-contamination)	Single positive LJ culture with <20 colonies or a single positive MGIT culture with MTB growth ≥28 days per patient
Exclusion (NTM)	Specimens with growth of mycobacteria other than MTB complex
Contaminated culture	LJ culture overgrown by fungal or bacterial contaminant or flagged positive MGIT culture without detection of AFB
Exclusion contamination	Both contaminated cultures for the same specimen
RIF resistant specimen	Determined according to MGIT phenotypic RIF analysis (see, chapter 3, section 8.3)
RIF susceptible specimen	Determined according to MGIT phenotypic RIF analysis (see, chapter 3, section 8.3)
Xpert MTB positive specimen	Any "MTB positive" result was considered positive. Any result needed repetition (see chapter 3, section 11.4) was considered as definite Xpert result after repetition
Xpert MTB RIF resistant specimen	Any "Rif resistance detected" result was considered RIF resistant
Exclusion (RIF resistance)	"Rif resistance indeterminate" result
Xpert MTB invalid	If Xpert result was "error", "invalid" or "no result", and this result remained after repetition this sample was excluded from analysis. Rate of invalid results was calculated separately.
Amplicor positive specimen	Instrument positive result
Amplicor indeterminate	MTB negative sample and negative internal control (see chapter 3 section 10.2)
Hain MTB positive specimen	TB positive result with positive AC and positive CC and any result for gene mutations
Hain MTB RIF resistant specimen	TB positive, at least one *rpoB* control missing and/or mutated gene present.
Hain MTB INH resistant specimen	TB positive, katG and/or inhA wt probe missing and/or mut gene probe present
Hain MTB TB indeterminate	No visible CC and/or AC band intensity stronger than TB probe
Hain MTB RIF/INH indeterminate	Locus control zone for *rpoB*, *inhA* or *katG* absent; wt and mut probe bands less intense than AC, reported separately

Table 14 Per sample analysis criteria

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Per patient analysis criteria	
Resolution	**Description**
Smear-positive, Culture-positive case	≥1 1+ positive smear or ≥2 scanty positive of 3 smears and ≥1 MTB positive culture of 4
Smear-negative, Culture-positive case	3 negative smears or 1 scanty positive smear and ≥1 MTB positive culture of 4
Patient exclusion on the basis of smear result	Smear positive, culture negative patients or <3 sputum specimens examined
Patient exclusion on the basis of culture result	Patient with ≥3 contaminated cultures or 2 NTM cultures
Culture negative case	Smear and culture negative case
Clinical TB positive case	Culture negative case, considered TB positive after FU by the expert panel review (see chapter 3, section 1.4)
Clinical TB negative case	Culture negative case, considered TB negative after FU by the expert panel review (see chapter 3, section 1.4)
Follow-up cases and controls	Smear negative culture negative case that is Xpert or Amplicor positive and the same number of Xpert and Amplicor negative controls
Exclusion after FU	Culture negative patient on TB treatment in the *MDR risk group* Cases and controls (see chapter 3, section 1.4) lost to follow up or passed away
RIF resistant case	MGIT RIF resistant case
RIF sensitive case	MGIT RIF sensitive case
Exclusion from RIF per patient susceptibility analysis	Discrepancy between phenotypic RIF results for samples S1 and S2
INH resistant case	MGIT INH resistant case
INH sensitive case	MGIT INH sensitive case
INH indeterminate case	Discrepant results between INH results for samples S1 and S2, were not excluded, but reported separately
Xpert MTB positive case	≥1 Xpert "TB positive" case. For S3 separate per patient analysis was carried out
Xpert MTB RIF resistant case	3 Xpert "RIF resistance detected" results. Any discrepant result between Xpert results and Xpert and culture was further analyzed by sequencing Xpert amplicon and culture isolate
Xpert MTB RIF sensitive case	≥1 Xpert "RIF resistance not detected"
Exclusion from RIF per patient susceptibility analysis	Discrepant Xpert RIF results confirmed after sequencing
Xpert MTB invalid case	3 results "invalid" or "error" or "no result" are analyzed and reported separately
Amplicor	(see chapter 3 section 10)
Hain	(see chapter 3 section 9)

Table 15 Per patient analysis criteria

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12.2 Statistical analysis

Numerical indicators of test performance were used to compare and analyse diagnostic accuracy of tests used in the study. The reference test for TB is the culture result. In this study diagnostic accuracy was analysed in respect to culture result and culture and clinical examination result.

Diagnostic test indicators		
Test indicator	**Formula**	**Description**
Sensitivity	TP/(TP + FN)	Proportion positive test results among diseased
Specificity	TN/(TN + FP)	Proportion negative test results among the disease-free
Positive predictive value (PPV)	TP/(TP + FP)	Proportion of diseased among subjects with a positive test result
Negative predictive value (NPV)	TN/(TN + FN)	Proportion of disease-free subjects among subjects with a negative test result
Positive likelihood ratio (LR+)	sensitivity/(1 - specificity)	Ratio of a positive test result among diseased to the same result among disease-free
Negative likelihood ratio (LR-)	(1 - sensitivity)/specificity	Ratio of a negative test result among diseased to the same result among disease-free
Diagnostic odds ratio (DOR)	(LR+)/(LR-)	Ratio of the odds of positivity in diseased to the odds of positivity in the disease-free

Table 16 Diagnostic test indicators

Comparison between a diagnostic test and its reference standard is represented as a 2 x 2 contingency table.

Contingency table			
		Reference test	
		Disease positive	Disease negative
Test	Test positive	TP	FP
	Test negative	FN	TN

Table 17 Contingency table; TP, FP, FN, and TN denote the number of true positives, false positives, false negatives, and true negatives, respectively

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Sensitivity and specificity give an idea of a test's potential to recognise subjects with disease or to exclude the condition of interest, respectively. Sensitivity and specificity analysis was performed using MS Office 2007 Excel with the data analysis pack.

PPV and NPV define the probability of disease in subjects with a positive test result or not having disease with a negative test, respectively. Predictive values are largely dependent on disease prevalence in the examined population. Therefore, predictive values from one study could not be compared to the predictive value result obtained in a population with a different prevalence of the disease.

LR+ is the indicator for ruling-in diagnosis. The higher the LR+ the more the test is indicative of a disease. Good diagnostic tests have LR+ > 10 and their positive result has a significant contribution to the diagnosis. LR- is the indicator for ruling-out the diagnosis. Good diagnostic tests have LR- < 0.1. The lower the LR- the more significant contribution of the test is in ruling-out disease. The confidence interval for LR+ and LR- was calculated using QuesGen Systems, Data Management for Clinical Research, Confidence interval - Likelihood Ratio.

DOR is used for comparison of diagnostic accuracies between two or more diagnostic tests. The value of a DOR ranges from zero to infinity, with higher values indicating better discriminatory test performance. A result for DOR=1 means that the test does not discriminate between patients with the disease and those without disease. Values lower than 1 point to improper test interpretation (more negative tests among the diseased) (Calculator for confidence intervals of odds ratio in an unmatched case control study).

The accuracy of each test within a specific group of subjects was compared using a confidence interval (CI) at 95%. This means that the probability is 0.95 of the result being within the confidence interval. The sensitivity, specificity, PPV and NPV confidence interval was calculated using a confidence interval for proportion calculation (Dimension Research, INC. Confidence interval for proportion calculator).

For specificity comparison between NAATs, the McNemar test was performed, in order to analyse whether there was a significant difference between two tests. The test is used for a sample size larger than 30. A null hypothesis is that two tests are equal and a two tailed result was used as final at a confidence level of 95% (Dimension Research, INC. McNemar test calculator)

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In order to analyse if there is a correlation between quantitative Xpert and smear result Fisher's exact two tailed test was used and p-value was calculated using GraphPad Prism.

Z – test is used to compare the proportions from two independent groups (early morning and spot samples) to determine if they are significantly different from one another.

13 Minimisation of bias

Patients presenting with typical symptoms of TB were screened for participating in the study. Participants meeting study criteria were included in the study.

In order to minimise measurement bias, samples were randomised. Since each patient provided three samples: two spot samples and one morning sample (generally an early morning sample is of the best quality) and since two samples underwent concentration before testing, while the other sample was tested directly, it was important to prevent bias in sample allocation.

Xpert results are generated directly by the software following real-time PCR analysis, and are not dependent on an operator's potentially subjective evaluation.

Laboratory technicians performing smear and culture analysis were blinded to the Xpert result. In addition, the Xpert instrument was in a separate laboratory from the one where the rest of the analysis took place.

The expert clinical evaluation team did not have access to the results of the three NAATs (Xpert, Amplicor and Hain). However, smear, culture and DST result was available to the research team performing NAATs.

Source documents were kept separately from Xpert result documents. The case report form did not contain Xpert results and hence could not influence patient diagnostic category assignment.

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IV Results

1 Patient characteristics

The recruitment period of the evaluation study lasted from June 2008 until February 2009. During that time 454 TB suspects were recruited. Of these, 364 met the study inclusion criteria, and provided three sputum samples. There were 90 patients excluded from the study because they failed to provide all three sputum samples, or sample volume was under 1.5ml.

Patient characteristics	
Age median, range	36, 18-80
Female gender %	113/333 (33.9%)
Patients with history of previous TB %	135/328 (41.2%)
HIV+ patients %	156/200 (78.0%)

Table 18 Patient characteristics

Patient demographics are shown in table 18. Patients recruited were mostly male in their thirties. Out of all recruited patients 41.2% had a history of previous TB and 78% were HIV positive. Distribution may be skewed toward HIV positivity, since patients with known HIV status were usually HIV positive. HIV results were only available in 60% of cases. The TB/HIV co-infection rate, determined after all results were completed, was 76%. This is similar to previous data from the same centre: the TB/HIV co-infection rate in the Khayelitsha area in 2006 was 67% (MSF report, 2007).

Final diagnostic categories after follow up	
Culture positive, smear positive cases %	96/364 (26.4%)
Culture positive, smear negative cases %	36/364 (9.9%)
Clinical TB cases, culture negative, clinically diagnosed as TB %	12/364 (3.3%)
Culture negative cases, clinically diagnosed as non-TB %	189/364 (51.9%)
Cases excluded from further analysis based on pre-stated criteria %	31/364 (8.5%)

Table 19 Final diagnostic categories

Patients were distributed among four diagnostic categories as per table 19. There were 8.5% (31/364) indeterminate cases after preliminary analysis and those were excluded from further analysis and results were not reported (Table 20). Therefore 333 patients met study analysis criteria.

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Criteria for patient exclusion from analysis	
Criteria	**Number of cases**
Lost to follow up	18
Culture negative MDR TB case on TB treatment	3
Discrepant phenotypic RIF result	2
Possible culture cross contamination	3
NTM	1
Smear positive culture negative cases	4
Total	31

Table 20 Numbers of excluded patients based on different criteria

Out of 18 patients lost to follow up, 11 patients died during the study period. Three patients in the MDR group, who had been on TB treatment at the time of recruitment, were culture negative after analysis, and those cases were excluded. Two cases had discrepant RIF susceptibility results after phenotypic analysis. Drug sensitivity was repeated and the result was the same.

After initial culture and NAAT results were obtained, patients with positive NAAT (either Xpert or Amplicor), but culture negative were invited to return for follow-up and provided a fourth sputum sample. The same number of culture- and NAAT-negative controls, selected from the central database as random controls, were invited for a follow-up visit.

Followed up cases	
All followed up patients	**27**
Cases	**12**
Initial diagnosis changed to TB after panel review	9/12 (75%)
Non-TB patients	3/12 (25%)
Controls	**15**
Initial diagnosis changed to TB after panel review	3/15 (20%)
Non-TB patients	12/15 (80%)

Table 21 Followed-up patients segregated in two groups: cases that were culture negative but Xpert and/or Amplicor positive and randomly selected culture and Xpert and Amplicor negative controls

An expert panel was assembled to examine followed-up cases and to determine whether there was a difference in CXR at enrolment and at follow-up, as described in chapter 3, section 1.4. In the case group, 75% were diagnosed with TB based on a decision of the expert panel. In the control group, 20% of initially symptomatic but culture and NAAT negative patients were diagnosed with TB after panel discussion. Nine patients in both case and control groups were lost to follow-up. For the remainder of this report, patients in both case and control categories,

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which were diagnosed as TB patients according to the panel decision, are referred to as “clinical TB patients”. There were 12 clinical TB patients; nine cases and three controls.

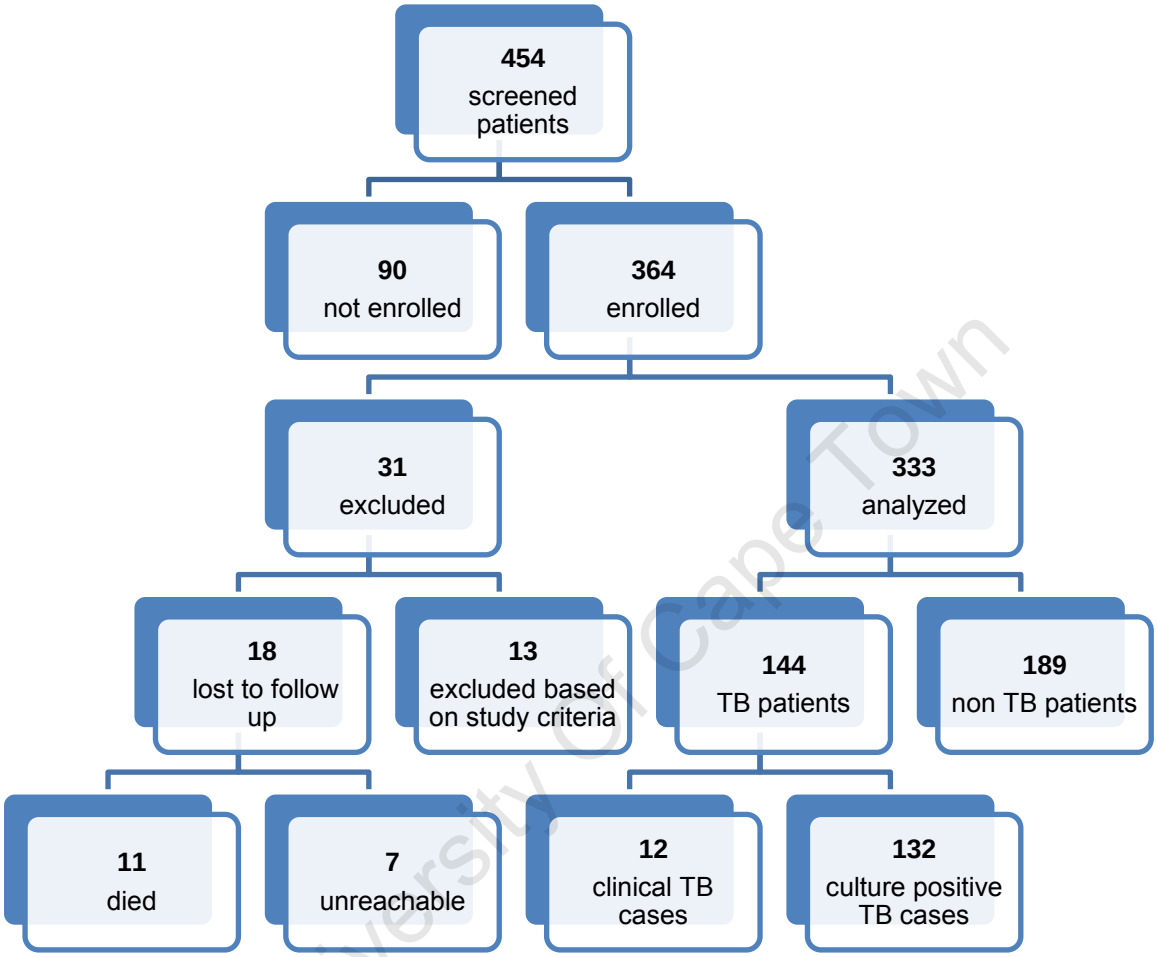

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Figure 13 Diagram of all screened patients and their outcomes

Patients were recruited at three recruitment centres: Khayelitsha Site B, Gugulethu HIV treatment centre and GF Jooste hospital.

