

Development and validation of a molecular diagnostic for tuberculosis

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DISCLAIMER

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-Romans 8:38-

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May God continue to bless you!

Isaac Mutingwende
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*I dedicate this thesis to
my family, my wife, Tariro, and my beloved children, Tadiwa and Isaac Jnr
for your constant support and unconditional love
I love you all dearly.*

ABSTRACT

Background: Tuberculosis is a curable disease that is still responsible for more than 1.4 million deaths annually. In the face of the HIV epidemic the modest gains that have been made in trying to control and eradicate the disease are under serious threat. Lack of affordable, accessible, and geographically available diagnostics is one of the main hindrances in controlling the disease in resource limited settings where the disease burden is highest. Molecular methods due to their good accuracy, short turnaround times (1 to 24 hours), relatively low cost and potential for automation promise to fill current diagnostic gaps.

Objectives: The main objective of this study was to develop and validate a low cost user-friendly molecular based test (known as NWU-TB) for tuberculosis diagnosis that provides a result on the same day.

Methods: After developing the initial NWU-TB prototype, a preliminary independent evaluation was conducted at the South African National Institute of Communicable Diseases (NICD) using 109 patient sputum samples. Concurrently, a blinded evaluation study was conducted at the North-West University by the development team using 548 patient sputum samples collected at Orkney-Westvaal hospital. Informed by results and recommendations from the two studies, improvements on the initial prototype were made. A new evaluation study was then conceived and executed in order to evaluate the performance of the new prototype using 176 new clinical sputum samples collected at Orkney-Westvaal hospital. This study was combined with a small pilot study that aimed to optimise and evaluate the potential use of propidium monoazide (PMA) to discriminate between live and dead mycobacteria in clinical samples. MGIT culture was used as the reference test (gold standard) in all 3 of the performance evaluation studies conducted. Except in the NICD study, MGIT culture, sputum smear microscopy (SSM) and Xpert MTB/Rif were performed by registered laboratory scientists stationed at Orkney-Westvaal hospital. Finally, the cost per test of the developed diagnostic was estimated from a laboratory perspective using the “ingredients approach” and a preliminary cost-effectiveness analysis based on a simple decision model was also conducted.

Results: In the NICD study, NWU-TB performance was compared with SSM against MGIT culture as the gold standard. NWU-TB showed a sensitivity of 67% and specificity of 88%, while SSM showed a sensitivity of 40% and specificity of 100%. For the initial NWU-TB prototype evaluation study conducted at the North-West University, comparisons were made to SSM and Xpert MTB/Rif using MGIT culture as gold standard. NWU-TB showed an overall sensitivity and specificity of 80.8% (95% CI: 75%–85.7%) and 75.6% (95% CI: 64.9%–84.4%) respectively, in comparison to 85.3% (95% CI: 79.9%– 89.6%) and 73.2% (95% CI: 62.2%–82.4%) respectively for Xpert MTB/Rif; and 62.1% (95% CI: 55.3%–68.4%) and 56.1% (95% CI: 44.7%–67%)

respectively for SSM. Sensitivity of NWU-TB was lower ($p=0.026$) than that of Xpert MTB/Rif but better ($p=0.001$) than that of SSM. However, specificities for all the three assays were significantly lower than expected. Clinical evaluation of the improved NWU-TB prototype showed that both sensitivity and specificity increased. NWU-TB showed a better sensitivity of 94.6% [95% CI: 89.1%; 97.8%) compared to Xpert MTB/Rif 84% ($p=0.0025$) and SSM 64.3% ($p<0.001$). Xpert MTB/Rif showed a better specificity of 100% [95% CI: 88.1%-100%), which was significantly higher than NWU-TB 86.2% ($p<0.001$) and SSM 62.1% ($p<0.001$). Overall, there was a strong correlation between Xpert MTB/Rif and NWU-TB results (Mc Nemar's $\chi^2=11.64$, p value = 0.0006).

Successful optimization of the PMA protocol was demonstrated using sputum samples. However, when samples were stratified and pooled based on whether individuals were receiving treatment or not, the results showed that there were no statistically significant differences between the two groups. Finally, the total cost per test for NWU-TB was estimated at \$5.36, with material cost per test contributing the highest (75%) and equipment cost contributing the least (1.31%). Also, in the preliminary cost-effectiveness analysis, incremental cost effectiveness ratios (ICERs) for community based TB screening within South African mines using NWU-TB and Xpert MTB/Rif were US\$50.3 per case detected and US\$181.52 per case detected respectively relative to the current base case scenario of passive case finding.

Conclusion: A relatively low cost (\$5.36 per test), same day (< 2 hours TAT) molecular test prototype for tuberculosis was developed and evaluated. Additional product development and longitudinal cohort studies are required in order to produce a final prototype that can be commercialised. Sensitivity of the NWU-TB was comparatively better than both Xpert MTB/Rif and SSM against MGIT culture as gold standard. However, the specificities of all the assays were significantly lower than expected. Since all the samples used in these studies (except for the NICD study) were collected at a single site, any disease dynamics unique to the site may have been replicated throughout the studies. Use of PMA to discriminate between live and dead mycobacteria was optimised and its use demonstrated some potential. This is supported by cases in which culture-negative samples that had tested positive with the NWU-TB test without PMA treatment would test negative after PMA treatment showing that DNA from dead mycobacteria was possibly responsible for the false positive result in the first instance. The pilot study was ill equipped to generate sufficient data that permits conclusive clinical decisions on the utility of PMA, but the developed protocol can be adopted to a larger longitudinal study where such firm conclusions can be drawn.

Key words: tuberculosis; NWU-TB; sputum; sensitivity; specificity; propidium monoazide

UITTREKSEL

Agtergrond: Tuberkulose is 'n geneeslike siekte wat steeds verantwoordelik is vir meer as 1,4 miljoen sterftes per jaar. Die opkoms van die gepaardgaande MIV epidemie dreig om die beskeie vordering wat gemaak is om die siekte te beheer en uit te roei, nietig te maak. Die gebrek aan bekostigbare, beskikbare en geografies toeganklike diagnostiese toetse is een van die grootste hindernisse in die beheer van tuberkulose in gebiede met beperkte infrastruktuur waar die voorkoms van die siekte die hoogste is. Molekulêre metodes, met hul goeie akkuraatheid, kort omkeertye (1 tot 24 uur), relatiewe lae koste en potensiaal vir toets outomatisering mag bydra om huidige gapings in die diagnostiese mark te vul.

Doelwitte: Die hoofdoel van die studie was om 'n lae koste en gebruikersvriendelike molekulêre toets wat tuberkulose binne een dag kan diagnoseer te ontwikkel en te bekragtig. Die toets staan bekend as NWU-TB.

Metodes: Na die ontwikkeling van die aanvanklike NWU-TB prototipe, is 'n voorlopige onafhanklike evaluering by die Suid-Afrikaanse Nasionale Instituut vir Oordraagbare Siektes (NIOS) op sputum monsters van 109 pasiënte uitgevoer. Terselfdertyd is 'n evalueringstudie deur die ontwikkelingspan by die Noordwes-Universiteit uitgevoer op 548 sputum monsters afkomstig van pasiënte van die Orkney-Wesvaal hospitaal. Die studie ontwerp was sodanig dat die monsteranalise blind was t.o.v. pasiënt data tot en met die interpretasie van die analyses. Die resultate en terugvoer van die twee studies, het gelei tot verbeteringe aan die aanvanklike prototipe, dus 'n tweede prototipe. Ten einde die verrigting van die nuwe prototipe te bepaal, is 'n soorgelyke blinde evalueringstudie op 176 kliniese sputum monsters ,verkry vanaf die Orkney-Wesvaal hospitaal, uitgevoer. Terselfdertyd is die potensiaal van die gebruik van propidium monoazide (PMA) om tussen lewendige en dooie mikobakterieë in kliniese monsters te onderskei in 'n klein loodsstudie ondersoek. MGIT kultuur is gebruik as verwysingstoets (goudstandaard) in al drie evalueringstudies. Behalwe vir die NIOS studie het geregistreerde laboratorium wetenskaplikes, gestasioneer by Orkney-Wesvaal hospitaal, alle MGIT kultuur, sputum smeer mikroskopie (SSM) en Xpert MTB/Rif uitgevoer. Ten slotte is die koste van die gebruik van die ontwikkelde diagnostiese toets vanuit 'n laboratorium perspektief met behulp van 'n "bestanddeel benadering" beraam en 'n koste-effektiwiteitsanalise uitgevoer op 'n eenvoudige besluitnemingsmodel.

Resultate: In die NIOS studie is die NWU-TB werksverrigting vergelyk met SSM teenoor MGIT kultuur as die verwysings- of goudstandaard. Die NWU-TB het 'n sensitiwiteit van 67% en spesifisiteit van 88% getoon, terwyl SSM 'n sensitiwiteit van 40% en spesifisiteit van 100% getoon het. Vir die eerste NWU-TB prototipe evalueringstudie wat by die Noordwes-Universiteit uitgevoer is, is die NWU-TB sisteem met SSM en Xpert MTB/Rif teenoor MGIT kultuur as die goudstandaard vergelyk. Die NWU-TB het 'n algehele sensitiwiteit en spesifisiteit van 80.8%

(95% VI: 75%-85.7%) en 75.6% (95% VI: 64.9%-84.4%) onderskeidelik getoon, in vergelyking met 85.3% (95% VI: 79.9 %-89.6%) en 73.2% (95% VI: 62.2%-82.4%) onderskeidelik vir Xpert MTB/Rif en 62.1% (95% VI: 55.3%-68.4%) en 56.1% (95% VI: 44.7%-67%) onderskeidelik vir SSM. Sensitiwiteit van die NWU-TB was laer ($p=0.026$) as dié van Xpert MTB/Rif, maar beter ($p=0.001$) as dié van SSM. Die spesifisiteit vir al drie die toetse was wel aansienlik laer as wat verwag is. Kliniese evaluering van die verbeterde NWU-TB prototipe het 'n toename in beide sensitiwiteit en spesifisiteit getoon. Die NWU-TB het 'n beter sensitiwiteit van 94.6% [95% VI: 89.1%- 97.8%) getoon in vergelyking met Xpert MTB/Rif 84% ($p = 0.0025$) en SSM 64.3% ($p < 0.001$). Xpert MTB/Rif het 'n beter spesifisiteit getoon van 100% [95% VI: 88.1% -100%), wat aansienlik hoër was as NWU-TB 86.2% ($p < 0.001$) en SSM 62.1% ($p < 0.001$). Algeheel was daar 'n sterk korrelasie tussen Xpert MTB/Rif en NWU-TB resultate (McNemar se chi-kwadraattoets = 11.64, p waarde = 0.0006).

Suksesvolle optimalisering van die PMA protokol is op sputum monsters gedemonstreer. Wanneer die monsters gestratifiseer en saam gegroepeer is op die basis van individue wat behandeling ontvang het al dan nie, het die resultate getoon dat daar geen statistiese beduidende verskil tussen die twee groepe bestaan nie. Ten slotte, die totale koste per toets vir die NWU-TB is beraam op US\$5.36, met die materiaal koste wat die hoogste bydra (75%) en toerusting koste die minste (1.31%) bydra lewer. In die voorlopige koste-effektiwiteitsanalise is die inkrementele koste-effektiwiteitsverhoudings (IKEVs) vir gemeenskapsgebaseerde TB sifting in Suid-Afrikaanse myne per geval bepaal as US\$50.3 vir die NWU-TB en US\$181.52 vir Xpert MTB/Rif relatief tot die huidige geval basis van passiewe identifikasie.

Gevolgtrekking: 'n Relatiewe lae koste (\$5.36 per toets), dieselfde dag (< 2 uur omkeertyd) molekulêre prototipe toets is vir tuberkulose ontwikkel en geëvalueer. Addisionele produk ontwikkeling en 'n longitudinale kohortstudie moet nog uitgevoer word ten einde 'n finale prototipe te ontwikkel wat kommersialiseer kan word. Die sensitiwiteit van die NWU-TB was relatief beter as die van beide Xpert MTB/Rif en SSM teenoor MGIT kultuur as die goudstandaard. Die spesifisiteit vir al drie die toetse was aansienlik laer as wat verwag is. Aangesien al die monsters gebruik in hierdie studies afkomstig van 'n enkele geografiese and sosiale versamelpunt was (behalwe vir die NICD studie), is dit moontlik dat 'n siekte dinamika wat uniek tot die area is weerspieël word in die resultate.

Die gebruik van PMA om tussen lewendige en dooie mikobakterieë te onderskei, is gedemonstreer en toon potensiaal. Hierdie potensiaal word ondersteun deur gevalle waar kultuur-negatiewe monsters positief met die NWU-TB-toets sonder PMA behandeling getoets het en dan negatief na afloop met PMA behandeling getoets het. Dit kan aandui dat DNA van dooie mikobakterieë moontlik verantwoordelik was vir die aanvanklike vals positiewe uitslae. Die loodsstudie waarin

die inisiëring en optimalisering van lewendig/dood onderskeiding gedoen is, was nie groot genoeg om voldoende inligting te bekom om die nut daarvan in 'n kliniese besluit te kan bevestig nie. Die ontwikkelde protokol kan met vrug in 'n groot longitudinale/moniterende studie toegepas word sodat definitiewe en gevalideerde gevolgtrekkings gemaak kan word.

Sleutelwoorde: tuberkulose; NWU-TB; sputum; sensitiwiteit; spesifisiteit; propidium monoazide

Table of Contents

Disclaimer	i
Acknowledgements	ii
Abstract	iv
Uittreksel	vi
Table of Contents	ix
List of Annexures	xii
List of Tables	xiii
List of Figures	xiv
List of Abbreviations	xvi

Chapter 1: Problem statement & study objectives

1.1 Background and Problem statement	2
1.2 Study Rationale	4
1.3 Aim of the Study	5
1.4 Study Objectives.....	5
1.5 Structure of the thesis	7
1.6 Dissemination of findings	7
1.7 Declarations.....	8
1.8 References	9

Chapter 2: Literature Review

1.1 Mycobacterium tuberculosis	13
1.2 Tuberculosis pathogenesis	13
1.3 Tuberculosis Burden	15
1.3.1 Paediatric tuberculosis	16
1.3.2 Drug-resistant TB burden.....	16
1.4 TB High Burden countries	17
1.5 TB treatment.....	18
1.6 TB in South Africa	19
1.7 Future targets for TB incidence rate	20
1.8 TB laboratory diagnosis challenges.....	21
1.9 Measurement of the accuracy for diagnostic tests.....	22
1.10 Ideal TB diagnostic tests	23
1.11 Current tuberculosis and MDR diagnosis	24

1.11.1 Smear microscopy.....	26
1.11.2 Culture methods.....	27
1.11.3 Nucleic Acid amplification tests.....	30
1.12 Future TB diagnostic systems	35
1.12.1 LoopAMP™ MTBC Detection Kit	39
1.12.2 EasyNAT TB Diagnostic kit	41
1.12.3 Epistem Genedrive.....	42
1.12.4 Trueant MTB	43
1.13 Evaluation of new diagnostic tests	44
1.14 Cost-effectiveness of diagnostic tests	45
1.14.1 Building decision analysis model	45
1.15 Conclusion.....	51
1.16 References	53

Chapter 3: Commentary on independent clinical evaluation

Abstract.....	78
Text.....	78
References.....	80

Chapter 4: Multiplex-PCR based system for tuberculosis diagnosis

Abstract.....	83
1. Introduction.....	83
2. Materials and Methods.....	84
2.1 Study population and specimens.....	84
2.2 Reference Standard tests.....	84
2.2.1 Sputum smear microscopy (SSM).....	84
2.2.2 MGIT Culture.....	84
2.2.3 GeneXpert.....	85
2.2.4 NWU-TB.....	85
2.2.5 Biosafety evaluation of NWU-TB cell lysis step.....	85
2.2.6 Statistical analysis.....	86
2.2.7 Ethics.....	86
3. Results.....	86
3.1 Patient population.....	86
3.2 Performance of NWU-TB in the individuals with no TB history.....	86

3.3 Performance of NWU-TB in cohort analysis.....	86
3.4 Frequency of non-tuberculosis mycobacteria.....	86
3.5 Biosafety evaluation.....	86
4. Discussion.....	86
5. Limitations of the study.....	88
References.....	88

Chapter 5: Detection of *M. tuberculosis* by an improved NWU-TB

Proof of submission to journal.....	91
Abstract.....	93
1. Introduction.....	94
2. Methods.....	95
2.1 Study Design.....	95
2.2 SSM, MGIT Culture and Xpert MTB/Rif.....	96
2.3 NWU-TB system.....	97
2.3.1 Sample preparation.....	97
2.3.2 Design and synthesis of primer and fluorogenic probe.....	97
2.3.3 Real-time PCR.....	97
2.4 Live/dead discrimination.....	98
2.5 Ethics.....	98
2.6 Statistical Analysis.....	98
3. Results.....	99
3.1 Study population.....	99
3.2 Correlation of NWU-TB CT values and smear grades.....	99
3.3 Comparison of NWU-TB system and MGIT culture.....	100
3.4 PMA treatment.....	101
4. Discussion.....	103
5. References.....	107

Chapter 6: Cost-effectiveness of NWU-TB in South Africa

Abstract.....	115
Introduction.....	115
Methods.....	117
NWU-TB costing.....	117
Material costs per test.....	117

Number of tests per day.....	117
Labour cost per test.....	118
Equipment costs and daily equivalents.....	118
Overhead cost per test	118
Cost-effectiveness.....	118
Results	121
NWU-TB costing.....	121
Material costs.....	121
Labour costs.....	121
Equipment cost per test.....	122
Total cost per test.....	123
Cost-effectiveness.....	123
Discussion.....	125
References.....	128

Chapter 7: Summary & future prospects

1. Introduction.....	145
2. Summary of study design, execution and results.....	145
3. Discussion.....	145
3.1 PMA use in live/dead discrimination.....	145
3.2 Specificity of SSM, Xpert MTB/Rif and NWU-TB.....	145
4. Future research focus.....	150
5. References.....	151

List of Annexures

“Annexure A”	153
“Annexure B”	155
“Annexure C”	168
“Annexure D”	171
“Annexure E”	193

LIST OF TABLES

Chapter 2: Literature Review

Table 1: Post-2015 Global TB strategy and targets.....	21
Table 2: Distance to nearest Medical Facility for the poorest 5th of the World population....	22
Table 3: Categorical classification of facilities where diagnostics may be implemented.....	23
Table 4: Proposed minimum specifications for new TB POC diagnostic tests.....	25
Table 5: Tuberculosis diagnostic products in later-stage development.....	36
Table 6: TB diagnostic technologies endorsed by the WHO, since 2007.....	37
Table 7: STARD checklist for reporting of studies of diagnostic accuracy.....	46

Chapter 4: Multiplex-PCR based system for tuberculosis diagnosis

Table 1: Sequences of primers used in multiplex PCR.....	84
Table 2: Demographic and microbiological information.....	86
Table 3: Performance of SSM, GeneXpert, and NWU-TB system.....	86
Table 4: Sensitivities of SSM, GeneXpert, and NWU-TB system.....	87
Table 5: Performance outcomes of SSM, GeneXpert, and NWU-TB stratified by HIV status, against MGIT culture as gold standard in the cohort.....	87
Table 6: Frequencies of non-tuberculosis mycobacteria found in the cohort.....	87
Table 7: Reported diagnostic performance of different NAATs in smear-negative sputum TB presumptive individuals in different countries.....	88

Chapter 5: Detection of *M. tuberculosis* by an improved NWU-TB

Table 1: Study population characteristics.....	99
Table 2: Performance of SSM, Xpert MTB/RIF and NWU-TB system.....	100
Table 3: Smear and culture results of 50 clinical sputum samples used in PMA testing.....	101
Table 4: Discrepant results from the four tests including PMA-treated results from NWU-TB.....	102

Chapter 6: Cost-effectiveness of NWU-TB in South Africa

Table 1: Model parameter values and ranges, and sources.....	120
Table 2: Material costs per NWU-TB test.....	121
Table 3: Medical Laboratory Technologist labour cost per NWU-TB test in South Africa...	122
Table 4: Equipment cost per test for NWU-TB.....	122
Table 5: Total cost per test for NWU-TB based on component costs.....	123
Table 6: Incremental cost-effectiveness ratios of the different diagnostic strategies	123

Chapter 7: Preliminary results on MDR-TB detection

Table 1: Comparison of Performance and features of NWU-TB to commonly used TB diagnostic tests in South Africa.....	137
Table 2: Sequences of primers and sloppy molecular beacons used in the study.....	141

LIST OF FIGURES

Chapter 1: Problem statement & study objectives

Figure 1: Map showing location of Orkney-Westvaal hospital, also showing its location relative to Potchefstroom and Johannesburg.....	6
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Chapter 2: Literature Review

Figure 1: Incidence rates of TB infection in Sub-Saharan Africa compared to other geographic regions.....	15
Figure 2: New TB cases in the 22 High-Burden Countries in 2013.....	18
Figure 3: Recent and projected trends in global TB incidence (cases) between 2015 and 2050.....	20
Figure 4: Characteristic serpentine structure of young <i>M. tuberculosis</i> colonies grown in Middlebrook 7H9 broth for MODS.....	29
Figure 5: Xpert MTB/RIF assay overview. Steps 1 through to 3 are manual, while the rest of the steps are automated.....	33
Figure 6: Hybridization patterns obtained with line probe strips.....	34
Figure 7: 2013 TB diagnostics funders' contributions.....	38
Figure 8: Current and emerging automated, semi-modular or non-integrated TB NAATs; their intended laboratory location and release or anticipated time.....	39
Figure 9: Schematic description of the workflow for LoopAMP MTBC.....	40
Figure 10: The XCP nucleic acid detection device (Ustar).....	42
Figure 11: Components of the Genedrive system developed by Epistem.....	43
Figure 12: Sputum is added to a red-capped sample bottle containing lyophilized reagents to liquefy the sputum.....	44
Figure 13: Schematic of the tuberculosis diagnostics development pathway.....	45
Figure 14: An example of a basic decision tree for decision analysis.....	48

Chapter 4: Multiplex-PCR based system for tuberculosis diagnosis

Figure 1: Lyser device and lysis microreactor used during cell lysis step in the NWU-TB system.....	84
Figure 2: Multiplex PCR amplification of IS6110 (123 bp), MPT64 (240 bp) and protein antigen b (419 bp).....	85
Figure 3: Patient flow, showing two arms for data analyses, namely the Cohort and Subgroup.....	85
Figure 4: Time to Positivity after MGIT culture of NWU-TB system lysed 10-fold serial dilutions of M.....	87
Figure 5: Aerial view of LMR showing a fitted filter to trap bioaerosols during heat lysis.....	88

Chapter 5: Detection of M. tuberculosis by an improved NWU-TB

Figure 1: Correlation between NWU-TB CT values and bacteriology results.....	100
Figure 2: Receiver operator characteristic curves for NWU-TB, SSM and GeneXpert compared to MGIT culture as gold standard.....	101
Figure 3: Comparison between the mean difference in threshold cycle (ΔC_t) obtained from sputum samples collected from patient either receiving anti-tuberculosis therapy (treatment group) or not receiving treatment (treatment naive).....	103

Chapter 6: Cost-effectiveness of NWU-TB in South Africa

Figure 1: Simplified schematic of decision tree used in the study.....	119
Figure 2: Cost-effectiveness analysis output graph.....	124
Figure 3: One-way sensitivity-analysis of key parameters used in the model.....	125

Chapter 7: Preliminary results on MDR-TB detection

Figure 1: Light exposure apparatus used in the PMA assay.....	125
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LIST OF ABBREVIATIONS

All abbreviations are indicated and explained where they first appear in the text, where after only the abbreviation is used.

ACF	Active case finding
AFB	Acid fast bacteria
AUC	Area under curve
BHQ	Black Hole Quencher
CEA	Cost-effectiveness analysis
CFU	Colony forming unit
CI	Confidence Interval
C _T	Cycle threshold
DALY	Disability-adjusted life year
DNA	Deoxyribonucleic acid
DOR	Diagnostic odds ratio
DST	Drug susceptibility testing
EPTB	Extrapulmonary tuberculosis
FAM	Fluorescein amidite
FDA	Food and Drug Administration
FIND	Foundation for Innovative New Diagnostics)
gDNA	genomic DNA
HBCs	High-burden countries
HEG	Hexathylene glycol
HIV	Human immunodeficiency virus
ICER	Incremental cost-effectiveness ratio
INH	Isoniazid
LAMP	Loop mediated isothermal amplification
LMR	Lysis microreactor
LTBI	Latent TB infection
MDR-TB	Multidrug resistant TB
MGIT	Mycobacterial growth indicator tube
MODS	Microscopic-observation drug-susceptibility assay
MOTT	Mycobacteria other than tuberculosis
M-PCR	Multiplex- Polymerase chain reaction
MTB	<i>Mycobacterium tuberculosis</i>

MTBC	<i>Mycobacterium tuberculosis</i> complex
NAATS	Nucleic acid amplification tests
NALC	N-acetyl-L-cysteine-sodium hydroxide
NaOH	Sodium hydroxide
NHLS	National Health Laboratory Service
NICD	National Institute of Communicable Diseases
NPV	Negative predictive value
NTM	Nontuberculous mycobacteria
NWU-TB	North-West University-TB
PCR	Polymerase chain reaction
PLHIV	People living with HIV
PMA	Propidium monoazide
POC	Point-of-care
PPV	Positive predictive value
RIF	Rifampin
RMP	Rifampin
RNA	Ribonucleic acid
RRDR	Rifampicin Resistance Determining Region
S ² D ²	Single step DNA diagnostics
SMB	Sloppy molecular beacon
SSM	Sputum smear microscopy
STARD	Standards for the Reporting of Diagnostic accuracy studies
TAT	Total assay time
TB	Tuberculosis
VAT	Value added tax
WHO	World Health Organization
WTP	Willingness to pay threshold
XDR-TB	Extensively drug-resistant TB
ZN	Ziehl-Neelsen

CHAPTER 1:

Problem statement & study objectives

This is an introductory chapter to the thesis, and it defines current challenges/problems associated with tuberculosis diagnosis and outlines the objectives of the study in order to address some of the challenges that have been highlighted.



Image (advertorial) reprinted from Colorado State University news article (2012)

1.1 Background and Problem statement

Tuberculosis is an ancient scourge that has plagued humankind throughout its known history (Daniel, 2006). In his famous 1882 *Die Aetiologie der Tuberculose* presentation to the Berlin Physiological Society, Robert Koch outlined the structure of the *tubercle bacillus*, which is the causative agent of *Mycobacterium Tuberculosis* (MTB). Since then, significant progress has been made on many fronts including discovery of potent drugs, a co-ordinated international response to control the spread of the disease and the development of diagnostic tests. However, in spite of these positive developments, an estimated one third of the world's population still has latent tuberculosis (TB) infection (Dagnew *et al.*, 2012). The rise of the colliding human immunodeficiency virus (HIV) epidemic also threatens to erode the modest gains made in the global fight against TB. In 2013 alone, there were an estimated 9.0 million new cases of TB (13% co-infected with HIV) and 1.5 million TB deaths globally (World Health Organization, 2014). In the same year, the proportion of new cases with multidrug resistant TB (MDR-TB) remained largely unchanged at 3.5% in comparison to recent years (World Health Organization, 2014).

Although TB is curable and treatment is freely available in low-income countries; the unavailability of rapid, affordable, highly sensitive and specific diagnostic tools is inhibiting initiation of appropriate therapy in a timely manner. Inappropriate treatment as a result of the diagnostic challenges, the associated treatment initiation delays and poor patient compliance are some of the main drivers of the recent increase of multidrug resistant-TB (MDR-TB) cases (Andrews *et al.*, 2007; Lange *et al.*, 2014; Li *et al.*, 2014). Correct diagnosis of TB is needed to improve treatment, reduce transmission, and control development of drug resistance. Despite spirited efforts within the scientific community and other stakeholders to develop better diagnostic

tests, the century old sputum smear microscopy (SSM) technique remains the mainstay of TB diagnosis, as it is the most widely used and often only available test in low-income countries (Weyer *et al.*, 2011). SSM is cheap (costs less than US\$ 3 per test) (Shah *et al.*, 2013) and has a short turnaround time (2-3 days) which makes it attractive, but it is handicapped by its poor and variable accuracy, the need for multiple specimens to make a diagnosis and the requirement of skilled microscopists (Pai *et al.*, 2004). Infection with HIV further reduces the sensitivity of SSM and increases the proportion of extrapulmonary disease cases. In a study of HIV-positive TB patients in Khayelitsha, South Africa, 49% of patients had negative smears and positive cultures (Coetzee *et al.*, 2004). Culture, another century old test, remains the gold standard for TB diagnosis despite its high cost and lengthy turnaround times (2-6 weeks) (Somoskovi *et al.*, 2000). The requirement for expensive technology, reliable electricity supply, biosafety infrastructure, and trained laboratory staff also limit its use in most high-burden settings (Niemz & Boyle, 2012). In addition, culture has been reported to miss as many as 20% of true TB cases among HIV-TB coinfecting patients and is prone to contamination (Chien *et al.*, 2000; Wilson *et al.*, 2006; Schito *et al.*, 2012). This also presents serious challenges when evaluating new diagnostic tests, as the globally accepted gold standard itself is not 100% accurate.

In the past two decades, much effort has been expended on bringing nucleic acid amplification tests (NAATS) to the market. DNA isolation from sputum introduces complexities due to the presence of contaminating fungi and bacteria, presence of PCR inhibitors, and identification of suitable molecular targets are some of the difficulties faced in the development of NAATs for point-of-care (POC) testing. In spite of these challenges, several commercial and in-house NAATs have been developed over the years with differing degrees of success. A meta-analysis study reported sensitivities and specificities of in-house NAATs to be 9.4%–100% and 5.6%–100% respectively (Flores *et al.*, 2005) compared to 36%–100% and 54%–100% (Ling *et al.*, 2008) for commercial assays. Despite the good accuracies for some of the commercial NAATs, their widespread adoption in resource-limited settings has been inhibited by high cost, cold chain requirements for reagents and level of technical skill required to operate them (McNerney *et al.*, 2015).

Recently, Xpert MTB/Rif (Cepheid, Sunnyvale, CA), launched an automated NAATs, which simultaneously detects MTB and resistance to rifampicin, and has been endorsed by the World Health Organization (WHO) (Weyer *et al.*, 2013). A single cartridge integrates sample processing, amplification, and detection of amplicons thus reducing the amount of manual manipulation and

level of technical expertise required (Boehme *et al.*, 2010). Results are available within 2 hours from receipt of patient specimen. South Africa was one of the first countries to roll out the Xpert MTB/Rif assay and by the end of 2011 the country had procured over half (330,540 cartridges out of 591,450 cartridges) of the cartridges produced by Cepheid (WHO, February 2012). Early clinical evaluation studies had predicted that the good sensitivity and specificity of the test would translate into dramatic increases in case detection and cure rates but emerging reports indicate that the test has not made significant impact on morbidity and mortality (McNerney & Zumla, 2015). Like all other available NAATs, Xpert MTB/Rif cannot be used in treatment monitoring due to its inability to distinguish between live and dead mycobacteria. Further weaknesses of the assay include high costs, stable electricity supply requirement and the need for annual calibration (McNerney *et al.*, 2012).

There is a debate on the definition of a POC test (Hicks *et al.*, 2001; Schito *et al.*, 2012). Traditional definitions of POC testing are mainly product-oriented (e.g. dipstick) but we support a goal-oriented view (i.e. rapid initiation of treatment). Thus POC testing is defined as “testing that will result in a clear, actionable management decision within the same clinical encounter” (Dowdy *et al.*, 2014). Cognisant of the diversity of requirements that an ideal POC test must possess, which are almost impossible to satisfy with a single test, developers have to make some trade-offs especially between sensitivity and specificity. Two possible scenarios with very different technical specification requirements exist: Developers may either concentrate on having a POC test suitable for community-based screening to eliminate infection reservoirs or focus on a confirmatory test based at the clinic. For example, high sensitivity is mandatory for a screening test and a lower specificity may be acceptable but in the case of a confirmatory test specificity becomes more important than sensitivity in order to avoid false positives which may lead to inappropriate treatment (Dowdy *et al.*, 2014).

1.2 Study Rationale

The TB diagnostics pipeline has expanded rapidly in recent years in response to the diagnostic gaps identified in the problem statement, but the ideal POC test continues to elude us. Limitations of current diagnostic tests are partly responsible for the estimated 3 million infectious cases that are missed annually and this has contributed to the current size of the epidemic (World Health Organization, 2014). A modelling study predicts that a rapid and widely accessible POC test requiring no laboratory infrastructure could reduce both TB prevalence and mortality and save as many as 400,000 lives annually (Keeler *et al.*, 2006). The World Health Organization has thus

identified the development of new diagnostic tools as one of the three key pillars in the post-2015 global TB strategy and the policy has been recently adopted by the World Health Assembly (World Health Organization, 2014).