Patient characteristics in different recruitment sites				
Category	**Recruitment sites**			
	K	**G**	**J**	**All**
No of patients	280	21	32	333
TB positive[1] %	124/280 (44.3%)	3/21 (14.3%)	17/32 (53.1%)	144/333 (43.2%)
C+S+[2] %	80/280 (28.6%)	0/21 (0.0%)	16/32 (50.0%)	96/333 (28.8%)
C+S-[3] %	33/280 (11.8%)	3/21 (14.3%)	0/32 (0.0%)	36/333 (10.8%)
Clinical TB patients[4] %	11/280 (3.9%)	0/21 (0.0%)	1/32 (3.1%)	12/333 (3.6%)
HIV-positive%	124/163 (76.1%)	19/19 (100%)	13/18 (72.2%)	156/200 (78.0%)
Previous TBx[5] %	108/275 (39.3%)	10/21 (47.6%)	17/32 (53.1%)	135/328 (41.2%)
No of MDR[6]	2	0	2	4
No of INH mono[7]	3	0	1	4

Table 22 Patient characteristics stratified by recruitment sites K (Khayelitsha Site B) G Gugulethu HIV treatment centre) and J (GF Jooste hospital), [1](TB positive cases based on culture and clinical diagnosis), [2](culture positive smear positive cases), [3](culture positive smear negative cases), [4](patients diagnosis based on clinical examination-clinical TB), [5](previous history of previous TB), [6](number of MDR cases), [7](number of INH mono-resistant cases)

Most of the patients were recruited in Khayelitsha: 84.1% (280/333) and the general study population was representative of this study site. The overall percentage of study participants with a TB diagnosis was 43.2% whilst 44.3% of study subjects recruited in Khayelitsha were diagnosed with TB

2 Isoniazid and rifampicin resistance

Patients were stratified into case detection group (61.9% of patients) and MDR risk group (38.1% of patients) as per the predefined criteria, based on their presenting history. RIF and INH resistance was based purely on phenotypic testing of two sputum samples. An MDR case was defined as resistant to RIF and INH.

Drug resistance	
RIF resistant/culture positive patients %	4/132 (3.0%)
INH resistant/culture positive patients %	8/132 (6.1%)
MDR cases/culture positive patients %	3/132 (2.3%)

Table 23 Drug resistance

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Out of all culture positive cases there were 2.3% MDR cases (3 cases) and this figure closely resembles an MSF MDR-TB survey result of 2.6% MDR cases among TB cases, conducted in March 2009 (MSF report, 2009).

3 Per patient analysis

Per patient results are summarised in diagrams below. This analysis focuses on agreement between culture, smear and NAAT results. The analysis is primarily based on a comparison between commercially available assays (Amplicor and Hain) and Xpert with emphasis on performance in culture positive - smear negative cases. Each case had one result for each Amplicor and Hain assay and three results for Xpert and was analysed according to the pre-stated criteria (see chapter 3, section 12.1).

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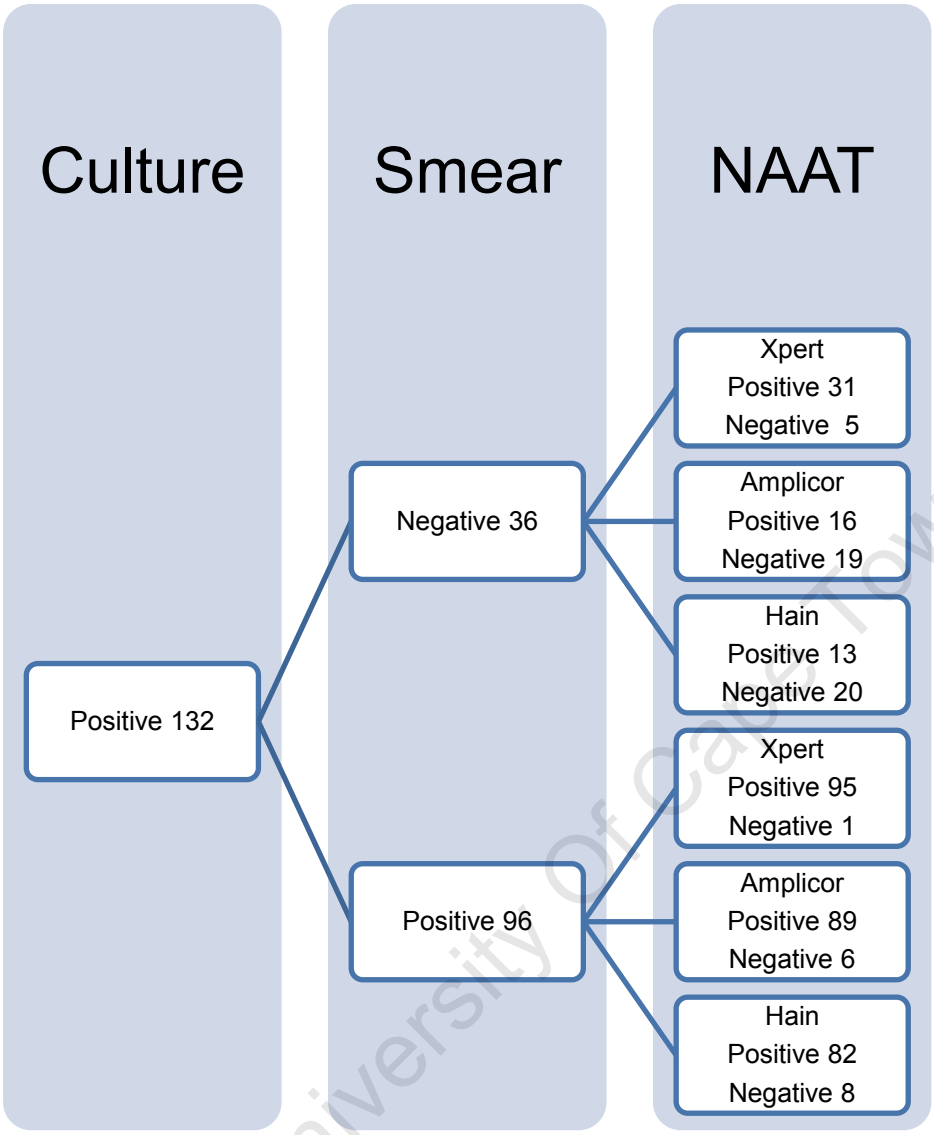

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Figure 14 Culture positive cases

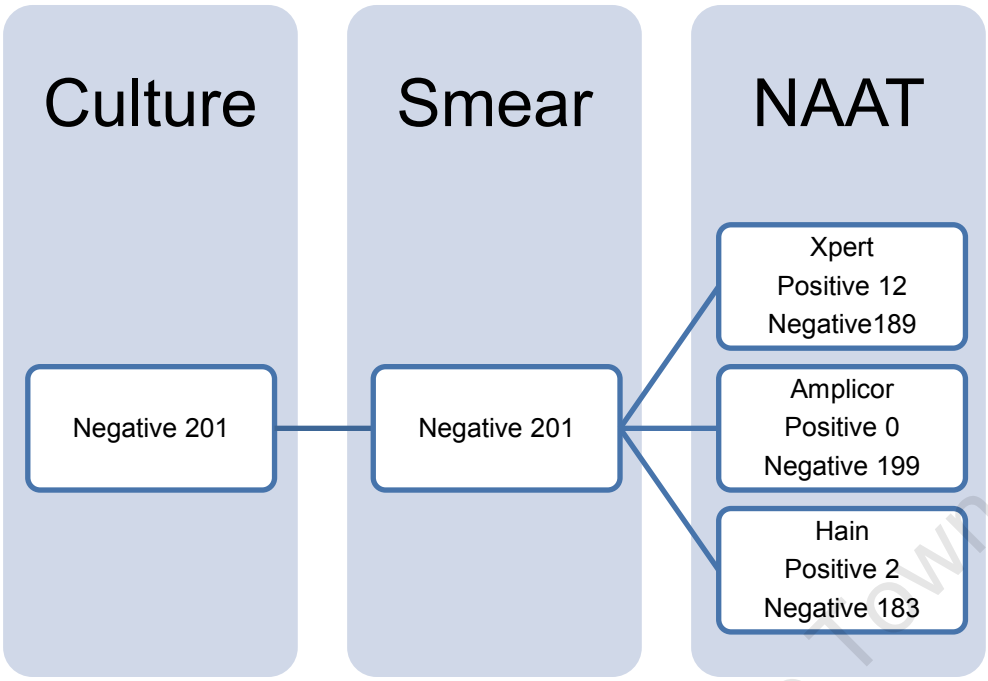

Figure 15 Culture negative cases

In the culture-positive group, TB was detected using smear in 96/132 (72.7%) cases. Xpert detected TB in 126/132 (95.5%) cases, Amplicor in 105/130 (80.8%) and Hain in 95/123 (77.2%) cases.

Per patient sensitivity analysis might favour Xpert in comparison with alternative NAATs, because Xpert was performed on three samples (two concentrated, and one direct sample), whereas alternative NAATs were performed on one concentrated sample, each. For the same reason specificity might be lower for Xpert.

Some samples did not have Amplicor and/or Hain testing performed, since sample volume was insufficient. This included 25 Hain results (six results missing in the culture positive smear positive group, three in culture positive smear negative group and 16 in culture negative group) and 4 Amplicor results (one result missing in the both culture positive smear negative and in smear positive groups and two in the culture negative group) . The sample volumes for Hain and Amplicor tests differ and volume was five times larger for Hain (0.5ml concentrated sample for Hain and 0.1 ml concentrated sample for Amplicor) so it was more likely to have a sample of insufficient volume to perform the Hain test after the sample had been used for Xpert analysis, as performing Xpert was a study priority.

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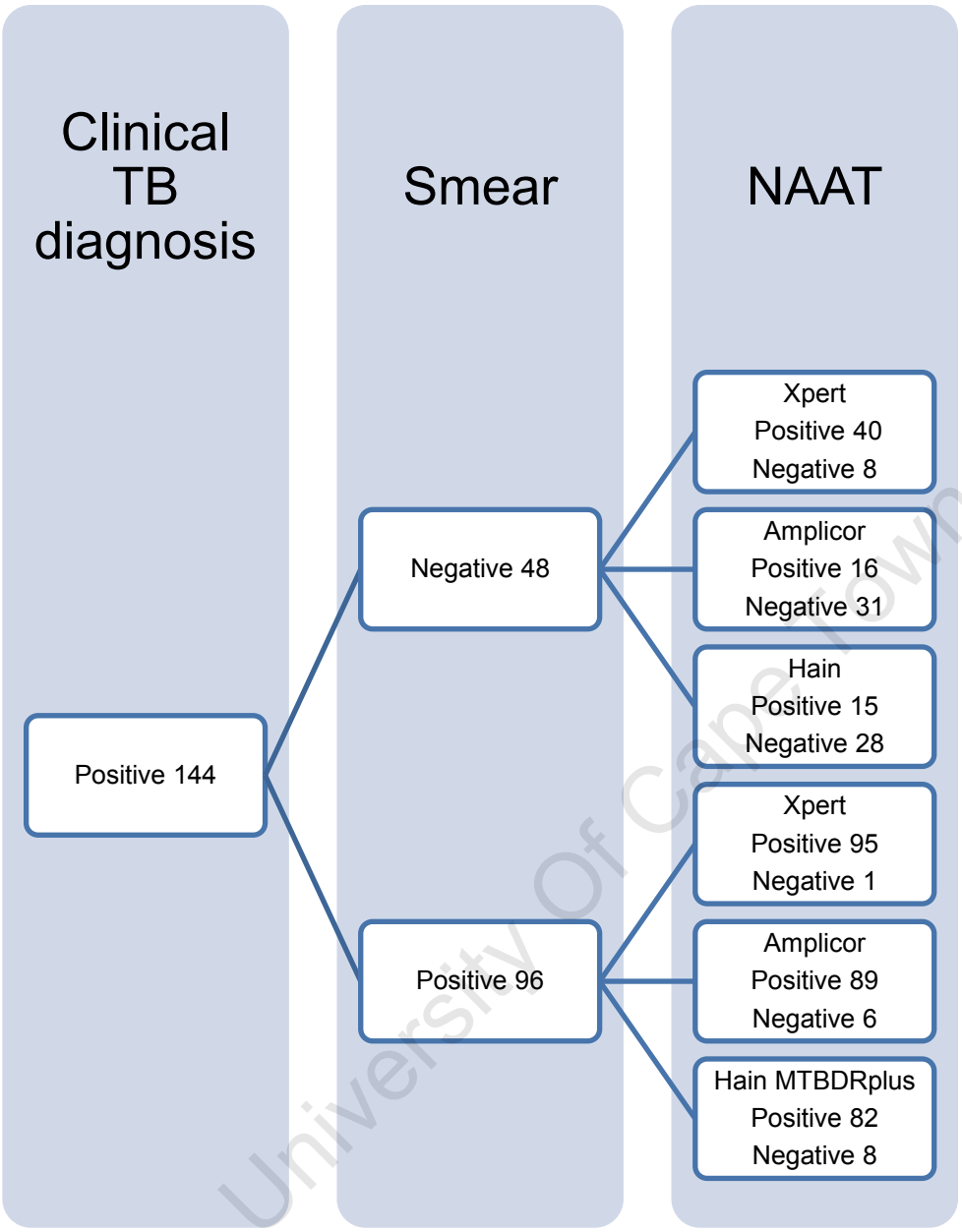