In view of the aforementioned TB diagnostic problems and global efforts to address them, this study was consummated in order to overcome or at least minimise the technical limitations and/or to provide a commercially viable alternative to the available tests. This study, which is part of a large study titled “Rapid Diagnosis of Tuberculosis in Resource poor settings”, was conceived and carried out under the guidance of Prof Anne F Grobler and Prof Hendrik Viljoen. The project was a collaborative study between the North-West University, Potchefstroom campus in South Africa and the University of Nebraska-Lincoln, Omaha, in the USA.

1.3 Aim of the Study

The aim of the study was to develop a new POC test for tuberculosis diagnosis that (i) provides results on the same day and is user-friendly (ii) can accurately diagnose infected individuals leading to treatment initiation on the same clinical encounter, (iii) suitable for TB diagnosis in adults within a high HIV prevalence setting (iv) ideal for the infrastructure level present at primary healthcare facilities and/or community-based screening, (v) can possibly detect drug resistance, and (vi) has a low cost.

1.4 Study Objectives

The first and major objective of this study was to develop a low cost, same day (1h turnaround time) test suitable for community based TB disease screening. The developed test was initially referred to as single step DNA diagnostics (S^2D^2) but the name was later changed to NWU-TB system (used in most of the chapters in this thesis). The second objective was to evaluate the performance of the test in clinical samples in comparison to other microbiological tests currently used in South Africa (sputum smear microscopy (SSM), culture, Xpert MTB/Rif). As mentioned earlier, HIV infection decreases the sensitivity of TB tests and the new test must have better sensitivity in HIV infected patients in order to make a significant impact in South Africa. Yearly, an estimated 30% of the global cases of HIV-associated TB occur in South Africa (Meintjes, 2014). Within South Africa, HIV-TB coinfection cases are highest among miners and prisoners (Stuckler *et al.*, 2011; Telisinghe *et al.*, 2014). In order to ensure that the sample size was sufficiently powered, clinical evaluation was conducted at AngloGold Ashanti Orkney-Westvaal Hospital which caters for the company’s mining personnel. Orkney is a gold mining town situated in the

Klerksdorp district of the North West province of South Africa and approximately 180 km from Johannesburg.

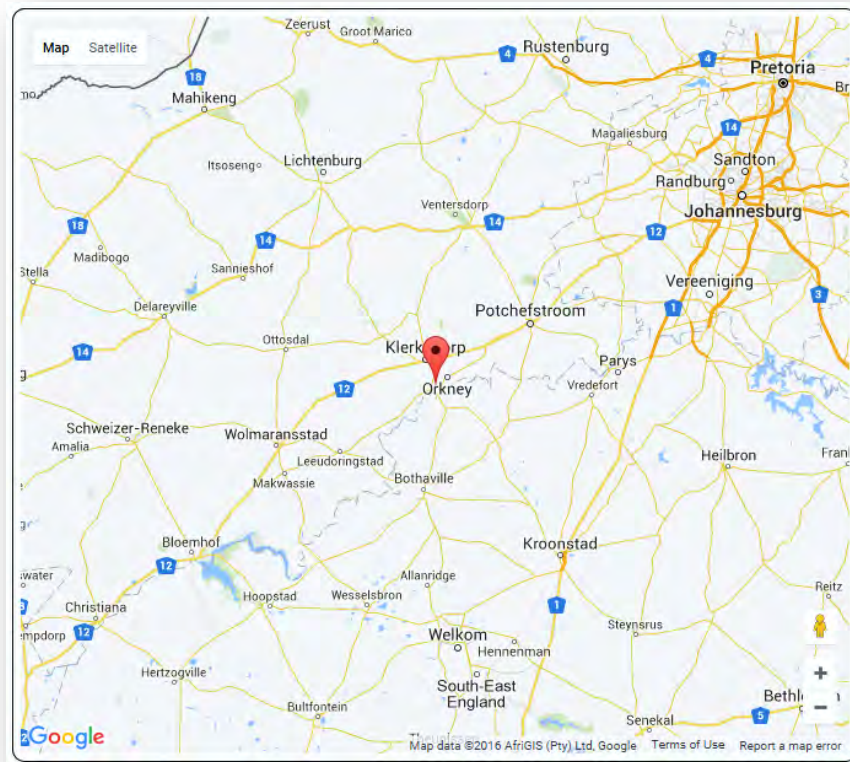


Figure 1: Map showing location of Orkney-Westvaal hospital, also showing its location relative to Potchefstroom and Johannesburg. Source: Google Earth, 2016. Accessed on 11 February 2016.

SSM and NAATs offer no information with regards to viability of mycobacteria thus both cannot be used to monitor treatment outcomes. Conventional culture is thus relied upon for treatment monitoring but due to its long turnaround time (2-6 weeks), routine bedside treatment decisions cannot be made. The third objective of this study aimed to address this limitation of NAATs by developing a strategy that enables NWU-TB to be used in treatment outcomes monitoring.

Diagnostic accuracy (sensitivity and specificity) of POC tests is an insufficient indicator of the potential impact on morbidity and mortality of the test after deployment. Recent studies are indicating that, despite the high accuracy of Xpert MTB/Rif in clinical evaluation studies, its impact on new case detection and patient survival has been minimal (Theron *et al.*, 2014; McNerney *et al.*, 2015; McNerney & Zumla, 2015). Instead of drawing conclusions about the potential impact of NWU-TB after deployment based entirely on diagnostic accuracy data, the fourth and final objective of the study was to estimate the cost per test for NWU-TB from a

healthcare provider's perspective and further determine its cost-effectiveness in community based screening of all miners in South Africa. By addressing the four objectives mentioned above, we will be able to develop a prototype of the NWU-TB system suitable for TB diagnosis and treatment monitoring.

1.5 Structure of the thesis

This thesis was submitted in full fulfilment of the requirements of a Doctor of Philosophy degree in Pharmaceutics at North-West University, Potchefstroom Campus, South Africa. The study forms part of the “Rapid Diagnosis of Tuberculosis in Resource poor settings” project and was financially supported by the South African Technology Innovation Agency (TIA), South Africa Medical Research Council - Strategic Health Innovation Partnerships (MRC-SHIP), the South African TB Research & Innovation Initiative (SATRII), and the North-West University. This thesis is submitted in a manuscript format in accordance with the General Academic Rules (A.7.5.7.4) of the North-West University. Each chapter is written in accordance with specific guidelines as stipulated by the journals intended for publication. Manuscript 1 has been accepted and published in the Journal of Microbiological Methods; manuscript 2 has been submitted to the Journal of Clinical Microbiology and is still under review; and manuscript 3 has also been submitted to BMC Infectious Diseases and is still under review.

1.6 Dissemination of findings

Findings from this study were presented to a broad audience including North West Provincial government leadership, clinicians, laboratory technologists, academics and managers from the Department of Health. The manuscripts emanating from this project were written to distribute the findings of the study and one of them has already been published and can be accessed online. The articles will mainly cater for the academic community, laboratory technologists and clinicians. I delivered presentations at local workshops/conferences especially in the North West Province in order to disseminate information to local stakeholders and government officials. I also presented part of the work at the point-of-care diagnostics world congress held on 18-19 September 2014 in San Diego, California, USA.

1.7 Declarations

The contribution for each author in the manuscripts is as follows:

I. Mutingwende (Candidate)

- Planning and design of the studies.
- Experimental work.
- Interpretation of results.
- Writing of the manuscripts and thesis.
- Corresponding author of manuscripts.

Prof. A.F. Grobler (Promoter)

- Conceptualisation of the main study within which this project falls.
- Supervision of the planning and design of the studies.
- Assistance in the interpretation of results.
- Supervision and critical review in the writing of the manuscripts and thesis.
- Corresponding author of manuscripts.
- Sourcing of funding for the study.

Prof. H. J. Viljoen (Co-promoter)

- Conceptualisation of the main study within which this project falls.
- Supervision of the planning and design of the studies.
- Assistance in the interpretation of results.
- Supervision and critical review in the writing of the manuscripts and thesis.

Prof. H.S. Steyn

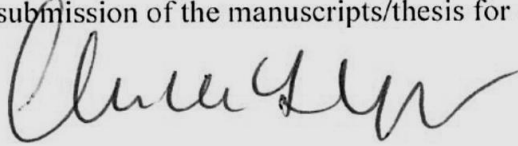
- Performed some of the statistical analysis
- Critical review in the writing of some of the manuscripts

Mr. Urban Vermeulen

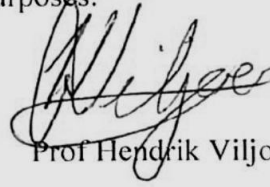
- Design and manufacturing of some of the special equipment used in this study.
- Assistance in the interpretation of results.
- Critical review in the writing of some of the manuscripts.

Declarations

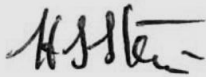
I do hereby solemnly declare that I have read and approved the manuscripts/thesis and that my role in the study as outlined above is a truly representative of my actual contribution. I also give my permission as author or co-author to Mr. Isaac Mutingwende (24662399) for submission of the manuscripts/thesis for degree purposes.



Prof Anne F Grobler



Prof Hendrik Viljoen



Prof Faans Steyn



Mr Urban Vermuelen

1.8 References

Andrews, J.R., Sarita Shah, N., Gandhi, N., Moll, T. & Friedland, G. 2007. Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis: Implications for the HIV Epidemic and Antiretroviral Therapy Rollout in South Africa. *Journal of Infectious Diseases*, 196(Supplement 3):S482-S490.

Boehme, C.C., Nabeta, P., Hillemann, D., Nicol, M.P., Shenai, S., Krapp, F., Allen, J., Tahirli, R., Blakemore, R., Rustomjee, R., Milovic, A., Jones, M., O'Brien, S.M., Persing, D.H., Ruesch-Gerdes, S., Gotuzzo, E., Rodrigues, C., Alland, D. & Perkins, M.D. 2010. Rapid molecular detection of tuberculosis and rifampin resistance. *New England Journal of Medicine*, 363(11):1005-1015.

Chien, H.P., Yu, M.C., Wu, M.H., Lin, T.P. & Luh, K.T. 2000. Comparison of the BACTEC MGIT 960 with Löwenstein-Jensen medium for recovery of mycobacteria from clinical specimens. *The International Journal of Tuberculosis and Lung Disease*, 4(9):866-870.

Coetzee, D., Hilderbrand, K., Goemaere, E., Matthys, F. & Boelaert, M. 2004. Integrating tuberculosis and HIV care in the primary care setting in South Africa. *Tropical Medicine & International Health*, 9(6):A11-15.

Dagnaw, A.F., Hussein, J., Abebe, M., Zewdie, M., Mihret, A., Bedru, A., Chanyalew, M., Yamuah, L., Medhin, G., Bang, P., Doherty, T.M., Hailu, A. & Aseffa, A. 2012. Diagnosis of

latent tuberculosis infection in healthy young adults in a country with high tuberculosis burden and BCG vaccination at birth. *BMC Research Notes*, 5:415.

Daniel, T.M. 2006. The history of tuberculosis. *Respiratory Medicine*, 100(11):1862-1870.

Dowdy, D.W., Houben, R., Cohen, T., Pai, M., Cobelens, F., Vassall, A., Menzies, N.A., Gomez, G.B., Langley, I., Squire, S.B., White, R. & for the, T.B.M.A.C.m.p. 2014. Impact and cost-effectiveness of current and future tuberculosis diagnostics: the contribution of modelling. *The International Journal of Tuberculosis and Lung Disease*, 18(9):1012-1018.

Flores, L., Pai, M., Colford, J. & Riley, L. 2005. In-house nucleic acid amplification tests for the detection of *Mycobacterium tuberculosis* in sputum specimens: meta-analysis and meta-regression. *BMC Microbiology*, 5(1):55.

Hicks, J.M., Haeckel, R., Price, C.P., Lewandrowski, K. & Wu, A.H.B. 2001. Recommendations and opinions for the use of point-of-care testing for hospitals and primary care: summary of a 1999 symposium. *Clinica Chimica Acta*, 303(1-2):1-17.

Keeler, E., Perkins, M.D., Small, P., Hanson, C., Reed, S., Cunningham, J., Aledort, J.E., Hillborne, L., Rafael, M.E., Girosi, F. & Dye, C. 2006. Reducing the global burden of tuberculosis: the contribution of improved diagnostics. *Nature*, 444 Suppl 1:49-57.

Lange, C., Abubakar, I., Alffenaar, J.-W.C., Bothamley, G., Caminero, J.A., Carvalho, A.C.C., Chang, K.-C., Codecasa, L., Correia, A., Crudu, V., Davies, P., Dedicoat, M., Drobniewski, F., Duarte, R., Ehlers, C., Erkens, C., Goletti, D., Günther, G., Ibraim, E., Kampmann, B., Kuksa, L., de Lange, W., van Leth, F., van Lunzen, J., Matteelli, A., Menzies, D., Monedero, I., Richter, E., Rüsç-Gerdes, S., Sandgren, A., Scardigli, A., Skrahina, A., Tortoli, E., Volchenkov, G., Wagner, D., van der Werf, M.J., Williams, B., Yew, W.-W., Zellweger, J.-P. & Cirillo, D.M. 2014. Management of patients with multidrug-resistant/extensively drug-resistant tuberculosis in Europe: a TBNET consensus statement. *European Respiratory Journal*, 44(1):23-63.

Li, Y., Ehiri, J., Oren, E., Hu, D., Luo, X., Liu, Y., Li, D. & Wang, Q. 2014. Are We Doing Enough to Stem the Tide of Acquired MDR-TB in Countries with High TB Burden? Results of a Mixed Method Study in Chongqing, China. *PLoS ONE*, 9(2):e88330.

Ling, D.I., Flores, L.L., Riley, L.W. & Pai, M. 2008. Commercial Nucleic-Acid Amplification Tests for Diagnosis of Pulmonary Tuberculosis in Respiratory Specimens: Meta-Analysis and Meta-Regression. *PLoS ONE*, 3(2):e1536.

McNerney, R., Cunningham, J., Hepple, P. & Zumla, A. 2015. New tuberculosis diagnostics and rollout. *The International Journal of Tuberculosis and Lung Disease*, 32:81-86.

McNerney, R., Maeurer, M., Abubakar, I., Marais, B., Mchugh, T.D., Ford, N., Weyer, K., Lawn, S., Grobusch, M.P., Memish, Z., Squire, S.B., Pantaleo, G., Chakaya, J., Casenghi, M., Migliori, G.-B., Mwaba, P., Zijenah, L., Hoelscher, M., Cox, H., Swaminathan, S., Kim, P.S., Schito, M., Harari, A., Bates, M., Schwank, S., O'Grady, J., Pletschette, M., Ditui, L., Atun, R. & Zumla, A. 2012. Tuberculosis Diagnostics and Biomarkers: Needs, Challenges, Recent Advances, and Opportunities. *Journal of Infectious Diseases*, 205(suppl 2):S147-S158.

McNerney, R. & Zumla, A. 2015. Impact of the Xpert MTB/RIF diagnostic test for tuberculosis in countries with a high burden of disease. *Current Opinion in Pulmonary Medicine*, 21(3):304-308.

Meintjes, G. 2014. Management challenges in tuberculosis and HIV. *South African Medical Journal*, 104(12):885-885.

Niemz, A. & Boyle, D.S. 2012. Nucleic acid testing for tuberculosis at the point-of-care in high-burden countries. *Expert review of molecular diagnostics*, 12(7):687-701.

Pai, M., McCulloch, M., Enanoria, W. & Colford, J.M. 2004. Systematic reviews of diagnostic test evaluations: What's behind the scenes? *Evidence-Based Medicine* 9:101 - 103.

Schito, M., Peter, T.F., Cavanaugh, S., Piatek, A.S., Young, G.J., Alexander, H., Coggin, W., Domingo, G.J., Ellenberger, D., Ermantraut, E., Jani, I.V., Katamba, A., Palamountain, K.M., Essajee, S. & Dowdy, D.W. 2012. Opportunities and Challenges for Cost-Efficient Implementation of New Point-of-Care Diagnostics for HIV and Tuberculosis. *Journal of Infectious Diseases*, 205(suppl 2):S169-S180.

Shah, M., Chihota, V., Coetzee, G., Churchyard, G. & Dorman, S. 2013. Comparison of laboratory costs of rapid molecular tests and conventional diagnostics for detection of tuberculosis and drug-resistant tuberculosis in South Africa. *BMC Infectious Diseases*, 13(1):352.

Somoskovi, A., Kodmon, C., Lantos, A., Bartfai, Z., Tamasi, L., Fuzy, J. & Magyar, P. 2000. Comparison of recoveries of mycobacterium tuberculosis using the automated BACTEC MGIT 960 system, the BACTEC 460 TB system, and Lowenstein-Jensen medium. *Journal of Clinical Microbiology*, 38(6):2395-2397.

Stuckler, D., Basu, S., McKee, M. & Lurie, M. 2011. Mining and Risk of Tuberculosis in Sub-Saharan Africa. *American Journal of Public Health*, 101(3):524-530.

Telisinghe, L., Fielding, K.L., Malden, J.L., Hanifa, Y., Churchyard, G.J., Grant, A.D. & Charalambous, S. 2014. High Tuberculosis Prevalence in a South African Prison: The Need for Routine Tuberculosis Screening. *PLoS ONE*, 9(1):e87262.

Theron, G., Zijenah, L., Chanda, D., Clowes, P., Rachow, A., Lesosky, M., Bara, W., Mungofa, S., Pai, M., Hoelscher, M., Dowdy, D., Pym, A., Mwaba, P., Mason, P., Peter, J. & Dheda, K. 2014. Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. *The Lancet*, 383(9915):424-435.

Weyer, K., Carai, S. & Nunn, P. 2011. Viewpoint TB Diagnostics: What Does the World Really Need? *Journal of Infectious Diseases*, 204(suppl 4):S1196-S1202.

Weyer, K., Mirzayev, F., Migliori, G.B., Van Gemert, W., D'Ambrosio, L., Zignol, M., Floyd, K., Centis, R., Cirillo, D.M., Tortoli, E., Gilpin, C., de Dieu Iragena, J., Falzon, D. & Raviglione, M. 2013. Rapid molecular TB diagnosis: evidence, policy making and global implementation of Xpert MTB/RIF. *European Respiratory Journal*, 42(1):252-271.

WHO. February 2012. Update on Xpert MTB/RIF roll out Xpert MTB/RIF February 2012.

Wilson, D., Nachega, J., Morroni, C., Chaisson, R. & Maartens, G. 2006. Diagnosing smear-negative tuberculosis using case definitions and treatment response in HIV-infected adults. *The International Journal of Tuberculosis and Lung Disease*, 10(1):31-38.

World Health Organization. 2014. Global tuberculosis report 2014. *World Health Organization: Geneva*. URL: http://www.who.int/tb/publications/global_report/gtbr14_main_text.pdf (Date of access: 17 January 2015).

World Health Organization. 2014. Global strategy and targets for tuberculosis prevention, care and control after 2015. *World Health Organization: Geneva*. URL: http://www.who.int/tb/post2015_strategy/en/ (Date of access: 11 November 2014).

CHAPTER 2:

Review of relevant TB diagnosis literature

This chapter reviews relevant literature on tuberculosis diagnosis. It specifically focuses on the disease burden and the current diagnostic landscape, highlighting the strengths and weaknesses of available tools, and identifying the gaps that new tools must address if global tuberculosis eradication by 2050 is to be achieved.

1.1 *Mycobacterium tuberculosis*

The genus *Mycobacterium* consists of more than 160 species, which are divided into three groups; *M. tuberculosis* complex (MTBC), *M. leprae*, and non-tuberculous mycobacteria (NTM) (also termed atypical mycobacteria or 'mycobacteria other than *M. tuberculosis* complex') (Rastogi *et al.*, 2001; Jang *et al.*, 2014). Tuberculosis (TB) is caused by one of the members of a group closely related mycobacterial species belonging to the MTBC. There are about nine mycobacterial species belonging to the complex. Although the complex members have different host ranges and phenotypic characteristics, they exhibit extreme genetic similarity, with 0.01%–0.03% synonymous nucleotide variation (Gutierrez *et al.*, 2005). In humans, TB infections are mainly caused by these mycobacterial species; *M. tuberculosis*, *M. africanum*, *M. canettii*, and *M. bovis* (Grange, 2001; de Jong *et al.*, 2010; Koeck *et al.*, 2010; Mireia *et al.*, 2013). *M. bovis* is the main cause of tuberculosis in cattle, and other domesticated mammals (Cole *et al.*, 1998). Other species causing TB disease in various mammalian hosts include: *M. microti*, *M. caprae*, *M. pinnipedii*, *M. suricattae* and *M. mungi* (Kremer *et al.*, 1998; Alexander *et al.*, 2010; Sabrina *et al.*, 2011; Parsons *et al.*, 2013; Loeffler *et al.*, 2014).

The *M. tuberculosis* genome is 4,411,529 base pairs (bp) long, with a G+C content of 65.6% (Kremer *et al.*, 1998). Molecular tools targeting various regions of this genome have been developed for identification and characterization purposes. Early molecular methods to detect or identify *M. tuberculosis* targeted the insertion sequences *IS6110* and *IS1081* (Thierry *et al.*, 1990; Collins & Stephens, 1991). With time, the panel of molecular targets increased to include: *rrs*, *gyrA*, *gyrB*, *hsp65*, *recA*, *rpoB*, *sodA* genes and 16S-23S internal transcribed spacer (ITS) genes (Radomski *et al.*, 2013).

1.2 Tuberculosis pathogenesis

M. tuberculosis is spread by air when an infectious person coughs or sneezes expelling infectious aerosol droplets 0.5 to 5.0 µm in diameter. An estimated 22% of those exposed to the infectious aerosol droplets become infected with *M. tuberculosis*. Ninety percent (90%) of those infected with *M. tuberculosis* remain asymptomatic, which is referred to as latent TB infection (LTBI) (Ducati *et al.*, 2006). In HIV uninfected individuals with LTBI, the lifetime risk of progressing to TB disease (symptomatic TB) ranges between 10% and 20% (Corbett *et al.*, 2003). However, in MTB/HIV coinfecting individuals, the annual risk of progressing to TB disease exceeds 10% (Corbett *et al.*, 2003).

In most cases TB disease progression follows the general four stage process described by Wallgren (Wallgren, 1948; Smith, 2003). In stage one, which occurs 3 to 8 weeks following exposure to *M. tuberculosis*, the inhaled infectious droplets lodge in the alveoli. These droplets are then disseminated via the lymphatic circulation to lymph nodes in the lungs leading to the formation of Ghon complex. The Ghon complex is subpleural lesion that also involves infection of the adjacent lymphatics and hilar lymph nodes. At this time, conversion to tuberculin reactivity occurs. In stage two, the mycobacteria are disseminated to other parts of the lungs and body organs via blood circulation. This stage lasts for about 3 months, and death due to tuberculous meningitis or miliary (disseminated) TB can occur during this stage. In the third stage, there is inflammation of the pleural surfaces leading to severe chest pains. Typically, the stage lasts for 3 to 7 months, but can be delayed for up to 2 years. Pleurisy occurs when TB antigens enter the pleural space thereby eliciting an intense immune response. Initially, neutrophils and macrophages are involved, followed by interferon (IFN)- γ -producing T-helper cell (Th) type 1 lymphocytes, which activates macrophages to overcome inhibition of phagolysosome maturation (MacMicking *et al.*, 2003; Trajman *et al.*, 2008). The activated macrophages process and present mycobacterial antigen to CD4⁺ lymphocytes, thereby initiating a cell-mediated response, which in turn results in the production of a number of cytokines such as TNF- α , IFN- γ , IL-6 and IL-12 (Lawn *et al.*, 2002). These cytokines attract and activate more macrophages leading to the formation of a granuloma whose main function is to ‘wall off’ bacteria in the host, resulting in containment or cure in 90% of individuals (Lawn *et al.*, 2002; Zuñiga *et al.*, 2012). In the last stage, the disease does not progress and this may last for about 3 years. During this time, some individuals develop extrapulmonary lesions in bones and joints and these usually present as chronic back pain.

Since the granuloma sit at the epicentre of TB immunopathogenesis and CD4⁺ lymphocytes are crucially involved in its formation, depletion of CD4⁺ lymphocytes in HIV infected individuals has an impact on the process. Studies suggest that CD4⁺ lymphocytes help maintain the architecture and integrity of the granuloma and thus the reactivation of latent *M. tuberculosis* infection, dissemination, and progression to active TB disease in co-infected individuals are prevented (Sonnenberg *et al.*, 2001; Diedrich & Flynn, 2011). As a result of the disruption in granuloma formation, there is poor containment of mycobacteria which then spread to the lungs and other parts of the body. This explains why the proportion of extrapulmonary tuberculosis (EPTB) cases is higher among HIV-infected individuals than among HIV-uninfected individuals. The consequent presence of fewer bacilli in sputum specimens from HIV/TB co-infected

individuals compared to HIV-uninfected individuals makes microbiological diagnosis of active TB disease challenging (Shankar *et al.*, 2014).

1.3 Tuberculosis Burden

Tuberculosis (TB) is the world's second leading cause of death from a single infectious agent, accounting for approximately 1.5 million deaths in 2013 (World Health Organization, 2014a). Although, there has been a 41% decline in TB prevalence since 1990, the war is not over and new challenges are emerging. The rise in multi-drug resistant tuberculosis (MDR-TB) cases, 3 million un-diagnosed TB patients and diagnostic complications associated with HIV/TB co-infection seek to erode those gains.

All regions of the world are affected by TB as shown in Figure , though about 80% of new cases occur in 22 high-burden countries (HBCs) yearly (World Health Organization, 2010). In 2013, an estimated 9 million people developed TB disease; approximately 56% of these were in South-East Asia and Western Pacific Regions; another 25% were in the African Region, which also had the highest incidence rates (280 cases per 100,000 population) and deaths relative to population. But among these 9 million people who developed TB in 2013, over a third (about 3 million cases) were not diagnosed, mainly due to shortcomings of the available TB diagnostic systems. These current systems are either ill-adapted to resource-limited settings or specific patient needs, or simply priced beyond the reach of the target population (UNITAID, 2015).

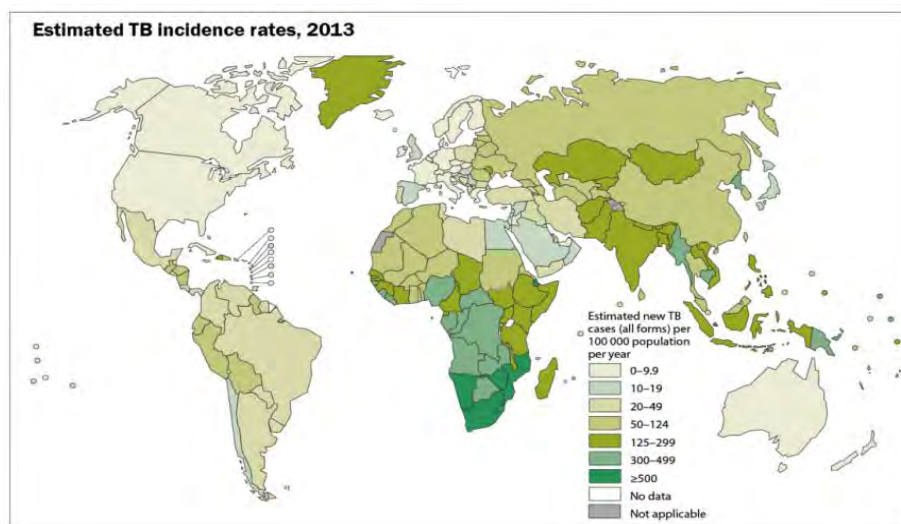


Figure 1: Incidence rates of TB infection in Sub-Saharan Africa compared to other geographic regions. The darker colours indicate regions with the highest incidence whereas the lighter colours indicate regions with the lowest incidence. Reprinted from (World Health Organization, 2014a), with permission from the World Health Organization.

Although TB is an airborne disease, it is not an equal opportunity disease as the route of transmission may suggest. For a number of reasons, some population groups are at a higher risk to develop active TB disease compared to the general population. These high risk groups include: HIV infected individuals, prisoners, miners, the elderly, children under 5 years, TB contacts, diabetics, homeless individuals and the urban poor (Glynn *et al.*, 2005; Parwati *et al.*, 2010; Prados-Torres *et al.*, 2013; Vinkeles Melchers *et al.*, 2013; Telisinghe *et al.*, 2014). In 2013, an estimated 1.1 million (13 %) of the 9 million people who developed TB were HIV-positive, making HIV seropositivity the biggest predisposing factor. Appropriate and affordable diagnostic tools for active case finding in these high risk groups should be prioritized, as every untreated person with active pulmonary TB may infect as many as 10–15 people every year (Nathanson *et al.*, 2010). Health-care providers should recognize the increased risk of TB in these populations and give special attention to surveillance and preventive services.

1.3.1 Pediatric tuberculosis

Childhood TB has been dubbed the hidden epidemic due to the difficulties in diagnosis in most children. In 2013, there were an estimated 530,000 TB cases (6 % of global total cases) among children under 15 years of age (World Health Organization, 2014a). Sputum smear microscopy (SSM) and culture, the two common tests for TB diagnosis, yield false negative results in most children and consequently the diagnosis is made based on clinical diagnosis (Zar *et al.*, 2010; Perez-Velez & Marais, 2012). However, signs and symptoms of childhood TB are non-classical and unreliable since they also appear in other diseases, thus making clinical diagnosis unreliable as well (Marais *et al.*, 2005; Bergamini *et al.*, 2009; Anderson *et al.*, 2014). Furthermore, adoption of unvalidated clinical scores in different settings leads to over-or-under diagnosis and thus often results in inappropriate treatment (Hesseling *et al.*, 2002; Hatherill *et al.*, 2010; Pearce *et al.*, 2012). Radiological examinations in childhood TB are nonspecific (Swingler *et al.*, 2005). Children also have difficulty expectorating sputum, the common TB test specimen. In conclusion, diagnosing childhood TB is different from that of adult TB, thus tests suitable for diagnosing childhood TB are urgently required.

1.3.2 Drug-resistant TB burden

Multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB are emerging global health threats (Zumla *et al.*, 2012). MDR-TB is defined as resistance to rifampicin and isoniazid, the two common first-line anti-TB drugs, while XDR-TB is defined as MDR-TB with additional resistance to a fluoroquinolone and one of the second-line injectable drugs (Migliori *et al.*, 2007). MDR-TB

treatment is complicated, lengthy, expensive and associated with very high morbidity and mortality. Recently, totally drug-resistant TB strains that are resistant to all currently available drugs have also emerged (Velayati *et al.*, 2009). Globally, in 2013, the WHO estimates that 450,000 people developed MDR-TB; of these, 170,000 people died. It is also estimated that less than one in four cases of MDR-TB were detected in 2012, thus highlighting the inadequacies of current diagnostics for MDR-TB detection. Progress towards targets for universal access to diagnosis and treatment of MDR-TB is far off-track and in many countries this now constitutes a public health crisis that is not being adequately recognized and addressed (World Health Organization, 2014a).

1.4 TB High Burden countries

Though TB is a global problem, for the past two decades, 80% of tuberculosis cases remain concentrated in 22 ‘high-burden’ countries (HBCs). India and China (Figure 2) had the highest number of reported cases in 2013; however, relative to the population of individual nations, South Africa (860 cases/ 100,000 population), jointly followed by Mozambique and Zimbabwe (552 cases/100,000 population) have the highest incidence rates in the world (World Health Organization, 2014a). The main drivers of TB in these regions include poverty, malnutrition, inadequate and unaffordable healthcare services, overcrowded and poorly ventilated houses, and unavailability of suitable diagnostic tests (Krieger & Higgins, 2002; Broekmans *et al.*, 2005). Countries like China and Brazil are experiencing a sustained gradual decline in annually reported cases, while in others the decline is painfully slow. The three assessment indicators used by the WHO are TB incidence rates, TB prevalence and TB mortality. Seven of the 22 HBCs have met the 2015 targets for reductions in TB cases and deaths, and a further four HBCs are on track to do so by 2015. However, South Africa, the Democratic Republic of the Congo and Afghanistan are the three countries that are completely off-track to meet the 2015 targets on all 3 indicators (World Health Organization, 2014a).