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Figure 16 Culture and CXR suggestive of TB

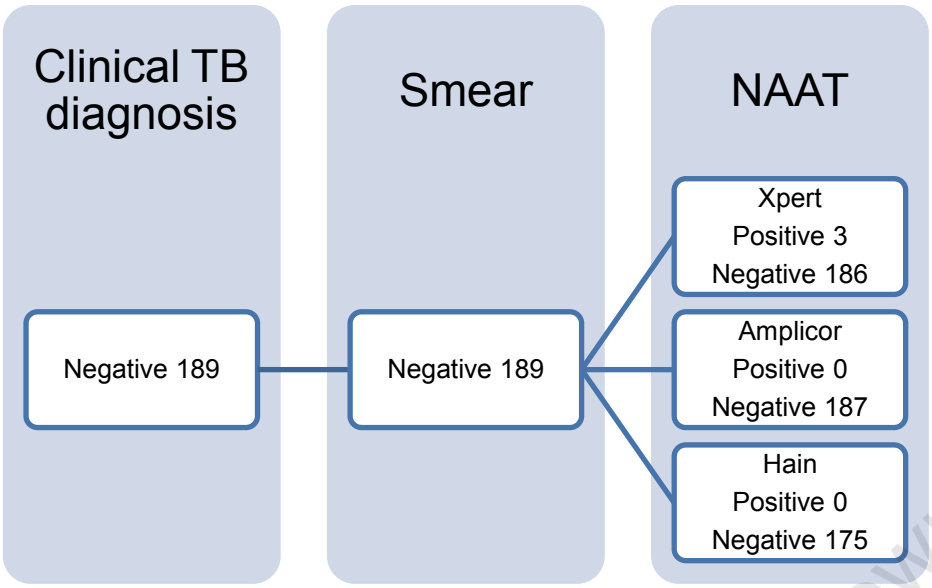

Figure 17 Diagram non-TB patients

After follow-up and expert panel evaluation of 27 cases and controls, 12 culture negative cases were diagnosed as TB patients based on panel assessment or follow-up culture. Those 12 clinical TB cases patients with 132 culture positive patients together make 144 TB patients recruited during the study. Eleven of the cases were reassigned based on panel assessment, whilst 1 of the 12 had a positive TB culture result on the 2-month follow-up sample (this patient had a positive Xpert result on an initial sample). Eight out of the 12 (67%) of clinical TB cases were HIV positive, one was HIV negative and in three cases HIV status was unknown.

Xpert detected TB in 75% (9/12) of cases in this group. Of those nine patients, all had a positive result on direct sputum (S3). None had a positive result when tested on Amplicor and two were positive for MTB complex on Hain.

3.1 Per patient analysis of performance of NAATs and smear

Performance of diagnostic tests was analysed, firstly using culture as the reference standard and then culture and expert panel assessment combined as the reference standard.

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Per patient performance of methods where culture is the reference test				
Performance	**Smear**	**Xpert**	**Amplicor**	**Hain**
Sensitivity% [CI]	72.7 [65.1-80.3]	95.5 [91.9-99.0]	80.8 [74.0-87.5]	77.2 [69.8-84.6]
Specificity% [CI]	100.0	94.0 [90.8-97.3]	100.0	98.9 [97.4-100.0]
LR+ [CI]	293.1 [18.4-4,679.5]	16.0 [9.2-27.2]	322.1 [20.2-5,138.9]	71.4 [17.9-284.5]
LR- [CI]	0.28 [0.21-0.36]	0.05 [0.02-0.11]	0.19 [0.14-0.28]	0.23 [0.17-0.32]
DOR [CI]	1,065.5 [64.7-17,544.1]	330.8 [121.0-904.1]	1,650.8 [99.5-27,387.0	310.4 [72.4-1,331.2]
PPV% [CI]	100.0	91.3 [86.8-96.0]	100.0	97.9 [95.1-100.0]
NPV% [CI]	84.8 [80.2-89.4]	96.9 [94.5-99.4]	88.8 [84.7-93.0]	86.7 [82.2-91.3]

Table 24 Performance of NAAT and smear where culture is a reference test, in case if specificity was 100%, i.e. one of the cells in the 2 x 2 contingency table contains zero, 0.5 was added to all the values in the table to calculate an approximate LR+, LR- and DOR (Haldane et al., 1955)

Sensitivity of Xpert was higher than other NAATs, resulting in a high NPV. Specificity (and hence PPV) of Xpert was lower than smear and other NAATs.

Per patient performance of methods based on culture and clinical diagnosis				
Performance	**Smear**	**Xpert**	**Amplicor**	**Hain**
Sensitivity% [CI]	66.7 [59.0-74.4]	93.8 [89.8-97.7]	73.9 [66.7-81.2]	72.9 [65.4-80.5]
Specificity% [CI]	100.0	98.4 [96.6-100]	100.0	100.0
LR+ [CI]	252.9 [15.8-4,038.2]	59.1 [19.2-181.6]	277.4 [17.4-4,426.2]	256.1 [16.1-4,086.5]
LR- [CI]	0.34 [0.27-0.42]	0.06 [0.03-0.12]	0.26 [0.20-0.35]	0.27 [0.21-0.36]
DOR	754.1 [46.0-12,362.1]	930.0 [247.1-3,500.0]	1,055 [64.1-17,357.0]	937.6 [56.9-15,445.4]
PPV% [CI]	100.0	97.8 [95.4-100.0]	100.0	100.0
NPV% [CI]	79.8 [74.6-84.9]	95.4 [92.9-100.0]	83.5 [78.6-88.3]	82.9 [77.9-88.0]

Table 25 Performance of NAAT and smear where diagnosis is based on culture result and clinical expert panel diagnosis

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Including culture negative, clinical TB patients, in the analysis reduced the sensitivity of smear, Amplicor and Hain, but increased specificity of Xpert (Table 25). Specificity and hence PPV in this analysis was 100% for all tests except Xpert.

It is of particular interest to analyse the performance of NAAT in the culture-positive, smear-negative group.

Per patient performance of methods stratified by culture and smear result			
Test	**Sensitivity all C+**	**Sensitivity C+ S+**	**Sensitivity C+ S-**
Xpert% [CI]	95.5 [91.9-99.0]	99.0 [96.9-100.0]	86.1 [74.8-97.4]
Amplicor% [CI]	80.8 [74.0-87.5]	93.7 [88.8-98.6]	45.7 [29.2-62.2]
Hain% [CI]	77.2 [69.8-84.7]	91.1 [85.2-97.0]	39.4 [22.7-56.1]

Table 26 Performance of NAAT stratified by C+ (culture positive), C+S+ (culture positive and smear positive) and C+S- (culture positive and smear negative) group

All tests showed satisfactory performance in the culture and smear positive group (Table 26), but in the smear negative group, Xpert demonstrated markedly increased sensitivity, but with a wide confidence interval since the sample size was relatively small (36 patients were diagnosed as culture-positive, smear-negative). It should also be noted that this is comparison with three Xpert results per patient as opposed to one result for Amplicor and Hain.

3.2 Performance of GeneXpert MTB/RIF on the direct sample

Xpert and direct smear results from sample S3 are shown in table 27. All Xpert-positive, smear-positive cases were culture-positive. In the Xpert-positive, smear-negative group (62 cases) there was only one non-TB case and nine clinical TB cases whilst the rest were culture-positive on samples S1 and S2. One Xpert-negative, smear-positive case was culture-positive and in the Xpert negative and smear negative group nine cases were culture-positive and three were clinical TB cases.

Direct smear analysis			
Smear result for direct smear S3	**Xpert result for direct smear S3**		
	MTB positive	MTB negative	*Error*
Positive	69	1	1
Negative	62	197	3

Table 27 Correlation between Xpert result and smear result for direct sample S3

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Using Fisher's exact two tailed test and excluding *error* as a result, association between smear and Xpert for direct sample was highly significant, with p-value < 0.0001.

Since there was no culture results for the direct sputum S3, Xpert results for direct sputum S3 were stratified in groups according to combined culture results from S1 and S2 and overall smear results from all three smear results.

Performance of Xpert on direct sputum						
Test	**Sensitivity C+**	**Sensitivity C+ S+**	**Sensitivity C+ S-**	**Specificity C-**	**Sensitivity Clin+ C+**	**Specificity Non-TB**
Xpert S3% [CI]	92.4 [87.8-96.9]	96.8 [93.3-100]	80.6 [67.7-93.5]	95.0 [91.9-98]	90.9 [86.2-95.6]	99.5 [98.4-100]

Table 28 Performance of Xpert and smear on direct sputum stratified by C+ (culture positive), C+S+ (culture positive and smear positive), C+S- (culture positive and smear negative) Clin+C+ (clinical TB and culture positive cases combined), Non-TB (clinically and culture negative).

Four Xpert results for direct sputum were not interpretable, since an *error* result was observed. A single Xpert test performed on direct sputum demonstrated high sensitivity and specificity. This is the important finding, since Xpert is intended to be used on direct sputum samples.

4 Per sample analysis

In the study 666 culture results were obtained for each solid and liquid culture method, since two samples were cultured per patient.

Culture results in the analysis				
Culture results for samples S1 and S2		**Liquid culture**		
		Positive	Negative	Contaminated
Solid culture	Positive	205	0	2
	Negative	18	400	4
	Contaminated	19	17	1

Table 29 Correlation between solid and liquid culture performance in samples S1 and S2 (sample 1 and sample 2)

Contamination rate for liquid culture was 1.1% and for solid culture 5.6% for both samples S1 and S2. Liquid culture was positive in an additional 18 cases, compared with solid culture, whilst there was no sample with a positive solid culture and negative liquid culture. A single liquid culture detected 91.7% culture-proven cases and a solid culture 78.4%.

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Next we compared the performance of Xpert on the two concentrated samples S1 and S2 with culture and smear results.

Xpert performance for concentrated samples S1 and S2		
Culture result per samples S1 and S2	**Xpert results for samples S1 and S2 combined**	
	Negative	Positive
C+S+	0	173
C+S-	18	52
C-	403	13
Sensitivity C+ % [CI]	92.6 [89.3-95.9]	
Sensitivity C+S+ % [CI]	100.0	
Sensitivity C+S- % [CI]	74.3 [64.1-84.5]	
Specificity C- % [CI]	96.9 [95.2-98.6]	

Table 30 Xpert performance for S1 and S2 combined (Xpert result for samples 1 and 2) stratified by C+ (culture positive), C+S+ (culture positive and smear positive) and C+S- (culture positive and smear negative) group

Seven concentrated samples out of 666 (1.05%) tested on Xpert were not interpretable. Sensitivity in the culture positive group is comparable to sensitivity of a single direct sample S3. Interestingly, the combined sensitivity of two Xpert tests on concentrated samples S1 and S2 (92.6%) was almost identical to that of a single Xpert on direct sputum S3 (92.4%).

Sample S1 was a concentrated sample tested for culture, smear Xpert and Amplicor. We directly compared the performance of Xpert and Amplicor in three diagnostic categories: culture and smear positive, culture-positive and smear-negative and culture-negative.

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Xpert and Amplicor per sample performance on sample S1				
Culture per sample S1	**Xpert sample S1**		**Amplicor sample S1**	
	Negative	Positive	Negative	Positive
C+S+	0	82	1	80
C+S-	11	28	16	23
C-	200	8	206	2
Sensitivity C+ % [CI]	90.9 [85.8-96.0]		85.8 [79.6-92.1]	
Sensitivity C+S+ % [CI]	100		98.8 [96.4-100]	
Sensitivity C+S- % [CI]	71.8 [57.7-85.9]		59.9 [43.5-74.4]	
Specificity % [CI]	96.2 [93.5-98.8]		99.0 [97.7-100]	

Table 31 Xpert and Amplicor performance for sample S1 stratified by C+ (culture positive), C+S+ (culture positive and smear positive) and C+S- (culture positive and smear negative))

One sample was excluded from the per sample analysis since it was smear positive and culture negative, based on study criteria (see chapter 3, section 12.1). Three Xpert results were invalid after repetition (3/332). In two cases Amplicor was not performed (2/332) and two Amplicor results were indeterminate (2/332).

Although, there was a trend towards improved sensitivity for Xpert, statistical analysis of culture positive patients showed no significant difference in sensitivity between Xpert and Amplicor at the 95% confidence level (p-value for 2 tailed test p=0.182). Specificity analysis at 95% confidence level showed that Amplicor performed better than Xpert and the difference is significant (p-value for 2 tailed test p=0.041).

Sample S2 was a concentrated sample tested with culture and smear as well as Xpert and Hain.

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Xpert and Hain per sample performance on sample S2				
Culture per sample S2	**Xpert sample S2**		**Hain Sample S2**	
	Negative	Positive	Negative	Positive
C+S+	0	91	4	80
C+S-	7	24	16	13
C-	203	5	190	3
Sensitivity C+ % [CI]	94.3 [90.1-98.4]		82.3 [75.3-89.3]	
Sensitivity S+C+ % [CI]	100		95.2 [90.7-99.8]	
Sensitivity S-C+ % [CI]	77.4 [62.7-92.1]		44.8 [26.7-62.9]	
Specificity % [CI]	97.6 [95.5-99.7]		98.5 [96.7-100]	

Table 32 Xpert and Hain performance for S2 (sample 2) stratified by C+ (culture positive), C+S+ (culture positive and smear positive) and C+S- (culture positive and smear negative) group

Two cases (2/333) were excluded, based on study criteria (see chapter 3, section 12.1). One was smear positive, culture negative and the other had both contaminated cultures. One Xpert result was invalid for sample 2 (1/331). There were 25 results for Hain (25/331) not available, since sample volume was insufficient to perform the test. Sensitivity in all culture positive samples was 94.3% for Xpert and 82.3% for Hain with a significant difference (p-value for 2 tailed test p=0.002). Specificity for Xpert was 97.6% and for Hain 98.5%. The McNemar test could not be performed to determine significance in difference in specificity, since there were no cases that were negative on both Xpert and Hain, but culture positive.

4.1 Performance of GeneXpert MTB/RIF depending on time of sample taken

Theoretically, an early morning sample may be of better quality, and should contain more MTB.

After randomization, only one early morning sample was analyzed as sample S1. Culture was not performed on sample S3, and culture results for sample S3 only refer to overall culture results for samples S1 and S2, both solid and liquid.

In order to compare percentage of positive and negative results on the Xpert and liquid culture when early morning sample or spot were analyzed only sample S2 should provide valuable information, since only in this case Xpert and liquid culture results were available in sufficient number for both early morning and smear sample (Table 33).