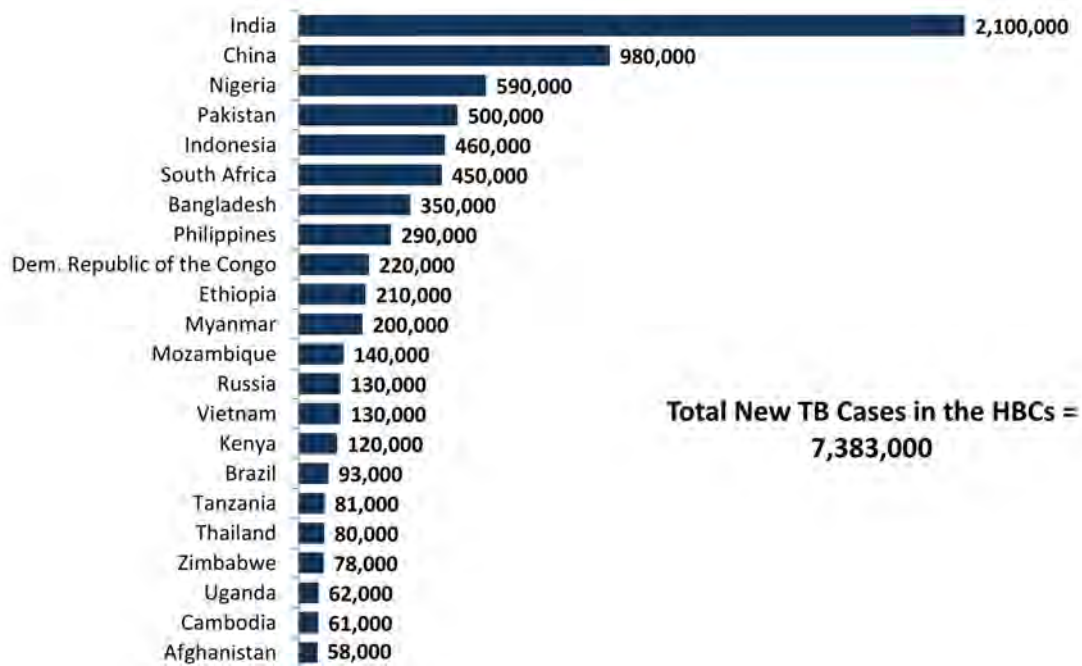


Figure 2: New TB cases in the 22 High-Burden Countries in 2013. India had the highest number of reported new TB cases in 2013. South Africa had the 6th highest number of reported new cases in the same year. Reprinted with permission from the Kaiser Family Foundation, <http://kff.org/globaldata/>

1.5 TB treatment

Tuberculosis is a treatable and curable disease. Early diagnosis is a key factor in ensuring treatment success. In endemic settings, there is a high rate of empirical therapy in which case patients are commenced on treatment based on clinical symptoms but with no microbiological test results (Theron *et al.*, 2014a). Such high levels of empirical therapy have been reported to lower the potential population level impact on treatment outcomes and mortality in settings where new rapid point of care TB tests have been adopted (Hanrahan *et al.*, 2013).

Rifampicin (RMP) and isoniazid (INH) form the backbone of first-line anti-tuberculosis treatment. New and recurrent cases of both pulmonary and extrapulmonary tuberculosis are usually treated with a combination of rifampicin, isoniazid, pyrazinamide, and ethambutol for two months and, in the second phase, a combination of isoniazid and rifampicin for a further four months (2RHZE/4RH regimen). First line drugs sometimes fail to cure tuberculosis due to several reasons. Patients with bacilli resistant to at least RMP and INH are classified as having multidrug-resistant tuberculosis and a combination regimen of streptomycin, ethambutol, terizidone, pyrazinamide, and one quinolone (levofloxacin or ofloxacin) is usually administered (Arbex *et al.*, 2010).

Increasingly, extensively drug-resistant TB (XDR-TB), which is defined as MDR-TB with resistance to a fluoroquinolone and an injectable agent (2nd line aminoglycoside or capreomycin), cases are also being reported (Dheda *et al.*, 2010; Gandhi *et al.*, 2010). Management of XDR-TB is very difficult with standard treatment of primary infection consisting of an initial 6-month intensive phase of 7 drugs (ethionamide, pyrazinimide, capreomycin, moxifloxacin, clofazimine, terizidone, para-aminosalicylic acid) followed by an 18-month continuation phase of 6 drugs (ethionamide, pyrazinimide, moxifloxacin, clofazimine, terizidone, para-aminosalicylic acid) (Pooran *et al.*, 2013). In spite of such a long and intense treatment regimen the treatment outcomes are still poor (Pietersen *et al.*, 2014). A strategy focusing on timely detection and treatment of drug susceptible tuberculosis will curb the increase in the number drug resistant cases which are difficult to treat.

1.6 TB in South Africa

South Africa had the highest per capita incidence of TB among the 22 HBCs, approaching 1% (9,800 per million) in 2013, with 62% of cases being HIV-positive. It also had the highest TB/HIV co-infection burden which is approximately 1.3 per 100 susceptible individuals per annum in 15–49 year olds (Rehle *et al.*, 2010). Thirty percent of the global incidence cases of TB-HIV co-infection occurred in SA, which roughly equates to 530,000 people being infected with TB per annum (World Health Organization, 2013; Creswell *et al.*, 2014). The combined mortality rate among HIV-positive and HIV-negative TB cases was 2,180 per million (0.2%) in 2013. Multidrug-resistant (MDR) tuberculosis accounted for 1.8% (95% confidence interval [CI], 1.4 to 2.3) of all new cases of tuberculosis and 6.7% (95% CI, 5.4 to 8.2) of retreatment cases (World Health Organization, 2013). Approximately 10% of the reported MDR tuberculosis cases were XDR tuberculosis cases making South Africa the country with the highest reported XDR tuberculosis cases in the world.

South Africa was not on track to meet the 2015 targets for reductions in disease burden, and TB incidence is projected to continue rising in the foreseeable future unless a new paradigm emerges. Confronted by poor TB statistics, South Africa was the first HBC to roll-out the Xpert MTB/RIF as the primary diagnostic test for all presumptive TB cases. Emerging data, however, shows limited impact of the Xpert MTB/Rif rollout on morbidity and mortality in high burdened settings (Theron *et al.*, 2014b; McNerney & Zumla, 2015). Instead of focusing on improving TB diagnosis alone, additional initiatives such as strengthening and improving quality of national TB treatment programmes, special health education initiatives targeting high risk populations, and better access

to anti-retroviral therapy in HIV/TB co-infected individuals will aid in reducing the TB burden in South Africa.

1.7 Future targets for TB incidence rate

The vision of the Stop TB Partnership and the World Health Organization (WHO) is to create a TB-free world (World Health Organization, 2011). To achieve this target, the incidence rate must fall at an average of 17% (Figure 3) annually between 2015 and 2050, much faster than the maximum of 10% per year that was achieved in Western Europe in the 1950s when anti-TB drugs became available (World Health Organization, 2014). If the present decline of 2% per year continues beyond 2015 (Figure 3), the incidence rate will still be 1000 times greater than the elimination threshold by 2050. The Post-2015 Global TB Strategy approved by the World Health Assembly in May 2014 aimed to reduce TB deaths by 95% and cut new cases by 90% between 2015 and 2035. The strategy also set interim milestones for 2025 and 2035 (Table 1).

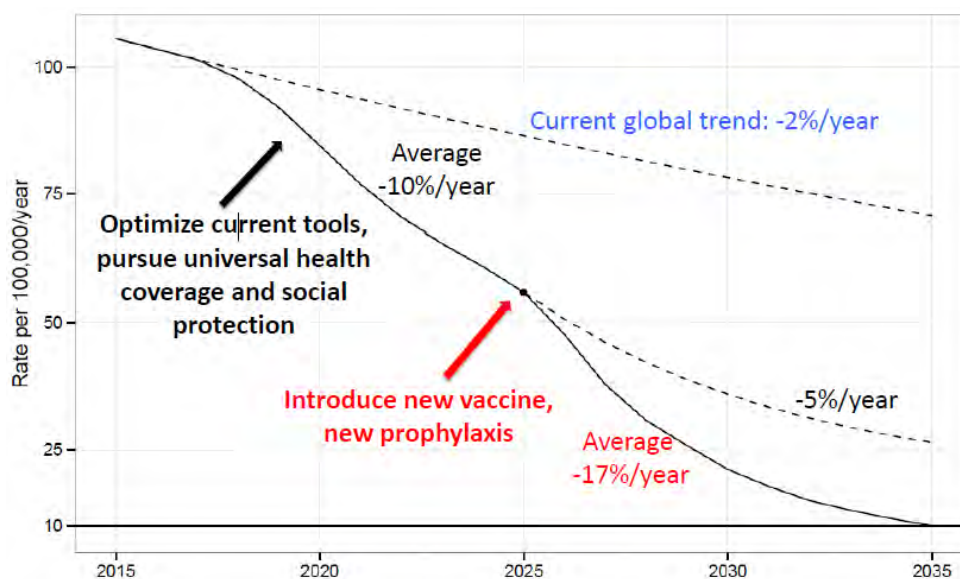


Figure 3: Recent and projected trends in global TB incidence (cases) between 2015 and 2050. To eliminate TB by 2050, novel technologies that can help achieve 20% reductions per year are a must. Currently, there is a 2% annual reduction in the number of new TB cases, which is insufficient. Reprinted from (Raviglione & Director, 2013), with permission from the World Health Organization.

Pragmatically however, with the current anti-TB arsenal and systems for delivering them, these targets are unattainable and have never been achieved on a global scale for any other epidemic. In order to eliminate TB by 2050, two things are critical: concurrent discovery of optimal vaccines,

drugs, diagnostics; and strengthening of health systems to ensure effective delivery of the new products (Raviglione & Director, 2013).

Table 1: Post-2015 Global TB strategy and targets

Vision	A world free of TB: zero deaths, disease and suffering due to TB
Goal	End the global TB epidemic
Milestones for 2025	75% reduction in TB deaths (compared with 2015) 50% reduction in TB incidence rate (compared with 2015) No affected families facing catastrophic costs due to TB
Targets for 2035	95% reduction in TB deaths (compared with 2015) 90% reduction in TB incidence rate (compared with 2015) No affected families facing catastrophic costs due to TB

Reprinted from (Raviglione & Director, 2013), with permission from the World Health Organization.

1.8 TB laboratory diagnosis challenges

In most developing countries, TB control relies on passive case finding among symptomatic individuals on routine visits to healthcare facilities, followed by either clinical diagnosis or sputum smear microscopy (SSM) laboratory diagnosis (Ogbudebe *et al.*, 2015). At least two sputum specimens are required on different days (Parsons *et al.*, 2011), necessitating repeated visits to the clinic for specimen delivery and collection of results, thereby increasing likelihood of patient dropout due to prohibitive costs associated with the visits as patients travel long distances to the nearest healthcare facilities (Table 2). Sadly, even after these delays the sensitivity of SSM has been reported to vary considerably (range, 20 to 80%) making it unreliable in high prevalence settings (Steingart *et al.*, 2006b), and often depending on the diligence with which specimens are collected. Also, quality assurance programs including quality control and external quality assessments (EQAs) are often lacking at most microscopy centers in burdened countries.

In addition, childhood TB is notoriously difficult to diagnose and all conventional TB tests perform poorly in this vulnerable population group (Zar, 2007; Lighter & Rigaud, 2009). HIV/TB co-infected individuals form the second most difficult population group to diagnose with TB due to the associated low bacillary load which makes SSM unreliable (Bruchfeld *et al.*, 2000; Worodria *et al.*, 2003; Steingart *et al.*, 2006b). Very few reference laboratories are relied upon in most of the HBCs as they are among the poorest nations in the world. Furthermore these reference laboratories

have inadequate infrastructure and outdated equipment, poor biosafety measures, and are incapable of reliably performing TB culture and drug susceptibility testing (DST) (Parsons *et al.*, 2011; Paglia *et al.*, 2012).

In conclusion, current TB diagnostics are not adapted for the above-mentioned specific patient groups or decentralized healthcare settings, and limited (or no) information on the quality of diagnostics is available to guide procurement. Thus, there is a critical need for new, sensitive, easy and rapid point-of-care diagnostics and also for sustained investments in laboratory infrastructure, quality assurance programs, and well-trained staff.

Table 2: Distance to nearest Medical Facility for the poorest 5th of the World population

Country	Distance (km)
Uganda	4.7
Tanzania	4.7
Benin	7.5
Haiti	8.0
Zimbabwe	8.6
Bolivia	11.8
Madagascar	15.5
Chad	22.9
Niger	26.9

Selected from the 2004 World Health Report, p.22

1. 9 Measurement of the accuracy of diagnostic tests

Diagnostic accuracy measures quantify the ability of a new test to discriminate between and/or predict disease and health when evaluated against a gold standard (Drobatz, 2009). These measures may either serve a discriminatory function (i.e. sensitivity and specificity) or serve a predictive function (i.e. predictive values, likelihood ratios, area under the receiver operating characteristic curve, overall accuracy and diagnostic odds ratio) (Okeh & Okoro, 2012). It is also important to note that these measures are not fixed indicators of a given test's quality and performance. Diagnostic measures are very sensitive to the characteristics of the population (e.g. disease prevalence) in which the test has been evaluated, genetic variation of the pathogen or host and the study design (Banoo *et al.*, 2008). Poor methodological designs of diagnostic evaluation studies can cause bias by over-estimating or under-estimating the performance of the indicator test and also make comparisons between different studies difficult (Eusebi, 2013).

The discriminatory function is concerned with correctly classifying people into two groups; those who are diseased and those who are not diseased (Eusebi, 2013). The main measures are sensitivity

and specificity and the results are of interest to particular to health policy experts. Sensitivity is the probability, expressed as a percentage, that a truly infected individual will test positive, while, specificity is the probability, expressed as a percentage, that a truly uninfected individual will test negative (Banoo *et al.*, 2008). Since these values are calculated in comparison to a “gold standard” test which is assumed to be 100% sensitive and specific, any shortcomings in the accuracy of the “gold standard” will lead to errors in calculating sensitivity and accuracy of the indicator test. The choice of “gold standard” is therefore very important in evaluating a new test.

The predictive function is concerned with estimating the post-test probability of a disease in a patient (Eusebi, 2013). These measures are of particular importance to clinicians as they provide an indication of test performance i.e. the trustworthiness of the result. Predictive values are often used for this task. A positive predictive value (PPV) tells the clinician what percent of patients with a positive test result truly has the disease. Negative predictive value (NPV) indicates the percentage of those patients with a negative test result do not have the disease (Drobatz, 2009).

1.10 Ideal TB diagnostic tests

Table 3, provides a list the infrastructure and human capital available at different healthcare levels for low and middle income countries. However, at the primary level (level 3) which serves most of the suspected TB patients, limited infrastructure and personnel to house and operate sophisticated TB diagnostic tests exist.

Table 3: Categorical classification of facilities where diagnostics may be implemented

	LEVEL 1	LEVEL 2	LEVEL 3
Power/AC	Available and reliable	Available	May not be available/reliable
Clean water	Available and reliable	Available	May not be available/reliable
Buildings	Well-equipped Laboratories with safety facilities	Smaller laboratory facilities offering less sophisticated facilities	Limited or no laboratory testing and safety facilities
Staff	Nurses, physicians, trained laboratory staff	Trained nurses, few physicians, few or variably trained laboratory staff	Nurses, healthcare workers, no/limited laboratory staff
Typical locations	Urban centres, large hospitals, teaching hospitals	District hospitals, urban health centres	Health clinics, usually local
Examples of current systems in each level	Xpert MTB/RIF assay, Bactec MGIT series	LAMP tests, Urinary LAM tests e.g Alere ClearView	Sputum smear microscopy (direct or concentrated)

These definitions can be applied to low and middle-income countries. Reprinted from (Batz *et al.*, 2011), with permission from the Treatment Action Group.

In 2009, a group of 34 representatives from various organizations including Treatment Action Group, Me'decins Sans Frontie`res, Partners in Health, and other agencies, developed minimum technical test specifications any new Point-of-Care (POC) TB test suitable for microscopy centers (level 3) must meet (Table 4). The meeting could not, however, reach a consensus on 3 test specifications; (i) sensitivity in smear-negative adults i.e. 60% versus 80% as the minimum; (ii) diagnosis of extrapulmonary TB in adults as a minimal requirement; and (iii) rejection of use of sputum as an appropriate sample. In the interim, the following points were agreed upon, (i) exclusion of sputum sample as highly desirable but not a minimal requirement and (ii) extrapulmonary diagnosis set as highly desirable but not a minimal requirement (Expert meeting "Defining Test Specifications for a TB POC Test" Paris, March 17-18 2009).

No existing POC TB test meets all of these specifications, although the Xpert MTB/RIF and Eiken-LAMP meet most of them. POC tests usually have lower sensitivity and specificity than laboratory-based equivalents. Commercial serodiagnostic tests, which have largely been marketed as POC tests, have had a dismal performance record, prompting the WHO to issue policy guidance against their use (World Health Organization, 2011). Basic scientific research to identify new biomarkers specifically for POC tests needs to be urgently supported. Currently, nucleic acid amplification testing (NAATs) is arguably the most appealing option for a POC TB diagnostic test. However, due to the technical complexity of DNA extraction, amplification and detection, NAAT technology is not easily translated into a POC device, as demonstrated by the lack of a successful POC NAAT for any disease (Weyer *et al.*, 2011).

1.11 Current tuberculosis and MDR diagnosis

In most of the high burdened countries, the public sector diagnostic infrastructure relies primarily on sputum smear microscopy (SSM) that cannot detect drug resistance. Currently, most national TB programmes do not offer universal DST, resulting in detection of less than one in four cases of MDR TB. The conventional phenotypic methods for assessing drug resistance are slow.

Table 4: Proposed minimum specifications for the design of any new TB POC diagnostic test

	Minimum specifications required
Medical decision	Treatment Initiation
Sensitivity - Adults (regardless of HIV status)	Pulmonary TB: Smear pos., culture positive: 95% Smear neg., culture positive: * [60-80%] [Detection of extrapulmonary TB being a preferred but not a minimal requirement]
Sensitivity - children (incl. EPTB; regardless of HIV status)	80% compared to culture of any specimen and 60% of probable TB
Specificity	Adults: 95% compared to culture Children: 95% compared to culture and 90% for culture neg., probable TB [problem of gold standard]
Time to results	Max. 3 hours (patient must get result the same day) [Desirable would be <15min]
Throughput	Min 20 test/day by 1 lab staff
Sample collection	Adult: urine, oral, breath, venous blood, sputum [Desired: NON-sputum based sample type and use of finger prick instead of venous blood] Child: urine, oral, finger/heel prick
Sample preparation	Safe – biosafety level 1 Max. 3 steps Approximate volumes of samples & reagents (no precise pipetting) Preparation not highly time sensitive
Number of samples	One sample per test
Read out	Easy, unambiguous to read with qualitative and simple ‘yes’, ‘no’, ‘invalid’ answer. Read out stays permanent for at least 1 hour
Waste disposal	Simple burning or dispose in sharp pit; no glass Environmentally acceptable disposal
Controls	Positive control included in the kit Quality control simpler and easier than with SSM
Reagents	All reagents contained in a self-contained kit Kit contains sample collection device, H ₂ O (if needed)
Storage/stability	Shelf life 24 months incl. for reagents Stable at 30°C, higher temp for shorter time periods [to be defined] Stable in high humidity environments
Instrumentation	If instrument needed, no maintenance required Instrument works in tropical conditions Acceptable replacement cost Must fit in backpack, shock resistant
Training time	1 day max. training time Can be performed by any health worker
Instrument Design	Can work on battery
Cost	Below US\$10 per test after scale up

Reprinted from (Batz *et al.*, 2011), with permission from the Treatment Action Group.

In order to avoid delays in both therapy and prevention of MDR transmission, various genotypic methods based on line probe assays, DNA sequencing or real-time PCR, have been proposed for detection of the mutations associated with resistance to anti-tuberculosis drugs. This section briefly discusses the techniques and clinical performance of current diagnostic tests for both tuberculosis and drug resistant tuberculosis (DR-TB).

1.11.1 Smear microscopy

Ziehl-Neelsen (ZN) smear microscopy remains the primary diagnostic test for pulmonary MTB in high-burden countries (HBCs) and also for TB treatment monitoring. In a recent study of the 22 HBCs, a total of 77.6 million sputum smears were performed annually at a value of US\$ 137 million in 42 827 microscopy centers (Kik *et al.*, 2014). It is affordable, the average cost of SSM is US\$ 1.77 (IQR US\$ 1.28–2.69) per smear and rapid (result in a day) (Kik *et al.*, 2014). However, it suffers from low and variable sensitivity, especially in paucibacillary HIV infected individuals, and more significantly, high operator dependence thereby increasing variability (Steingart *et al.*, 2006b; Steingart *et al.*, 2007). The effectiveness of microscopy is further diminished by the conditions in which laboratory staff operate i.e. poor microscopes, inferior reagents, intermittent electricity supply, insufficient time to examine slides properly, and a poor work ethic (Kivihya-Ndugga *et al.*, 2003; Steingart *et al.*, 2007; Kik *et al.*, 2014).

The original method is now over 130 years old and involves staining the mycobacterial cell wall with colorimetric or fluorescent dyes for subsequent visualization via microscopy. A positive smear test indicates the presence of acid fast bacteria (AFB), but does not discriminate between members of the MTB complex and other non-tuberculous mycobacteria (NTM). Two or three sputum specimens collected on different days are required in SSM algorithms. The specimens are either processed directly, but concentration of MTB via centrifugation or bleach sedimentation may improve sensitivity (Steingart *et al.*, 2006b). Centrifugation, however, adds an extra biosafety requirement for sealed rotor buckets to minimize risk from aerosols. This requirement is not easily implementable in resource limited settings.

In most high burden settings, SSM uses a conventional light binocular microscope and the ZN staining method where AFBs are stained red with carbol fuchsin. A 40x and 100x magnification objective with oil immersion on the microscope is used. Visible AFB units are scored to determine the bacterial load based on the number of AFB viewed per field and classified according to the WHO/International Union Against Tuberculosis and Lung Disease guidelines with scoring as

follows; “0” for absence of AFB, scanty 1-9, 1+, 2+, or 3+ (Kantor de Narvaiz *et al.*, 1998). Fully trained microscopists can read a maximum of 25–30 slides a day.

Fluorescence microscopy (FM) has led to an improvement over conventional ZN as AFB observation is easier and faster. Staining of AFB with fluorescent dyes is more rapid than the ZN method and most importantly each field can be read at a lower magnification thus it is easier for microscopists to identify the AFB. Consequently, a trained microscopist can read double the number of slides per day compared to conventional SSM. A number of studies report an average of 10% better sensitivity of light emitting diode (LED)-FMs used for SSM compared to light microscopy but with similar specificity (Trusov *et al.*, 2009; Albert *et al.*, 2013; Xia *et al.*, 2013).

The major drawback of SSM is its poor and highly variable sensitivity (20–80%), especially in paucibacillary specimens (common with poor-quality sputum samples or HIV co-morbidity) (Steingart *et al.*, 2006b). SSM cannot be used for drug resistance detection and its specificity may be reduced by the presence of NTMS due to the test’s inability to distinguish MTB from NTMs. Nevertheless, a major systematic review analysing data from 30 articles reported an average specificity of 97% for SSM, indicating that the assay is generally specific (Steingart *et al.*, 2006a). The reading of slides is time-consuming, tedious, operator dependent and a repetitive task that requires diligence to be effective. Due to this low sensitivity, it is estimated that 17% of TB transmission is from patients who are SSM-negative (Behr *et al.*, 1999), highlighting the shortcomings of SSM and the consequences thereof. With effective external quality assurance (EQA) plans some of the performance defaults can be addressed but the resources to maintain and coordinate such systems can also be extensive and even prohibitive.

1.11.2 Culture methods

Mycobacterial culture is the current gold standard for MTB diagnosis in most HBCs despite MTB being a slow growing microorganism with an extended generation time (20 to 22 hours) compared to other bacteria (40 to 60 minutes). Decontamination of patient sputum samples is an essential step in the isolation of mycobacteria as contaminating bacteria and fungi may further slow the growth of *M. tuberculosis* (Chatterjee *et al.*, 2013). A number of decontamination procedures exist and to yield the best results a procedure must be mild but sufficiently control the level of contaminants. The common decontamination methods include: the sodium hydroxide (NaOH) based Petroff, the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) and the Kudoh (Falodun *et al.*, 2012). NaOH reduces the viability of *M. tuberculosis* and has been reported to kill up to

60% of the bacilli in clinical samples (Asmar & Drancourt, 2015). This reduces the sensitivity of culture methods especially in high HIV prevalence settings due to paucibacillary disease.

After successful decontamination, the samples are cultivated in an appropriate media. A variety of such media have been developed in an attempt to speed up the growth rate of *M. tuberculosis*. Solid media or liquid media or two-phase media (having a liquid phase and a solid phase) may be used in culturing *Mycobacterium tuberculosis* complex (MTBC). Lowenstein-Jensen medium, containing variable concentrations of malachite green is the most widely used solid media but growth of TB bacilli on such traditional solid medium requires 4-8 weeks. Liquid culture systems reduce the delays in obtaining results to days rather than weeks. A number of automated commercial liquid-culture based systems are available on the market. These include the BACTEC MGIT 960 system (Becton Dickinson), the BacT/Alert 3D System (bioMérieux), and the microscopic-observation drug-susceptibility assay (MODS; Hardy Diagnostics). The following sections discuss these systems in more detail.

1.11.2.1 Bactec MGIT 960

The BACTEC Mycobacteria Growth Indicator Tube System (MGIT 960) (Becton Dickinson Microbiology Systems, Sparks, Md.) is a nonradiometric, fully automated, continuous monitoring system, introduced as an alternative to the radiometric BACTEC 460 for growth and detection of mycobacteria. Currently, MGIT 960 is considered the gold standard in liquid culture systems and the top performer with regard to sensitivity (Palomino, 2005). MGIT 960 has a high sensitivity (80% - 98.1%) and high specificity (89.6%-100%), with a cost per test of between US\$16-US\$32 (Molicotti *et al.*, 2014). The main advantage of the MGIT 960 system over the more sensitive solid culture systems is its shorter time to positivity of 10-14 days. The MGIT 960 system is also used extensively for *in vitro* antibiotic sensitivity testing, and data from several studies have shown that it is an accurate (sensitivity and specificity >98%), quick and effective system to determine sensitivity to anti-tuberculosis drugs of a tubercular isolated strain (Ardito *et al.*, 2001; Kontos *et al.*, 2004).

1.11.2.2 BacT/Alert 3D

The BacT/ALERT 3D system (bioMérieux, Durham, NC, USA), is a fully automated system based on the detection of CO₂ released into the liquid media by actively proliferating mycobacteria. CO₂ lowers the pH in the media, which in turn produces a color change in a sensor in the vial, detected by a reflectometric unit in the instrument. Several studies have reported comparable performance

of the BacT/Alert 3D with that of MGIT 960 for both diagnosis and DST of MTB (Brunello & Fontana, 2000; Bemer *et al.*, 2004; Guney *et al.*, 2007). The BacT/Alert 3D with its computerized data management system is an acceptable method for diagnosis of MTB but has some disadvantages, for example, being prone to contamination, longer turnaround time [13.2 days (range 7-40 days)], high instrument cost (US\$ 63,264.30), and high cost per test (\$7.91 to \$37.35 depending on sample volume) (Rohner *et al.*, 1997). Based on currently available information, it is apparent that further studies are required to fully evaluate the performance of the BacT/ALERT 3D system.

1.11.2.3 Microscopic-observation drug-susceptibility assay (MODS)

The MODS test simultaneously detects viable mycobacteria and performs drug susceptibility testing of both isoniazid and rifampicin, providing results in 7 to 9 days, compared to 21 to 26 days for standard culture (Moore *et al.*, 2006b; Shiferaw *et al.*, 2007). Each decontaminated sputum sample is divided into 12 aliquots and Middlebrook 7H9 culture medium (containing different antimicrobial agents and supplements) is placed into each of the wells. Four of the wells are drug-free and the other eight have low and high concentrations of anti-TB medications. After incubation at room temperature, plates are read under a microscope. Positive cultures are identified in drug-free control wells by cord formation, characteristic of *M. tuberculosis* growth in liquid medium (Figure). Concurrent growth in drug-containing wells indicates resistance.

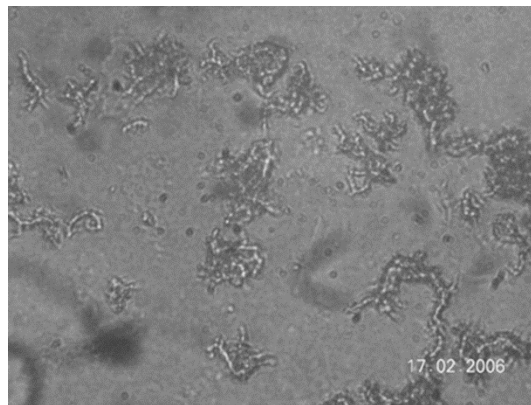


Figure 4: Characteristic serpentine structure of young *M. tuberculosis* colonies grown in Middlebrook 7H9 broth for MODS, as seen under an inverted-light microscope (original magnification, $\times 20$). Reprinted from (Shiferaw *et al.*, 2007), with permission from the American Society for Microbiology.

MODS does not require expensive equipment apart from an inverted microscope (approximately \$2,500 USD) and costs only \$2 per test (Caviedes & Moore, 2007). So far, several studies comprising more than 3000 adults, including HIV-infected persons, and 5600 sputum specimens

have evaluated MODS (Moore *et al.*, 2006b; Arias *et al.*, 2007; Shiferaw *et al.*, 2007). These multi-center studies have demonstrated that MODS possess similar performance characteristics as the other more expensive liquid culture systems, but slightly faster than traditional culture methods. Overall sensitivity of MODS in sputum is reported to range from 86 to 100% (Moore *et al.*, 2006b; Arias *et al.*, 2007; Shiferaw *et al.*, 2007; Minion *et al.*, 2010) for TB diagnosis and 92 to 97% for MDR TB (Mello *et al.*, 2007; Shiferaw *et al.*, 2007; Coronel *et al.*, 2014; Huang *et al.*, 2014). Specificity was reported to range between 94 and 99 % (Moore *et al.*, 2006a; Huang *et al.*, 2014). Despite its promising performance in clinical evaluations, some limitations of MODS still exist. First, the qualitative nature of the MODS assay limits a resistance proportion evaluation of cultures. Secondly, the possibility of cross-contamination as in other culture methods still exists though it has been reported to be infrequent and very low (Moore *et al.*, 2006a; Coronel *et al.*, 2014).

1.11.3 Nucleic Acid amplification tests

Nucleic acid amplification tests (NAATs) are increasingly becoming available and popular in the mycobacteriology laboratory. NAATs are based on detection of unique regions of the MTB genome, and specific mutations that confer antibiotic resistance. These tests offer a number of advantages over conventional diagnostics: Turnaround times are shorter (results available within a few hours after receipt of specimens), results are highly accurate, and systems are more affordable and accessible (Couturier *et al.*, 2014; McNerney *et al.*, 2015). Despite these great benefits, some limitations of NAATs include inability to distinguish between live and dead bacilli, inhibition of amplification, and variable sensitivity (5.6%-100%) in both commercial and in-house NAAT systems, especially in the problematic smear negative-culture positive group (Flores *et al.*, 2005; Ling *et al.*, 2008; Greco *et al.*, 2009). The common amplification targets include insertion element *IS 6110*, *cyp141*, *16S rDNA*, *rpoB* gene encoding the β -subunit of the RNA polymerase, the gene coding for the 32 kD protein, the *recA* gene, the *hsp65* gene, *mpt64*, *dnaJ* gene, *sodA* gene and *16S-23S rRNA ITS* (Verma *et al.*, 1994; Yamada-Noda *et al.*, 2007; Boehme *et al.*, 2010; Nimesh *et al.*, 2013; Costa *et al.*, 2014; Ou *et al.*, 2014; Farzam *et al.*, 2015). Commercial NAAT systems on the market include the Amplified MTD® (Gen-Probe Incorporated, San Diego, CA) Amplicor MTB (Roche Diagnostic Systems, Somerville, NJ), Xpert MTB/Rif (Cepheid, Sunnyvale, CA), and Genotype MTBDRplus (Hain Life sciences GmbH, Nehren, Germany).

1.11.3.1 Amplified MTD® test

In 1995, the United States Food and Drug Administration (FDA) approved the Amplified Mycobacterium Tuberculosis Direct Test (MTD) (GenProbe San Diego, CA, USA), for smear-

positive specimens and an enhanced MTD in 1999 for both smear-positive and smear-negative specimens. AMTD is an isothermal (42°C) transcription-mediated amplification nucleic acid probe test. The AMTD test provides detection of *M. tuberculosis* complex rRNA within 2.5 to 3.5 hours after starting the test procedure. Thus, while the AMTD test cannot be used to ascertain drug susceptibility, it can be used to perform rapid and reliable diagnosis of *M. tuberculosis*. A high sensitivity (96.9%) and specificity (100%) when the test is used for AFB smear positive specimens has been reported by the manufacturer (Hologic Gen-Probe inc). In a meta-analysis of AMTD studies, sensitivity of 97% and specificity of 96% among smear-positive respiratory specimens was found, and a sensitivity of 76% and specificity of 97% among smear-negative specimens (Greco *et al.*, 2006). AMTD costs on average \$50 per test (MAG Mutual Healthcare Solutions, 2008), and is not cost-effective (Dowdy *et al.*, 2003). Thus, while the assay has offered some benefits, it has not successfully provided a practical solution for all levels of clinical and public health laboratories.