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Performance of Xpert and liquid culture on early morning sample and spot sample						
	X negative	**X positive**	**X NA**	**C negative**	**C positive**	**C contam**
Sample 1						
EM	1/1 (100%)	NA	NA	1/1 (100%)	NA	NA
Spot	211/332 (63.6%)	118/332 (35.3%)	5/332 (1.5%)	209/332 (63.0%)	120/332 (36.1%)	3/332 (0.9%)
Sample 2						
EM	122/186 (65.6%)	63/186 (33.9%)	1/186 (0.5%)	124/186 (66.7%)	60/186 (36.1%)	2/186 (1.1%)
Spot	89/147 (60.5%)	58/147 (39.5%)	NA	83/147 (56.5%)	62/147 (44.0%)	NA
Sample 3						
EM	81/141 (57.4%)	58/141 (41.1%)	2/141 (1.4%)	79/141 (56.0%)	62/141 (44.0%)	NA
Spot	117/192 (60.9%)	73/192 (38.0%)	2/192 (1.0%)	122/192 (63.5%)	70/192 (36.5%)	NA

Table 33 Performance of Xpert and liquid culture on early morning sample and spot sample X (Xpert), X NA (Xpert not available), C (culture), C contam (culture contaminated), EM (early morning sample), Spot (spot sample)

Z – test was used to compare the proportions from two independent groups: Xpert and liquid culture positive and negative result, to determine if they were significantly different from one another.

Z value for early morning sample at 95% confidence level for positive test results, Xpert and liquid culture were 0.22 and for negative test results were 0.11. Z value for spot sample at 95% confidence level for positive test results Xpert and liquid culture were 0.356 and for both negative test results were 0.592. There was no significant difference in any group.

The difference between early morning sample and spot sample for Xpert negative and Xpert positive results is not significant for one and two tailed test, with Z value of 0.835 and 0.938 respectively. The difference between early morning sample and spot sample is significant for one tailed Z – test for both culture negative and culture positive results with Z value of 1.794 and 1.751 and not significant for two tailed test.

5 Performance of NAAT for detection of TB in HIV-infected patients

Sensitivity and specificity of NAATs were analysed with respect to culture and smear result and stratified according to HIV status.

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NAAT performance according to HIV status								
NAATs	**C+**		**C+S+**		**C+S-**		**C-**	
	HIV +	**HIV-**	**HIV+**	**HIV-**	**HIV+**	**HIV-**	**HIV+**	**HIV-**
Xpert % [CI]	95.4 [90.3-100]	100	100	100	87.0 [73.2-100]	100 (3/3)*	98.8 [96.5-100]	96.2 [88.8-100]
Amplicor % [CI]	80.3 [70.3-90.3]	85.7 [67.4-100]	92.7 [84.7-100]	90.9 [73.9-100]	43.5 [23.2-63.7]	66.7 (2/3)*	100	100
Hain % [CI]	75.4 [64.6-86.2]	85.7 [67.4-100]	94.7 [87.6-100]	90.9 [73.9-100]	55.0 [33.2-76.8]	66.7 (2/3)*	100	100

Table 34 NAATs performance according to the HIV status and culture and smear result, C+ (culture positive), C+S+ (culture positive and smear positive), C+S- (culture positive and smear negative) and C- (culture negative) group, (*)case numbers were not high enough for CI calculation

There were 200 patients with known HIV status and 156 were HIV positive. Xpert sensitivity was significantly better among HIV-positive patients than Amplicor and Hain, particularly in the culture-positive, smear-negative group.

6 GeneXpert MTB/RIF semi-quantitative analysis

Xpert software displays results as MTB positive or negative with a semi-quantitative determinate, calculated on the basis of the cycle threshold (Ct). Ct is defined as the number of cycles required for the fluorescent signal to cross the threshold (exceed background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (the lower the Ct level the greater the amount of target nucleic acid in the sample) (see chapter 1, section 8.2.2).

Xpert semi-quantitative results are displayed as: very low, low, medium and high. Smear result is quantified according to the number of bacilli visualized on smear and assigned as: scanty, 1+, 2+ and 3+.

Semi-quantitative Xpert and smear results					
Smear results	**Xpert results for samples S1 S2 and S3**				
	Negative	Very Low	Low	Medium	High
Negative	619	71	42	13	1
Scanty	0	9	17	8	2
1+	2	9	28	37	13
2+	0	3	17	25	10
3+	0	0	1	35	29

Table 35 Semi-quantitative smear result and Xpert result

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In order to analyse the association, results for smear and Xpert were combined into two groups of response “low” and “high”. Very low and low Xpert results were grouped as “low”, while medium and high were grouped as “high”. Scanty and +1 smear result were grouped as “low” and 2+ and 3+ as “high”.

Semi-quantitative Xpert and smear contingency table		
Smear	**Xpert**	
	Low	High
Low	63	97
High	21	99

Table 36 Contingency table for smear and Xpert quantitative result

The qualitative value of Xpert and smear results were strongly associated (Fisher’s exact test $p<0.0001$), however it may be observed from the table 36 that a significant proportion of those samples with a “low” smear result had a “high” Xpert result.

7 GeneXpert MTB/RIF invalid and repeated results

If Xpert could not determine a result (internal control had high Ct or MTB result was borderline) it reported an *invalid* result. If the reaction was not completed (e.g. due to blockage within the cartridge) Xpert reported *error*. In both cases the test was repeated if there was enough sample (≥0.5ml of sample without Xpert sample reagent or 2ml of sample with sample reagent).

During the study *error* was reported 25 times. In five cases there was not enough sample to repeat the test, in two cases *error* was reported after repetition, and in 18 cases interpretable results were obtained after repetition. Most of the error messages (16/25) were reported for direct sample S3.

An *invalid* result was reported in five cases. After repetition, four samples had an interpretable result and one result out of five was not repeated.

In two cases Xpert reported RIF result as *Rif resistance indeterminate.* One result was for sample S2 and the other for S3. In both cases samples were culture positive. In one case Xpert result for S1 was TB positive RIF sensitive and for S2 MTB negative. In the other case Xpert detected RIF sensitivity for samples S1 and S3.

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8 Detection of rifampicin resistance

8.1 Per patient rifampicin resistance analysis

Out of 132 culture positive cases, all were tested for first-line drug resistance. Xpert detects RIF resistance and those results were compared with phenotypic RIF resistance results.

Per patient RIF resistance (Xpert vs. Phenotypic DST)			
RIF DST result for S1 and S2	**Xpert per patient result**		
	RIF resistant	RIF sensitive	RIF indeterminate
RIF resistant	4	0	1
RIF sensitive	0	119	2

Table 37 Xpert and DST performance for RIF resistance per patient

Sensitivity of Xpert for RIF was 100% in the phenotypically confirmed RIF resistant group. Specificity was also 100%. Six samples were phenotypically RIF sensitive but Xpert TB negative.

There were three cases with discordant Xpert drug sensitivity (these were excluded from the above analysis according to predefined criteria), and these were classified as indeterminate Xpert results for RIF resistance. One case was phenotypically RIF resistant and Xpert RIF resistant in two samples and the third sample (S1) was RIF sensitive. Two phenotypically RIF sensitive samples were both sensitive when tested on Xpert for samples S2 and S3 and resistant for sample S1.

Samples with indeterminate Xpert results were sent to New Jersey Medical School, University of Medicine and Dentistry of New Jersey, where both culture isolates and amplicons from Xpert cartridges were sequenced. Results after sequencing are presented in table 38.

Discrepant RIF Xpert and phenotypic results									
Case	**DST**	**Xpert**			**Sequencing culture S1**	**Sequencing culture S2**	**Sequencing amplicon S1**	**Sequencing amplicon S2**	**Sequencing amplicon S3**
		S1	S2	S3					
Case 1	RIF resistant	sens	res	res	mut (531 TTG) and wt	mut (531 TTG, 512 CGC)	wt	mut (512 CGC)	mut (531 TTG, 512 CGC) and wt
Case 2	RIF sensitive	res	sens	sens	mut (533 CCG) and wt	mut (533 CCG) and wt	mut (533 CCG) and wt	mut (533 CCG) and wt	mut (533 CCG) and wt
Case 3	RIF sensitive	res	sens	sens	wt	wt	wt		

Table 38 RIF resistance resolution after sequencing for three Xpert RIF indeterminate cases, sens (sensitive), res (reisistant), wt (wild type), mut (mutated gene)

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Cases 1 and 2 were resolved as mixed type infection (both wt and mut genotype found) and case 3 was proven to be RIF sensitive after sequencing (false-positive Xpert RIF resistance on sample S1).

The Hain test was also performed in those three cases. In case 1 Hain result was not interpretable for RIF resistance since bands were too pale. In case 2 Hain result was negative and in case 3 Hain was not performed, since there was not enough decontaminated sample left for analysis.

The Hain test was also compared with standard DST results. Hain was reported as indeterminate if RIF resistance was not interpretable, if control bands did not appear or they were too pale to reliably read.

Per patient RIF resistance (Hain vs. Phenotypic DST)			
RIF DST result for S1 and S2	Hain result for sample S2		
	RIF resistant	RIF sensitive	RIF indeterminate
RIF resistant	3	0	0
RIF sensitive	0	82	10

Table 39 Hain and DST performance for RIF resistance per patient

Nine results out of 132 culture positive results were not analysed on Hain since there was not enough sample for processing. Two phenotypically RIF resistant cases were negative (no TB detected) on Hain and 26 phenotypically RIF sensitive results were negative. Sensitivity and specificity for Hain RIF resistance detection are 100% if the negative cases were excluded.

In the culture-negative group Hain detected TB in two cases. One case was RIF sensitive and the other indeterminate (bands were too pale for interpretation). Both cases were clinical TB cases. The rest of culture negative cases were also Hain negative and 16 cases were not analysed since there was insufficient sample volume.

8.2 Per sample rifampicin resistance analysis

Analysis of tests for detection of RIF resistance on a per sample basis was performed for sample S1 for Xpert and for sample S2 for Xpert and Hain.

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RIF resistance per sample S1		
RIF DST result for S1	**Xpert result for S1**	
	RIF resistant	RIF sensitive
RIF resistant	3	1
RIF sensitive	2	99

Table 40 Xpert performance for RIF resistance per sample S1

One case was excluded from analysis since it was smear-positive, culture-negative. There were 210 culture negative cases for S1. Of these, Xpert was negative in 200 cases, two Xpert results were reported as error, one case was RIF resistant and seven were RIF sensitive.

Xpert sensitivity in the per sample RIF resistance analysis was 75% (3/4) and specificity was 98% with confidence interval [95.3-100]. In the culture-positive group, eight DST results were not available. Xpert result was negative in eight cases and in one case Xpert result was error.

RIF resistance was analysed per sample S2 for Xpert and Hain.

RIF resistance per sample S2				
RIF DST result for S2	**Xpert result for S2**		**Hain result for S2**	
	RIF resistant	RIF sensitive	RIF resistant	RIF sensitive
RIF resistant	5	0	3	0
RIF sensitive	0	106	0	78

Table 41 Xpert and Hain performance for RIF resistance per sample S2

Two cases were excluded from per sample analysis, one had both solid and liquid cultures contaminated and the other was smear-positive, culture-negative. Three DST results were not available, since DST was not performed on those samples, but DST was available for S1, so those results were reported in the per patient analysis.

Sensitivity and specificity for Xpert was 100%. Out of 114 RIF sensitive cases Xpert was negative in seven cases, indeterminate in one and sensitive in 106 cases.

Hain sensitivity and specificity in RIF resistance analysis for S2 was 100%. Out of five phenotypically RIF resistant cases, Hain detected RIF resistance in three cases and was negative (no TB detected) in two cases. Out of 114 phenotypically RIF sensitive cases, nine Hain results were not available, 18 were negative, nine were indeterminate and 78 were RIF sensitive.

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9 Detection of isoniazid resistance

Hain detects INH resistance as well as RIF resistance. Since the main purpose of this analysis is to determine RIF resistance and compare phenotypic and Xpert results, discrepant phenotypic INH cases were not excluded from the study, as was the case for RIF resistance. DST for INH therefore represents drug susceptibility results for two samples S1 and S2. An indeterminate Hain result means that bands for INH resistance were not interpretable but the strip was positive for TB.

INH resistance (Hain vs. Phenotypic DST)			
INH DST result for S1 and S2	**Hain result for sample S2**		
	INH resistant	INH sensitive	INH indeterminate
INH resistant	6	0	0
INH sensitive	2	78	7
INH discrepant	1	1	0

Table 42 Performance of Hain test for INH resistance

Sensitivity of the test for INH resistance was 100% and specificity was 97.5% (95% CI 94.1-100). Seven results were indeterminate since bands determining INH resistance were pale or did not show at all. Nine results were not available since sample volume necessary to perform the test was too low (less than 0.5ml of processed sputum). Two phenotypically resistant samples were TB negative on Hain. Out of 112 INH sensitive cases based on DST 25 were TB negative on Hain.

Out of 201 culture negative cases there were two positive Hain results. Both culture negative cases were clinically diagnosed TB cases based on panel expert decision after FU. One was INH indeterminate and the other was INH sensitive.

The Hain test provides information regarding type of mutation for both RIF and INH. A mutation in *katG* corresponds to high level INH resistance and mutation in *inhA* corresponds to low INH resistance.

Genes involved in INH resistance				
INH DST result for S2	**Hain result for sample S2**			
	INH R *katG*	INH R *inhA*	INH S	INH I
INH resistant	3	4	0	0
INH sensitive	0	2	75	6

Table 43 Hain INH resistance results stratified by DST culture result for S2 INH R katG *(high resistance established by* katG *mutation) INH R* inhA *(low resistance detected by* inhA *mutation), INH S (INH sensitive) as INH sensitive or INH resistant (indeterminate),*

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One case had two culture samples contaminated and was excluded from analysis, and one case was culture-negative, smear-positive and was excluded from per sample analysis.

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V Discussion

We performed an evaluation study of the performance of Xpert as a novel TB/RIF resistance detection system amongst TB suspects with a high rate of HIV co-infection. In 2007, the case notification rate for TB in the region was 1,500 per 100,000 per year, which is amongst the highest TB incidence rates in the world (WHO report, 2009). The spread of TB through the population is influenced by overcrowding, malnutrition and HIV (Jaramillo, 1999). Presumably most TB transmission occurs before the disease is diagnosed, hence a rapid diagnosis method and effective treatment would reduce the spread of the disease especially among the most vulnerable, the HIV-infected population.

In the study, patients were recruited during routine visits to the clinic or hospital. At the major recruitment site, Khayelitsha, the diagnosis of TB is largely dependent on smear microscopy, with CXR and culture only performed if smear is negative and there is a strong clinical suspicion of TB.

Culture-based methods for diagnosing TB were chosen in order to achieve the "gold standard" with two MGIT results and two LJ results per patient. The most cited commercially available NAAT methods (Amplicor and Hain) were selected in order to compare the performance of Xpert with the most frequently used competitor assays.

Performance of smear microscopy and routine culture:

Auramine smear performed remarkably well (sensitivity of 72.7% for culture-confirmed cases) in this population considering the high HIV prevalence. In field settings where smear microscopy is routinely used, performance is often considerably lower (40% - 60%). In HIV-positive patents smear sensitivity can be as low as 20% (FIND Diagnostics for tuberculosis, 2006). The high sensitivity of smear microscopy during this study may be related to laboratory resources, as testing was performed in a well equipped academic TB laboratory, with highly experienced staff performing the auramine test. In addition two out of three samples were decontaminated and concentrated; since concentration improves the sensitivity of smear microscopy, this may explain the high sensitivity of this method.

The per sample analysis of solid and liquid culture in comparison with pooled culture results gives some indication of the relative performance of solid vs. liquid culture. The sensitivity of a single liquid culture was 91.7% and of solid culture 78.4% for culture-proven disease. Surprisingly, the contamination rate for liquid culture was low (1.1%). The contamination rate for

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solid culture was higher, 5.6%. Culture contamination rates are usually higher for liquid culture than for solid, typically being around 8% and 2% respectively (Paramasivan et al, 2010). The low rate of contamination of liquid cultures in this study may be the result of over-vigorous decontamination of samples prior to MGIT inoculation. The overall contamination rate of MGIT cultures in this laboratory is 8% - it is unclear why this was substantially different in this study. False-negative cultures, as a result of over-decontamination, may have resulted in a reduced specificity of NAAT tests.

Performance of NAAT testing:

The performance of Xpert was impressive, with the highest sensitivity (95.5%) compared with Amplicor (80.8%) and Hain (77.2%) amongst culture positive patients. In particular, the performance of Xpert in the smear-negative, culture-positive patient group (sensitivity 86.1%) was substantially better than that of Amplicor (sensitivity 45.7%) and Hain (sensitivity 39.4%). In HIV-infected patients with culture-positive, smear-negative TB the sensitivity of Xpert was 87%, Amplicor 43.5% and Hain 55%.

One needs to bear in mind that this comparison is between 3 Xpert tests and one Amplicor or Hain test. Data from previous studies showed a strong correlation between test performance and the number of tested specimens for each patient (Piersimoni et al., 2003, Cohen et al., 1998, Rajalahti et al., 1998). In order to control for this, we performed a per sample analysis, which demonstrated increased sensitivity for Xpert (90.9%) compared with Amplicor (85.8%), but the difference was not significant at the 95% confidence level (p-value for 2 tailed test p=0.182). Similarly, in the per sample analysis, Xpert was more sensitive than Hain (94.3% vs. 82.3% respectively; p-value for 2 tailed test p=0.002).

In contrast, Xpert appeared to have the lowest specificity (94.0%) in comparison with smear (100%), Amplicor (100%) and Hain (98.9%) in per-patient analysis. One possible reason for relatively reduced specificity of Xpert is that there were three Xpert results for each patient and only one for Hain and Amplicor. If, however, the relatively enhanced specificity of Amplicor was related to sample number, one would expect this difference to disappear on the per sample analysis. This was not the case, with specificity of Amplicor (99%) remaining higher than that of Xpert (96.2%) on per sample analysis and this was significantly different at the 95% confidence level (p-value for 2 tailed test p=0.041).

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An important issue is whether the Xpert-positive, culture-negative patients had false positive Xpert results or false negative culture results. When culture and smear negative but clinically diagnosed TB patients were included in the analysis, the sensitivity and NPV of all methods was reduced but Xpert performance was less affected (sensitivity reduced from 95.5% to 93.8% and NPV from 96.9% to 95.4%) when compared with Amplicor (sensitivity reduced from 80.8% to 73.9% and NPV from 88.8% to 83.5%) and Hain (sensitivity reduced from 77.2% to 72.9% and NPV from 86.7% to 82.9%). In contrast, specificity and hence the PPV value for Xpert improved noticeably (specificity from 94.0% to 98.4% and PPV from 91.3% to 97.8%). The implication of these results is that, in a high burden setting, such as South Africa, a positive Xpert test is highly likely to indicate true TB, whilst a negative test will rule out TB with 95% certainty.

Since Xpert is intended to be used for unprocessed samples, the most relevant finding is the performance of Xpert on direct sputum S3. When the performance of a single Xpert test on direct sputum, was analysed, a single Xpert test was almost as sensitive (92.4%) as three Xpert tests (95.5%), or two Xpert tests on concentrated sputum (92.6%). Specificity in the non-TB group (culture and clinically TB negative patient group) for Xpert on direct sputum was 99.5%. These results lead to the conclusion that concentrating sample prior to Xpert does not influence performance of the test. The implication is that Xpert can be performed directly on sputum at a microscopy level laboratory, without necessity for the BSL3 facilities required for sputum concentration. Further, a single Xpert test performed on direct sputum is highly sensitive.

There was an association between the semi-quantitative Xpert result and the degree of smear positivity, suggesting that Xpert may be useful for monitoring response to therapy, although this needs further study.

Performance of NAAT for detection of drug resistance:

The proportion of MDR cases amongst TB suspects in this area was previously documented to be 2.6% (MSF report, 2009). We identified MDR TB in 2.3% of study participants, in line with this previous report.

The sensitivity and specificity of Xpert for detection of RIF resistance on per patient analysis was 100% in the phenotypically confirmed RIF resistant group. Three samples were Xpert indeterminate due to discordant Xpert results (and hence excluded from the primary analysis). After sequencing, two of these were resolved as mixed infections (RIF resistant and sensitive)

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for both culture and Xpert amplicons, whilst one showed a single false-resistant RIF result on Xpert testing. Sensitivity and specificity for Hain RIF resistance were both 100%.

Due to the small number of RIF resistant cases in this study, the conclusions that may be drawn from this data are limited.

Sensitivity of the Hain test for INH resistance was 100% and specificity was 97.5% in the per patient analysis and 100% and 97.4% in the per sample analysis. Again, interpretation is limited by small numbers of INH resistant cases.

Logistic considerations and ease of use

Amplicor

The Amplicor test has an automated PCR-detection step, and the result is not operator dependent, but it takes considerable logistical organisation in order to achieve cost effectiveness in reagent usage.

The sample used for the Amplicor test must be decontaminated and concentrated. Sample preparation for PCR must be performed in a BSL 3 laboratory. Sample preparation involves centrifugation in order to obtain a pellet following the sample washing step. This is usually an undesirable step, even in a BSL 3 laboratory, due to safety concerns over the process. Besides safety, removing supernatant from a pelleted sample requires a lot of skill, since if the pellet is aspirated, MTB DNA is lost and the sample could be false negative, so removing supernatant is a crucial step in the Amplicor test. The pellet is very light and feathery, often not even visible.

Reagents required for sample preparation are provided by manufacturer, Roche, and should be kept at 4-8^{0}C. The sample can be prepared a day in advance, but positive and negative control must be prepared just prior to the test, and do not require BSL 3 facilities.

For each run on the Amplicor instrument, 12 different reagents are used. Some of them are kept at room temperature, some at 4^0 - 8^{0}C. Each of them has a different expiration time (16h, 2 weeks, 28 days or 30 days) and their volumes differ, so each reagent vial is used for a different number of samples (32, 75, 100 samples). Also if the test is not run at full capacity, reagents use is inefficient.

Each run takes 6.5h - 7.5h depending on the number of samples, with the maximum number of samples being 20, plus positive and negative control. The test could not be performed

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overnight, since the operator should immediately after the run return unused reagents to the storage temperature.

The instrument is not particularly robust and requires considerable space. It also requires weekly maintenance. We encountered instrument malfunction on two occasions, and eventually had to replace the instrument.

Hain

The Hain test was most time consuming, among NAATs. DNA isolation takes 1h, but samples can be prepared in batches. Making batches of DNA samples also enables the researcher to more easily follow a uni-directional flow of pre-PCR, especially since DNA isolation requires BSL 3 facilities, separated from a DNA-free area, which logistically makes pre-PCR sample manipulation more complicated.

PCR is the crucial step for the Hain test. The sample decontamination and DNA isolation procedure can influence test outcomes; the concentration of Mg^{2+} in the master mix and PCR amplification profile may make a significant difference to the test's sensitivity.

Hain Lifescience GmbH provides reagents necessary for post-PCR analysis of product, but each DNA isolation and PCR is less well standardised, especially with regard to reagents use and amplification profile. The major published performance evaluation of this test was carried out using HotStarTag polymerase from Qiagen. We elected to use the same polymerase in our study.

Hain Lifescience GmbH has two devices for post-PCR analysis of product, which must be used in order to obtain valid results for clinical sample analysis:

- TwinCubator® for Hybridization
- GT-Blot 48 for Hybridization

In our study we used both instruments. The price difference between those instruments is considerable in favour of TwinCubator. TwinCubator is a semi-automatic device, essentially an over-expensive dry heating block, enabling proper temperature and mixing of products through the hybridization step. Hybridization steps require consecutive manual washings and the adding of reagents, making the procedure prone to cross-contamination, and offering very little hands-off time, since each washing step is between 15 and 30 minutes apart, which actually oblige the operator to stay next to the instrument, preparing for the next hybridization step.

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GT-Blot 48 is fully automatic, but only after samples, detection strips and all reagents are loaded onto the instrument. Automatic washings and reagent aspiration and addition, make this method more favourable considering the threat of cross-contamination. This instrument also gives the operator 2.5h hands-off time. But the price and maintenance are considerably higher.

During the study there were no maintenance problems with either of the instruments, but GT-Blot 48 can have considerable drawbacks: the hybridization buffer is added after being pre-warmed at 37^0 - 45^0C, and this makes salt crystals dissolve. The buffer is placed in a container and a silicon tube is placed into the container, leading to a mobile arm with a syringe. If the buffer temperature is not optimal, or the tube is cold due to the environmental temperature, buffer salts can clog the tube or the syringe, and those parts need to be replaced. Since the manufacturer is based in Germany, it takes a few weeks until the instrument is operational again, leading to a huge backlog in sample flow.

It takes an experienced and accustomed operator to read results. Resulting hybridization strips have a wide range of intensities, and result reading and interpretation of results is solely operator dependent. Resulting strips fade in time, and they should be kept in a dark place if subsequent reading is needed. Strips are also very frail, and should be handled and stored accordingly. Storage practically means that they should be glued onto a paper log.

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Xpert

Xpert was the easiest to use and time efficient. It required 30-45 minutes of sample preparation with incubation, depending on the number of samples. When used directly on sputum (not on concentrated sediment), the sample preparation step would only involve reagent addition to the direct sample and 15 minutes of incubation, before transferring the sample to the disposable cartridge. During the study, we followed safety procedures, described by the IIDMM, medical microbiology, BSL 3 safety manual, which suggests that all samples containing airborne pathogens must be processed in a BSL 3 laboratory, which made sample preparation more demanding in terms of time consumption and disposing of infectious material.

Two Xpert instruments with four modules were placed in a molecular laboratory where routine laboratory work involves DNA and RNA manipulation.

There were no major difficulties while operating the Xpert instrument. Software is easily accessible and manageable. Xpert is relatively robust and extraordinarily simple. Training of an operator usually takes an hour to get familiar with system.

Problems with cartridge loading were encountered after an electrical current fluctuation, even though the instrument was connected to an uninterrupted power supply. We had to replace three modules during the study. The modules in question gave more error messages than the others. On one occasion a cartridge was unable to unload from the instrument. Module changing did not interrupt the study flow, since all modules function individually, and even if one was not working, others were available and fully functioning.

Module replacement was a very simple procedure, involving opening the instrument box and connecting a new module.

Since each module within the Xpert instrument operates independently, user can test each sputum sample as it arrives in the laboratory instead of saving samples for batch processing. This important feature can potentially result in dramatically reduced turnaround times.

Cost implications

A direct comparison of Xpert with Amplicor and Hain was not possible, since pricing structures for the Xpert test have not yet been released. A preferential pricing agreement is in place for Xpert; however the details of this arrangement are not yet available.

Limitations of this study

Cases invited for follow-up visits were Xpert and/or Amplicor positive and culture negative, with the same number of Xpert and/or Amplicor negative, culture negative controls. In this way, Xpert specificity is likely to have been artificially increased, since this gave the opportunity for Xpert-positive, culture-negative cases to be reviewed and recategorized.

The drop-out rate during the study, based on the patient's inability to provide three sputum samples, was 20% (90/454). One of the reasons for this drop-out rate is that routine workup in South Africa, according to WHO recommendations, requires two samples for TB testing and this was therefore a change to routine clinic practice.

The HIV result was not available in 45% (164/364) of case study participants, and therefore many HIV infected participants are likely to have been missed.

A very small number of RIF resistant cases was identified during the study, as a result we were unable to draw conclusions regarding the performance of Xpert for detection of RIF resistance.

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Similarly, the number of culture positive, smear negative cases was relatively low, and this resulted in wide confidence intervals around the point estimates of performance for this patient group.

Future use of Xpert

An ongoing study is aiming to compare turnaround time for Xpert, smear, culture and DST result and analyze the cost effectiveness of Xpert, implemented at microscopy centre level.

Moreover since the Xpert instrument is a closed system there is a possibility of implementing it at the level of community clinic where tuberculosis is detected, registered, treated, and followed up. In this way, there is no need for patient to come back for the result, since result could be readily obtained while patient is still at the clinic.

Whilst this study did not include children and was not specifically designed to address performance in HIV-infected patients, since Xpert showed satisfactory sensitivity in smear negative samples, it is likely to be useful for diagnosis in these patient populations. Two studies including these specific groups are ongoing, and results should be available in the near future.

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VI Conclusions

1. The key features of this evaluation of Xpert are a high degree of accuracy for diagnosis of TB, rapid time to result and ease of use. A single Xpert test on direct sputum detected 90.9% of culture- and clinically-proven TB cases with very high specificity (99.5%).
2. Xpert is an innovative system that is suitable for testing for both TB diagnosis and drug resistance detection at levels of the health system where many seek care.
3. Xpert has potential value as a replacement for smear microscopy, since it requires very basic laboratory infrastructure and was significantly more sensitive than smear microscopy.
4. Since Xpert appears to detect RIF resistance accurately, whilst simultaneously detecting the presence of TB, it may be a cost-effective and feasible alternative to smear microscopy followed by Hain testing of all smear-positive samples, which is the proposed standard algorithm for South Africa, and recommended by the WHO
5. Cost is likely to be the critical factor determining the widespread uptake of this test. A preferential pricing agreement for high burden countries has been negotiated; however the details of this remain unclear.