1.11.3.2 Roche Amplicor Mtb PCR

The FDA-approved Amplicor MTB Test (Roche Molecular Systems, Basel, Switzerland) amplifies a 584-bp DNA fragment within a larger region encoding for 16S rRNA using biotinylated primers. An internal control, which acts as a safeguard against false negative results arising from inhibition of the polymerase, is included in the assay. It also has a built-in system (uracil-N-glycosylase) that secures against contamination by amplicons from previous runs, which is the most common cause of false positive results. The enzyme, added to the samples before amplification, destroys any amplicon resulting from previous amplifications without damaging the uracil-free target DNA. Turnaround time for the system is 6-7 hours. The reported specificity is close to 100 % while sensitivity ranges from 90 % to 100 % in smear-positive samples and from 50 % to 95.9 % in smear negative specimens (Soini & Musser, 2001). In 2009, the manufacture of the AMPLICOR MTB assay was discontinued, possibly due to the launch of the COBAS® TaqMan® MTB Test by the same company (Safianowska *et al.*, 2012).

1.11.3.3 Xpert MTB/Rif

The Xpert MTB/Rif assay (Cepheid, Sunnyvale, CA) received FDA approval in July 2013 (Food and Drug Administration, 2013). It is a self-contained, integrated real-time PCR platform that offers minimal hands-on time, with low potential for PCR contamination. The assay, simultaneously, detects an 81-bp “core” region of the *rpoB* gene for MTB identification and utilizes five molecular beacons to probe mutations associated with rifampicin resistance in this

rifampicin resistance determining region (RRDR). A short description of the Xpert MTB/Rif workflow is provided in Figure . It has revolutionized TB diagnostics due to its rapid and sensitive detection of *M. tuberculosis* and rifampicin (RMP) resistance (Boehme *et al.*, 2010; Helb *et al.*, 2010; Moure *et al.*, 2011).

When implemented as a replaced strategy for SSM, Xpert MTB/Rif has been reported to have a pooled sensitivity of 89% (95% Credible Interval (CrI) [85% - 92%]) and pooled specificity of 99% (95% CrI [98% - 99%]) (Steingart *et al.*, 2014). The same report also indicated that pooled sensitivity for Xpert MTB/Rif in paucibacillary HIV/TB coinfecting individuals was 79% (95% CrI [70% to 86%]) as compared to 86% in HIV negative individuals. In another recent study conducted in South Africa, evaluating the performance of Xpert MTB/Rif for diagnosing extrapulmonary TB using a variety of biological samples, an overall sensitivity of 59% (95% CI, [53% - 65%]) and specificity of 92% (95% CI [90% - 94%]) was reported (Scott *et al.*, 2014). This good performance of Xpert MTB/Rif is encouraging and has demonstrated that rapid and accurate diagnosis of TB is achievable.

However, in spite of such a high potential impact of Xpert MTB/Rif, wide-scale implementation has only occurred in South Africa while the other 21 HBCs still rely on SSM (Qin *et al.*, 2015). In these countries, selected use of Xpert MTB/Rif in patients either at risk of MDR-TB or HIV co-infection is recommended. The low rate of implementation and usage is mainly due to the high installation, operational and maintenance costs (World Health Organization, 2014b). A recent multicentre study has also reported that Xpert MTB/Rif deployment in high burden settings has not significantly reduced tuberculosis related morbidity, partly due to high levels of empirical therapy (Theron *et al.*, 2014b). The instrument also depends on a stable supply of electricity and requires annual maintenance which makes it not a true POC test (Denkinger *et al.*, 2013).

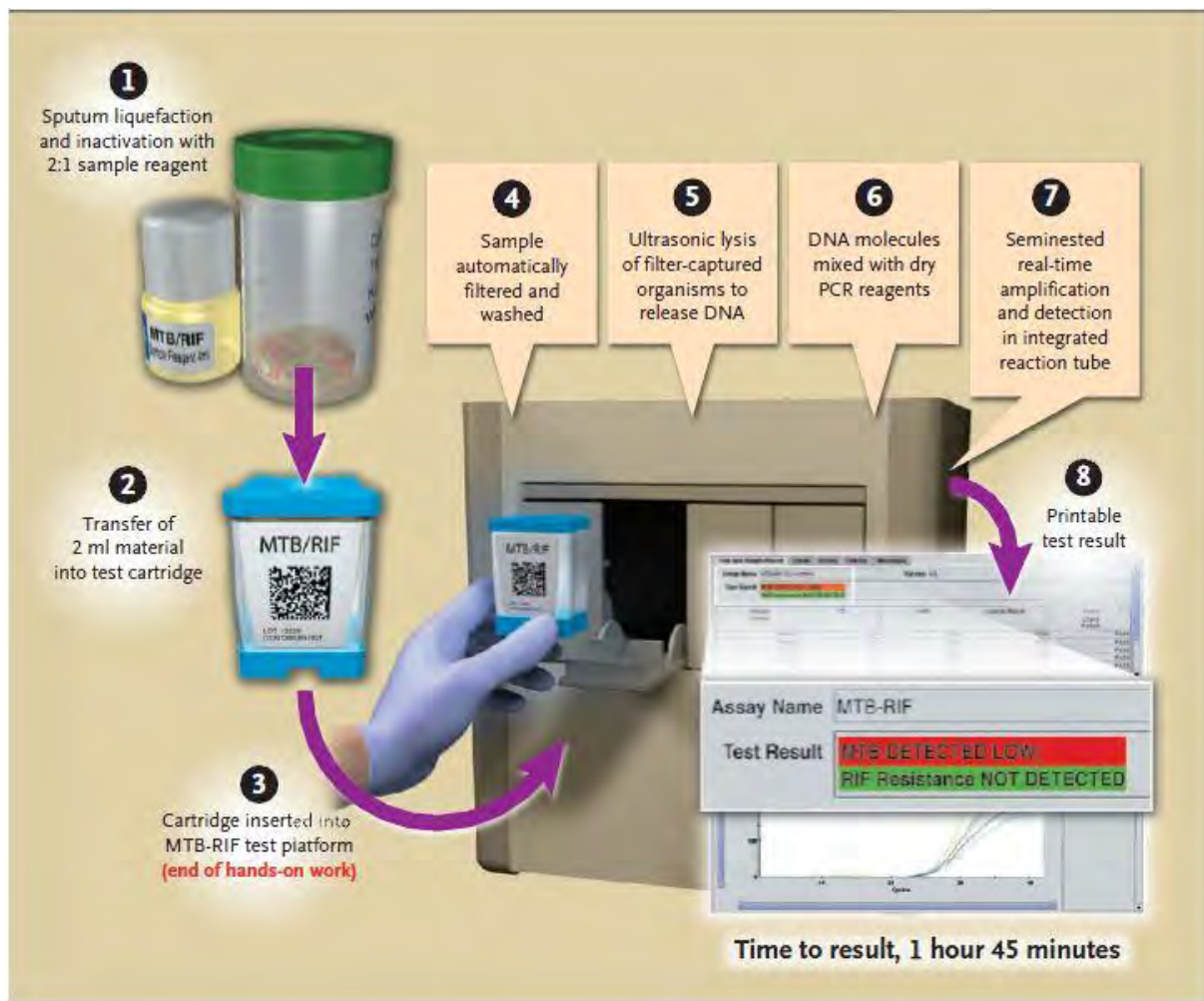


Figure 5: Xpert MTB/RIF assay overview. Steps 1 through to 3 are manual, while the rest of the steps are automated. The instrument automatically calls MTB results and in positive individuals it also reports the rifampicin resistance status permitting appropriate treatment within 2 hours.

Advertorial source:

http://www.finddiagnostics.org/programs/tb/find_activities/automated_naot.html (accessed 25/09/2015).

1.11.3.4 Molecular Line Probe Assays

Rapid line probe assays (LPAs) have recently been implemented to screen patients at high risk for drug-resistant tuberculosis. These tests include the INNO-LiPARif.TB assay (Innogenetics), the GenoType MTBDRplus assay (Hain Lifescience) and the new AID line probe assay (AID Diagnostika, Germany). Both are quick tests to detect the TB bacterium and to check if it is resistant (RMP and INH) to drugs within a day. Systematic reviews on the performance of LPAs in comparison to conventional DST methods have shown that LPAs are highly sensitive (97%) and specific (99%) for the detection of rifampicin resistance alone or in combination with INH (sensitivity, 90%; specificity, 99%) (Barnard *et al.*, 2008; World Health Organization, 2008). The

WHO has recommended the use of LPAs based on data from studies in South Africa, which showed the feasibility of introducing LPAs in high-volume public health laboratories. Costing data in the same study showed that by using the LPAs in routine diagnostic algorithms, a 30% to 50% cost reduction compared to conventional DST methods can be achieved (World Health Organization, 2008).

The INNO-LiPARif.TB kit (Innogenetics, Zwijndrecht, Belgium) is a commercially available line probe assay (LiPA) able to identify the *M. tuberculosis* complex and simultaneously detect genetic mutations in the *rpoB* gene region related to rifampicin resistance (Rossau *et al.*, 1997). The LiPA kit contains 10 oligonucleotide probes (one specific for the *M. tuberculosis* complex, five overlapping wild-type S probes, and four R probes for detecting specific mutations of resistant genotypes) immobilized on nitrocellulose paper strips (Rossau *et al.*, 1997). DNA is extracted from mycobacterial cultures or directly from clinical samples, followed by PCR amplification of the *rpoB* gene RRDR. Biotinylated PCR products are then hybridized with immobilized probes, and results are determined by colorimetric development. The *M. tuberculosis* isolate is considered rifampicin susceptible if all of the wild-type susceptible (S) probes give a positive signal and all of the resistant (R) probes react negatively (Figure).

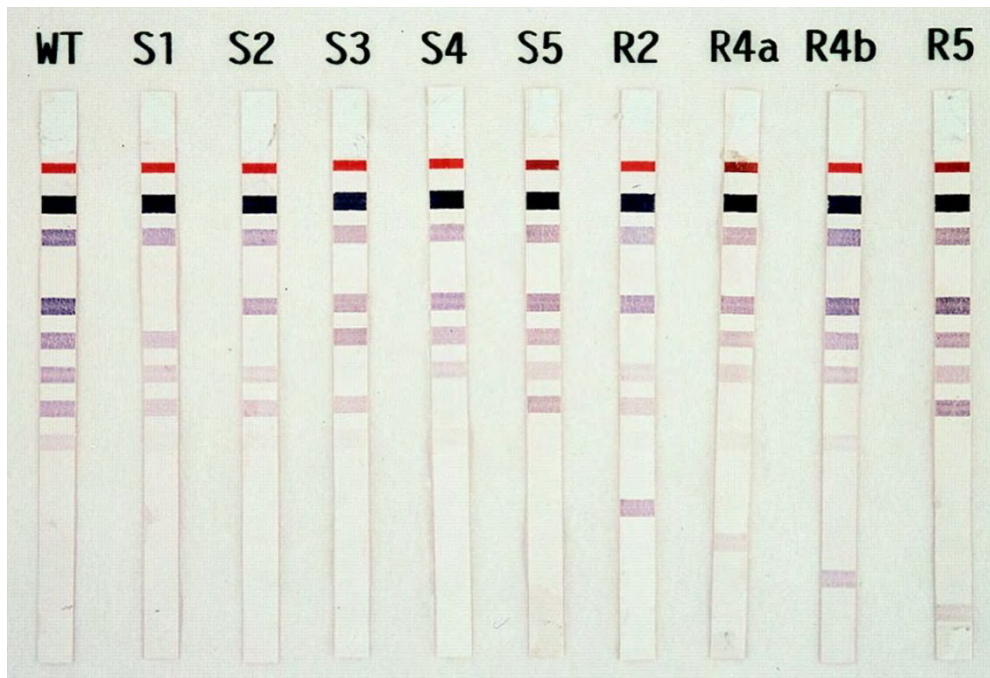


Figure 6: Hybridization patterns obtained with line probe strips. S1 through S5 are probes for wild-type (WT) sequences in the 75-bp hyper variable region of *rpoB* gene. Probes for specific mutations were as follows: R2, Asp-516-Val; R4a, His-526-Tyr; R4b, His-526-Asp; R5, Ser-531-

Leu. Reprinted from (Hirano *et al.*, 1999), with permission from the American Society for Microbiology.

The GenoType MTBDRplus is a DNA strip assay (Hain Lifescience, Germany) developed for the rapid detection of gene mutations associated with rifampicin and isoniazid resistance (*rpoB* and *katG*) in clinical isolates (Hillemann *et al.*, 2007). Evaluations of the assay conducted by several research groups report high accuracy for rifampicin resistance but lower accuracy for isoniazid (Makinen *et al.*, 2006; Miotto *et al.*, 2006; Hillemann *et al.*, 2007; Bwanga *et al.*, 2009; Yadav *et al.*, 2013). The GenoType MTBDRplus instrument costs 23,790€ and the assay costs approximately 3.50€ per test. The turnaround time is 48 hours (Yadav *et al.*, 2013).

The AID line probe assay detects the most prevalent chromosomal mutations associated with resistance to first-line (isoniazid and rifampin) and second-line (streptomycin, amikacin, capreomycin, fluoroquinolones, and ethambutol) TB drugs (Ritter *et al.*, 2014). This LPA has a total turnaround time of one day. Its design consists of three test modules termed INH/RIF, FQ/EMB, and AG, with the third module detecting mutations related to resistance to the aminoglycosides (AG) (kanamycin, amikacin and streptomycin), and capreomycin (Ritter *et al.*, 2014). Each LPA strip has four control probes (conjugate control, amplification control, mycobacterium genus control and *M. tuberculosis* complex control) in order to verify the test procedures. For results to be considered valid all four control bands should test positive; otherwise, the results are considered invalid. Two studies have reported on the performance of AID in clinical samples; both showed that AID is in agreement (> 91.5% for all other drugs, and 72.9% for ethambutol) with reference tests such as sequencing and DST (Ritter *et al.*, 2014; Molina-Moya *et al.*, 2015). However, Ritter and co-workers mostly used clinical isolates instead of actual patient samples (Ritter *et al.*, 2014). Thus definitive conclusions on the clinical usefulness of AID should be drawn with care, since a ratio of mutant DNA to wild-type DNA below 10% in clinical samples is known to reduce the sensitivity of any molecular assay (Molina-Moya *et al.*, 2015). More studies that evaluate AID in clinical samples are needed in order to determine the clinical usefulness of this assay.

1.12 Future TB diagnostic systems

Though smear microscopy and culture still form the backbone of the typical TB mycobacteriology laboratory in the HBCs, emerging technologies are rapidly changing the landscape and providing hope in the fight against TB. There are more than 81 manufacturers and developers working on

191 TB diagnostic products (tests or associated hardware); 144 of these are already commercially available and 47 are in late- or mid-stage development (Daniels, 2014). A few examples of new TB diagnostics at various stages of development that are under the WHO pre-qualification are listed in Table 5.

Table 5: Tuberculosis diagnostic products in later-stage development or on track for evaluation by the World Health Organization

Test	Type	Sponsor	Status
MeltPro TB/INH	Closed-tube real-time PCR for INH drug resistance	Zeesan Biotech	3-site evaluation using 1,096 clinical isolates; Chinese FDA-approved
Alere Q	fully automated nucleic acid testing platform	Alere	Early stage development; test for TB case detection and drug resistance
Q-TB test	Cartridge-based asymmetric PCR amplification (Q-POC™ platform)	QuantuMDx	Early stage development; permits MTBC and MDR assays simultaneously on the handled device
MeltPro TB/STR	Closed-tube real-time PCR for streptomycin drug resistance	Zeesan Biotech	3-site evaluation using 1,096 clinical isolates
PURE-LAMP	Manual NAAT by loop-mediated isothermal amplification for MTB detection	Eiken	A multisite showed average sensitivity of 97.2% (95% CI, 94.3% to 98.2%) in culture-positive samples (Gray <i>et al.</i> , 2016)
RealTime MTB/TB MDx m2000	Automated real-time for MTB	Abbott	Lower limit of detection than Roche Cobas assay;
GeneChip	Real-time PCR for RMP and INH resistance	CapitalBio	Chinese Center for Disease Control and Prevention in collaboration with; On the market
REBA MTB-XDR	Line-probe assay for fluoroquinolone and second-line injectable drugs	YD Diagnostics	Initial evaluation study in 2015, Marketed
BD MAX MTB assay	Real-time PCR for MTB in automated BD MAX	Becton Dickson	100% sensitivity and specificity for smear-positive samples
CAD 4TB	Digital chest X-ray for TB screening	Delft Imaging systems	Used in ZAMSTAR study
Giant African pouched rats (<i>Cricetomys gambianus</i>)	Trained sniffer rates to detect MTB in sputum	Apopo Foundation	Rats detected 80% of MTB species while ignoring <i>Mycobacterium avium/intracellulare</i>

Test	Type	Sponsor	Status
Lateral flow urine lipoarabinomannan assay (LF-LAM)	Urine dipstick for TB LAM protein	Alere	Approved by WHO in 2015 for diagnosis and screening of active tuberculosis in people living with HIV (World Health Organization, 2015b)
RPA	Isothermal based Recombinase Polymerase Amplification (RPA) using three core enzymes	TwistDx	Sensitivity of 91.4% (95%CI: 85, 97.9) and specificity of 100% (Boyle <i>et al.</i> , 2014)
VerePLEX Lab-On-Chip	Microarray technology Rifampicin and isoniazid resistance	Veredus Laboratories	Released for research use (McNerney <i>et al.</i> , 2015)

Source: Adapted from (Harrington & Benjamin, 2015) with some minor modifications.

In addition, since 2007, several tools with improved sensitivity and speed of diagnosis have been endorsed by the WHO Strategic and Technical Advisory Group for Tuberculosis (STAG-TB) (Table 6). However, in 2014, no TB technologies underwent review. Other technologies not shown in Table 6 were also reviewed and some received negative policy recommendations (e.g. commercial serological antibody-based TB tests; interferon-gamma release assays [IGRAs] for detection of active TB) (World Health Organization, 2011), while others were not endorsed due to lack of adequate evidence (e.g. LAMP); LPAs for second-line drugs (World Health Organization, 2013). The only WHO-endorsed NAATs are the first-generation LPAs and the more recent Xpert® MTB/Rif assay. A significant deterrent to widespread application of NAATs is the need for appropriate field evaluation of newer tests. Currently, there have been limited independent, multicentre evaluations in high-burden settings of the next-generation NAATs, with only two evaluations of LoopAMP™ MTBC Detection Kit and EasyNAT™, and one of each for Genedrive®, Truelab™ and FluoroCycler® technologies (Harrington & Benjamin, 2015).

Table 6: TB diagnostic technologies endorsed by the WHO, since 2007

Year	Technology reviewed by WHO
2007	Commercial liquid culture and DST tools, and rapid speciation strip tests
2008	Molecular LPAs for first-line anti-TB drug resistance detection
2010	LED microscopy
2010	Selected non-commercial DST methods (MODS, CRI and NRA)
2010	Xpert MTB/Rif
2013	Policy update on Xpert MTB/Rif, with extension to childhood and EPTB

Reprinted from (UNITAID, 2015), with permission from the World Health Organization.

Another major deterrent in the development of diagnostic products is the shallow funding landscape in which researchers are asked to confront such a sizeable epidemic with shrinking resources. The 2011–2015 Global Plan calls for annual investments of \$340 million in research to develop new TB diagnostics. In 2013, various funders contributed a total of \$67.8 million to diagnostics research (Figure), leaving a gap of \$272.2 million (Frick, 2014). The major funders of diagnostics research were the Gates Foundation and the US-National Institute of Allergy and Infectious Diseases (NIAID) with investments of \$16.0 and \$15.8 million respectively. Only two companies rank among the top five diagnostics funders in 2013: Company Y contributing \$5.0 million, and Qiagen with \$4.1 million. Alere is another private-sector company with 2013 investments of \$2 million and ranking 10th. However, the figures are not very accurate as the South African government through its various departments and agencies provides significant funding to TB research.

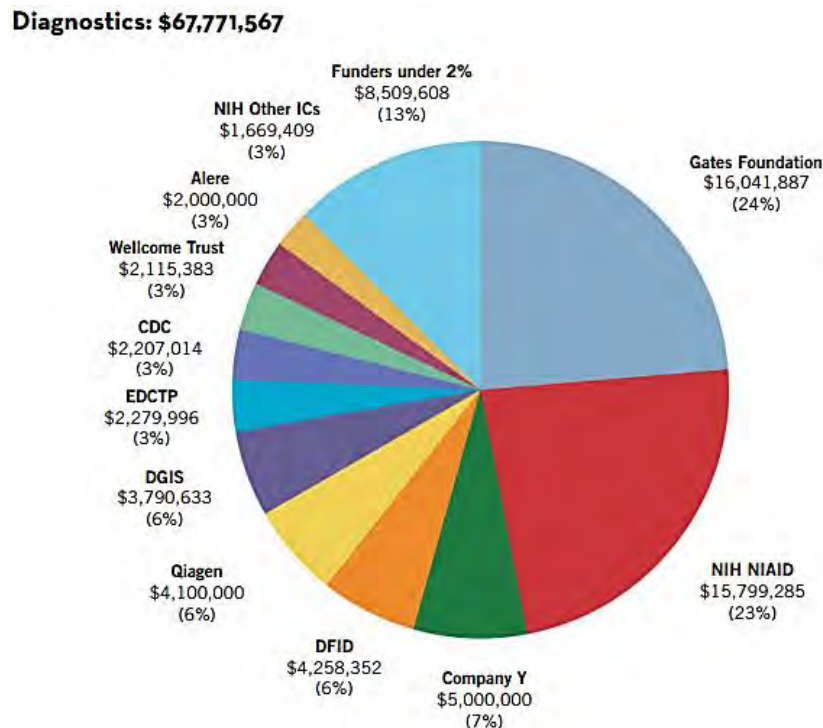


Figure 7: 2013 TB diagnostics funders' contributions. The South African Medical Research Council, which is the only African funding organization on the list, contributed \$15,238. All figures are in United States dollars. Reprinted from (Frick, 2014), with permission the Treatment Action Group.

Despite all these challenges a number of NAATs at various stages of development (Figure 8) are in the pipeline. The assays under development include LoopAMP™ MTBC Detection Kit (Eiken Chemical Company; Japan), EasyNAT™ TB kit (Ustar, China), Genedrive® MTB assay

(Epistem; United Kingdom), and Trueant MTB (Molbio; India). The principles, current status and prospects of some NAATs that can be deployed at microscopy centers will be discussed in the following sections. However, the absence of a dipstick type POC TB test continues to be a gaping hole in the pipeline. This is mainly due to insufficient progress in biomarker research and lack of strong industry interest in TB (Wilson & Palriwala, 2011).

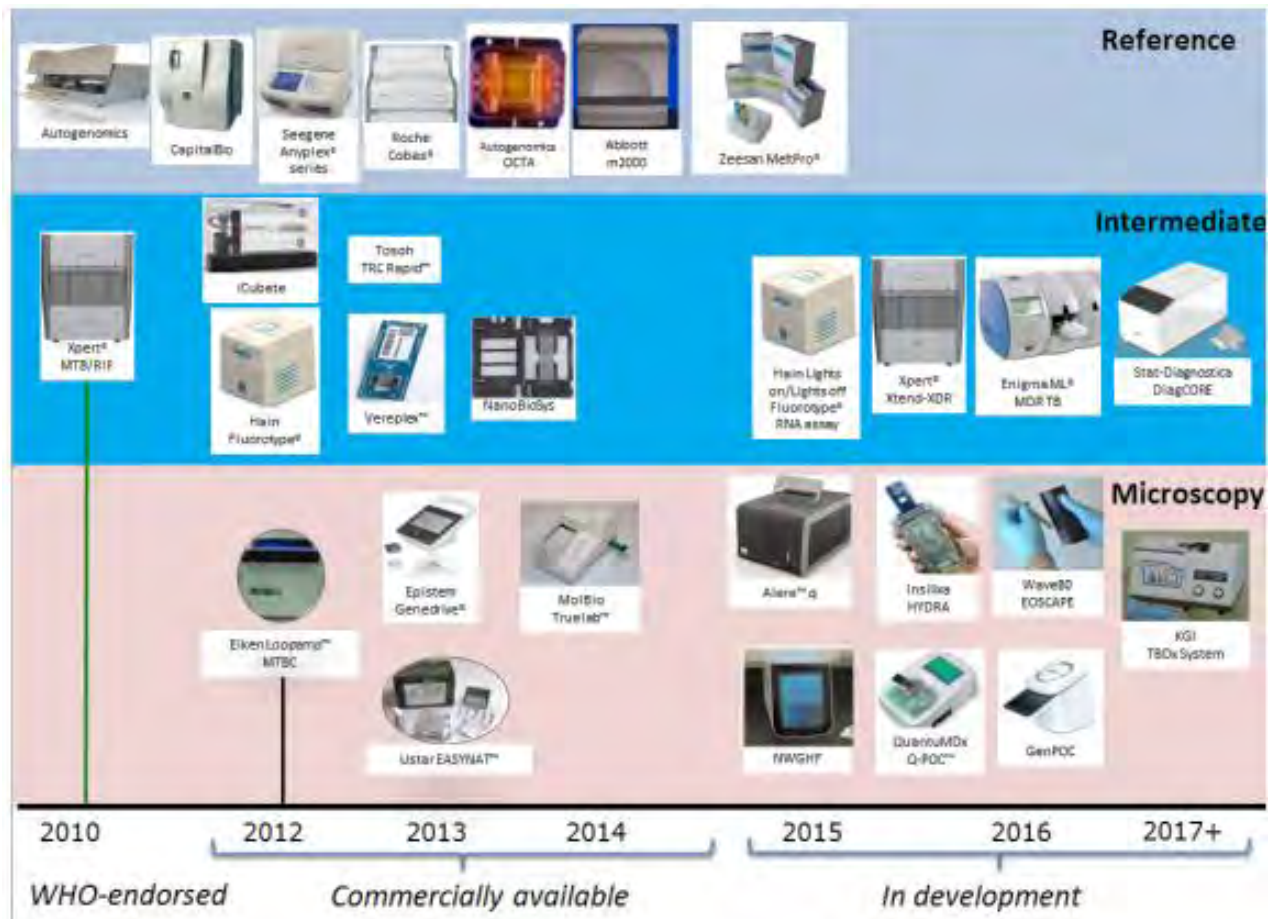


Figure 8: Current and emerging automated, semi-modular or non-integrated TB NAATs; their intended laboratory location and release or anticipated time. Reprinted from (UNITAID, 2014), with permission from the World Health Organization.

1.12.1 LoopAMP™ MTBC Detection Kit

In 2000, Notomi and coworkers first described a molecular method that had the ability to accurately amplify a few copies of DNA 10^9 - 10^{10} times in 15-60 minutes under isothermal conditions (about 65°C) with great accuracy (Notomi *et al.*, 2000). A detailed description of the assay can be found elsewhere (Tomita *et al.*, 2008), but briefly it involves (i) auto-cycling strand displacement DNA synthesis which is performed by a DNA polymerase with high-strand displacement activity and (ii) a set of four specially designed primers that identify a total of six distinct sequences in the target DNA (Figure 9). Eiken Chemical Co. Ltd, Tokyo, Japan has a

commercially available modified LAMP assay, which has a simplified DNA extraction process. In their methodology, raw sputum is treated with two extraction solutions and, following heating for 10 min at 100°C, the sputum is briefly centrifuged at 2,000g. The target of the Eiken LAMP assay is an *M. tuberculosis* specific region of the *gyrB* gene, encoding DNA gyrase subunit B. The amplified product can be seen by direct visual inspection of turbidity or fluorescence in the tube (Neonakis *et al.*, 2011).

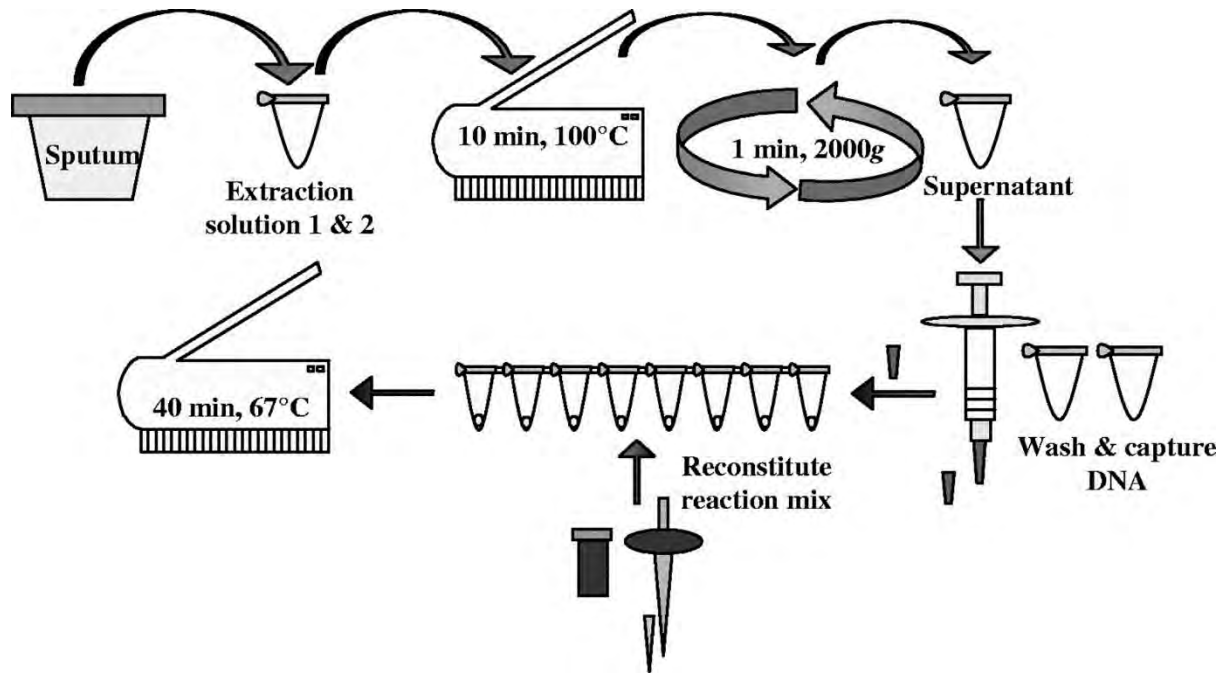


Figure 9: Schematic description of the workflow for LoopAMP MTBC. Currently it is a manual procedure and results are determined by fluorescent signal detection. Reprinted from (UNITAID, 2012), with permission from the World Health Organization.

The first clinical evaluation of the LAMP assay for the detection of pulmonary tuberculosis showed a sensitivity of 97.7% in smear-positive sputum samples and 48.8% in smear-negative samples, and a specificity of 99% (Boehme *et al.*, 2007). A recent study has confirmed that the Eiken-LAMP assay has comparable diagnostic performance to that of the original LAMP (Mitarai *et al.*, 2011). However, the specificity of the TB-LAMP assay remains a major concern especially when the TB prevalence falls below 10%. Also the poor performance (48% sensitivity) in smear-negative patients (Boehme *et al.*, 2010), reduces its utility in some population groups with high rates of smear-negative tuberculosis, such as HIV-positive individuals and children. Feasibility studies in microscopy centres have demonstrated that minimal technical training was required suggesting that the platform is applicable for use in peripheral laboratory settings (Boehme *et al.*, 2007). Further advantages include rapidity, high sensitivity, ease of application and cost-effectiveness.

If Eiken-LAMP is to be adopted as a replacement to smear microscopy especially in high HIV prevalence settings where the sensitivity of sputum smear microscopy is reduced, more head-to-head comparison studies with TB-LAMP and LED microscopy are needed in different geographic regions. Currently, insufficient field evaluation data exists for Eiken-LAMP to receive a recommendation either in favor of, or against by the WHO Expert Review Group (UNITAID, 2015).

1.12.2 EasyNAT TB Diagnostic kit

EasyNAT TB Diagnostic kit (Ustar, China) is another novel assay for diagnosis of active pulmonary TB based on isothermal amplification-Lateral Flow. It was approved by the China Food and Drug Administration (CFDA) on 26th February 2014 but limited peer-reviewed information is available on this system. Briefly, 1 mL of liquefied sputum is heated to lyse the mycobacteria. The lysed sample is then transferred into a DNA purification device, for standard solid-phase extraction using a DNA affinity membrane and a series of wash steps, followed by elution. For MTB detection, the DNA extract is mixed with cross-priming amplification (CPA) reagents, and incubated at 63°C for 1h. Six to eight primers target the *gyrB* gene of *M. tuberculosis* bacilli. The detection of amplified products is performed on a lateral flow strip housed in a sealed XCP nucleic acid detection device (Figure) to minimize cross-contamination of amplicons (Fang *et al.*, 2009; Niemz & Boyle, 2012).

In a landmark clinical evaluation study, the sensitivity of EasyNAT was 87.5% (28/32) in smear-negative sputum samples and 96.9% (63/65) in smear-positive specimens (Fang *et al.*, 2009). Though the sample size was too small, it showed good sensitivity in the problematic smear-negative, culture-positive group, which would make it an ideal replacement of smear microscopy. Screening of 13 non-tuberculous mycobacterial species showed that the assay is 100% specific for TB. The total assay time is approximately 90 minutes at an estimated cost of US \$4 to 5 per test (Mhimbara *et al.*, 2015). The current sample processing step for the EasyNAT system is fairly complicated, and to become a true POC a simplified sputum specimen processing method that shortens total assay time (TAT), minimizes biological risk, and decreases the risk of sample contamination must be developed. Another drawback of the system is its inability to detect TB drug resistance, which is an important requirement in HBCs.