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References

Abu-Amero KK, Halablab MA, **2004**. Evaluation of the COBAS AMPLICOR MTB test for the detection of Mycobacterium tuberculosis complex. East Mediterr Health J. 10: 329-335.

Abu-Raddad LJ, Sabatelli L, Achterberg JT, Sugimoto JD, Longini IM Jr, Dye C, Halloran ME, **2009**. Epidemiological benefits of more-effective tuberculosis vaccines, drugs, and diagnostics. Proc Natl Acad Sci U S A.106: 13980-13985.

Akane A, Matsubara K, Nakamura H, Takahashi S, Kimura K, **1994**. Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci. 39: 362-372.

Alfaresi MS, Abdulsalam AI, Elkoush AA, **2006**. Comparison of the automated Cobas Amplicor Mycobacterium tuberculosis assay with the conventional methods for direct detection of Mycobacterium tuberculosis complex in respiratory and extrapulmonary specimens. Saudi Med J. 27: 1346-1351.

Ali A, Hasan Z, Tanveer M, Siddiqui AR, Ghebremichael S, Kallenius G, Hasan R, **2007**. Characterization of Mycobacterium tuberculosis Central Asian Strain 1 using mycobacterial interspersed repetitive unit genotyping.
BMC Microbiol. 7:76.

Almeda J, García A, González J, Quintó L, Ventura PJ, Vidal R, Rufí G, Martínez JA, Jiménez de Anta MT, Trilla A, Alonso PL, **2000**. Clinical evaluation of an in-house IS6110 polymerase chain reaction for diagnosis of tuberculosis. Eur J Clin Microbiol Infect Dis.19: 859-867.

Arora J, Singh UB, Suresh N, Rana T, Porwal C, Kaushik A, Pande JN, **2009**. Characterization of predominant Mycobacterium tuberculosis strains from different subpopulations of India. Infect Genet Evol. 9: 832-839.

Arya M, Shergill IS, Williamson M, Gommersall L, Arya N, Patel HR, **2005**. Basic principles of real-time quantitative PCR. Expert Rev Mol Diagn. 5: 209-219.

Ausina V, Gamboa F, Gazapo E, Manterola JM, Lonca J, Matas L, Manzano JR, Rodrigo C, Cardona PJ, Padilla E.J, **1997**. Evaluation of the semiautomated Abbott LCx Mycobacterium tuberculosis assay for direct detection of Mycobacterium tuberculosis in respiratory specimens. Clin Microbiol. 35: 1996-2002.

Bannister BA, Begg NT, Gillespie SH, **1996**. Infectious Disease. Blackwell Science

Barksdale L, Kim KS, **1977**. Mycobacterium. Bacteriol Rev. 41: 217-372

Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME, **2008**. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am J Respir Crit Care Med. 177: 787-792.

Baron EJ Clin Infect Dis. **2006**. Implications of new technology for infectious diseases practice. 43: 1318-23.

Beggs CB, Noakes CJ, Sleigh PA, Fletcher LA, Siddiqi K, **2003**. The transmission of tuberculosis in confined spaces: an analytical review of alternative epidemiological models. Int J

University Of Cape Town

Tuberc Lung Dis. 7: 1015-1026.

Behr MA, Warren SA, Salamon H, Hopewell PC, Ponce de Leon A, Daley CL, Small PM, **1999**. Transmission of Mycobacterium tuberculosis from patients smear-negative for acid-fast bacilli. Lancet. 353: 444-449.

Bergmann JS, Yuoh G, Fish G, Woods GL, J Clin Microbiol. **1999**. Clinical evaluation of the enhanced Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test for rapid diagnosis of tuberculosis in prison inmates. 37: 1419-1425.

Bergmann JS, Keating WE, Woods GL, **2000**. Clinical evaluation of the BDProbeTec ET system for rapid detection of Mycobacterium tuberculosis. J Clin Microbiol. 38: 863-865.

Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN, **2002**. Global dissemination of the Mycobacterium tuberculosis W-Beijing family strains.Trends Microbiol. 10: 45-52.

Biketov S, Mukamolova GV, Potapov V, Gilenkov E, Vostroknutova G, Kell DB, Young M, Kaprelyants AS, **2000**. Culturability of Mycobacterium tuberculosis cells isolated from murine macrophages: a bacterial growth factor promotes recovery. FEMS Immunol Med Microbiol. 29: 233-240.

Boehme C, Molokova E, Minja F, Geis S, Loscher T, Maboko L, Koulchin V, Hoelscher M, **2005**. Detection of mycobacterial lipoarabinomannan with an antigen-capture ELISA in unprocessed urine of Tanzanian patients with suspected tuberculosis. Trans R Soc Trop Med Hyg. 99: 893-900.

Boehme CC, Nabeta P, Henostroza G, Raqib R, Rahim Z, Gerhardt M, Sanga E, Hoelscher M, Notomi T, Hase T, Perkins MD, **2007**. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. J Clin Microbiol. 45: 1936-1940.

Borgdorff MW, Small PM, **2009**. Scratching the surface of ignorance on MDR tuberculosis. Lancet. 373:1822-1824.

Boshoff HI, Barry CE 3rd, **2005**. Tuberculosis - metabolism and respiration in the absence of growth. Nat Rev Microbiol. 3: 70-80.

Broccolo F, Scarpellini P, Locatelli G, Zingale A, Brambilla AM, Cichero P, Sechi LA, Lazzarin A, Lusso P, Malnati MS, **2003**. Rapid diagnosis of mycobacterial infections and quantitation of Mycobacterium tuberculosis load by two real-time calibrated PCR assays. J Clin Microbiol. 41: 4565-4572.

Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parsons LM, Pym AS, Samper S, van Soolingen D, Cole ST, **2002**. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl Acad Sci U S A. 99: 3684-3689.

Cambau E, Wichlacz C, Truffot-Pernot C, Jarlier V, **1999**. Evaluation of the new MB redox system for detection of growth of mycobacteria. J Clin Microbiol. 1999 37: 2013-2015.

Caws M, Thwaites G, Dunstan S, Hawn TR, Lan NT, Thuong NT, Stepniewska K, Huyen MN, Bang ND, Loc TH, Gagneux S, van Soolingen D, Kremer K, van der Sande M, Small P, Anh PT, Chinh NT, Quy HT, Duyen NT, Tho DQ, Hieu NT, Torok E, Hien TT, Dung NH, Nhu NT, Duy

University of Cape Town

PM, van Vinh Chau N, Farrar J, **2008**. The influence of host and bacterial genotype on the development of disseminated disease with Mycobacterium tuberculosis. PLoS Pathog. 4: e1000034

CDC, 2005. Guidelines for the Investigation of Contacts of Persons with Infectious Tuberculosis. www.cdc.gov/mmwr/preview/mmwrhtml/rr5415a1.htm [Accessed 17.03.2010]

CDC, 2000. Notice to Readers: Update: Nucleic Acid Amplification Tests for Tuberculosis. www.cdc.gov/mmwr/preview/mmwrhtml/mm4926a3.htm [Accessed 10.03.2010]

CDC, Updated Guidelines for the Use of Nucleic Acid Amplification Tests in the Diagnosis of Tuberculosis, 2009 www.cdc.gov/mmwr/preview/mmwrhtml/mm5801a3.htm [Accessed 10.03.2010]

CDC, 1996. Notices to Readers Nucleic Acid Amplification Tests for Tuberculosis. www.cdc.gov/mmwr/preview/mmwrhtml/00044313.htm [Accessed 10.03.2010]

CDC, TB Treatment of Drug-Susceptible Tuberculosis Disease in HIV-infected persons www.cdc.gov/tb/publications/factsheets/treatment/treatmentHIVpositive.pdf [Accessed 05.02. 2010]

CDC, TB Treatment of Drug-Susceptible Tuberculosis Disease in Persons Not Infected with HIV www.cdc.gov/tb/publications/factsheets/treatment/ treatmentHIVnegative.pdf [Accessed 05.02. 2010]

Charles M, Pape JW, **2006**. Tuberculosis and HIV: Implications in the developing world. Curr HIV/AIDS Rep. 3: 139-144.

Chatterjee D, **1997**. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. Curr Opin Chem Biol. 1: 579-588

Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, Mosteller F. **1994**. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA. 271: 698-702.

Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG, **1998**. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. Nature. 393: 537-544

Cole ST, Eisenach KD, McMurray DN, Jacobs WR Jr, **2005**. Tuberculosis and the Tubercle Bacillus. ASM Press

Coleman WB, Tsongalis GJ, **2006** Molecular Diagnostics: For the Clinical Laboratorian.. 2nd edition, Humana press

Cooper AM, Solache A, Khader SA, **2007**. Interleukin-12 and tuberculosis: an old story revisited. Curr Opin Immunol. 19: 441-447

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

University Of Cape Town

Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, Dye C, **2003**. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. 163: 1009-1021.

Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, Mengoli C, **2004**. Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria. J Clin Microbiol. 2004 42: 2321-2325.

Cruz AT, Starke JR, **2007**. Clinical manifestations of tuberculosis in children. Paediatr Respir Rev. 8: 107-117.

Dalovisio JR, Montenegro-James S, Kemmerly SA, Genre CF, Chambers R, Greer D, Pankey GA, Failla DM, Haydel KG, Hutchinson L, Lindley MF, Nunez BM, Praba A, Eisenach KD, Cooper ES, **1996**. Comparison of the amplified Mycobacterium tuberculosis (MTB) direct test, Amplicor MTB PCR, and IS6110-PCR for detection of MTB in respiratory specimens. Clin Infect Dis. 23: 1099-1106.

Davies PD, Pai M, **2008**. The diagnosis and misdiagnosis of tuberculosis. Int J Tuberc Lung Dis. 12: 1226-1234.

De Wit D, Wootton M, Dhillon J, Mitchison DA, **1995** The bacterial DNA content of mouse organs in the Cornell model of dormant tuberculosis. Tuber Lung Dis. 76: 555-562.

Desmond Tutu HIV Foundation, Hannan Crusaid Treatment Centre www. Desmondtutuhivcentre .org.za /en/section/community-projects/hannan-crusaid-treatment-centre [Accessed 18.03.2010]

Diagnostic Standards and Classification of Tuberculosis in Adults and Children. **2000** [No authors listed] Am J Respir Crit Care Med. 161: 1376-1395.

DiDomenico N, Link H, Knobel R, Caratsch T, Weschler W, Loewy ZG, Rosenstraus M, **1996**. COBAS AMPLICOR: fully automated RNA and DNA amplification and detection system for routine diagnostic PCR. Clin Chem. 1996 42: 1915-1923.

Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, Waugh N, Drobniewski F, Lalvani A, **2007**. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. Health Technol Assess.11: 1-196.

Donoghue HD, Spigelman M, Greenblatt CL, Lev-Maor G, Bar-Gal GK, Matheson C, Vernon K, Nerlich AG, Zink AR, **2004.** Tuberculosis: from prehistory to Robert Koch, as revealed by ancient DNA. Lancet Infect Dis. 4: 584-592.

Drobniewski F, **2005**. Non-pulmonary mycobacterial infection Med. 33: 109-111.

Dye C, **2006**. Global epidemiology of tuberculosis. Lancet. 367: 938-940.

Dye C, **2006**. Global epidemiology of tuberculosis.Lancet. 367:938-940.

Ehrt S, Schnappinger D, **2007**. Mycobacterium tuberculosis virulence: lipids inside and out. Nat Med. 13: 284-285.

El-Sayed Zaki M, Abou-el Hassan S. **2008**. Clinical evaluation of Gen-Probe's amplified mycobacterium tuberculosis direct test for rapid diagnosis of Mycobacterium tuberculosis in

University Of Cape Town

Egyptian children at risk for infection. Arch Pathol Lab Med. 132: 244-247.

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

Eyangoh SI, Torrea G, Tejiokem MC, Kamdem Y, Piam FF, Noeske J, Van Deun A, **2008**. HIV-related incremental yield of bleach sputum concentration and fluorescence technique for the microscopic detection of tuberculosis. Eur J Clin Microbiol Infect Dis. 9: 849-855.

Feldman WH, Hinshaw HC, **1948**. Streptomycin: a valuable anti- tuberculosis agent. Br Med J. 1: 87-92.

FIND, Diagnostics for tuberculosis: global demand and market potential, report. **2006**.

FIND press release: BD and FIND Help Address Emergence of XDR-TB With New Pricing Agreement for Liquid Culture System. 2007. www.finddiagnostics.org/media/press/ 071105.html [Accessed 25.02.2010]

FIND Demonstration Projects of Rapid Detection of MDR TB. 2008 www.euro.who.int/document/TUB/ pl4s2_Rapid_Detection_OBrien_eng.pdf [Accessed 19.03.2010]

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

Floss HG, Yu TW, **2005**. Rifamycin-mode of action, resistance, and biosynthesis. Chem Rev. 105: 621-32.

Forbes BA, **1997**. Critical assessment of gene amplification approaches on the diagnosis of tuberculosis. Immunol Invest. 26: 105-116.

Friedman, **2000.** Tuberculosis: current concepts and treatment, 2nd edition, CRC press

Frothingham R, Stout JE, Hamilton CD, **2005**. Current issues in global tuberculosis control. Int J Infect Dis. 9: 297-311.

Gamboa F, Fernandez G, Padilla E, Manterola JM, Lonca J, Cardona PJ, Matas L, Ausina V, **1998.** Comparative evaluation of initial and new versions of the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test for direct detection of Mycobacterium tuberculosis in respiratory and nonrespiratory specimens. J Clin Microbiol. 36: 684-689.

Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G, **2006**. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 368: 1575-1580.

Gandhi NR, Shah NS, Andrews JR, Vella V, Moll AP, Scott M, Weissman D, Marra C, Lalloo UG, Friedland GH, **2010.** HIV coinfection in multidrug- and extensively drug-resistant tuberculosis results in high early mortality. Am J Respir Crit Care Med.18: 80-86.

Ganeswrie R, Chui CS, Balan S, Puthucheary SD, **2004**. Comparison of BACTEC MGIT 960 system and BACTEC 460 TB system for growth and detection of Mycobacteria from clinical specimens. Malays J Pathol. 26: 99-103.

University Of Cape Town

García de Viedma D, **2003.** Rapid detection of resistance in Mycobacterium tuberculosis: a review discussing molecular approaches. Clin Microbiol Infect. 9: 349-359.

Garrino MG, Glupczynski Y, Degraux J, Nizet H, Delmée M, **1999**. Evaluation of the Abbott LCx Mycobacterium tuberculosis assay for direct detection of Mycobacterium tuberculosis complex in human samples. J Clin Microbiol. 37: 229-232.