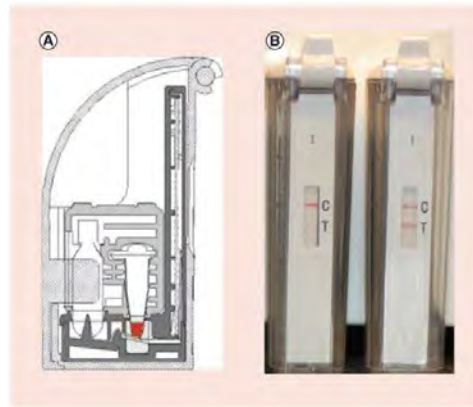


Figure 10: The XCP nucleic acid detection device (Ustar). (A) The schematic cross-section, showing the amplicon cartridge (B) indicates a positive cross-priming amplification test result in addition to control line. Reprinted from (Niemz & Boyle, 2012), with permission from Taylor & Francis publishing group.

1.12.3 Epistem Genedrive

Epistem (Manchester, UK) has developed the Genedrive system, a lightweight (560 g) and portable instrument that enables rapid PCR amplification with real-time detection of MTB from sputum or urine samples in <45 min. Initially, cell lysis and DNA extraction is performed using a novel composite paper-based approach from raw or liquefied sputum. Three 20 µl aliquots of the extract are transferred into the test cartridge (Figure 2) containing lyophilized reagents for PCR amplification. The test cartridge is then inserted into the Genedrive instrument (Figure 2) for PCR amplification targeting *REP13E12* and *rpoB* genes. The instrument automatically reports results at the end of amplification cycles. The instrument performs rapid PCR amplification with probe-based real-time fluorescence detection and high resolution melt curve analysis to verify target amplicon production, and enable genotyping of the *rpoB* RRDR. This US \$4000 instrument can be operated on a 12V DC battery and the anticipated cartridge price will be between US\$ 10 and US\$ 17.



Figure 2: Components of the Genedrive system developed by Epistem. Test cartridge (left) and Genedrive instrument (right). Images (advertorial) reprinted from Epistem (UK). <https://www.genedrive.com/>. Date of access: 6 January 2016

In a recent study conducted in Spain using spiked sputum samples, sensitivity of 90.8% in comparison to culture for MTBC detection and 72.3% for *rpoB* detection was reported (Castan *et al.*, 2014). In the SSM scanty or negative samples characteristic of paubacillary patients, the performance of Genedrive was comparable to GeneXpert. However, these results should be treated with extreme caution as clinical sputum samples were not used in this study. The small sample volume, portability, battery power operation, low cost and performance of the Genedrive make it an ideal POC test for MTBC detection and rifampicin resistance in low resource settings. Multisite studies on clinical samples should be urgently conducted to evaluate this promising system.

1.12.4 Trueant MTB

Trueant MTB (Molbio/Bigtec Diagnostics, India) is a semi-automated, battery-powered, and portable chip based real time PCR device for MTB diagnosis. It involves four simple processes that can be completed within an hour. These processes are (i) sample collection, (ii) sample preparation, (iii) automatic analysis, and (iv) result reporting, respectively (Figure 3). The total assay time is approximately one hour, enabling rapid detection of *M. tuberculosis* DNA.

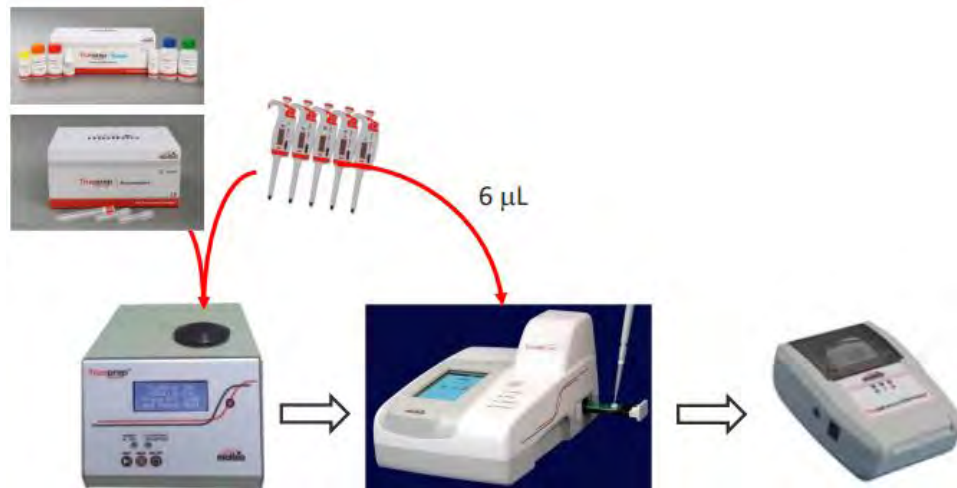


Figure 3: Sputum is added to a red-capped sample bottle containing lyophilized reagents to liquefy the sputum. After DNA extraction 6 µL is aliquoted into a disposable reaction cartridge, which is then entered into the Truelab™ Uno analyser for processing and analysis. Images (advertorial) reprinted from Bigtech Private Ltd (India).

The Truenat MTB test was reported to have sensitivity and specificity of 91.1% (CI: 86.1–94.7) and 100% (CI: 90.0–100) respectively (Nikam *et al.*, 2013). Limited diagnostic performance data is available and larger studies in different geographic regions are urgently required to evaluate the Truenat MTB test.

1.13 Evaluation of new diagnostic tests

After developing, optimizing and obtaining initial proof-of-concept data on the prototype, the next phase involves evaluating its accuracy, reliability and performance outcomes. Figure 4 outlines the TB diagnostics development pathway, formulated by the Stop Tuberculosis Partnership's New Diagnostics Working group (Stop TB Partnership's New Diagnostics Working Group, 2009; Wallis *et al.*, 2010). In diagnostic accuracy studies, outcomes from the new test are compared with outcomes from the reference standard—both measured in the same cohort of TB suspects. Flaws in study design can lead to biased and optimistic estimates of diagnostic accuracy during evaluation. As several potential threats to the internal and external validity of a study on diagnostic accuracy exist, Standards for the Reporting of Diagnostic accuracy studies (STARD) (Table 7) were designed to (i) improve the accuracy and completeness of diagnostic studies, (ii) permit users to evaluate the potential bias in a study, and assess whether results can be extrapolated to a broader population (Bossuyt *et al.*, 2003).

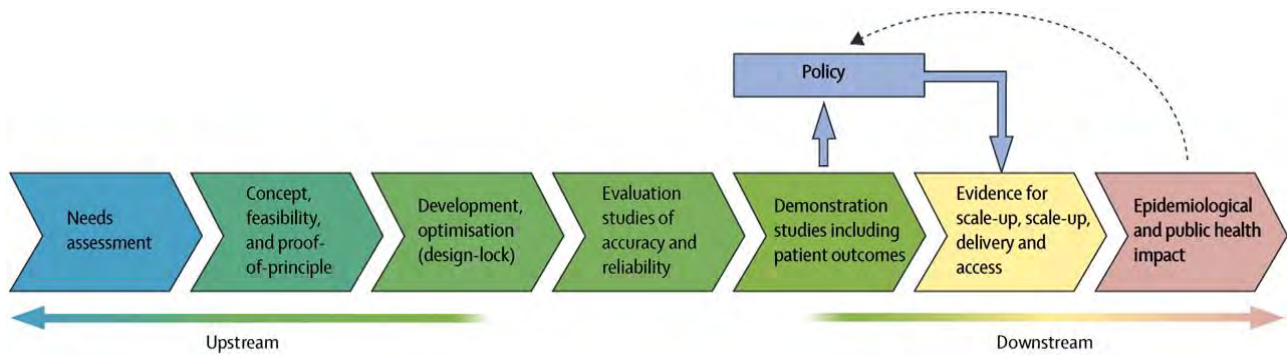


Figure 4: Schematic of the tuberculosis diagnostics development pathway. It must be noted that the process is not completely unidirectional, but there is movement back and forth between the various developmental stages. The schematic was formulated by the Stop tuberculosis Partnership's New Diagnostics Working group. Reprinted from (Wallis *et al.*, 2010), with permission from Elsevier.

Although accuracy of diagnostic tests is an important attribute, it should never be considered in isolation from other benchmarks. The 2004 Health Development Report cited the lack of access and unaffordability as two major reasons why new diagnostics fail (Travis *et al.*, 2004). Some accurate TB diagnostics are available on the market but they are neither affordable nor accessible to most patients in the developing world where disease burden is the greatest. Therefore, research and development of tuberculosis diagnostic tools should not only focus on diagnostic accuracy but they must be also easy to operate, accessible and cost-efficient to be adopted in endemic areas.

1.14 Cost-effectiveness of diagnostic tests

Cost-effectiveness analysis (CEA) is one of a number of economic techniques for assessing gains in health relative to the costs of alternative health interventions (Robinson, 1993). These competing alternatives can be independent or mutually exclusive, but in both cases policy makers still need to rationalize the scarce resources available (Earnshaw & Lewis, 2008). In literature the terms cost-benefit, cost-effectiveness and cost-utility are often used interchangeably but strictly speaking these terms are distinct terms, though related.

Table 7: STARD checklist for reporting of studies of diagnostic accuracy

Section and Topic	Item #	
TITLE/ABSTRACT/KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.
METHODS		
<i>Participants</i>	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?
<i>Test methods</i>	7	The reference standard and its rationale.
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available.
<i>Statistical methods</i>	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).
	13	Methods for calculating test reproducibility, if done.
RESULTS		
<i>Participants</i>	14	When study was performed, including beginning and end dates of recruitment.
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).
<i>Test results</i>	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.

Section and Topic	Item #	
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.
	20	Any adverse events from performing the index tests or the reference standard.
<i>Estimates</i>	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).
	22	How indeterminate results, missing data and outliers of the index tests were handled.
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.
	24	Estimates of test reproducibility, if done.
DISCUSSION	25	Discuss the clinical applicability of the study findings.

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A cost-benefit analysis involves assigning a monetary value to both the cost of the intervention and the benefit (health in this case) derived from such an intervention (Levin & McEwan, 2001). This requirement of placing a direct monetary value on human health (life) is ethically problematic, thus rendering cost-benefit analyses less important in health economics. The main difference between cost-effectiveness analyses (CEA) and cost-utility analysis (CUA) lies in how the benefits are measured and valued. If benefits are measured as life-years gained (LYGs), this will be called CEA and its main output is known as an incremental cost-effectiveness ratio (ICER) (Jakubiak-Lasocka & Jakubczyk, 2014). If instead of LYGs, Quality-Adjusted Life Years (QALYs), the analysis will be known as a cost-utility analysis (CUA) and its main output parameter referred to as an incremental cost-utility ratio (ICUR) (Jakubiak-Lasocka & Jakubczyk, 2014). ICURs are controversial because when calculating QALYs the “years of life” are weighted by a utility score that measures patients’ preferences for a particular state of health, which is highly subjective as individuals or societies have different perceptions on life. Thus in spite of the weaknesses of ICERs to be discussed more fully later, ICERs are considered a more objective measure of effectiveness and is the focus of cost analysis in this thesis. ICER basically refers to the cost differential between two alternative interventions, divided by their effectiveness differential. The following parameters are involved in the computation:

$$ICER = \Delta C / \Delta E = \text{Cost new test} - \text{cost current test} / \text{Effect new test} - \text{effect current test}.$$

1.14.1. Building decision analysis models

The determination of an ICER is a complicated process that requires careful development and validation of appropriate models and collection of appropriate data to input into the model. The stages involved in creating a decision analysis model and the key considerations thereof will be discussed in the following sections.

1.14.1.1 Define the problem

The first and most important step in decision analysis is to define the problem, to identify the possible alternatives available and their consequences. A decision tree (Figure 5), a visual representation of all the possible options and the consequence of each option, assists the modeler in clearly defining the problem. In a decision tree, a decision node (represented by a square) is a point where several alternatives are possible, while chance node (represented by a circle) is a point in a decision tree where chance determines which event will occur. The sum of probabilities for all branches emanating from a chance node must equal 1.0.

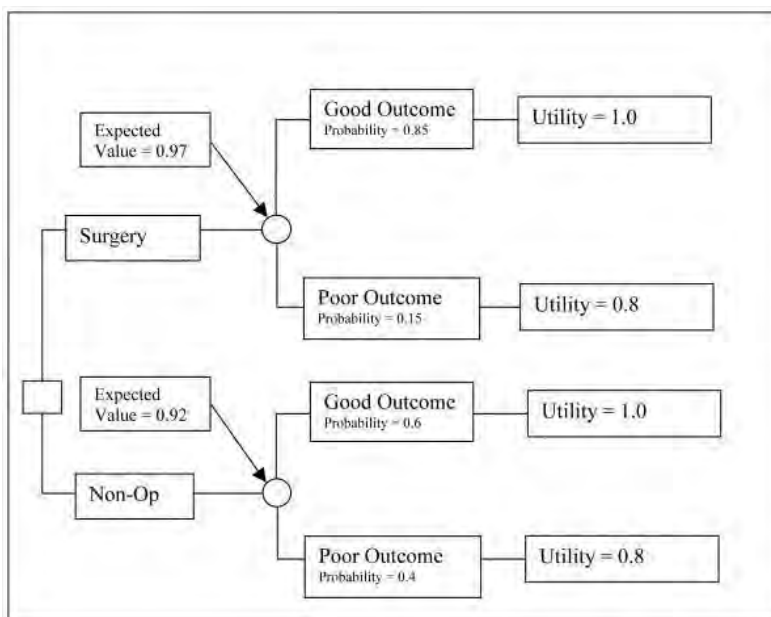


Figure 5: An example of a basic decision tree that can assist in defining the problem in decision analysis.

1.14.1.2 Define the perspective

The perspective determines the components to be included in the costing of the interventions and the manner in which they are valued (Neumann, 2009). A range of perspectives can be used, including the societal, patient, and provider perspectives (Ryder *et al.*, 2009). In the societal perspective all medical and non-medical costs associated of an intervention are relevant, even

though the analyst may not be able to measure or ascribe a value to some of them. For the provider (government/health insurer) perspective, certain categories of costs may not be relevant, such as patient time and travel costs, and indirect costs. The societal perspective requires the broadest approach to characterizing costs and health outcomes of treatment, and is the recommended perspective in public policy applications (Russell et al., 1996). However, due to the difficulties in computing the costs of the societal perspective, health economists often use the provider perspective. It is important to recognize that what may be cost-effective from a societal perspective may not be cost-effective from the perspective of a patient.

1.14.1.3 Define the base case(s) of analysis

This stage involves evaluating both the intended and unintended consequences of the different alternatives in the decision tree. The probabilities of each of those consequences are also considered. The sources of such data include published peer-reviewed articles, meta-analyses, ongoing study data, in country expert opinion, and other trusted sources such as UNAIDS and WHO life tables.

1.14.1.4 Specify the time horizon, and define thresholds for analysis

In decision tree models, events and outcomes are modelled over a specified time horizon or period of time during which the benefits of the intervention are expected to materialise. This is critical as short-term vs. long-term costs/effects may be very different and thus can alter the C/E ratio of a given intervention depending on the time horizon used in the analysis. Ideally the time horizons used range from 1 year to 10 years (Ramsey *et al.*, 2005).

In order for an analyst to make objective recommendations from ICER results emanating from different studies/settings there is need for a reference point to enable proper comparison. For example, how does one decide whether US\$ 450 per QALY gained in Brazil or US\$ 27 per QALY gained in the Tanzania represents good use of money for the national health-care system? Three approaches have been put forward to address this challenge: (i) thresholds based on per capita national incomes; (ii) benchmark interventions and (iii) league tables. Of the three approaches the use of thresholds has gained popularity. There are a number of widely referenced thresholds that are used to define the boundaries of a cost-effective intervention. Arbitrary thresholds such as \$50,000/QALY in the United States or £20,000/QALY in the United Kingdom are used sometimes but these are highly controversial because they are not generalizable across settings and are not empirically-derived (Diamond & Kaul, 2009).

Recently, the use of thresholds that are based on per capita gross domestic product (GDP) has gained prominence in cost-effectiveness analyses studies and is promoted by the World Health Organization in that organization's publication: Choosing Interventions that are Cost-Effective (WHO-CHOICE) project (World Health Organization, 2015a). Generally an intervention is considered to be highly cost-effective when the cost per DALY averted is below the WHO-CHOICE country-specific willingness-to-pay (WTP) threshold. This threshold value is dependent on economic status and cultural factors, thus is variable between nations or even regions (Francis *et al.*, 2013).

Another important consideration in cost-effectiveness analysis is the selection of an appropriate discount rate. Discounting means the conversion of future costs to their present value. The concept is informed by the fact that individuals and society have general preferences to consume something now over consumption in the future. For comparison across studies, it is important that analyses are performed using a common discount rate. In this regard the WHO-CHOICE uses a discount rate of 3%. However as with many other issues in cost-effectiveness analyses, this practice is widely debated (Van Hout, 1998; Gravelle & Smith, 2001; Reinschmidt, 2002).

1.14.1.5 Populate the model with probabilities, costs, and effectiveness data

Once all of the components of the model have been gathered from the various sources, this data can now be populated in the model and then perform the decision analysis using mathematical equations. However, nowadays a variety of software packages (e.g. Tree Age Pro and Decision Tools Suite) are available which can calculate the ICER and sensitivity analyses based on the formulas.

1.14.1.6 Conduct sensitivity analyses

Since estimates of costs and effects are used in decision analysis, these are often bound to vary with location, season and a multiple other factors. Sensitivity analyses tests show how robust the model is to these uncertainties by varying model inputs and evaluating their effect on the results from the model. Sensitivity analysis also serves as an alternative to confidence intervals which do not have value in decision analysis. One-way, two-way and three-way sensitivity analyses are often reported based on how many variables are allowed to fluctuate at a time; one, two or three respectively (Werner *et al.*, 2013).

1.14.2 Weaknesses of cost-effectiveness analysis models

Cost-effectiveness analysis has a number of limitations that are well recognized but that does not diminish its value in health economics. Rather, it simply requires that the “consumers” of this information (i.e, guideline committees, policy makers, and health insurers) be properly informed about its strengths and limitations (Weintraub & Cohen, 2009). First and foremost, the quality of the data fed into the model determines the quality of the outputs. If the literature search and background data gathering is biased then no matter how excellent the research questions and decision model are, the outcomes will be biased. In recent years standardized methods for conducting these analyses have been developed in order to minimize bias. However, at times it is unrealistic to perfectly adhere to these standards and compromises that are scientifically justifiable are often made (Russell *et al.*, 1996).

Cost-effectiveness analyses will certainly always require a number of assumptions to be made, as it is not possible to quantitatively measure all the parameters necessary for such a model. Even in cases where measurements are available, they may not adequately represent values appropriate for the analysis at hand. Therefore the rationale behind these assumptions also influences the quality of the outputs. Also, quality of life estimation (e.g QALYS) that involves making considerable assumptions may present with several methodological errors. Practically it is impossible to reduce such a qualitative phenomenon (quality of life) that incorporates diverse personal, cultural and psychological beliefs into a single numerical value (Francis *et al.*, 2013). Although disability weights/utilities are well studied and widely used in quality of life assessment (Salomon *et al.*, 2013), they are still an imperfect method of quantifying such a qualitative outcome. Notwithstanding, these limitations, cost-effectiveness analysis remains a useful tool for guiding clinicians, researchers, and policy makers in making delicate decisions related to the allocation of scarce resources. It is however of equal importance to understand that it only represents a limited dimension of decision-making.

1.15 Conclusion

This chapter has reviewed literature on tuberculosis epidemiology, current diagnostic tests and their limitations and finally looked at some tuberculosis diagnostic tests in the pipeline. The magnitude of the tuberculosis burden in the world, especially among 22 high burden countries is huge and is compounded by the HIV epidemic. The accuracy of diagnostic tests is lower in HIV/TB coinfecting individuals. Sputum smear microscopy, which is the test mostly relied upon in the majority of high burden settings, has sensitivity as low as 20% in HIV/TB coinfecting

individuals due to the low bacillary load. It will be a catastrophe to allow such a scenario to persist. New TB diagnostics with high sensitivity and specificity in paucibacillary HIV infected individuals are urgently needed.

Since TB infections predominantly occur in low resource settings due to poor living conditions, new tests should be affordable, cost-effective and rapidly provide results to allow initiation of appropriate therapy during the same clinical encounter. Patient dropout due to the need to make multiple visits to the clinic to provide specimens and collect results when using tests that have long turnaround times or require multiple samples has been cited as one of the drivers in the spread of both MTB and MDR-TB. Nucleic acid amplification based tests have a short turnaround time providing results within a few hours after receiving samples. This allows patients to be commenced on appropriate therapy on the same day thus reducing the dropout rate. Sensitive detection of pathogens is dependent on the availability of quality DNA or RNA templates. Thus efficient sample processing methods that provide quality templates are critical. Traditional methods for DNA/RNA extraction are labour intensive, lengthy, and require highly specialized skill, and this hindered the adoption of molecular methods at the primary/district levels. Recent TB tests such as the Xpert MTB/Rif and the Genedrive rely on the use of cartridges. Sample extraction, amplification and detection are all carried out within the self-contained, single use cartridge. This eliminates the need for intensive manual labour and highly specialized skill but this comes at a huge cost (Choi *et al.*, 2013). Cost-effective sample processing methods that offer similar advantages to cartridges will improve access to nucleic acid based TB tests.

As highlighted earlier in this chapter, a number of TB tests exist but there is little data from large multi-site evaluation studies to guide policy makers in choosing the most appropriate technologies. After developing any new tests, innovators must create the necessary collaborations to evaluate the tests in appropriate settings. These studies should follow STARD guidelines for conducting evaluation studies in order to avoid bias and allow comparison of data from different studies. After tests with better performance (sensitive and specific) have been identified, cost-effectiveness analyses should be prioritized before implementation. This approach will minimize post-market failure of new tests due to market related factors such as willingness to pay, availability of funding for national TB programs, infrastructure, and size of the market.

It remains a challenge to evaluate the comparative qualitative and quantitative value of these tests and the use of a number of value parameters have been described and will be applied to the NWU TB diagnostic system in Chapter 6 of the thesis.

1.16 References

- Albert, H., Nakiyingi, L., Sempa, J., Mbabazi, O., Mukkada, S., Nyesiga, B., Perkins, M.D. & Manabe, Y.C. 2013. Operational Implementation of LED Fluorescence Microscopy in Screening Tuberculosis Suspects in an Urban HIV Clinic in Uganda. *PLoS ONE*, 8(9):e72556.
- Alexander, K.A., Laver, P.N., Michel, A.L., Williams, M., van Helden, P.D., Warren, R.M. & Gey van Pittius, N.C. 2010. Novel Mycobacterium tuberculosis Complex Pathogen, *M. mungi*. *Emerging Infectious Diseases*, 16(8):1296-1299.
- Anderson, S.T., Kaforou, M., Brent, A.J., Wright, V.J., Banwell, C.M., Chagaluka, G., Crampin, A.C., Dockrell, H.M., French, N., Hamilton, M.S., Hibberd, M.L., Kern, F., Langford, P.R., Ling, L., Mlotha, R., Ottenhoff, T.H., Pienaar, S., Pillay, V., Scott, J.A., Twahir, H., Wilkinson, R.J., Coin, L.J., Heyderman, R.S., Levin, M. & Eley, B. 2014. Diagnosis of childhood tuberculosis and host RNA expression in Africa. *New England Journal of Medicine*, 370(18):1712-1723.
- Arbex, M.A., Varella, M.d.C.L., Siqueira, H.R.d. & Mello, F.A.F.d. 2010. Drogas antituberculose: interações medicamentosas, efeitos adversos e utilização em situações especiais - parte 1: fármacos de primeira linha. *Jornal Brasileiro de Pneumologia*, 36:626-640.
- Ardito, F., Posteraro, B., Sanguinetti, M., Zanetti, S. & Fadda, G. 2001. Evaluation of BACTEC Mycobacteria Growth Indicator Tube (MGIT 960) Automated System for Drug Susceptibility Testing of Mycobacterium tuberculosis. *Journal of Clinical Microbiology*, 39(12):4440-4444.
- Arias, M., Mello, F.C.Q., Pavón, A., Marsico, A.G., Alvarado-Gálvez, C., Rosales, S., Carvalho Pessôa, C.L., Pérez, M., Andrade, M.K., Kritski, A.L., Fonseca, L.S., Chaisson, R.E., Kimerling, M.E. & Dorman, S.E. 2007. Clinical evaluation of the microscopic-observation drug-susceptibility assay for detection of tuberculosis. *Clinical Infectious Diseases*, 44(5):674-680.
- Asmar, S. & Drancourt, M. 2015. Chlorhexidine decontamination of sputum for culturing Mycobacterium tuberculosis. *BMC Microbiology*, 15(1):1-6.
- Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P.G., Sriram, N., Wongsrichanalai, C., Linke, R., O'Brien, R., Perkins, M., Cunningham, J., Matsoso, P., Nathanson, C.M., Olliaro, P., Peeling, R.W. & Ramsay, A. 2008. Evaluation of diagnostic tests for infectious diseases: general principles. *Nature Reviews Microbiology* S16-S28. doi:10.1038/nrmicro1523.

- Barnard, M., Albert, H., Coetzee, G., O'Brien, R. & Bosman, M.E. 2008. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. *American Journal of Respiratory and Critical Care Medicine* 177(7):787-792.
- Batz, H.-G., Cooke, G.S. & Reid, S.D. 2011. Towards lab-free tuberculosis diagnosis. *Treatment Action Group, the TB/HIV Working Group of the Stop TB Partnership, Imperial College, MSF. Geneva.*
- Behr, M.A., Warren, S.A., Salamon, H., Hopewell, P.C., de Leon, A.P., Daley, C.L. & Small, P.M. 1999. Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli. *The Lancet*, 353(9151):444-449.
- Bemer, P., Bodmer, T., Munzinger, J., Perrin, M., Vincent, V. & Drugeon, H. 2004. Multicenter Evaluation of the MB/BACT System for Susceptibility Testing of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, 42(3):1030-1034.
- Bergamini, B.M., Losi, M., Vaienti, F., D'Amico, R., Meccugni, B., Meacci, M., De Giovanni, D., Rumpianesi, F., Fabbri, L.M., Balli, F. & Richeldi, L. 2009. Performance of Commercial Blood Tests for the Diagnosis of Latent Tuberculosis Infection in Children and Adolescents. *Pediatrics*, 123(3):e419-e424.
- Boehme, C.C., Nabeta, P., Henostroza, G., Raqib, R., Rahim, Z., Gerhardt, M., Sanga, E., Hoelscher, M., Notomi, T., Hase, T. & Perkins, M.D. 2007. Operational feasibility of using loop-mediated isothermal amplification for diagnosis of pulmonary tuberculosis in microscopy centers of developing countries. *Journal of Clinical Microbiology*, 45(6):1936-1940.
- Boehme, C.C., Nabeta, P., Hillemann, D., Nicol, M.P., Shenai, S., Krapp, F., Allen, J., Tahirli, R., Blakemore, R., Rustomjee, R., Milovic, A., Jones, M., O'Brien, S.M., Persing, D.H., Ruesch-Gerdes, S., Gotuzzo, E., Rodrigues, C., Alland, D. & Perkins, M.D. 2010. Rapid molecular detection of tuberculosis and rifampin resistance. *New England Journal of Medicine*, 363(11):1005-1015.

Bossuyt, P.M., Reitsma, J.B., Bruns, D.E., Gatsonis, C.A., Glasziou, P.P., Irwig, L.M., Moher, D., Rennie, D., de Vet, H.C.W. & Lijmer, J.G. 2003. The STARD Statement for Reporting Studies of Diagnostic Accuracy: Explanation and Elaboration. *Clinical Chemistry*, 49(1):7-18.

Boyle, D.S., McNerney, R., Teng Low, H., Leader, B.T., Pérez-Osorio, A.C., Meyer, J.C., O'Sullivan, D.M., Brooks, D.G., Piepenburg, O. & Forrest, M.S. 2014. Rapid Detection of *Mycobacterium tuberculosis* by Recombinase Polymerase Amplification. *PLoS ONE*, 9(8):e103091.

Broekmans, J., Caines, K. & Paluzzi, J.E. 2005. Investing in strategies to reverse the global incidence of TB. London: Earthscan.

Bruchfeld, J., Aderaye, G., Palme, I.B., Bjorvatn, B., Kallenius, G. & Lindquist, L. 2000. Sputum concentration improves diagnosis of tuberculosis in a setting with a high prevalence of HIV. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 94(6):677-680.

Brunello, F. & Fontana, R. 2000. Reliability of the MB/BacT System for Testing Susceptibility of *Mycobacterium tuberculosis* Complex Isolates to Antituberculous Drugs. *Journal of Clinical Microbiology*, 38(2):872-873.

Bwanga, F., Hoffner, S., Haile, M. & Joloba, M. 2009. Direct susceptibility testing for multi drug resistant tuberculosis: A meta-analysis. *BMC Infectious Diseases*, 9(1):67.

Castan, P., dePablo, A., Fernández-Romero, N., Rubio, J., Cobb, B.D., Mingorance, J. & Toro, C. 2014. Point-of-Care System for Detection of *Mycobacterium tuberculosis* and Rifampin Resistance in Sputum Samples. *Journal of Clinical Microbiology*, 52(2):502-507.

Caviedes, L. & Moore, D.A.J. 2007. Introducing mods: A low-cost, low-tech tool for high-performance detection of tuberculosis and multidrug resistant tuberculosis. *Indian Journal of Medical Microbiology*, 25(2):87-88.

Chatterjee, M., Bhattacharya, S., Karak, K. & Dastidar, S.G. 2013. Effects of different methods of decontamination for successful cultivation of *Mycobacterium tuberculosis*. *The Indian Journal of Medical Research*, 138(4):541-548.

- Choi, H.W., Miele, K., Dowdy, D. & Shah, M. 2013. Cost-effectiveness of Xpert® MTB/RIF for diagnosing pulmonary tuberculosis in the United States. *The International Journal of Tuberculosis and Lung Disease* 17(10):1328-1335.
- Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S.V., Eiglmeier, K., Gas, S., Barry, C.E., 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, M.A., Rajandream, M.A., Rogers, J., Rutter, S., Seeger, K., Skelton, J., Squares, R., Squares, S., Sulston, J.E., Taylor, K., Whitehead, S. & Barrell, B.G. 1998. Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature*, 393(6685):537-544.
- Collins, D.M. & Stephens, D.M. 1991. Identification of an insertion sequence, IS1081, in Mycobacterium bovis. *FEMS Microbiology Letters* 67(1):11-15.
- Corbett, E.L., Watt, C.J., Walker, N. & et al. 2003. The growing burden of tuberculosis: Global trends and interactions with the hiv epidemic. *Archives of Internal Medicine*, 163(9):1009-1021.
- Coronel, J., Roper, M.H., Herrera, C., Bonilla, C., Jave, O., Gianella, C., Sabogal, I., Huancaré, V., Leo, E., Tyas, A., Mendoza-Ticona, A., Caviedes, L. & Moore, D.A.J. 2014. Validation of microscopic observation drug susceptibility testing for rapid, direct rifampicin and isoniazid drug susceptibility testing in patients receiving tuberculosis treatment. *Clinical Microbiology and Infection*, 20(6):536-541.
- Costa, P., Amaro, A., Ferreira, A.S., Machado, D., Albuquerque, T., Couto, I., Botelho, A., Viveiros, M. & Inácio, J. 2014. Rapid identification of veterinary-relevant Mycobacterium tuberculosis complex species using 16S rDNA, IS6110 and Regions of Difference-targeted dual-labelled hydrolysis probes. *Journal of Microbiological Methods*, 107(0):13-22.
- Couturier, B.A., Weight, T., Elmer, H. & Schlager, R. 2014. Antepartum Screening for Group B Streptococcus by Three FDA-Cleared Molecular Tests and Effect of Shortened Enrichment Culture on Molecular Detection Rates. *Journal of Clinical Microbiology*, 52(9):3429-3432.

Creswell, J., Sahu, S., Sachdeva, K.S., Ditiu, L., Barreira, D., Mariandyshev, A., Mingting, C. & Pillay, Y. 2014. Tuberculosis in BRICS: challenges and opportunities for leadership within the post-2015 agenda. *Bulletin of the World Health Organization*, 92(6):459-460.

Daniels, C. 2014. The tuberculosis diagnostics pipeline 2014. New York. URL: <http://www.pipelinereport.org/2014/tb-diagnostics>. Date of access: 11 February 2015.

de Jong, B.C., Antonio, M. & Gagneux, S. 2010. *Mycobacterium africanum*—Review of an Important Cause of Human Tuberculosis in West Africa. *PLoS Neglected Tropical Diseases*, 4(9):e744.