Gennaro ML, **2000.** Immunologic diagnosis of tuberculosis. Clin Infect Dis.30 Suppl 3:S243-236.

Gillespie SH, **2002**. Evolution of drug resistance in Mycobacterium tuberculosis: clinical and molecular perspective. Antimicrob Agents Chemother. 46: 267-274.

Goessens WH, de Man P, Koeleman JG, Luijendijk A, te Witt R, Endtz HP, van Belkum A. **2005**. Comparison of the COBAS AMPLICOR MTB and BDProbeTec ET assays for detection of Mycobacterium tuberculosis in respiratory specimens. J Clin Microbiol. 43: 2563-2566.

Gordon SV, Bottai D, Simeone R, Stinear TP, Brosch R, **2009.** Pathogenicity in the tubercle bacillus: molecular and evolutionary determinants. Bioessays. 31: 378-388.

Grandjean L, Moore DA, **2008**. Tuberculosis in the developing world: recent advances in diagnosis with special consideration of extensively drug-resistant tuberculosis. Curr Opin Infect Dis. 21: 454-461.

Greco S, Girardi E, Navarra A, Saltini C, **2006**. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. Thorax. 61: 783-790.

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

Gutierrez MC, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, Supply P, Vincent V, **2005**. Ancient origin and gene mosaicism of the progenitor of Mycobacterium tuberculosis. PLoS Pathog. 1: e5

Haldane J. **1955** The estimation and significance of the logarithm of a ratio of frequencies. Ann Hum Genet 20: 309–314.

Hanekom M, van der Spuy GD, Gey van Pittius NC, McEvoy CR, Hoek KG, Ndabambi SL, Jordaan AM, Victor TC, van Helden PD, Warren RM, **2008**. Discordance between mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing and IS6110 restriction fragment length polymorphism genotyping for analysis of Mycobacterium tuberculosis Beijing strains in a setting of high incidence of tuberculosis.J Clin Microbiol. 46: 3338-3345.

Harries AD, **2000.** Issues facing TB control (6). Tuberculosis control in sub-Saharan Africa in the face of HIV and AIDS. Scott Med J. 45: 47-50

Harries AD, Maher D, Graham S, **2004.** TB/HIV: a clinical manual. 2nd edition, WHO

Health Systems Trust. Health statistics HIV www.hst.org.za/healthstats/13/data [Accessed 20.01.2010]

Health Systems Trust. Health statistics, Tuberculosis www.hst.org.za/healthstats/16/data. [Accessed 20.01.2010]

University Of Cape Town

Heid CA, Stevens J, Livak KJ, Williams PM, **1996**. Real time quantitative PCR. Genome Res. 6: 986-994.

Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, Kop J, Owens MR, Rodgers R, Banada P, Safi H, Blakemore R, Lan NT, Jones-López EC, Levi M, Burday M, Ayakaka I, Mugerwa RD, McMillan B, Winn-Deen E, Christel L, Dailey P, Perkins MD, Persing DH, Alland D, **2010**. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J Clin Microbiol. 48: 229-237.

Hermans PW, Schuitema AR, Van Soolingen D, Verstynen CP, Bik EM, Thole JE, Kolk AH, van Embden JD.c, **1990** Specific detection of Mycobacterium tuberculosis complex strains by polymerase chain reaction. J Clin Microbiol. 28: 1204-1213.

Higuchi R, Dollinger G, Walsh PS, Griffith R, **1992**. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y). 10: 413-417.

Higuchi R, Fockler C, Dollinger G, Watson R, **1993**. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N Y).11: 1026-1030.

Hillemann D, Rüsch-Gerdes S, Richter E, **2005**. Application of the Capilia TB assay for culture confirmation of Mycobacterium tuberculosis complex isolates. Int J Tuberc Lung Dis. 9: 1409-1411.

Hillemann D, Rüsch-Gerdes S, Richter E, **2007**. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of Mycobacterium tuberculosis strains and clinical specimens. J Clin Microbiol. 25: 2635-2640.

Huang WL, Chen HY, Kuo YM, Jou R, **2009.** Performance assessment of the GenoType MTBDRplus test and DNA sequencing in detection of multidrug-resistant Mycobacterium tuberculosis. J Clin Microbiol. 47: 2520-2524.

Iseman, **1994**. Evolution of drug-resistant tuberculosis: A tale of two species. Proc. Nati. Acad. Sci. USA 91: 2428-2429.

Jacob JT, Mehta AK, Leonard MK, **2009**. Acute forms of tuberculosis in adults. Am J Med. 122: 12-17.

Jo EK, **2008** Mycobacterial interaction with innate receptors: TLRs, C-type lectins, and NLRs. Curr Opin Infect Dis. 21: 279-286.

Johnson R, Streicher EM, Louw GE, Warren RM, van Helden PD, Victor TC, **2006**. Drug Resistance in *Mycobacterium tuberculosis* Curr. Issues Mol. Biol. 8: 97-112.

Keeler E, Perkins MD, Small P, Hanson C, Reed S, Cunningham J, Aledort JE, Hillborne L, Rafael ME, Girosi F, Dye C, **2006**. Reducing the global burden of tuberculosis: the contribution of improved diagnostics. Nature 444: 49-57.

Kevany S, **2005**. An Economic Evaluation of the Impact of Widespread Antiretroviral Treatment on Secondary Hospitals in South Africa: Case Study of the GF Jooste Hospital Antiretroviral Referral Unit. Health Systems Trust

Kiepiela P, Bishop KS, Smith AN, Roux L, York DF, **2000.** Genomic mutations in the katG, inhA and aphC genes are useful for the prediction of isoniazid resistance in Mycobacterium tuberculosis isolates from Kwazulu Natal, South Africa. Tuber Lung Dis. 80: 47-56.

University Of Cape Town

Knottnerus JA, van Weel C, Muris JW. **2002**. Evaluation of diagnostic procedures. BMJ. 324: 477-480.

Koch, R., Die Aetiogie der Tuberculose. Berl. Klin. Wochenschr **1882**. 19: 221-230.

Korbel DS, Schneider BE, Schaible UE, **2008**. Innate immunity in tuberculosis: myths and truth. Microbes Infect. 10: 995-1004.

Kubica GP, Dye WE, Cohn ML, Middlebrook G, **1963**. Sputum digestion and decontamination with N-acetyl-L-cysteine-sodium hydroxide for culture of mycobacteria. Am Rev Respir Dis. 87: 775-779.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N, **2006**. The real-time polymerase chain reaction. Mol Aspects Med. 27: 95-125.

Lacoma A, Garcia-Sierra N, Prat C, Ruiz-Manzano J, Haba L, Rosés S, Maldonado J, Domínguez J, **2008**. GenoType MTBDRplus assay for molecular detection of rifampin and isoniazid resistance in Mycobacterium tuberculosis strains and clinical samples. J Clin Microbiol. 46: 3660-3667.

Lalvani A, Nagvenkar P, Udwadia Z, Pathan AA, Wilkinson KA, Shastri JS, Ewer K, Hill AV, Mehta A, Rodrigues C. **2001**. Enumeration of T cells specific for RD1-encoded antigens suggests a high prevalence of latent Mycobacterium tuberculosis infection in healthy urban Indians. J Infect Dis.183: 469-477

Lalvani A, **2007**. Diagnosing tuberculosis infection in the 21st century: new tools to tackle an old enemy. Chest. 131: 1898-906.

Lawn SD, Bekker LG, Middelkoop K, Myer L, Wood R, **2006**. Impact of HIV infection on the epidemiology of tuberculosis in a peri-urban community in South Africa: the need for age-specific interventions. Clin Infect Dis. 42: 1040-1047.

Lawn SD, Edwards DJ, Kranzer K, Vogt M, Bekker LG, Wood R, **2009**. Urine lipoarabinomannan assay for tuberculosis screening before antiretroviral therapy diagnostic yield and association with immune reconstitution disease. AIDS. 23: 1875-1880.

Lienhardt C, Fielding K, Sillah JS, Bah B, Gustafson P, Warndorff D, Palayew M, Lisse I, Donkor S, Diallo S, Manneh K, Adegbola R, Aaby P, Bah-Sow O, Bennett S, McAdam K, **2005.** Investigation of the risk factors for tuberculosis: a case-control study in three countries in West Africa. Int J Epidemiol. 34: 914-923.

Ling DI, Flores LL, Riley LW, Pai M, **2008.** Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: meta-analysis and meta-regression. PLoS One. 3:e1536.

Lipsitch M, Levin BR, **1998**. Population dynamics of tuberculosis treatment: mathematical models of the roles of non-compliance and bacterial heterogeneity in the evolution of drug resistance. Int J Tuberc Lung Dis. 2: 187-199.

Longo MC, Berninger MS, Hartley JL, **1990**. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene. 93: 125-128.

University of Cape Town

Mendelson M, **2007**. Diagnosing tuberculosis in HIV-infected patients: challenges and future prospects. Br Med Bull. 81-82: 149-165.

Marais BJ, Pai M, **2007**. New approaches and emerging technologies in the diagnosis of childhood tuberculosis. Paediatr Respir Rev. 8: 124-133

Martin A, Portaels F, Palomino JC. **2007**. Colorimetric redox-indicator methods for the rapid detection of multidrug resistance in Mycobacterium tuberculosis: a systematic review and meta-analysis. J Antimicrob Chemother. 59: 175-183.

Martin A, Munga Waweru P, Babu Okatch F, Amondi Ouma N, Bonte L, Varaine F, Portaels F, **2009.** Implementation of the thin layer agar method for diagnosis of smear-negative pulmonary tuberculosis in a setting with a high prevalence of human immunodeficiency virus infection in Homa Bay, Kenya. J Clin Microbiol. 47: 2632-2634

Martino A, **2008**. Mycobacteria and innate cells: critical encounter for immunogenicity. J Biosci. 33: 137-144.

Mathur ML, Gaur J, Sharma R, Solanki A, **2009.** Rapid culture of Mycobacterium tuberculosis on blood agar in resource limited setting. Dan Med Bull. 56: 208-210.

McDonough KA, Kress Y, Bloom BR, **1993**. Pathogenesis of tuberculosis: interaction of Mycobacterium tuberculosis with macrophages. Infect Immun. 61: 2763-2773

McPhee SJ, Papadakis MA, **2008** Current Medical Diagnosis and Treatment. McGraw Hill

Meltzer SJ, **1998.** PCR in bioanalysis. Humana press

Migliori GB, Matteelli A, Cirillo D, Pai M, **2008**. Diagnosis of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis: Current standards and challenges.
Can J Infect Dis Med Microbiol. 19: 169-172.

Modi S, Buff AM, Lawson CJ, Rodriguez D, Kirking HL, Lipman H, Fishbein DB, **2009**. Reporting patterns and characteristics of tuberculosis among international travelers, United States, June 2006 to May 2008. Clin Infect Dis. 49: 885-891.

Modi S, Buff AM, Lawson CJ, Rodriguez D, Kirking HL, Lipman H, Fishbein DB, **2009**. Reporting patterns and characteristics of tuberculosis among international travelers, United States, June 2006 to May 2008. Clin Infect Dis. 49: 885-891.

Monis PT, Giglio S, Saint CP, **2005**. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. Anal Biochem. May 340: 24-34.

Moore DF, Guzman JA, Mikhail LT, **2005**. Reduction in turnaround time for laboratory diagnosis of pulmonary tuberculosis by routine use of a nucleic acid amplification test. Diagn Microbiol Infect Dis. 52: 247-254.

MSF report, 2007 Report on the Integration of TB and HIV Services in *Ubuntu* clinic (Site B), Khayelitsha http://www.msf.or.jp/info/pressreport/pdf/2009_hiv02.pdf [Accesed 15.03.2010]

MSF, A patient-centred approach to drug resistant tuberculosis treatment in the community: a pilot project in Khayelitsha, South Africa, 2009. www.msf.org.za/Docs/Khayelitsha/ MSF_DR_TB_Report_March_2009.pdf [Accessed 10.03.2010]

University Of Cape Town

Muhumuza J, Asiimwe BB, Kayes S, Mugyenyi R, Whalen C, Mugerwa RD, Boom H, Eisenach KD, Joloba ML, **2006**. Introduction of an in-house PCR for routine identification of M. tuberculosis in a low-income country. Int J Tuberc Lung Dis. 10: 1262-1267.

Neonakis IK, Gitti Z, Baritaki S, Petinaki E, Baritaki M, Spandidos DA, **2009**. Evaluation of GenoType mycobacteria direct assay in comparison with Gen-Probe Mycobacterium tuberculosis amplified direct test and GenoType MTBDRplus for direct detection of Mycobacterium tuberculosis complex in clinical samples. J Clin Microbiol. 47: 2601-2603.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T, **2000**. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28: E63.

Ongut G, Ogunc D, Gunseren F, Ogus C, Donmez L, Colak D, Gultekin M, **2006**. Evaluation of the ICT Tuberculosis test for the routine diagnosis of tuberculosis. BMC Infect Dis. 27: 6:37.

Ozkutuk A, Kirdar S, Ozden S, Esen N, **2006**. Evaluation of Cobas Amplicor MTB test to detect Mycobacterium tuberculosis in pulmonary and extrapulmonary specimens. New Microbiol. 29: 269

Pai M, Kalantri S, Dheda K. **2006** .New tools and emerging technologies New tools and emerging technologies for the diagnosis of tuberculosis: part II. Active tuberculosis and drug resistance. Expert Rev Mol Diagn. 6: 423-432.

Pai M, Ramsay A, O'Brien R, **2008**. Evidence-based tuberculosis diagnosis. PLoS Med. 5: e156.

Pai M, Minion J, Sohn H, Zwerling A, Perkins MD, **2009**. Novel and improved technologies for tuberculosis diagnosis: progress and challenges. Clin Chest Med. 30: 701-716.

Palomino JC, **2005**. Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. Eur Respir J.26: 339-350.

Palomino JC, Leão SC, RitaccoV, **2007.** Tuberculosis 2007. From basic science to patient care. TuberculosisTextbook.com

Palomino JC, Martin A, Von Groll A, Portaels F, **2008**. Rapid culture-based methods for drug-resistance detection in Mycobacterium tuberculosis. J Microbiol Methods. 75: 161-166.

Palomino JC, **2009**. Molecular detection, identification and drug resistance detection in Mycobacterium tuberculosis. FEMS Immunol Med Microbiol. 56: 103-111.

Peres RL, Maciel EL, Morais CG, Ribeiro FC, Vinhas SA, Pinheiro C, Dietze R, Johnson JL, Eisenach K, Palaci M, **2009**. Comparison of two concentrations of NALC-NaOH for decontamination of sputum for mycobacterial culture. Int J Tuberc Lung Dis. 13: 1572-1575.