Denkinger, C.M., Nicolau, I., Ramsay, A., Chedore, P. & Pai, M. 2013. Are peripheral microscopy centres ready for next generation molecular tuberculosis diagnostics? *European Respiratory Journal*, 42(2):544-547.

Dheda, K., Warren, R.M., Zumla, A. & Grobusch, M.P. 2010. Extensively drug-resistant tuberculosis: epidemiology and management challenges. *Infectious Disease Clinics of North America*, 24(3):705-725.

Diamond, G.A. & Kaul, S. 2009. Cost, effectiveness, and cost-effectiveness. *Circulation: Cardiovascular Quality and Outcomes*, 2(1):49-54.

Diedrich, C.R. & Flynn, J.L. 2011. HIV-1/*Mycobacterium tuberculosis* Coinfection Immunology: How Does HIV-1 Exacerbate Tuberculosis? *Infection and Immunity*, 79(4):1407-1417.

Dowdy, D., Maters, A., Parrish, N., Beyrer, C. & Dorman, S. 2003. Cost-effectiveness of the Gen-Probe amplified *Mycobacterium tuberculosis* direct test as used routinely on smear-positive respiratory specimens. *Journal of Clinical Microbiology*, 41:948–953.

Drobatz, K.J. 2009. Measures of accuracy and performance of diagnostic tests. *Journal of Veterinary Cardiology*, 11, Supplement 1:S33-S40.

Ducati, R.G., Ruffino-Netto, A., Basso, L.A. & Santos, D.S. 2006. The resumption of consumption : a review on tuberculosis. *Memórias do Instituto Oswaldo Cruz*, 101:697-714.

- Earnshaw, J. & Lewis, G. 2008. NICE guide to the methods of technology appraisal. *Pharmacoeconomics*, 26(9):725-727.
- Eusebi, P. 2013. Diagnostic Accuracy Measures. *Cerebrovascular Diseases*, 36(4):267-272.
- Falodun, O.I., Adesokan, H.K. & Cadmus, S.I.B. 2012. Recovery rates of Mycobacterium tuberculosis using five decontamination methods. *African journal of medicine and medical sciences*, 41 Suppl:181-185.
- Fang, R., Li, X., Hu, L., You, Q., Li, J., Wu, J., Xu, P., Zhong, H., Luo, Y., Mei, J. & Gao, Q. 2009. Cross-Priming Amplification for Rapid Detection of Mycobacterium tuberculosis in Sputum Specimens. *Journal of Clinical Microbiology*, 47(3):845-847.
- Farzam, B., Imani Fooladi, A.A., Izadi, M., Hossaini, H.M. & Feizabadi, M.M. 2015. Comparison of cyp141 and IS6110 for detection of Mycobacterium tuberculosis from clinical specimens by PCR. *Journal of Infection and Public Health*, 8(1):32-36.
- Flores, L., Pai, M., Colford, J. & Riley, L. 2005. In-house nucleic acid amplification tests for the detection of Mycobacterium tuberculosis in sputum specimens: meta-analysis and meta-regression. *BMC Microbiology*, 5(1):55.
- Food and Drug Administration. 2013. FDA permits marketing of first US test labeled for simultaneous detection of tuberculosis bacteria and resistance to the antibiotic rifampin. *Clinical Infectious Diseases*, 57.
- Francis, S.A., Daly, C., Heydari, B., Abbasi, S., Shah, R.V. & Kwong, R.Y. 2013. Cost-effectiveness analysis for imaging techniques with a focus on cardiovascular magnetic resonance. *Journal of Cardiovascular Magnetic Resonance*, 15(1):1-11.
- Frick, M. 2014. Tuberculosis Research and development: 2014 Report on Tuberculosis Research Funding Trends, 2005–2013. URL: http://www.treatmentactiongroup.org/sites/g/files/g450272/f/201505/TAG_2014_TB_Funding_Report_2nd_Ed.pdf. Date of access: 28 February 2015.

- Gandhi, N.R., Nunn, P., Dheda, K., Schaaf, H.S., Zignol, M., van Soolingen, D., Jensen, P. & Bayona, J. 2010. Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis. *The Lancet*, 375(9728):1830-1843.
- Glynn, J.R., Crampin, A.C., Yates, M.D., Traore, H., Mwaungulu, F.D., Ngwira, B.M., Ndlovu, R., Drobniewski, F. & Fine, P.E.M. 2005. The Importance of Recent Infection with *Mycobacterium tuberculosis* in an Area with High HIV Prevalence: A Long-Term Molecular Epidemiological Study in Northern Malawi. *Journal of Infectious Diseases*, 192(3):480-487.
- Grange, J.M. 2001. *Mycobacterium bovis* infection in human beings. *Tuberculosis* 81(1-2):71-77.
- Gravelle, H. & Smith, D. 2001. Discounting for health effects in cost–benefit and cost-effectiveness analysis. *Health economics*, 10(7):587-599.
- Gray, C.M., Katamba, A., Narang, P., Giraldo, J., Zamudio, C., Joloba, M., Narang, R., Paramasivan, C.N., Hillemann, D., Nabeta, P., Amisano, D., Alland, D., Cobelens, F. & Boehme, C.C. 2016. Feasibility and Operational Performance of Tuberculosis Detection by Loop-Mediated Isothermal Amplification Platform in Decentralized Settings: Results from a Multicenter Study. *Journal of Clinical Microbiology*, 54(8):1984-1991.
- Greco, S., Girardi, E., Navarra, A. & Saltini, C. 2006. The current evidence on diagnostic accuracy of commercial based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. *Thorax*, 61:783–790.
- Greco, S., Rulli, M., Girardi, E., Piersimoni, C. & Saltini, C. 2009. Diagnostic Accuracy of In-House PCR for Pulmonary Tuberculosis in Smear-Positive Patients: Meta-Analysis and Metaregression. *Journal of Clinical Microbiology*, 47(3):569-576.
- Guney, C., Suel, A., Karagoz, T. & Yorulmaz, Y. 2007. P1642 Evaluation of the BACT/ALERT 3D system for testing susceptibility of *Mycobacterium tuberculosis* to first-line antituberculosis agents: comparison with BacTec MGIT 960 and Lowenstein-Jensen proportion method. *International Journal of Antimicrobial Agents*, 29, Supplement 2(0):S463.

Gutierrez, M.C., 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 Pathogens*, 1(1):e5.

Hanrahan, C.F., Selibas, K., Deery, C.B., Dansey, H., Clouse, K., Bassett, J., Scott, L., Stevens, W., Sanne, I. & Van Rie, A. 2013. Time to Treatment and Patient Outcomes among TB Suspects Screened by a Single Point-of-Care Xpert MTB/RIF at a Primary Care Clinic in Johannesburg, South Africa. *PLoS ONE*, 8(6):e65421.

Harrington, M. & Benjamin, W. 2015. The Tuberculosis Diagnostics Pipeline. *2015 PIPELINE REPORT*:153. URL:

<http://www.pipelinereport.org/sites/g/files/g575521/f/201507/TB%20Diagnostics.pdf>. Date of access: 16 December 2015.

Hatherill, M., Hanslo, M., Hawkrigde, T., Little, F., Workman, L., Mahomed, H., Tameris, M., Moyo, S., Geldenhuys, H., Hanekom, W., Geiter, L. & Hussey, G. 2010. Structured approaches for the screening and diagnosis of childhood tuberculosis in a high prevalence region of South Africa. *Bulletin of the World Health Organization*, 88(4):312-320.

Helb, D., Jones, M., Story, E., Boehme, C., Wallace, E., Ho, K., Kop, J., Owens, M.R., Rodgers, R., Banada, P., Safi, H., Blakemore, R., Lan, N.T.N., Jones-López, E.C., Levi, M., Burday, M., Ayakaka, I., Mugerwa, R.D., McMillan, B., Winn-Deen, E., Christel, L., Dailey, P., Perkins, M.D., Persing, D.H. & Alland, D. 2010. Rapid Detection of *Mycobacterium tuberculosis* and Rifampin Resistance by Use of On-Demand, Near-Patient Technology. *Journal of Clinical Microbiology*, 48(1):229-237.

Hesseling, A.C., Schaaf, H.S., Gie, R.P., Starke, J.R. & Beyers, N. 2002. A critical review of diagnostic approaches used in the diagnosis of childhood tuberculosis. *The International Journal of Tuberculosis and Lung Disease*, 6(12):1038-1045.

Hillemann, D., Rüsç-Gerdes, S. & Richter, E. 2007. Evaluation of the GenoType MTBDRplus Assay for Rifampin and Isoniazid Susceptibility Testing of *Mycobacterium tuberculosis* Strains and Clinical Specimens. *Journal of Clinical Microbiology*, 45(8):2635-2640.

Hirano, K., Abe, C. & Takahashi, M. 1999. Mutations in the rpoB Gene of Rifampin-Resistant Mycobacterium tuberculosis Strains Isolated Mostly in Asian Countries and Their Rapid Detection by Line Probe Assay. *Journal of Clinical Microbiology*, 37(8):2663-2666.

Hologic Gen-Probe inc. Amplified MTD Test Performance Data. <http://www.gen-probe.com/products-services/amplified-mtd> Date of access: 13 March 2015

Huang, Z., Li, G., Chen, J., Li, W., Xu, X., Luo, Q., Xiong, G., Sun, J. & Li, J. 2014. Evaluation of MODS assay for rapid detection of Mycobacterium tuberculosis resistance to second-line drugs in a tertiary care tuberculosis hospital in China. *Tuberculosis*, 94(5):506-510.

Jakubiak-Lasocka, J. & Jakubczyk, M. 2014. Cost-effectiveness versus Cost-Utility Analyses: What Are the Motives Behind Using Each and How Do Their Results Differ?—A Polish Example. *Value in Health Regional Issues*, 4:66-74.

Jang, M.-A., Koh, W.-J., Huh, H.J., Kim, S.-Y., Jeon, K., Ki, C.-S. & Lee, N.Y. 2014. Distribution of Nontuberculous Mycobacteria by Multigene Sequence-Based Typing and Clinical Significance of Isolated Strains. *Journal of Clinical Microbiology*, 52(4):1207-1212.

Kantor de Narvaiz, I., Kim Jae, S., Frieden, T., Laszlo, A., Luelmo, F., Norval, P.-Y., Rieder, H., Valenzuela, P. & Weyer, K. 1998. Laboratory services in tuberculosis control, part II: Microscopy. Geneva, Switzerland. URL: [http://apps.who.int/iris/bitstream/10665/65942/1/WHO_TB_98.258_\(part1\).pdf](http://apps.who.int/iris/bitstream/10665/65942/1/WHO_TB_98.258_(part1).pdf). Date of access: 11 June 2014.

Kik, S.V., Denking, C.M., Chedore, P. & Pai, M. 2014. Replacing smear microscopy for the diagnosis of tuberculosis: what is the market potential? *European Respiratory Journal*, 43(6):1793-1796.

Kivihya-Ndugga, L.E.A., van Cleeff, M.R.A., Githui, W.A., Nganga, L.W., Kibuga, D.K., Odhiambo, J.A. & Klatser, P.R. 2003. A comprehensive comparison of Ziehl-Neelsen and fluorescence microscopy for the diagnosis of tuberculosis in a resource-poor urban setting. *The International Journal of Tuberculosis and Lung Disease*, 7(12):1163-1171.

- Koeck, J.L., Fabre, M., Simon, F., Daffé, M., Garnotel, é., Matan, A.B., Gérôme, P., Bernatas, J.J., Buisson, Y. & Pourcel, C. 2010. Clinical characteristics of the smooth tubercle bacilli 'Mycobacterium canettii' infection suggest the existence of an environmental reservoir. *Clinical Microbiology and Infection*, 17(7):1013-1019.
- Kontos, F., Maniati, M., Costopoulos, C., Gitti, Z., Nicolaou, S., Petinaki, E., Anagnostou, S., Tselentis, I. & Maniatis, A.N. 2004. Evaluation of the fully automated Bactec MGIT 960 system for the susceptibility testing of Mycobacterium tuberculosis to first-line drugs: a multicenter study. *Journal of Microbiological Methods*, 56(2):291-294.
- Kremer, K., van Soolingen, D., van Embden, J., Hughes, S., Inwald, J. & Hewinson, G. 1998. Mycobacterium microti: More Widespread than Previously Thought. *Journal of Clinical Microbiology*, 36(9):2793-2794.
- Krieger, J. & Higgins, D. 2002. Housing and Health: Time Again for Public Health Action. *American Journal of Public Health* 92(5):758–768.
- Lawn, S.D., Butera, S.T. & Shinnick, T.M. 2002. Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to Mycobacterium tuberculosis. *Microbes and Infection*, 4(6):635-646.
- Levin, H.M. & McEwan, P.J. 2001. Cost-effectiveness analysis: Methods and applications. Vol. 4: Sage Publications.
- Lighter, J. & Rigaud, M. 2009. Diagnosing childhood tuberculosis: traditional and innovative modalities. *Current Problems in Pediatric and Adolescent Health Care*, 39(3):61-88.
- Ling, D.I., Flores, L.L., Riley, L.W. & Pai, M. 2008. Commercial Nucleic-Acid Amplification Tests for Diagnosis of Pulmonary Tuberculosis in Respiratory Specimens: Meta-Analysis and Meta-Regression. *PLoS ONE*, 3(2):e1536.
- Loeffler, S.H., de Lisle, G.W., Neill, M.A., Collins, D.M., Price-Carter, M., Paterson, B. & Crews, K.B. 2014. The seal tuberculosis agent, Mycobacterium pinnipedii, infects domestic cattle in New Zealand: epidemiologic factors and DNA strain typing. *Journal of Wildlife Diseases*, 50(2):180-187.

MacMicking, J.D., Taylor, G.A. & McKinney, J.D. 2003. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science*, 302(5645):654-659.

MAG Mutual Healthcare Solutions. 2008. 2009 physicians' fee & coding guide. Duluth (GA), USA.

Makinen, J., Marttila, H.J., Marjamaki, M., Viljanen, M.K. & Soini, H. 2006. Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology*, 44(2):350-352.

Marais, B., Gie, R., Obihara, C., Hesselning, A., Schaaf, H. & Beyers, N. 2005. Well defined symptoms are of value in the diagnosis of childhood pulmonary tuberculosis. *Archives of Disease in Childhood*, 90(11):1162-1165.

McNerney, R., Cunningham, J., Hepple, P. & Zumla, A. 2015. New tuberculosis diagnostics and rollout. *International Journal of Infectious Diseases*, 32:81-86.

McNerney, R. & Zumla, A. 2015. Impact of the Xpert MTB/RIF diagnostic test for tuberculosis in countries with a high burden of disease. *Current Opinion in Pulmonary Medicine*, 21(3):304-308.

Mello, F.C., Arias, M.S., Rosales, S., Marsico, A.G., Pavon, A., Alvarado-Galvez, C., Pessoa, C.L., Perez, M., Andrade, M.K., Kritski, A.L., Fonseca, L.S., Chaisson, R.E., Kimerling, M.E. & Dorman, S.E. 2007. Clinical evaluation of the microscopic observation drug susceptibility assay for detection of *Mycobacterium tuberculosis* resistance to isoniazid or rifampin. *Journal of Clinical Microbiology*, 45(10):3387-3389.

Mhimbira, F.A., Bholla, M., Sasamalo, M., Mukurasi, W., Hella, J.J., Jugheli, L. & Reither, K. 2015. Detection of *Mycobacterium tuberculosis* by EasyNAT Diagnostic Kit in Sputum Samples from Tanzania. *Journal of Clinical Microbiology*, 53(4):1342-1344.

Migliori, G.B., Besozzi, G., Girardi, E., Kliiman, K., Lange, C., Toungoussova, O.S., Ferrara, G., Cirillo, D.M., Gori, A., Matteelli, A., Spanevello, A., Codecasa, L.R. & Raviglione, M.C. 2007.

Clinical and operational value of the extensively drug-resistant tuberculosis definition. *European Respiratory Journal*, 30(4):623-626.

Minion, J., Leung, E., Menzies, D. & Pai, M. 2010. Microscopic-observation drug susceptibility and thin layer agar assays for the detection of drug resistant tuberculosis: a systematic review and meta-analysis. *The Lancet Infectious Diseases*, 10(10):688-698.

Miotto, P., Piana, F., Penati, V., Canducci, F., Migliori, G.B. & Cirillo, D.M. 2006. Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strains isolated in Italy. *Journal of Clinical Microbiology*, 44(7):2485-2491.

Mireia, C., Astrid, L., Sonja, M., Kerstin, M.-R., Sébastien, C.-S., Andreas, N., Piotr Wojtek, D., Aleksandar, R., Stefan, N., Julian, P., Emmanuel, C.-H., Julia, F., Iñaki, C., Christophe, B., Sebastien, G. & Fabian, H.L. 2013. Novel *Mycobacterium tuberculosis* Complex Isolate from a Wild Chimpanzee. *Emerging Infectious Diseases* 19(6):969.

Mitarai, S., Okumura, M., Toyota, E., Yoshiyama, T., Aono, A., Sejimo, A., Azuma, Y., Sugahara, K., Nagasawa, T., Nagayama, N., Yamane, A., Yano, R., Kokuto, H., Morimoto, K., Ueyama, M., Kubota, M., Yi, R., Ogata, H., Kudoh, S. & Mori, T. 2011. Evaluation of a simple loop-mediated isothermal amplification test kit for the diagnosis of tuberculosis. *The International Journal of Tuberculosis and Lung Disease*, 15(9):1211-1217, i.

Molicotti, P., Bua, A. & Zanetti, S. 2014. Cost-effectiveness in the diagnosis of tuberculosis: choices in developing countries. *The Journal of Infection in Developing Countries*, 8(01):24-38.

Molina-Moya, B., Lacoma, A., Prat, C., Diaz, J., Dudnyk, A., Haba, L., Maldonado, J., Samper, S., Ruiz-Manzano, J., Ausina, V. & Dominguez, J. 2015. AID TB resistance line probe assay for rapid detection of resistant *Mycobacterium tuberculosis* in clinical samples. *Journal of Infection*, 70(4):400-408.

Moore, D.A.J., Caviedes, L., Gilman, R.H., Coronel, J., Arenas, F., LaChira, D., Salazar, C., Carlos Saravia, J., Oberhelman, R.A., Holm-Delgado, M.-G., Escombe, A.R., Evans, C.A.W. & Friedland, J.S. 2006a. Infrequent MODS TB culture cross-contamination in a high-burden resource-poor setting. *Diagnostic Microbiology and Infectious Disease*, 56(1):35-43.

- Moore, D.A.J., Evans, C.A.W., Gilman, R.H., Caviedes, L., Coronel, J., Vivar, A., Sanchez, E., Piñedo, Y., Saravia, J.C., Salazar, C., Oberhelman, R., Hollm-Delgado, M.-G., LaChira, D., Escombe, A.R. & Friedland, J.S. 2006b. Microscopic-Observation Drug-Susceptibility Assay for the Diagnosis of TB. *New England Journal of Medicine*, 355(15):1539-1550.
- Moure, R., Muñoz, L., Torres, M., Santin, M., Martín, R. & Alcaide, F. 2011. Rapid Detection of Mycobacterium tuberculosis Complex and Rifampin Resistance in Smear-Negative Clinical Samples by Use of an Integrated Real-Time PCR Method. *Journal of Clinical Microbiology*, 49(3):1137-1139.
- Nathanson, E., Nunn, P., Uplekar, M., Floyd, K., Jaramillo, E., Lonnroth, K., Weil, D. & Raviglione, M. 2010. MDR tuberculosis--critical steps for prevention and control. *New England Journal of Medicine*, 363(11):1050-1058.
- Neonakis, I.K., Spandidos, D.A. & Petinaki, E. 2011. Use of loop-mediated isothermal amplification of DNA for the rapid detection of Mycobacterium tuberculosis in clinical specimens. *European Journal of Clinical Microbiology & Infectious Diseases*, 30(8):937-942.
- Neumann, P.J. 2009. Costing and perspective in published cost-effectiveness analysis. *Medical care*, 47(7_Supplement_1):S28-S32.
- Niemz, A. & Boyle, D.S. 2012. Nucleic acid testing for tuberculosis at the point-of-care in high-burden countries. *Expert Review of Molecular Diagnostics*, 12(7):687-701.
- Nikam, C., Jagannath, M., Narayanan, M.M., Ramanabhiraman, V., Kazi, M., Shetty, A. & Rodrigues, C. 2013. Rapid Diagnosis of *Mycobacterium tuberculosis* with Truenat MTB: A Near-Care Approach. *PLoS ONE*, 8(1):e51121.
- Nimesh, M., Joon, D., Pathak, A.K. & Saluja, D. 2013. Comparative study of diagnostic accuracy of established PCR assays and in-house developed sdaA PCR method for detection of Mycobacterium tuberculosis in symptomatic patients with pulmonary tuberculosis. *Journal of Infection*, 67(5):399-407.
- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28(12):e63.

- Ogbudebe, C.L., Chukwu, J.N., Nwafor, C.C., Meka, A.O., Ekeke, N., Madichie, N.O., Anyim, M.C., Osakwe, C., Onyeonoro, U., Ukwaja, K.N. & Oshi, D.C. 2015. Reaching the underserved: Active tuberculosis case finding in urban slums in southeastern Nigeria. *International Journal of Mycobacteriology*, 4(1):18-24.
- Okeh, U. & Okoro, C. 2012. Evaluating Measures of Indicators of Diagnostic Test Performance: Fundamental Meanings and Formulas. *Journal of Biometrics & Biostatistics*, 3(132).
- Ou, X., Song, Y., Zhao, B., Li, Q., Xia, H., Zhou, Y., Pang, Y., Wang, S., Zhang, Z., Cheng, S., Liu, C. & Zhao, Y. 2014. A multicenter study of Cross-Priming Amplification for tuberculosis diagnosis at peripheral level in China. *Tuberculosis*, 94(4):428-433.
- Paglia, M.G., Bevilacqua, N., Haji, H.S., Vairo, F., Girardi, E., Nicastrì, E., Muhsin, J., Racalbutto, V., Jiddawi, M.S. & Ippolito, G. 2012. Improvement of Tuberculosis Laboratory Capacity on Pemba Island, Zanzibar: A Health Cooperation Project. *PLoS ONE*, 7(8):e44109.
- Palomino, J.C. 2005. Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. *European Respiratory Journal*, 26(2):339-350.
- Parsons, L.M., Somoskövi, Á., Gutierrez, C., Lee, E., Paramasivan, C.N., Abimiku, A.I., Spector, S., Roscigno, G. & Nkengasong, J. 2011. Laboratory Diagnosis of Tuberculosis in Resource-Poor Countries: Challenges and Opportunities. *Clinical Microbiology Reviews*, 24(2):314-350.
- Parsons, S.D.C., Drewe, J.A., Gey van Pittius, N.C., Warren, R.M. & van Helden, P.D. 2013. Novel Cause of Tuberculosis in Meerkats, South Africa. *Emerging Infectious Diseases*, 19(12):2004-2007.
- Parwati, I., van Crevel, R. & van Soolingen, D. 2010. Possible underlying mechanisms for successful emergence of the Mycobacterium tuberculosis Beijing genotype strains. *The Lancet Infectious Diseases*, 10(2):103-111.
- Pearce, E.C., Woodward, J.F., Nyandiko, W.M., Vreeman, R.C. & Ayaya, S.O. 2012. A systematic review of clinical diagnostic systems used in the diagnosis of tuberculosis in children. *AIDS Research and Treatment*, 2012:401896.

- Perez-Velez, C.M. & Marais, B.J. 2012. Tuberculosis in Children. *New England Journal of Medicine*, 367(4):348-361.
- Pietersen, E., Ignatius, E., Streicher, E.M., Mastrapa, B., Padanilam, X., Pooran, A., Badri, M., Lesosky, M., van Helden, P., Sirgel, F.A., Warren, R. & Dheda, K. 2014. Long-term outcomes of patients with extensively drug-resistant tuberculosis in South Africa: a cohort study. *The Lancet*, 383(9924):1230-1239.
- Pooran, A., Pieterse, E., Davids, M., Theron, G. & Dheda, K. 2013. What is the Cost of Diagnosis and Management of Drug Resistant Tuberculosis in South Africa? *PLoS ONE*, 8(1):e54587.
- Prados-Torres, A., Calderón-Larrañaga, A., Hanco-Saavedra, J., Poblador-Plou, B. & van den Akker, M. 2013. Multimorbidity patterns: a systematic review. *Journal of Clinical Epidemiology*, 67(3):254-266.
- Qin, Z.Z., Pai, M., Van Gemert, W., Sahu, S., Ghiasi, M. & Creswell, J. 2015. How is Xpert MTB/RIF being implemented in 22 high tuberculosis burden countries? *European Respiratory Journal*, 45(2):549-554.
- Radomski, N., Roguet, A., Lucas, F.S., Veyrier, F.J., Cambau, E., Accrombessi, H., Moilleron, R., Behr, M.A. & Moulin, L. 2013. *atpE* gene as a new useful specific molecular target to quantify *Mycobacterium* in environmental samples. *BMC Microbiology*, 13(1):1-13.
- Ramsey, S., Willke, R., Briggs, A., Brown, R., Buxton, M., Chawla, A., Cook, J., Glick, H., Liljas, B. & Petitti, D. 2005. Good research practices for cost-effectiveness analysis alongside clinical trials: the ISPOR RCT-CEA task force report. *Value in health*, 8(5):521-533.
- Rastogi, N., Legrand, E. & Sola, C. 2001. The mycobacteria: an introduction to nomenclature and pathogenesis. *Scientific and Technical Review*, 20(1):21-54.
- Raviglione, M. & Director, G.T.B. 2013. Global strategy and targets for tuberculosis prevention, care and control after 2015: Geneva, World Health Organization. URL: http://www.who.int/tb/post_2015_tb_presentation.pdf?ua=1. Date of access: 11 August 2015.

- Rehle, T.M., Hallett, T.B., Shisana, O., Pillay-van Wyk, V., Zuma, K., Carrara, H. & Jooste, S. 2010. A Decline in New HIV Infections in South Africa: Estimating HIV Incidence from Three National HIV Surveys in 2002, 2005 and 2008. *PLoS ONE*, 5(6):e11094.
- Reinschmidt, K.F. 2002. Aggregate social discount rate derived from individual discount rates. *Management Science*, 48(2):307-312.
- Ritter, C., Lucke, K., Sirgel, F.A., Warren, R.W., van Helden, P.D., Böttger, E.C. & Bloemberg, G.V. 2014. Evaluation of the AID TB Resistance Line Probe Assay for Rapid Detection of Genetic Alterations Associated with Drug Resistance in Mycobacterium tuberculosis Strains. *Journal of Clinical Microbiology*, 52(3):940-946.
- Robinson, R. 1993. Cost-effectiveness analysis. *The British Medical Journal*, 307(6907):793-795.
- Rohner, P., Ninet, B., Metral, C., Emler, S. & Auckenthaler, R. 1997. Evaluation of the MB/BacT system and comparison to the BACTEC 460 system and solid media for isolation of mycobacteria from clinical specimens. *Journal of Clinical Microbiology*, 35(12):3127-3131.
- 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. *Antimicrobial Agents and Chemotherapy*, 41(10):2093-2098.
- Russell, L.B., Gold, M.R., Siegel, J.E., Daniels, N. & Weinstein, M.C. 1996. The role of cost-effectiveness analysis in health and medicine. *Journal of the American Medical Association*, 276(14):1172-1177.
- Ryder, H.F., McDonough, C., Tosteson, A.N.A. & Lurie, J.D. 2009. Decision Analysis and Cost-effectiveness Analysis. *Seminars in Spine Surgery*, 21(4):216-222.
- Sabrina, R., Javier, B., Beatriz, R., Lucía de, J., Julio, Á., Elena, C., Nuria, M., Francisco, L., Javed, M.T., José, L.S.-L., Ernesto, L., Ana, M., Lucas, D., Alicia, A. & Monitoring of Animal, T. 2011. *Mycobacterium caprae* Infection in Livestock and Wildlife, Spain. *Emerging Infectious Diseases*, 17(3):532.

- Safianowska, A., Walkiewicz, R., Nejman-Gryz, P. & Grubek-Jaworska, H. 2012. The use of selected commercial molecular assays for the microbiological diagnosis of tuberculosis. *Pneumonologia i Alergologia Polska*, 80:6-12.
- Salomon, J.A., Vos, T., Hogan, D.R., Gagnon, M., Naghavi, M., Mokdad, A., Begum, N., Shah, R., Karyana, M. & Kosen, S. 2013. Common values in assessing health outcomes from disease and injury: disability weights measurement study for the Global Burden of Disease Study 2010. *The Lancet*, 380(9859):2129-2143.
- Scott, L.E., Beylis, N., Nicol, M., Nkuna, G., Molapo, S., Berrie, L., Duse, A. & Stevens, W.S. 2014. Diagnostic Accuracy of Xpert MTB/RIF for Extrapulmonary Tuberculosis Specimens: Establishing a Laboratory Testing Algorithm for South Africa. *Journal of Clinical Microbiology*, 52(6):1818-1823.
- Shankar, E.M., Vignesh, R., Ellegård, R., Barathan, M., Chong, Y.K., Bador, M.K., Rukumani, D.V., Sabet, N.S., Kamarulzaman, A., Velu, V. & Larsson, M. 2014. HIV–Mycobacterium tuberculosis co-infection: a ‘danger-couple model’ of disease pathogenesis. *Pathogens and Disease*, 70(2):110-118.
- Shiferaw, G., Woldeamanuel, Y., Gebeyehu, M., Girmachew, F., Demessie, D. & Lemma, E. 2007. Evaluation of microscopic observation drug susceptibility assay for detection of multidrug-resistant Mycobacterium tuberculosis. *Journal of Clinical Microbiology*, 45(4):1093-1097.
- Smith, I. 2003. Mycobacterium tuberculosis Pathogenesis and Molecular Determinants of Virulence. *Clinical Microbiology Reviews*, 16(3):463-496.
- Soini, H. & Musser, J.M. 2001. Molecular diagnosis of mycobacteria. *Clinical Chemistry*, 47(5):809-814.
- Sonnenberg, P., Murray, J., Glynn, J.R., Shearer, S., Kambashi, B. & Godfrey-Faussett, P. 2001. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. *The Lancet*, 358(9294):1687-1693.

Steingart, K.R., Henry, M., Ng, V., Hopewell, P.C., Ramsay, A., Cunningham, J., Urbanczik, R., Perkins, M., Aziz, M.A. & Pai, M. 2006a. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *The Lancet Infectious Diseases*, 6(9):570-581.

Steingart, K.R., Ng, V., Henry, M., Hopewell, P.C., Ramsay, A., Cunningham, J., Urbanczik, R., Perkins, M.D., Aziz, M.A. & Pai, M. 2006b. Sputum processing methods to improve the sensitivity of smear microscopy for tuberculosis: a systematic review. *The Lancet Infectious Diseases*, 6(10):664-674.

Steingart, K.R., Ramsay, A. & Pai, M. 2007. Optimizing sputum smear microscopy for the diagnosis of pulmonary tuberculosis. *Expert review of anti-infective therapy* 5(3):327-331.

Steingart, K.R., Schiller, I., Horne, D.J., Pai, M., Boehme, C.C. & Dendukuri, N. 2014. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev*, 1.

Stop TB Partnership's New Diagnostics Working Group. 2009. Pathways to better diagnostics for tuberculosis: a blueprint for the development of TB diagnostics. Geneva: World Health Organization. URL:

http://www.stoptb.org/wg/new_diagnostics/assets/documents/blueprinttb_annex_web.pdf.

Date of access: 14 February 2014.

Swingler, G.H., du Toit, G., Andronikou, S., van der Merwe, L. & Zar, H.J. 2005. Diagnostic accuracy of chest radiography in detecting mediastinal lymphadenopathy in suspected pulmonary tuberculosis. *Archives of Disease in Childhood*, 90(11):1153-1156.

Telisinghe, L., Fielding, K.L., Malden, J.L., Hanifa, Y., Churchyard, G.J., Grant, A.D. & Charalambous, S. 2014. High tuberculosis prevalence in a South African prison: the need for routine tuberculosis screening. *PLoS One*, 9(1):e87262.

Theron, G., Peter, J., Dowdy, D., Langley, I., Squire, S.B. & Dheda, K. 2014a. Do high rates of empirical treatment undermine the potential effect of new diagnostic tests for tuberculosis in high-burden settings? *The Lancet Infectious Diseases*, 14(6):527-532.

- Theron, G., Zijenah, L., Chanda, D., Clowes, P., Rachow, A., Lesosky, M., Bara, W., Mungofa, S., Pai, M., Hoelscher, M., Dowdy, D., Pym, A., Mwaba, P., Mason, P., Peter, J. & Dheda, K. 2014b. Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. *The Lancet*, 383(9915):424-435.
- Thierry, D., Brisson-Noel, A., Vincent-Levy-Frebault, V., Nguyen, S., Guesdon, J.L. & Gicquel, B. 1990. Characterization of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis. *Journal of Clinical Microbiology*, 28(12):2668-2673.
- Tomita, N., Mori, Y., Kanda, H. & Notomi, T. 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature protocols* 3:877-882.
- Trajman, A., Pai, M., Dheda, K., van Zyl Smit, R., Zwerling, A.A., Joshi, R., Kalantri, S., Daley, P. & Menzies, D. 2008. Novel tests for diagnosing tuberculous pleural effusion: what works and what does not? *European Respiratory Journal*, 31(5):1098-1106.
- Travis, P., Bennett, S., Haines, A., Pang, T., Bhutta, Z., Hyder, A.A., Pielemeier, N.R., Mills, A. & Evans, T. 2004. Overcoming health-systems constraints to achieve the Millennium Development Goals. *The Lancet*, 364(9437):900-906.
- Trusov, A., Bumgarner, R., Valijev, R., Chestnova, R., Talevski, S., Vragoterova, C. & Neeley, E.S. 2009. Comparison of Lumin LED fluorescent attachment, fluorescent microscopy and Ziehl-Neelsen for AFB diagnosis. *The International Journal of Tuberculosis and Lung Disease*, 13(7):836-841.
- UNITAID. 2012. Tuberculosis: diagnostic technology landscape: Semi-annual update. www.unitaid.eu/images/marketdynamics/. Date of access: 14 July 2014.
- UNITAID. 2015. Tuberculosis diagnostics technology and market landscape,. Geneva: World Health Organization. https://c-path.org/wp-content/uploads/2015/11/Tuberculosis_diagnostics_technology_and_market_landscape_4th_edition_Oct_2015.pdf. Date of access: 16 July 2014.

- Van Hout, B.A. 1998. Discounting costs and effects: a reconsideration. *Health economics*, 7(7):581-594.
- Velayati, A.A., Masjedi, M.R., Farnia, P., Tabarsi, P., Ghanavi, J., Ziazarifi, A.H. & Hoffner, S.E. 2009. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in iran. *Chest*, 136(2):420-425.
- Verma, A., Rattan, A. & Tyagi, J. 1994. Development of a 23S rRNA-based PCR assay for the detection of mycobacteria. *Indian Journal of Biochemistry and Biophysics* 31:288 - 294.
- Vinkeles Melchers, N.V., van Elsland, S.L., Lange, J.M., Borgdorff, M.W. & van den Hombergh, J. 2013. State of affairs of tuberculosis in prison facilities: a systematic review of screening practices and recommendations for best TB control. *PLoS One*, 8(1):e53644.
- Wallgren, A. 1948. The time-table of tuberculosis. *Tubercle*, 29(11):245-251.
- Wallis, R.S., Pai, M., Menzies, D., Doherty, T.M., Walzl, G., Perkins, M.D. & Zumla, A. 2010. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. *The Lancet*, 375(9729):1920-1937.
- Weintraub, W.S. & Cohen, D.J. 2009. The limits of cost-effectiveness analysis. *Circulation: Cardiovascular Quality and Outcomes*, 2(1):55-58.
- Werner, E.F., Wheeler, S. & Burd, I. 2013. Creating Decision Trees to Assess Cost-Effectiveness in Clinical Research. *Journal of Biometrics & Biostatistics*, 2012.
- Weyer, K., Carai, S. & Nunn, P. 2011. Viewpoint TB Diagnostics: What Does the World Really Need? *Journal of Infectious Diseases*, 204(suppl 4):S1196-S1202.
- Wilson, P. & Palriwala, A. 2011. Prizes for global health technologies. URL: <http://healthresearchpolicy.org/assessments/prizes-global-health-technologies>. Date of access: 15 November 2015
- World Health Organization. 2014a. Global tuberculosis report 2014. Geneva Switzerland. URL: http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf. Date of access: 16 August 2015

World Health Organization. 2014b. Xpert MTB/RIF Implementation Manual: Technical and Operational 'How-To'; Practical Considerations. Geneva: World Health Organization. URL: http://apps.who.int/iris/bitstream/10665/112469/1/9789241506700_eng.pdf. Date of access: 7 October 2015

World Health Organization. 2008. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). *Policy statement June*. URL: http://www.who.int/tb/features_archive/policy_statement.pdf. Date of access: 14 May 2014

World Health Organization. 2010. Global tuberculosis control: WHO report 2010: World Health Organization: Geneva. URL: http://reliefweb.int/sites/reliefweb.int/files/resources/F530290AD0279399C12577D8003E9D65-Full_Report.pdf. Date of access: 17 April 2014

World Health Organization. 2011. Global tuberculosis control. *WHO Library Cataloguing-in-Publication Data*. URL: http://apps.who.int/iris/bitstream/10665/44728/1/9789241564380_eng.pdf. Date of access: 25 September 2014.

World Health Organization. 2013. Global tuberculosis report. WHO Report; 2013: WHO Press: Geneva, Switzerland. URL: http://apps.who.int/iris/bitstream/10665/91355/1/9789241564656_eng.pdf. Date of access: 27 September 2014

World Health Organization. 2014. Global strategy and targets for tuberculosis prevention, care and control after 2015. Geneva: World Health Organization. URL: http://www.who.int/tb/post2015_strategy/en/. Date of access: 16 May 2015.

World Health Organization. 2015a. Choosing interventions that are cost effective [Internet]. Geneva: World Health Organization. URL: http://www.who.int/tb/post2015_tbstrategy.pdf. Date of access: 17 April 2015

World Health Organization. 2015b. The use of lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis and screening of active tuberculosis in people living with HIV. Geneva:

World	Health	Organization.	URL:
http://apps.who.int/iris/bitstream/10665/193633/1/9789241509633_eng.pdf . Date of access: 10 August 2016.			

Worodria, W., Okot-Nwang, M., Yoo S, D. & Aisu, T. 2003. Causes of lower respiratory infection in HIV-infected Ugandan adults who are sputum AFB smear-negative. *The International Journal of Tuberculosis and Lung Disease*, 7(2):117-123.

Xia, H., Song, Y.Y., Zhao, B., Kam, K.M., O'Brien, R.J., Zhang, Z.Y., Sohn, H., Wang, W. & Zhao, Y.L. 2013. Multicentre evaluation of Ziehl-Neelsen and light-emitting diode fluorescence microscopy in China. *The International Journal of Tuberculosis and Lung Disease*, 17(1):107-112.

Yadav, R.N., Singh, B.K., Sharma, S.K., Sharma, R., Soneja, M., Sreenivas, V., Myneedu, V.P., Hanif, M., Kumar, A., Sachdeva, K.S., Paramasivan, C.N., Vollepore, B., Thakur, R., Raizada, N., Arora, S.K. & Sinha, S. 2013. Comparative Evaluation of GenoType MTBDRplus Line Probe Assay with Solid Culture Method in Early Diagnosis of Multidrug Resistant Tuberculosis (MDR-TB) at a Tertiary Care Centre in India. *PLoS ONE*, 8(9):e72036.

Yamada-Noda, M., Ohkusu, K., Hata, H., Shah, M.M., Nhung, P.H., Sun, X.S., Hayashi, M. & Ezaki, T. 2007. Mycobacterium species identification – A new approach via dnaJ gene sequencing. *Systematic and Applied Microbiology*, 30(6):453-462.

Zar, H. 2007. Diagnosis of pulmonary tuberculosis in children – what's new? . *South African Medical Journal*, 97:983-985.

Zar, H.J., Connell, T.G. & Nicol, M. 2010. Diagnosis of pulmonary tuberculosis in children: new advances. *Expert Review of Anti-infective Therapy* 8(3):277-288.

Zumla, A., Abubakar, I., Raviglione, M., Hoelscher, M., Ditiu, L., McHugh, T.D., Squire, S.B., Cox, H., Ford, N., McNerney, R., Marais, B., Grobusch, M., Lawn, S.D., Migliori, G.B., Mwaba, P., O'Grady, J., Pletschette, M., Ramsay, A., Chakaya, J., Schito, M., Swaminathan, S., Memish, Z., Maeurer, M. & Atun, R. 2012. Drug-resistant tuberculosis--current dilemmas, unanswered

questions, challenges, and priority needs. *The Journal of Infectious Diseases*, 205 Suppl 2:S228-240.

Zuñiga, J., Torres-García, D., Santos-Mendoza, T., Rodriguez-Reyna, T.S., Granados, J. & Yunis, E.J. 2012. Cellular and Humoral Mechanisms Involved in the Control of Tuberculosis. *Clinical and Developmental Immunology*, 2012:18.

CHAPTER 3:

Commentary on independent clinical evaluation of NWU-TB test

This chapter covers results from an independent preliminary evaluation of the NWU-TB system that was conducted by the Centre for Tuberculosis at the National Institute for Communicable Diseases (NICD), which is a division of the National Health Laboratory Service (NHLS) of South Africa. The aim of this pilot study was for the system to be assessed by independent experts and use their recommendations to make improvements on system design and performance. The chapter is written in the form of a commentary. A report of the study that was produced by the NICD can be found in “Annexure A”.

Commentary: A preliminary independent evaluation of the NWU-TB system

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Abstract

An independent evaluation of the NWU-TB system was carried out at the National Institute for Communicable Diseases in South Africa in order to assess the sensitivity and specificity of the assay in clinical sputum samples. One hundred and nine (109) sputum samples were used in this pilot study, and the sensitivity and specificity of NWU-TB was 67% and 88% respectively, relative to MGIT culture as gold standard. There were some weaknesses in the study design that made it difficult to validate the results; these include small sample size and the use of lysed frozen samples for PCR. This is a deviation from the standard workflow of the system where PCR is carried out immediately after lysis using the fresh lysates. However, some recommendations from the study were incorporated during further development of the system.

Text

During the early stages of development of the NWU-TB system, the funders of the project requested an independent preliminary evaluation of the performance of the initial prototype. The results of the evaluation were used to guide downstream developmental efforts. This evaluation was conducted by the Centre for Tuberculosis at the National Institute for Communicable Diseases (NICD), a division of the National Health Laboratory Service (NHLS) of South Africa. The NICD is a national public health institution with a number of centres focusing on surveillance, reference and research on specific diseases such as meningitis, HIV/AIDS, tuberculosis and malaria. The study was conducted between February and March 2014 at the Centre for Tuberculosis which is located in Pretoria, South Africa.

NWU-TB is a system that allows rapid diagnosis of *Mycobacterium tuberculosis* (MTB), giving a result in less than 2 hours. The method relies on rapid (within 10 minutes) cell lysis through mechanical, thermal and chemical means in one reaction (Grobler *et al.*, 2012); followed by amplification of specific target sequences unique to MTB using PCR (within 20 minutes). The PCR products are then visualized using agarose gel electrophoresis (within 15 minutes) (Mutingwende *et al.*, 2015).

The proposed study design for the independent evaluation included one hundred and fifty (150) smear-positive and one hundred (100) smear-negative sputum samples that were to be tested using sputum smear microscopy (SSM), MGIT culture, Xpert MTB/Rif and NWU-TB. Since the NICD is a national reference laboratory, they receive samples from all the provinces of South Africa and thus it can be assumed that the samples are representative of all the MTB strains occurring in the

country. However, only 109 samples were later used in the evaluation due to limited availability of samples during that time. Furthermore, the sample volumes were too small to allow testing with all the 4 tests (MGIT culture, Xpert MTB/Rif, SSM, and NWU-TB), and as a result the comparison with the Xpert MTB/Rif assay, which was the real purpose of the study, was not done in the evaluation. MGIT culture was used as the “gold standard” in the evaluation. The 109 sputum samples were therefore all tested using MGIT culture, SSM and NWU-TB.

To eliminate bias, the results of MGIT culture and SSM were kept blind to the scientist performing the index test and results were to be separately passed to a consulting biostatistician for analysis. To determine reproducibility and margin of operator error, two NICD scientists were to perform the index test separately on the same sputum samples. However, due to the changes in the study design (i.e. sample size and exclusion of Xpert MTB/Rif) a biostatistician was not involved in the data analysis; the analysis was done by scientists at the NICD. Furthermore, only one scientist carried out the tests using the NWU-TB, thus reproducibility of the assay could not be determined. In light of these weaknesses in the study design, the validity of some of the study outcomes could not be properly ascertained. For example, although SSM is reported to have a high specificity (Desikan, 2013), a 100% specificity was not expected considering the high prevalence of non-tuberculosis mycobacteria (NTMs) in South Africa due to HIV co-infection (Corbett *et al.*, 1999; Sookan & Coovadia, 2014).

In spite of the mentioned shortcomings on the study design, the results could not be ignored without further investigation. The NICD made two valid recommendations: the need to carry out independent assessments of the sample processing step, and migration to a real time PCR platform. It was noted that the 109 patient samples used in the study were lysed using the NWU-TB processing procedure and frozen at -20 °C for 48 hours due to problems with the primer set used in the PCR MasterMix (as highlighted in Annexure A). A fresh batch of primers was ordered and reconstituted for the study and quality control tests on the new batch were performed; this was assumed to be the source of the amplification problems. Therefore a previously used batch of the primer set was eventually used in the study. The use of previously lysed and frozen samples for PCR amplification was a deviation from the standard NWU-TB protocol where samples are lysed and amplified by PCR immediately. Later studies (data not shown) revealed that use of lysed and frozen samples reduces the sensitivity of NWU-TB, as some samples that tested positive when immediately amplified would test negative when amplified after storage. This is possibly due to the presence of polysaccharides that partition with the aqueous soluble DNA (Belisle &

Sonnenberg, 1998) and therefore interfere with PCR reactions (Olson & Morrow, 2012). Since the NWU-TB sample processing procedure uses lysed samples that are not processed to remove contaminants, storage of samples after lysis can promote the interaction of mycobacterial polysaccharides and DNA, leading to lower sensitivity when lysed and frozen samples are used in the PCR reaction.

Despite these problems inherent in the study design of the independent evaluation, the study highlighted some key weaknesses of the NWU-TB system that needed to be addressed. These include the lack of an internal amplification control and the need to run agarose gels after amplification. It is inconceivable for healthcare personnel at primary healthcare centres to run agarose gels on daily basis. The system was therefore subsequently modified to take into account these important recommendations.

References

- Belisle, J.T. & Sonnenberg, M.G. 1998. Isolation of genomic DNA from mycobacteria. *Mycobacteria Protocols*:31-44.
- Corbett, E.L., Blumberg, L., Churchyard, G.J., Molo, N., Mallory, K., Clayton, T.I.M., Williams, B.G., Chaisson, R.E., Hayes, R.J. & De Cock, K.M. 1999. Nontuberculous Mycobacteria. *American Journal of Respiratory and Critical Care Medicine*, 160(1):15-21.
- Desikan, P. 2013. Sputum smear microscopy in tuberculosis: Is it still relevant? *The Indian Journal of Medical Research*, 137(3):442-444.
- Grobler, A., Levanets, O., Whitney, S., Booth, C. & Viljoen, H. 2012. Rapid cell lysis and DNA capture in a lysis microreactor. *Chemical Engineering Science*, 81(0):311-318.
- Mutingwende, I., Vermeulen, U., Steyn, F., Viljoen, H. & Grobler, A. 2015. Development and evaluation of a rapid multiplex-PCR based system for Mycobacterium tuberculosis diagnosis using sputum samples. *Journal of Microbiological Methods*, 116(0):37-43.
- Olson, N.D. & Morrow, J.B. 2012. DNA extract characterization process for microbial detection methods development and validation. *BMC Research Notes*, 5:668-668.

Sookan, L. & Coovadia, Y.M. 2014. A laboratory-based study to identify and speciate non-tuberculous mycobacteria isolated from specimens submitted to a central tuberculosis laboratory from throughout KwaZulu-Natal Province, South Africa. *South African Medical Journal*, 104(11):766-768.

CHAPTER 4:

Multiplex-PCR based system for tuberculosis diagnosis

This chapter consists of a full length article that has been accepted and published in the *Journal of Microbiological Methods*. The author guidelines followed in submitting to the Journal can be found in “Annexure B” and the manuscript is presented in this thesis as it appears online (<http://www.sciencedirect.com/science/article/pii/S0167701215001852>). The chapter addresses the first and second objectives of the study as outlined in Chapter 1.



Development and evaluation of a rapid multiplex-PCR based system for *Mycobacterium tuberculosis* diagnosis using sputum samples



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ABSTRACT

Global tuberculosis (TB) control and eradication is hampered by the unavailability of simple, rapid and affordable diagnostic tests deployable at low infrastructure microscopy centers. We have developed and evaluated the performance of a nucleic acid amplification test for detection of *Mycobacterium tuberculosis* (MTB), the NWU-TB test, in clinical sputum specimens from 306 patients with suspected pulmonary tuberculosis. The test involves sputum sample processing using a Lyser device within 7 min, followed by rapid multiplex-PCR on a fast thermal cycler within 25 min, and amplicon resolution on agarose gel electrophoresis. Samples were also examined for presence of MTB using smear microscopy, GeneXpert and MGIT culture. Results were assessed in comparison to a MGIT culture as gold standard. Of the 306 patients, 174 had a previous TB history or already on treatment, and 132 were TB naïve cases. The NWU-TB system was found to have an overall sensitivity and specificity of 80.8% (95% CI: 75–85.7) and 75.6% (95% CI: 64.9–84.4) respectively, in comparison to 85.3% (95% CI: 79.9–89.6) and 73.2% (95% CI: 62.2–82.4) respectively for GeneXpert; and 62.1% (95% CI: 55.3–68.4) and 56.1% (95% CI: 44.7–67) respectively for smear microscopy. The study has shown that the NWU-TB system allows detection of TB in less than two hours and can be utilized at low infrastructure sites to provide quick and accurate diagnosis at a very low cost.

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1. Introduction

One hundred and thirty three years after Robert Koch recognized *Mycobacterium tuberculosis* (MTB) as the causative agent of tuberculosis (TB), it still poses an enormous global public health burden today (Koch, 1882). It is estimated that a third of the world's population, is infected with MTB, but most never develop active TB disease (Sudre et al., 1992). This is not the case, however, for persons living with human immunodeficiency virus (HIV) who contract TB infection. People with HIV-infection are 26 to 31 times more likely to become sick with TB in a given year compared to HIV-negative persons (World Health Organization, 2014). Worldwide, an estimated one-third of people living with HIV/AIDS are co-infected with TB. In 2013, there were an estimated 9.0 million new cases of TB (13% co-infected with HIV) and 1.4 million TB deaths globally (World Health Organization, 2014). In the same year, of the 27 countries that reported their multi-drug resistant tuberculosis (MDR-TB) data, the cases reached 450,000; of these, 170,000 people died (World Health Organization, 2014).

South Africa is home to the highest number (6.1 million individuals) of people living with HIV/AIDS (UNAIDS, 2013). This has led to an increase in the number of TB cases over the past decade, with the estimated TB incidence approaching 715/100,000 (World Health Organization, 2014). HIV-positive patients with smear-negative pulmonary TB (SN-PTB) are generally more severely immunosuppressed than those with smear-positive TB. Outcomes for them are poorer, especially when drug regimens do not contain rifampicin (Corbett et al., 2003). In a study of HIV-positive TB patients in Khayelitsha, South Africa, 49% of patients on TB treatment had negative smears on direct microscopy but their sputum cultures were positive (Coetzee et al., 2004). This makes smear microscopy, the most commonly used TB diagnostic test at low infrastructure sites, unreliable in Sub-Saharan Africa where HIV prevalence is high.

Global partnerships to improve disease diagnosis and appropriate treatment are a priority to prevent further global spread of TB. Complications due to HIV co-infection and emergence of drug resistant MTB strains seek to erode the gains that have been made so far to eradicate TB globally (Sharma and Mohan, 2006; Small and Pai, 2010). One critical impediment in the global fight against TB is the lack of rapid, affordable and effective diagnostics tests that can be performed at low-infrastructure sites, right at the doorsteps of most of the vulnerable communities (Urdea et al., 2006). Without better diagnostic tools for

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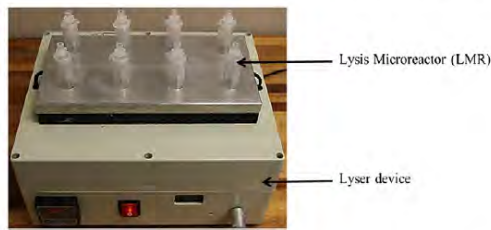


Fig. 1. Lyser device and lysis microreactor used during cell lysis step in the NWU-TB system.

TB/MDR-TB and effective strategies for their implementation, transmission will not be interrupted, mortality will not be checked, and TB will not be controlled especially in areas where HIV infection is prevalent.

Culture remains the gold standard for TB diagnosis despite its long turnaround time of between 2 and 6 weeks due to slow mycobacterial growth (Pai et al., 2004a). Ziehl-Neelsen sputum smear microscopy (SSM) is the most widely method at the primary level in Sub-Saharan Africa (SSA) due to its low installation and per sample costs. However it has poor and variable sensitivity (20–60%) especially in HIV/TB co-infected patients, and cannot distinguish MTB from other non-tuberculosis mycobacteria (NTM) (Pai et al., 2004b; Steingart et al., 2006). The costs to patients associated with multiple visits to clinics to provide specimens before treatment starts are oftentimes prohibitive and cause infectious individuals to drop out of the treatment program. Recently, GeneXpert, an automated user-friendly real-time polymerase chain reaction (PCR) assay able to simultaneously detect *M. tuberculosis* (MTB) and rifampicin resistance has been widely deployed and adopted in South Africa (Lawn et al., 2013; Nicol et al., 2011). It has a total assay time (TAT) of 2 h, high sensitivity and specificity in sputum samples but the test is expensive with a current unsubsidized cost of US\$16 per test, which the majority of people in Africa cannot afford. Given the background of these concerns, current research must focus on simplifying test protocols, decreasing cost and improving accuracy. Moreover, for sustainable solutions to the TB/HIV burden to become a reality, Africa as the greatest bearer of that burden should be part of the whole supply chain equation, right from product development to patient use. Without building and supporting diagnostic tests, development initiatives in Africa to solve diseases particular to that region, the list of manageable diseases that continues to unnecessarily kill millions of people yearly, is sure to keep growing uncurbed.

Several studies have documented the risk of mycobacterial infection of people working in diagnostic and research laboratories (Garber et al., 2003; Grist and Emslie, 1985; Miller et al., 1987; Muller, 1988). In spite of current knowledge on precautions and safety guidelines in TB laboratories, in 2003 TB conversion among healthcare workers (HCWs) in New York ranged from 2 to 6.6% (Garber et al., 2003). Some reports even suggest underreporting of the cases due to the social stigma attached and slow disease progression, which may result in HCW retiring before becoming symptomatic (Collins and Kennedy, 1999; Pike, 1976, 1979). Manipulation of liquid clinical specimens involves generation of infectious aerosols and exposure to these aerosols represents one of the most serious hazards encountered in the laboratory (Miller et al., 1987; Muller, 1988). Any new TB diagnosis system must have a proven safety profile to the health workers that operate it.

In an effort to have a simple, affordable, rapid and safe TB diagnostic system, a simple DNA diagnostic system for MTB detection (herein referred to as NWU-TB system) was developed, which can be readily applied to some other diseases. This molecular based system aims to possess the high accuracy associated with other molecular based diagnostic systems, but with the low cost structure of SSM. It is envisaged that such a system may replace SSM in SSA. However, before deployment of the NWU-TB system, there is a need to conduct a number of independent

studies to evaluate its performance using clinical samples. In the present study we describe and evaluate the performance and biosafety of NWU-TB system in clinical sputum samples.

2. Materials and methods

Specimen collection, SSM, MGIT culture, and Xpert MTB/Rif were performed by independent staff at Orkney-Westvaal Hospital, North West Province, South Africa. The Orkney-Westvaal Hospital is wholly owned and privately operated by AngloGold Ashanti to treat its gold miners. The NWU-TB system development and testing was performed at the North-West University, Potchefstroom Campus, South Africa.

2.1. Study population and specimens

This was a single site, blinded, prospective study conducted between January 2013 and October 2014 to evaluate the performance of the NWU-TB system in patients with suspected pulmonary TB in comparison to standard tests. Sputum samples were obtained under the supervision of trained nurses from presumptive TB patients on routine visit to the hospital. Standard TB tests (SSM, MGIT culture, GeneXpert) were performed on all patients, however, due to the large sputum volume required to perform all three tests it was not possible to perform GeneXpert for some patients. Left-over sputum samples after standard TB tests were frozen at -20°C and transferred to the North-West University every fortnight where they were stored under similar conditions until testing. To avoid bias, sputum samples from both TB treatment naive individuals and those at various anti-TB treatment stages were included in the study. Relevant patient information [including the TB patient history (for reinfection cases)] was only received at the data analysis stage. Sample storage time at the North-West University ranged from 2 weeks to 1 year before performing the indicator test.

HIV testing was performed as part of routine care for consenting patients according to standard guidelines (World Health Organization, 2004).

2.2. Reference standard tests

2.2.1. Sputum smear microscopy (SSM)

Two drops of NaOH-NALC decontaminated sample pellet were used for smear microscopy (ZN staining), according to standard protocol (World Health Organization, 1998). The grading of the AFB results was done in accordance with the WHO/International Union Against Tuberculosis and Lung Disease guidelines and scored as "0" for absence of AFB, scanty 1–9, 1+, 2+, or 3+ (World Health Organization, 1998).

2.2.2. MGIT culture

Samples were processed according to the manufacturers' guidelines for MGIT 960 cultures (Becton Dickinson and Company, 2013). Briefly, N-Acetyl-L-cysteine–1% sodium hydroxide (final concentration after mixing with the specimen) was used to decontaminate the sputum sample. Specimens were concentrated by centrifugation at $3000 \times g$, and pellets were resuspended in 0.5 ml of sterile phosphate buffer. 0.5 ml of the processed samples was inoculated into pre-prepared mycobacterial growth indicator tubes (MGITs) (Becton Dickinson, Sparks, Maryland), and incubated at 37°C for 6 weeks using the BACTEC

Table 1
Sequences of primers used in multiplex PCR.

Primer name	Sequence	fragment
Pab f	5'-ACC ACC GAG CGG TTC GCC TGA-3'	419 bp
Pab r	5'-GAT CTG CGG GTC GTC CCA GGT-3'	
IS1	5'-CCT GCG AGC GTA GGC GT-3'	123 bp
IS2	5'-CTC GTC CAG GCC CGC TTC GG-3'	
MPB1	5'-TCC GCT GCC AGT CGT CTT CC-3'	240 bp
MPB2	5'-GTC CTC GCG AGT CTA GGC CA-3'	

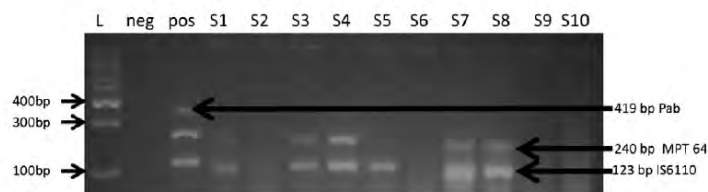


Fig. 2. Multiplex PCR amplification of IS6110 (123 bp), MPT64 (240 bp) and protein antigen b (419 bp). L – 100 bp marker; neg – negative control; pos – positive control; and S1 to S10 – patient sputum samples.

MGIT 960 instrument. The instrument automatically monitors and calls a positive result. Culture was considered negative after six weeks if the instrument did not detect mycobacterial growth except in cases where AFB smear was positive: then cultures were held for a total of 8 weeks. Qualitative confirmation of *Mycobacterium tuberculosis* complex (MTBC) in culture positive MGIT tubes was performed using the MGIT BD MGIT TBc Identification Test (TBc ID) (Becton, Dickinson & Company, Sparks, MD, USA) according to the manufacturers' instructions (BD Diagnostics, 2013).

2.2.3. GeneXpert

GeneXpert was performed according to the manufacturer's standard operating procedure (SOP) (Cepheid, USA). Briefly, in a 2:1 ratio, the sample reagent (Cepheid) was mixed with NaOH–NALC decontaminated sputum sample and incubated at room temperature for 15 min. Two milliliters of the mixture were transferred into an Xpert MTB/Rif cartridge and inserted into the instrument. The instrument automatically called a result.

2.2.4. NWU-TB

The NWU-TB system consists of three sequential steps; cell lysis within 7 min using a Lyser device, followed by Multiplex-PCR within 25 min and amplicon visualization on an agarose gel. Stored frozen sputum samples were thawed overnight at 4 °C. 250 µl of sample was mixed with an equal volume of lysis buffer [of 20 mM DTT (Dithiothreitol, Sigma-Aldrich, Inc., St. Louis, MO) and 20 × TE (Tris–EDTA, Sigma-Aldrich, Inc., St. Louis, MO)] in a lysis microreactor (LMR) (Fig. 1). The LMR was placed on a preset (115 °C and 5000 rpm) Lyser device (Fig. 1) for 7 min. Bacterial cell lysis is concurrently achieved through chemical, thermal and mechanical means (Grobler et al., 2012). 4 µl of lysate was used in the subsequent Multiplex-PCR (M-PCR) step. Each M-PCR reaction in a final volume of 20 µl contained 0.4 mg/ml BSA (Ambion, Foster City, CA, USA), 200 mM of each dNTP, 3.5 mM

MgSO₄, 1 U KOD Hotstart DNA polymerase in appropriate 10 × buffer (Novagen, San Diego, USA), and 200 nM of each of the previously described primers as shown in Table 1 (Kusum et al., 2011). The multiplex primers used target three separate genes in the *M. tuberculosis* complex genome which are IS6110, protein antigen b and MPB 64. M-PCR was performed on a Philisa rapid thermal cycler (Streck Inc., Omaha, NE) using the following protocol: Initial enzyme activation at 95 °C for 120 s, followed by 40 cycles of 95 °C for 10 s, 66 °C for 15 s and 72 °C for 6 s, and a final extension step at 72 °C for 120 s. PCR products were resolved by electrophoresis (Fig. 2) on a 2% agarose gel stained with gelred nucleic acid stain (Biotium, Hayward, CA, USA). Any specimen yielding a negative result in the NWU-TB system was re-tested if there was sufficient residual sputum and the latter result was considered in the analysis.

2.2.5. Biosafety evaluation of NWU-TB cell lysis step

MTB clinical isolates were inoculated into 12 MGIT tubes and incubated using the BACTEC-MGIT 960 automated culture system until the instrument automatically called a positive result, which occurred on day 12. This has been reported to correspond with approximately 10⁶ CFU/ml (Castan et al., 2014). Tween-80 (0.05%) was added to all MGIT tubes before transferring the contents into separate 15 ml sterile conical tubes followed by three successive rounds of vortexing each lasting about 7 min to declump the mycobacteria.

Confirmed smear negative-culture negative samples from two individuals were pooled together and used as diluent in preparing 10 fold serial dilutions (10⁶, 10⁵, 10⁴, 10³, 10², and 10¹ CFU/ml) in quintuples. For each dilution, the contents of 3 of the 5 tubes were subjected to lysis, and the other two tubes were not lysed (used as positive controls). Lysis was performed as per standard procedure in the NWU-TB system (described above). Pre-prepared MGIT tubes were inoculated with 500 µl of appropriate sample (dilutions described above) and incubated using the BACTEC-MGIT-960 for 42 days.

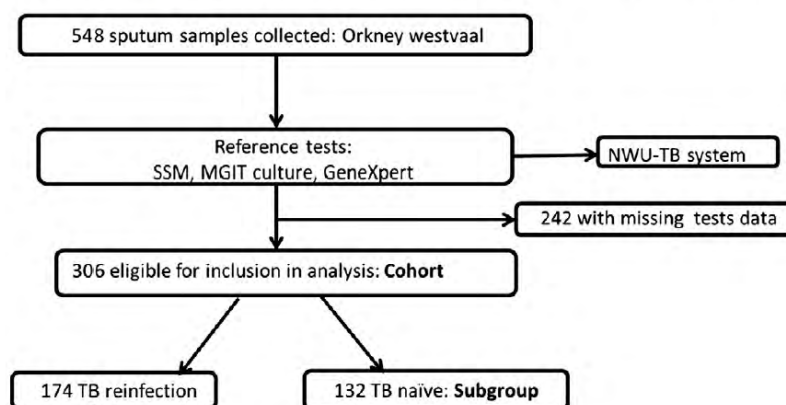


Fig. 3. Patient flow, showing the two arms for data analyses, namely the Cohort and Subgroup.

2.2.6. Statistical analysis

Accuracy measures (sensitivity, specificity, positive and negative predictive values) of the evaluated tests for *M. tuberculosis* detection were determined using MGIT culture as the gold standard. Only samples that had all smear, culture, Xpert MTB/Rif, and NWU-TB system results were included in the analysis. Statistical analyses were performed using STATA 11 (StataCorp, College Station, TX, USA). Due to the high frequency of TB re-infections (28.4%) in our cohort, a subgroup analysis (herein after referred to as subgroup) of patients with no known TB history and treatment TB naive was carried out.

2.2.7. Ethics

This study was approved by the North-West University Research Ethics Committee under ethics number: NWU-00005-11-A5. NWU-TB system results were not used in clinical decision making.