Perkins MD, Roscigno G, Zumla A, **2006**. Progress towards improved tuberculosis diagnostics for developing countries. Lancet. 367: 942-943.

Piersimoni C, Nista D, Bornigia S, De Sio G, **1998**. Evaluation of a new method for rapid drug susceptibility testing of Mycobacterium avium complex isolates by using the mycobacteria growth indicator tube. J Clin Microbiol. 36: 64-67.

Piersimoni C, Scarparo C, **2003**. Relevance of commercial amplification methods for direct detection of Mycobacterium tuberculosis complex in clinical samples. J Clin Microbiol. 41: 5355-5365.

University Of Cape Town

Piersimoni C, Olivieri A, Benacchio L, Scarparo C, **2006**. Current perspectives on drug susceptibility testing of Mycobacterium tuberculosis complex: the automated nonradiometric systems. J Clin Microbiol. 44: 20-28.

Del Portillo P, Murillo LA, Patarroyo ME, **1991**. Amplification of a species-specific DNA fragment of Mycobacterium tuberculosis and its possible use in diagnosis. J Clin Microbiol. 29: 2163-2168.

Pottumarthy S, Wells VC, Morris AJ, **2000**. A comparison of seven tests for serological diagnosis of tuberculosis. J Clin Microbiol. 38: 2227-2231.

Pym AS, Brodin P, Brosch R, Huerre M, Cole ST, **2002**. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines Mycobacterium bovis BCG and Mycobacterium microti. Mol Microbiol. 46: 709-717.

Quast TM, Browning RF, **2006**. Pathogenesis and clinical manifestations of pulmonary tuberculosis. Dis Mon. 52: 413-419.

Rafi W, Venkataswamy MM, Ravi V, Chandramuki A, **2007**. Rapid diagnosis of tuberculous meningitis: a comparative evaluation of in-house PCR assays involving three mycobacterial DNA sequences, IS6110, MPB-64 and 65 kDa antigen. J Neurol Sci. 252: 163-168.

Ramachandran R, Paramasivan CN, **2003**. What is new in the diagnosis of tuberculosis. Ind. J. Tub. 50: 133-151

Reddy KP, Brady MF, Gilman RH, Coronel J, Navincopa M, Ticona E, Chavez G, Sánchez E, Rojas C, Solari L, Valencia J, Pinedo Y, Benites C, Friedland JS, Moore DA, **2010**. Microscopic observation drug susceptibility assay for tuberculosis screening before isoniazid preventive therapy in HIV-infected persons. Clin Infect Dis. 50: 988-996.

Reiling N, Ehlers S, Hölscher C, **2008**. MyDths and un-TOLLed truths: sensor, instructive and effector immunity to tuberculosis. Immunol Lett. 116: 15-23.

Reischl U, Lehn N, Wolf H, Naumann L, **1998**. Clinical evaluation of the automated COBAS AMPLICOR MTB assay for testing respiratory and nonrespiratory specimens.
Clin Microbiol. 36: 2853-2860.

Robledo J, Mejia GI, Paniagua L, Martin A, Guzmán A. **2008**. Rapid detection of rifampicin and isoniazid resistance in Mycobacterium tuberculosis by the direct thin-layer agar method. Int J Tuberc Lung Dis. 12: 1482-1484.

Rossney AS, Herra CM, Brennan GI, Morgan PM, O'Connell B, **2008** Evaluation of the Xpert methicillin-resistant Staphylococcus aureus (MRSA) assay using the GeneXpert real-time PCR platform for rapid detection of MRSA from screening specimens. J Clin Microbiol. 46: 3285-3290.

Rossau R, Traore H, De Beenhouwer H, Mijs W, Jannes G, De Rijk P, Portaels F, **1997**. Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of Mycobacterium tuberculosis complex and its resistance to rifampin. Antimicrob Agents Chemother. 41: 2093-2098.

Roux KH, **1995**. Optimization and troubleshooting in PCR. Genome Res. 4: S185-S194.

University of Cape Town

Rustad TR, Sherrid AM, Minch KJ, Sherman DR, **2009**. Hypoxia: a window into Mycobacterium tuberculosis latency. Cell Microbiol. 11: 1151-1159

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N, **1985**. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science. 230: 1350-1354.

Schaaf HS, Moll AP, Dheda K, **2009**. Multidrug- and extensively drug-resistant tuberculosis in Africa and South America: epidemiology, diagnosis and management in adults and children. Clin Chest Med. 30: 667-683.

Schoch OD, Rieder P, Tueller C, Altpeter E, Zellweger JP, Rieder HL, Krause M, Thurnheer R, **2007**. Diagnostic yield of sputum, induced sputum, and bronchoscopy after radiologic tuberculosis screening. Am J Respir Crit Care Med. 175: 80-86.

Schirm J, Oostendorp LA, Mulder JG, **1995**. Comparison of Amplicor, in-house PCR, and conventional culture for detection of Mycobacterium tuberculosis in clinical samples. J Clin Microbiol. 33: 3221-3224.

Schwenk A, Macallan DC, **2000**. Tuberculosis, malnutrition and wasting. Curr Opin Clin Nutr Metab Care. 3: 285-291.

Seme K, Mocilnik T, Komlos KF, Doplihar A, Persing DH, Poljak M, **2008**. GeneXpert enterovirus assay: one-year experience in a routine laboratory setting and evaluation on three proficiency panels. J Clin Microbiol. 46: 1510-1513.

Siddiqi K, Lambert ML, Walley J, **2003**. Clinical diagnosis of smear-negative pulmonary tuberculosis in low-income countries: the current evidence. Lancet Infect Dis. 2003 3: 288-296.

Slayden RA, Barry CE 3rd, **2000**. The genetics and biochemistry of isoniazid resistance in mycobacterium tuberculosis. Microbes Infect. 2: 659-669.

Small PM, Shafer RW, Hopewell PC, Singh SP, Murphy MJ, Desmond E, Sierra MF, Schoolnik GK, **1993**. Exogenous reinfection with multidrug-resistant Mycobacterium tuberculosis in patients with advanced HIV infection. N Engl J Med. 328: 1137-1144.

Small PM, Shafer RW, Hopewell PC, Singh SP, Murphy MJ, Desmond E, Sierra MF, Schoolnik GK, **1993**. Exogenous reinfection with multidrug-resistant Mycobacterium tuberculosis in patients with advanced HIV infection. N Engl J Med. 328: 1137-1144.

Somosközi A, Hotaling JE, Fitzgerald M, O'Donnell D, Parsons LM, Salfinger M, **2001**. Lessons from a proficiency testing event for acid-fast microscopy. Chest. 120: 250-257.

Speers DJ, **2006**. Clinical applications of molecular biology for infectious diseases. Clin Biochem Rev. 27: 39-51.

Steingart KR, Ng V, Henry M, Hopewell PC, Ramsay A, Cunningham J, Urbanczik R, Perkins MD, Aziz MA, Pai M, **2006**. Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. Lancet Infect Dis. 6: 664-674.

Steingart KR, Henry M, Laal S, Hopewell PC, Ramsay A, Menzies D, Cunningham J, Weldingh K, Pai M, **2007**. A systematic review of commercial serological antibody detection tests for the diagnosis of extrapulmonary tuberculosis.Thorax. 62: 911-918.

University of Cape Town

Stinear TP, Seemann T, Harrison PF, Jenkin GA, Davies JK, Johnson PD, Abdellah Z, Arrowsmith C, Chillingworth T, Churcher C, Clarke K, Cronin A, Davis P, Goodhead I, Holroyd N, Jagels K, Lord A, Moule S, Mungall K, Norbertczak H, Quail MA, Rabbinowitsch E, Walker D, White B, Whitehead S, Small PL, Brosch R, Ramakrishnan L, Fischbach MA, Parkhill J, Cole ST, **2008**. Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. Genome Res. 18: 729-741

Suffys P, Palomino JC, Cardoso Leão S, Espitia C, Cataldi A, Alito A, Velasco M, Robledo J, Fernandez J, da Silva Rosa P, Romano MI, **2000**. Evaluation of the polymerase chain reaction for the detection of Mycobacterium tuberculosis. Int J Tuberc Lung Dis. 4: 179-183.

Talbot EA, Hay Burgess DC, Hone NM, Iademarco MF, Mwasekaga MJ, Moffat HJ, Moeti TL, Mwansa RA, Letsatsi P, Gokhale NT, Kenyon TA, Wells CD, **2004**. Tuberculosis serodiagnosis in a predominantly HIV-infected population of hospitalized patients with cough, Botswana, 2002. Clin Infect Dis. 39: e1-7.

Valentine-Thon E, van Loon AM, Schirm J, Reid J, Klapper PE, Cleator GM. **2001**. European proficiency testing program for molecular detection and quantitation of hepatitis B virus DNA. Clin Microbiol. 39: 4407-4412.

Toman K Frieden T, **2004.** Toman's tuberculosis case detection treatment, and monitoring: question and answers. 2nd edition, WHO

Treatment of Tuberculosis: Guidelines, **2009**. 4th edition, WHO

Tuberculosis**.** Clinical diagnosis and management of tuberculosis and measures for its prevention and control. **2006**. Royal College of Physicians of London.

Valway SE, Sanchez MP, Shinnick TF, Orme I, Agerton T, Hoy D, Jones JS, Westmoreland H, Onorato IM, **1998**. An outbreak involving extensive transmission of a virulent strain of Mycobacterium tuberculosis. N Engl J Med. 338: 633-639

Van Crevel R, Ottenhoff TH, van der Meer JW, 2002. Innate immunity to Mycobacterium tuberculosis. Clin Microbiol Rev. 15: 294-309

Van Deun A, Maug AK, Cooreman E, Hossain MA, Chambuganj N, Rema V, Marandi H, Kawria A, Portaels F, **2000**. Bleach sedimentation method for increased sensitivity of sputum smear microscopy: does it work? J Tuberc Lung Dis. 4: 371-376.

Van Deun A, Martin A, Palomino JC. **2010**. Diagnosis of drug-resistant tuberculosis: reliability and rapidity of detection. Int J Tuberc Lung Dis.14: 131-140.

Mechanisms of drug resistance in Mycobacterium tuberculosis. Wade MM, Zhang Y, **2004**. Front Biosci. 9: 975-994.

Waksman SA, Reilly HC, Schatz A, **1945**. Strain Specificity and Production of Antibiotic Substances: V. Strain Resistance of Bacteria to Antibiotic Substances, Especially to Streptomycin. Proc Natl Acad Sci USA. 31:157-164

Walker GT, Fraiser MS, Schram JL, Little MC, Nadeau JG, Malinowski DP, **1992**. Strand displacement amplification--an isothermal, in vitro DNA amplification technique.
Nucleic Acids Res. 20: 1691-1696.

University Of Cape Town

Walker GT, **1993.** Empirical aspects of strand displacement amplification. PCR Methods Appl. 3: 1-6.

Wang JY, Lee LN, Lai HC, Hsu HL, Jan IS, Yu CJ, Hsueh PR, Yang PC. **2007.** Performance assessment of the Capilia TB assay and the BD ProbeTec ET system for rapid culture confirmation of Mycobacterium tuberculosis.
Diagn Microbiol Infect Dis.59: 395-399.

Wells CD, Cegielski JP, Nelson LJ, Laserson KF, Holtz TH, Finlay A, Castro KG, Weyer K, **2007**. HIV infection and multidrug-resistant tuberculosis: the perfect storm. J Infect Dis. 196: S86-107

WHO AIDS epidemic update, 2009. Efforts towards universal access to HIV prevention, treatment, care and support are bringing AIDS out of isolation. data.unaids.org/pub/Report/2009 /JC1700_Epi_Update_2009_en.pdf [Accessed 15.03.2010.]

WHO report, Global tuberculosis control: epidemiology, strategy, financing, 2009. whqlibdoc.who.int/publications/2009/9789241563802_eng.pdf [Accessed 18.01.2010]

WHO, A research agenda for childhood tuberculosis. 2007 Improving the management of childhood tuberculosis within national tuberculosis programmes: research priorities based on a literature review whqlibdoc.who.int/hq/2007/ WHO_HTM_TB_2007.381_eng.pdf [Accessed 17.02.2010]

WHO, Acute Respiratory Infections, Update September 2009
www.who.int/vaccine_research/diseases/ari/en/index4.html [Accessed 17.03.2010]

WHO, TB and Children, 2006 www.searo. who.int/en/Section 10/Section2097/ Section2106_ 10681. htm [Accessed 18.05.2010]

WHO, Molecular line probe assay for rapid screening of patients at risk of MDR TB, 2008 www.who.int/tb/features_archive/policy_statement.pdf [Accessed 13.03.2010]

WHO, New laboratory diagnostic tools for tuberculosis control. 2008 www.tbevidence.org/ documents/tbdxpl/Diagnostic_ Brochure_ Print_Dec_22_3_3.pdf [Accessed 18.10.2010]

WHO, Reduction of number of smears for the diagnosis of pulmonary TB www.who.int/tb/dots/laboratory/policy/en/index2.html [Accessed 15.03.2010]

WHO, Tuberculosis facts, 2008 www.who.int/tb [Accessed 15.01.2010]

WHO, TB/HIV facts, 2009 www.who.int/tb/challenges/hiv/factsheet_hivtb_2009update.pdf [Accessed 19.05.2010]

WHO, Use of Liquid TB Culture and Drug Susceptibility Testing in Low and Medium Income Settings, 2007 report www.who.int/tb/dots/laboratory/Use%20of%20Liquid%20TB%20 Culture_Summary%20Report.pdf [Accessed 15.03.2010]

Wilson SM, McNerney R, Nye PM, Godfrey-Faussett PD, Stoker NG, Voller A, **1993** Progress toward a simplified polymerase chain reaction and its application to diagnosis of tuberculosis. J Clin Microbiol. 31: 776-782.

Wirth T, Hildebrand F, Allix-Béguec C, Wölbeling F, Kubica T, Kremer K, van Soolingen D, Rüsch-Gerdes S, Locht C, Brisse S, Meyer A, Supply P, Niemann S. **2008**. Origin, spread and

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demography of the Mycobacterium tuberculosis complex.
PLoS Pathog.4: e1000160.

Wright A, Zignol M, Van Deun A, Falzon D, Gerdes SR, Feldman K, Hoffner S, Drobniewski F, Barrera L, van Soolingen D, Boulabhal F, Paramasivan CN, Kam KM, Mitarai S, Nunn P, Raviglione M, **2009**. Epidemiology of antituberculosis drug resistance 2002-07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance.
Lancet. 373: 1861-1873

Zhang Y, Yew WW, **2009**. Mechanisms of drug resistance in Mycobacterium tuberculosis.
Int J Tuberc Lung Dis. 13:1320-30

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