3. Results

3.1. Patient population

548 sputum samples were supplied to the North-West University research team by Orkney-Westvaal Hospital over the indicated period; however for various reasons such as inadequate sample volumes to perform GeneXpert test or culture contamination, only 306 samples had results for all four tests and were thus included in the analysis. After unblinding and considering TB patient history, two data analysis arms were decided upon. Of the 306 (referred to as cohort) samples, 132 met the subgroup inclusion criteria as highlighted in the statistical analysis section (Fig. 3).

General demographic and microbiological characteristics of the study population are shown in Table 2. The median age of the cohort and subgroup was 43.9 and 46.0 years respectively. The cohort is predominantly male (96.4%), which is above the 2:1 (male: female) gender inequality seen in adult TB. This is because the hospital caters only for miners, who are mainly males. Of the 160 patients tested for HIV in the cohort, 122 (73.7% prevalence) were positive, which is similar to 75.3% in the subgroup. Based on SSM and culture classification, smear positive-culture positives were the highest: 50% and 45.4% in the subgroup and cohort respectively.

3.2. Performance of NWU-TB in the individuals with no TB history (referred to as subgroup analysis)

When analyzed against MGIT culture as gold standard, the sensitivities of SSM, GeneXpert and NWU-TB were 62.9% [95% CI: 52.9, 72.1]; 83.3% [95% CI: 75.3, 90.3]; and 87.6% [95% CI: 79.8, 93.2], respectively (Table 3). NWU-TB was more sensitive than SSM ($p = 0.001$) and had comparable sensitivity to GeneXpert ($p = 0.135$). The specificity of both GeneXpert and NWU-TB was significantly lower than expected as reported in other studies.

Positive predictive values (PPV), negative predictive values (NPV), and Diagnostic odds ratio (DOR) of SSM, GeneXpert, and NWU-TB are provided in Table 3. In all the indicators NWU-TB is better than SSM and comparable to GeneXpert.

The sensitivity of NWU-TB in the paucibacillary smear negative-culture positive group was 76.1% [95% CI: 60.7, 88.9]. Interestingly, in the culture negative group, 13 samples had positive results with the other 3 tests contributing to the unexpected high sensitivities for both NWU-TB and GeneXpert in smear positive-culture negative and smear negative-culture negative groups (Table 4). This raises a number of questions as to the sensitivity of culture in a high TB prevalence setting (mining/prisons) and the impact on diagnostic test evaluation in such a cohort.

Table 2

Demographic information and microbiological characteristics of subgroup and cohort in the analysis arms.

	Subgroup	Cohort
No. of TB suspects	132	306
Median age in years (95% CI)	43.9 (42.4–45.4)	46.0 (39.0–51.0)
HIV prevalence (n) %	(64/85) 75.3	(122/160) 73.7
Sex		
Male n (%)	127 (95.5)	295 (96.4)
Female n (%)	5 (4.5)	11 (3.6)
Race		
Black n (%)	132 (100)	306 (100)
Previous TB history n (%)	0 (0)	174 (56.9)
Smear and culture grading		
Smear ⁺ culture ⁺ n (%)	66 (50.0)	139 (45.4)
Smear ⁺ culture ⁺ n (%)	39 (29.5)	85 (27.7)
Smear ⁺ culture [−] n (%)	20 (15.2)	36 (11.8)
Smear [−] culture [−] n (%)	7 (5.3)	46 (15.0)

3.3. Performance of NWU-TB in cohort analysis

The performance of NWU-TB against MGIT culture performed on the same sputum samples is shown in Table 5 alongside outcomes for SSM and GeneXpert. The overall sensitivity in NWU-TB at 95% confidence interval was 80.8% (75%–85.7%). This was lower than GeneXpert with a sensitivity of 85.3% (79.9%–89.6%) ($p = 0.026$), but better than SSM 62.1% (55.3%–68.4%) ($p = 0.001$). Specificity of NWU-TB and GeneXpert was still lower than expected as in the subgroup analysis.

For the NWU-TB system, when stratified by HIV status, there was better sensitivity in the HIV negative group 86.8% (71.9%–95.6%) compared to the HIV positive group 78.7% (70.4%–85.6%), though not statistically significant ($p = 0.190$). Similar results with no statistical significance ($p = 0.519$) were also observed with GeneXpert 89.5% (75.2%–92.7%) in the HIV seronegative group compared to 86.1% (78.6–91.7%) in the HIV seropositive group.

3.4. Frequency of non-tuberculosis mycobacteria

Though this was not part of the initial study design, the high frequency of non-tuberculosis mycobacteria (NTM) (Table 6) in our cohort warrants reporting on.

3.5. Biosafety evaluation

No growth was observed in any of the lysed cultures, independent of the concentration after 42 days of MGIT culture (Fig. 4). There was growth in all positive controls and time to positivity was inversely proportional to starting mycobacteria concentration.

4. Discussion

Diagnostics tests are important decision support tools for physicians and nurses in deciding on the most appropriate drug regimens for various diseases. Toward this end, clinicians need rapid, accurate, affordable and accessible diagnostic tests before prescribing appropriate medication. Despite the recent upsurge in the number of MTB detection

Table 3

Performance of SSM, GeneXpert, and NWU-TB system against MGIT culture as gold standard.

Parameter	SSM	GeneXpert	NWU-TB
Sensitivity % (95% CI)	62.9 (52.9–72.1)	83.8 (75.3–90.3)	87.6 (79.8–93.2)
Specificity % (95% CI)	74.1 (53.7–88.9)	70.4 (49.8–86.2)	88.9 (70.8–97.6)
PPV % (95% CI)	90.4 (81.2–96.1)	91.7 (84.2–96.3)	96.8 (91–99.3)
NPV % (95% CI)	33.9 (22.1–47.4)	52.8 (35.5–69.6)	64.9 (47.5–79.8)
DOR	4.84 (1.91–12.2)	12.3 (4.7–32.1)	56.6 (15.6–201)

Table 4
Sensitivities of SSM, GeneXpert, and NWU-TB in various SSM and culture result groups.

Smear-culture grade	GeneXpert % (95% CI)	NWU-TB % (95% CI)
Smear-positive, culture-positive	96.7 (89.5–99.6)	93.9 (85.2–98.3)
Smear-negative, culture-positive	61.5 (44.6–76.6)	76.1 (60.7–88.9)
Smear-positive, culture-negative	15.0 (3.21–37.9)	0.0 (0.0–16.8)
Smear-negative, culture-negative	71.4 (29.4–96.3)	42.9 (9.9–81.6)

systems, improved or novel systems suitable for resource limited settings are still a high priority. Urdea et al. (2006) states that a rapid TB diagnostic test requiring no laboratory infrastructure, with at least 85% pooled sensitivity for smear-positive and smear-negatives cases, and 97% specificity, could save approximately 400,000 lives annually (Urdea et al., 2006). However, even the most promising diagnostic test will have only limited impact if it does not reach the patients who need it. A real-world evaluation of one such system under development, aiming to address those concerns and more especially the affordability aspect has been conducted.

The NWU-TB system performance in the both the subgroup and cohort analysis is comparable to that of GeneXpert, which is also a molecular based system. The observed sensitivity of GeneXpert in this study is within the range previously reported in similar studies conducted in other high HIV prevalence settings (Chang et al., 2012; Scott et al., 2011; Theron et al., 2011). However, the specificity is lower than that reported in literature: From our data we observed 13 samples in which all 3 of the other tests were positive except culture. Marlowe et al. (2011) also reported true positive results with the GeneXpert system in culture-negative clinical specimens. Furthermore, although, MGIT culture is considered the current gold standard for MTB diagnosis, it is not 100% sensitive itself, thus some of the MGIT culture negatives are likely to be true positives (McCarthy et al., 2012; Muchwa et al., 2012). Culture negative active TB has also been shown to be characterized by clinical disease and highly suggestive Chest X-ray (CXR) abnormalities (Maher, 2009).

The high prevalence of NTMs in our cohort is bound to have contributed to the poor specificity, as the NTM-positive participants possibly also had *M. tuberculosis*, which might have been outcompeted in MGIT culture (Dinnes et al., 2007; Ssengooba et al., 2014). The usefulness of GeneXpert in culture negative samples, especially in HIV seropositive patients, warrants urgent investigation in order to address the increasingly observed MGIT culture limitations in this group. Future studies with more robust identification methods such as 16S rDNA sequence

Table 5
Performance outcomes of SSM, GeneXpert, and NWU-TB stratified by HIV status, against MGIT culture as gold standard in the cohort.

Parameter	SSM % (95% CI)	GeneXpert % (95% CI)	NWU-TB % (95% CI)
Sensitivity			
Cohort (n = 306)	62.1 (55.3–68.4)	85.3 (79.9–89.6)	80.8 (75.0–85.7)
HIV positive (n = 167)	62.3 (53.1–70.9)	86.1 (78.6–91.7)	78.7 (70.4–85.6)
HIV negative (n = 54)	63.2 (46.0–78.2)	89.5 (75.2–97.1)	86.8 (71.9–95.6)
Specificity			
Cohort (n = 306)	56.1 (44.7–67.0)	73.2 (62.2–82.4)	75.6 (64.9–84.4)
HIV positive (n = 167)	51.1 (35.8–66.3)	71.1 (55.7–83.6)	73.3 (58.1–85.4)
HIV negative (n = 54)	50.0 (24.7–75.3)	75.0 (47.6–92.7)	81.3 (54.4–96.0)
PPV			
Cohort (n = 306)	79.4 (72.7–85.2)	89.7 (84.8–93.4)	90.0 (85.1–93.8)
HIV positive (n = 167)	77.6 (68.0–85.4)	89.0 (81.9–94.0)	88.9 (81.4–94.1)
HIV negative (n = 54)	75.0 (56.6–88.5)	89.5 (75.2–97.1)	91.7 (77.5–98.2)
NPV			
Cohort (n = 306)	35.1 (27.0–43.9)	64.5 (53.9–74.2)	59.0 (49.0–68.5)
HIV positive (n = 167)	33.3 (22.4–45.7)	65.3 (50.4–78.3)	55.9 (42.4–68.8)
HIV negative (n = 54)	36.4 (17.2–59.3)	75.0 (47.6–92.7)	72.2 (46.5–90.3)

All values are in percentages (95% CI).

Table 6
Frequencies of non-tuberculosis mycobacteria found in the cohort.

NTM	Frequency	Growth rate
<i>M. abscessus</i>	1	Rapid grower
<i>M. avium</i>	24	Slow grower
<i>M. chelonae</i>	3	Rapid grower
<i>M. fortuitum</i> complex	4	Rapid grower
<i>M. intracellulare</i>	1	Slow grower
<i>M. kansasii</i>	25	Slow grower
<i>M. simiae</i>	1	Slow grower

based identification (Patel et al., 2000) on negative cultures are also recommended to eliminate the possibility of culture false-negatives.

The smear positive-culture positive group is associated with high mycobacterial burden and most TB detection systems show high sensitivity (94–100%) in this group. Sensitivities for GeneXpert and NWU-TB in both subgroup and cohort analyses were within the reported ranges of other molecular based systems in literature (Boehme et al., 2011; Castan et al., 2014; Marlowe et al., 2011). However, in the problematic, smear negative-culture positive group, NWU-TB showed a marginally better sensitivity compared to GeneXpert in the subgroup and cohort analyses. GeneXpert had a similar performance to that reported in another South Africa study with a comparable HIV prevalence (Table 7) (Scott et al., 2011). NWU-TB performed better (Table 7) than other molecular based MTB diagnosis in literature in the smear negative-culture positive group.

Tuberculosis is a disease more commonly seen in developing countries and is typically associated with poverty. Although the GeneXpert system is simple with good performance even in high HIV prevalence settings, its high costs ultimately leads to limited use (Helb et al., 2010). In this study, the NWU-TB system shows comparable performance to GeneXpert and due to the simplicity of the technology we estimate the cost per test to be less than US\$4 compared to US\$18 for GeneXpert (Lawn and Nicol, 2011). One of the main advantages of the NWU-TB system is the small sputum sample volume (250 µL) required to obtain an accurate result compared to GeneXpert, which requires a minimum volume of 0.7 mL (Boehme et al., 2010). This is beneficial for some patient populations such as the elderly, children, and HIV infected individuals who have difficulties in expectorating sputum (A. Harries et al., 1998; A.D. Harries et al., 1998; Zar et al., 2005). The sputum sample matrix is also known for its complex PCR inhibitors, and our ability to directly amplify (without need of a nucleic acid extraction step) lysed sputum samples with sensitivity comparable to that of GeneXpert is significant.

The total assay time (TAT) for NWU-TB system is about 1 h 30 min consisting of a 7 minute cell lysis step, 25 min PCR cycling, 30 min agarose gel processing and 30 min for manipulation procedures in between the steps. The semi-automation of the NWU-TB system is one of the next steps in its development and this will further reduce TAT to about 1 h and should lower cost even further. This short TAT as in

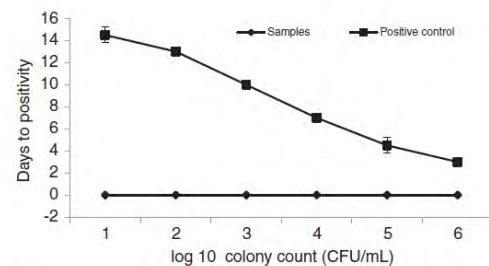


Fig. 4. Time to positivity after MGIT culture of NWU-TB system lysed 10-fold serial dilutions of *M. tuberculosis* over a period of 42 days.

Table 7

Reported diagnostic performance of different NAATs in smear-negative sputum TB presumptive individuals in different countries.

Location	Test	HIV prevalence	Sensitivity % (95% CI)	Reference
Brazil	Gen-Probe A-MTD	100%	70.8 (48.0–87.3)	Barreto et al. (2014)
Zambia	Gen-Probe A-MTD	75%	60.0 (43.0–87.3)	Kambashi et al. (2001)
Zambia	IS6110 PCR	75%	40.0 (25.0–57.0)	Kambashi et al. (2001)
South Africa	GeneXpert	70%	61	Scott et al. (2011)
South Africa	GeneXpert	75.3%	61.5	This study
South Africa	NWU-TB	75.3%	76.1	This study

GeneXpert (2 h) will reduce patient dropout rates ensure timely initiation of effective treatment for infected patients and timely quarantine of infected individuals, which has a significant role in controlling spread of tuberculosis.

Potential dangers during mycobacterial culture are well known and widely reported in literature (Bemer-Melchior and Drugeon, 1999; Cruciani et al., 2004). For SSM, there is limited epidemiologic data linking smear preparation with tuberculosis risk; however, most national guidelines recommend sputum handling and AFB smear preparation inside a biological safety cabinet (BSC) (Chedore et al., 2002; Kent, 1985). An ideal POC-TB test should carry less risk than SSM and should aim to eliminate all infectious risk. Safety during lysis and handling of lysates for health care workers is of primary concern and must be thoroughly investigated before deployment of any system. There is contrasting evidence as to the efficiency of heat of inactivation of *M. tuberculosis*. For example, Zwadik et al. reported the occurrence of living microorganisms after 30 min at 95 °C (Zwadyk et al., 1994). In NWU-TB, rapid cell lysis is achieved through a combination of thermal, chemical and mechanical means (Grobler et al., 2012). The 7 minute heat lysis step of the NWU-TB system may point toward a biohazard risk due to incomplete inactivation of the mycobacteria. However, the inclusion of a chemical lysis buffer which weakens the bacterial cell wall enable use of such a short lysis time as demonstrated by zero mycobacterial growth after 42 days of MGIT culture in this preliminary study. The LMR used for lysis is fitted with a filter (Fig. 5) to trap bioaerosols formed during heating at the high temperatures. Although smoke-tests were performed, in-depth studies using a Biosampler to trap aerosols exiting the LMR filter during lysis step are needed before reaching final conclusion on the biosafety. From the preliminary assessment it can be concluded that there is complete mycobacterial inactivation after the 7 minute lysis step. Bacillary load has been reported to be one of the main variables in causing incomplete mycobacteria inactivation but in this study there was complete inactivation independent of the bacillary load (Castro et al., 2009).

In conclusion, the NWU-TB system has shown good sensitivity and specificity results comparable to GeneXpert, which is the best in class molecular based TB detection test. The system is also safe, and based on a simple and efficient sputum sample preparation method. Further development of the NWU-TB system is ongoing.

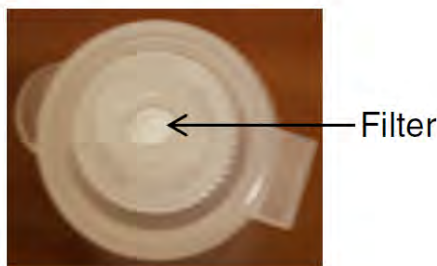


Fig. 5. Aerial view of LMR showing a fitted filter to trap bioaerosols during heat lysis.

5. Limitations of the study

The patient cohort does not represent the normal population and the high accuracy reported in this study could be due to the high prevalence of MTB strains with multiple copies of the insertion sequence IS6110 in this cohort (Alonso et al., 2013). Fresh sputum samples were used for GeneXpert, SSM and MGIT culture, but frozen samples stored for up to a year were used for NWU-TB analysis. Association between the use of frozen sputum samples and reduced the sensitivity of another (GeneXpert) molecular test has been previously reported (Steingart et al., 2013). The use of frozen samples may have altered the performance of NWU-TB and future evaluation studies must include fresh samples. Like all other current molecular based systems, our system does not distinguish between live and dead mycobacteria, which could partly explain the lower specificity especially in such a high TB prevalence setting.

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Conflict of interest

The authors declare that they have no conflicts of interest. All rights in patents were assigned to North-West University.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.mimet.2015.06.007>.

References

- Alonso, H., Samper, S., Martin, C., Ota, I., 2013. Mapping IS6110 in high-copy number *Mycobacterium tuberculosis* strains shows specific insertion points in the Beijing genotype. *BMC Genomics* 14, 422.
- Barreto, L.B.P.F., Lourenço, M.C.d.S., Rolla, V.C., Veloso, V.G., Huf, G., 2014. Use of amplified *Mycobacterium tuberculosis* direct test in respiratory samples from HIV-infected patients in Brazil. *J. Bras. Pneumol.: publicação oficial da Sociedade Brasileira de Pneumologia e Tisiologia* 40 (2), 148–154.
- BD Diagnostics, 15 September 2013. BD MGIT™ TBc identification test Vol. 2014 Date accessed.
- Becton Dickinson and Company, 2013. BBL™MGIT™Mycobacteria Growth Indicator Tube 7 mL With BACTEC™MGIT™960 Supplement Kit (Date accessed 15 September).
- Bemer-Melchior, P., Drugeon, H.B., 1999. Inactivation of *Mycobacterium tuberculosis* for DNA typing analysis. *J. Clin. Microbiol.* 37, 2350–2351.
- Boehme, C.C., Nabeta, P., Hillemann, D., Nicol, M.P., Shenai, S., Krapp, F., Allen, J., Tahirli, R., Blakemore, R., Rustomjee, R., Milovic, A., Jones, M., O'Brien, S.M., Persing, D.H., Ruesch-Gerdes, S., Gotuzzo, E., Rodrigues, C., Alland, D., Perkins, M.D., 2010. Rapid molecular detection of tuberculosis and rifampin resistance. *N. Engl. J. Med.* 363, 1005–1015.
- Boehme, C.C., Nicol, M.P., Nabeta, P., Michael, J.S., Gotuzzo, E., Tahirli, R., Gler, M.T., Blakemore, R., Worodria, W., Gray, C., Huang, L., Caceres, T., Mehdiyev, R., Raymond,

- L, Whitelaw, A., Sagadevan, K., Alexander, H., Albert, H., Cobelens, F., Cox, H., Alland, D., Perkins, M.D., 2011. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. *Lancet* 377, 1495–1505.
- Castan, P., dePablo, A., Fernández-Romero, N., Rubio, J., Cobb, B.D., Mingorance, J., Toro, C., 2014. Point-of-care system for detection of *Mycobacterium tuberculosis* and rifampin resistance in sputum samples. *J. Clin. Microbiol.* 52, 502–507.
- Castro, C., González, L., Roza, J.C., Puerto, G., Ribón, W., 2009. Biosafety evaluation of the DNA extraction protocol for *Mycobacterium tuberculosis* complex species, as implemented at the Instituto Nacional de Salud, Colombia. *Biomédica* 29, 561–566.
- Chang, K., Lu, W., Wang, J., Zhang, K., Jia, S., Li, F., Deng, S., Chen, M., 2012. Rapid and effective diagnosis of tuberculosis and rifampicin resistance with Xpert MTB/RIF assay: a meta-analysis. *J. Infect.* 64, 580–588.
- Chedore, P., Th'ing, C., Nolan, D.H., Churchwell, G.M., Sieffert, D.E., Hale, Y.M., Jamieson, F., 2002. Method for inactivating and fixing unstained smear preparations of *Mycobacterium tuberculosis* for improved laboratory safety. *J. Clin. Microbiol.* 40, 4077–4080.
- Coetzee, D., Hilderbrand, K., Goemaere, E., Matthys, F., Boelaert, M., 2004. Integrating tuberculosis and HIV care in the primary care setting in South Africa. *Trop. Med. Int. Health* 9, A11–A15.
- Collins, C.H., Kennedy, D.A., 1999. Laboratory Acquired Infections. Laboratory Acquired Infections: History, Incidence, Causes and Prevention. Butterworth-Heinemann, Boston.
- Corbett, E.L., Charalambous, S., Fielding, K., Clayton, T., Hayes, R.J., De Cock, K.M., Churchyard, G.J., 2003. Stable incidence rates of tuberculosis (TB) among human immunodeficiency virus (HIV)-negative South African gold miners during a decade of epidemic HIV-associated TB. *J. Infect. Dis.* 188, 1156–1163.
- Cruciani, M., Scarpato, C., Malena, M., Bosco, O., Serpelloni, G., Mengoli, C., 2004. Meta-analysis of BACTEC MGIT 960 and BACTEC 460TB, with or without solid media, for detection of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 42, 2321–2325.
- Dinnes, J., Deeks, J., Kunst, H., Gibson, A., Cummins, E., Waugh, N., Drobniewski, F., Lavan, A., 2007. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. *Health Technology Assessment (Winchester, England)* 11 pp. 1–196.
- Garber, E., San, G.P., Lambert, L., Saiman, L., 2003. A survey of latent tuberculosis infection among laboratory healthcare workers in New York City. *Infect. Control Hosp. Epidemiol.* 24, 801–806.
- Grist, N.R., Emslie, J.A.N., 1985. Infections in British clinical laboratories. *J. Clin. Pathol.* 40, 826–829 (1982–3).
- Grobler, A., Levanets, O., Whitney, S., Booth, C., Viljoen, H., 2012. Rapid cell lysis and DNA capture in a lysis microreactor. *Chem. Eng. Sci.* 81, 311–318.
- Harries, A., Banda, H., Boeree, M., Welby, S., Wirima, J., Subramanyam, V., Maher, D., Nunn, P., 1998a. Management of pulmonary tuberculosis suspects with negative sputum smears and normal or minimally abnormal chest radiographs in resource-poor settings. *Int. J. Tuberc. Lung Dis.* 2, 999–1004.
- Harries, A.D., Maher, D., Nunn, P., 1998b. An approach to the problems of diagnosing and treating adult smear-negative pulmonary tuberculosis in high-HIV-prevalence settings in sub-Saharan Africa. *Bull. World Health Organ.* 76, 651–662.
- Helb, D., Jones, M., Story, E., Boehme, C., Wallace, E., Ho, K., Kop, J., Owens, M.R., Rodgers, R., Banada, P., Safi, H., Blakemore, R., Lan, N.T.N., Jones-López, E.C., Levi, M., Burday, M., Ayakaka, I., Mugerwa, R.D., McMillan, B., Winn-Deen, E., Christel, L., Dailey, P., Perkins, M.D., Persing, D.H., 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.
- Kambashi, B., Mbulo, G., McNeerney, R., Tembwe, R., Kambashi, A., Tihon, V., Godfrey-Faussett, P., 2001. Utility of nucleic acid amplification techniques for the diagnosis of pulmonary tuberculosis in sub-Saharan Africa. *Int. J. Tuberc. Lung Dis.* 5, 364–369.
- Kent, P.T., 1985. Public Health Mycobacteriology: A Guide for the Level III Laboratory. In: Kubica, G.P. (Ed.), U.S. Dept. of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Ga.
- Koch, R., 1882. Die Aetiologie der Tuberkulose. *Berl. Klin. Wochenschr.* 15, 221–230.
- Kusum, S., Aman, S., Pallab, R., Kumar, S., Manish, M., Sudesh, P., Subhash, V., Meera, S., 2011. Multiplex PCR for rapid diagnosis of tuberculous meningitis. *J. Neurol.* 258, 1781–1787.
- Lawn, S.D., Nicol, M.P., 2011. Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. *Future Microbiol.* 6, 1067–1082.
- Lawn, S.D., Mwaba, P., Bates, M., Piatek, A., Alexander, H., Marais, B.J., Cuevas, L.E., McHugh, Z.A., Zijenah, L., Kapata, N., Abubakar, I., McNeerney, R., Hoelscher, M., Memish, Z.A., Migliori, G.B., Kim, P., Maeurer, M., Schito, M., Zumla, A., 2013. Advances in tuberculosis diagnostics: the Xpert MTB/RIF assay and future prospects for a point-of-care test. *Lancet Infect. Dis.* 13, 349–361.
- Maher, D., 2009. Clinical Features and Index of Suspicion in Adults (HIV-negative and HIV-positive). *Tuberculosis: A Comprehensive Clinical Reference*. Saunders, Philadelphia, pp. 164–168.
- Marlowe, E.M., Novak-Weekley, S.M., Cumpio, J., Sharp, S.E., Momeny, M.A., Babst, A., Carlson, J.S., Kawamura, M., Pandori, M., 2011. Evaluation of the Cepheid Xpert MTB/RIF assay for direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens. *J. Clin. Microbiol.* 49, 1621–1623.
- McCarthy, K.D., Cain, K.P., Winthrop, K.L., Udomsantisuk, N., Lan, N.T.N., Sar, B., Kimerling, M.E., Kanara, N., Lynen, L., Monkongdee, P., Tasaneeyapan, T., Varma, J.K., 2012. Nontuberculous mycobacterial disease in patients with HIV in Southeast Asia. *Am. J. Respir. Crit. Care Med.* 185, 981–988.
- Miller, C.D., Songer, J.R., Sullivan, J.F., 1987. A twenty-five year review of laboratory-acquired human infections at the National Animal Disease Center. *Am. Ind. Hyg. Assoc. J.* 48, 271–275.
- Muchwa, C., Akol, J., Etwom, A., Morgan, K., Onkiriza, P., Mumbowa, F., Odong, P., Kateete, D., Eisenach, K., Joloba, M., 2012. Evaluation of Capilia TB assay for rapid identification of *Mycobacterium tuberculosis* complex in BACTEC MGIT 960 and BACTEC 9120 blood cultures. *BMC Res. Notes* 5, 44.
- Muller, H.E., 1988. Laboratory-acquired mycobacterial infection. *Lancet* 2, 331.
- Nicol, M.P., Workman, L., Isaacs, W., Munro, J., Black, F., Eley, B., Boehme, C.C., Zemanay, W., Zar, H.J., 2011. Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: a descriptive study. *Lancet Infect. Dis.* 11, 819–824.
- Pai, M., Flores, L.L., Hubbard, A., Riley, L.W., Colford, J.M., 2004a. Nucleic acid amplification in the diagnosis of tuberculous pleuritis: a systematic review and meta-analysis. *BMC Infect. Dis.* 4, 6.
- Pai, M., McCulloch, M., Enanoria, W., Colford, J.M., 2004b. Systematic reviews of diagnostic test evaluations: what's behind the scenes? *Evid. Based Med.* 9, 101–103.
- Patel, J.B., Leonard, D.G.B., Pan, X., Musser, J.M., Berman, R.E., Nachamkin, I., 2000. Sequence-based identification of *Mycobacterium tuberculosis* species using the MicroSeq 500 16S rDNA bacterial identification system. *J. Clin. Microbiol.* 38, 246–251.
- Pike, R.M., 1976. Laboratory-associated infections: summary and analysis of 3921 cases. *Health Lab. Sci.* 13, 105–114.
- Pike, R.M., 1979. Laboratory-associated infections: incidence, fatalities, causes, and prevention. *Annu. Rev. Microbiol.* 33, 41–66.
- Scott, L.E., McCarthy, K., Gous, N., Nduna, M., Van Rie, A., Sanne, I., Venter, W.F., Duse, A., Stevens, W., 2011. Comparison of Xpert MTB/RIF with other nucleic acid technologies for diagnosing pulmonary tuberculosis in a high HIV prevalence setting: a prospective study. *PLoS Med.* 8, e1001061.
- Sharma, S.K., Mohan, A., 2006. Multidrug-resistant tuberculosis*: A menace that threatens to destabilize tuberculosis control. *Chest* 130, 261–272.
- Small, P.M., Pai, M., 2010. Tuberculosis diagnosis – time for a game change. *N. Engl. J. Med.* 363, 1070–1071.
- Ssengendo, W., Nakiyingi, L., Armstrong, D.T., Cobelens, F.G., Alland, D., Manabe, Y.C., Dorman, S.E., Ellner, J.J., Joloba, M.L., 2014. Clinical utility of a novel molecular assay in various combination strategies with existing methods for diagnosis of HIV-related tuberculosis in Uganda. *PLoS One* 9, e107595.
- Steingart, K.R., Ng, V., Henry, M., Hopewell, P.C., Ramsay, A., Cunningham, J., Urbanczik, R., Perkins, M.D., Aziz, M.A., 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, K.R., Sohn, H., Schiller, I., Kloda, L.A., Boehme, C.C., Pai, M., Dendukuri, N., 2013. Xpert(R) MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst. Rev.* 1, CD009593.
- Sudre, P., ten Dam, G., Kochi, A., 1992. Tuberculosis: a global overview of the situation today. *Bull. World Health Organ.* 70, 149–159.
- Theron, G., Peter, J., van Zyl-Smit, R., Mishra, H., Streicher, E., Murray, S., Dawson, R., Whitelaw, A., Hoelscher, M., Sharma, S., Pai, M., Warren, R., Dheda, K., 2011. Evaluation of the Xpert MTB/RIF assay for the diagnosis of pulmonary tuberculosis in a high HIV prevalence setting. *Am. J. Respir. Crit. Care Med.* 184, 132–140.
- UNAIDS, 2013. Report on the Global AIDS Epidemic 2013. Joint United Nations Programme on HIV/AIDS.
- Urdea, M., Penny, L.A., Olmsted, S.S., Giovanni, M.Y., Kaspar, P., Shepherd, A., Wilson, P., Dahl, C.A., Buchsbaum, S., Moeller, G., Hay Burgess, D.C., 2006. Requirements for high impact diagnostics in the developing world. 444 (Suppl 1), 73–79.
- World Health Organization, 1998. Laboratory Services in Tuberculosis Control, Part 2: Microscopy Publication Number. World Health Organization, Geneva, Switzerland.
- World Health Organization, 2004. Rapid HIV Tests: Guidelines for Use in HIV Testing and Counselling Services in Resource-Constrained Settings. WHO, Geneva.
- World Health Organization, 2014. Global Tuberculosis Report 2014. WHO, Geneva Switzerland.
- Zar, H.J., Hanslo, D., Apolles, P., Swingle, G., Hussey, G., 2005. Induced sputum versus gastric lavage for microbiological confirmation of pulmonary tuberculosis in infants and young children: a prospective study. *Lancet* 365, 130–134.
- Zwadyk, P., Down, J.A., Myers, N., Dey, M.S., 1994. Rendering of mycobacteria safe for molecular diagnostic studies and development of a lysis method for strand displacement amplification and PCR. *J. Clin. Microbiol.* 32, 2140–2146.

CHAPTER 5:

Detection of M. tuberculosis by an improved NWU-TB test

This chapter consists of a full length article that has been submitted to the *Journal of Clinical Microbiology*. The author guidelines for submitting to the Journal can be found in “Annexure D”, and these guidelines were followed in writing the manuscript. Two exceptions are made in following the guidelines: first, for easy reading figures, schemes and tables are inserted at their logical places as they would appear in the printed version and second, 1.5 line spacing was used to ensure consistency with the rest of the thesis. This chapter is connected to objective 1 covered in chapter 3 as it is an improvement of the NWU-TB system and also deals with the third objective of the study. The third objective aimed to develop a feasible strategy that can enable the NWU-TB system (and consequently other NAATs) to be used for tuberculosis treatment outcomes monitoring. The improved system discussed in this chapter uses a real-time PCR platform and a strategy to distinguish between live and dead mycobacteria. The ability to distinguish between live and dead mycobacteria will allow the use of a PCR based assay to monitor a patient’s response to therapy, which is not currently possible in clinical practice with any of the commercial PCR based MTB assays. Though a full prospective longitudinal study could not be conducted in order to evaluate the utility of the strategy for treatment outcomes monitoring, results presented in this chapter demonstrate that with further optimization the NWU-TB assay can potentially be used for treatment monitoring.

Proof of submission: Journal of Clinical Microbiology

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to me, isaacmutingwende@yahoo.com

Dear Mr. Mutingwende,

On February 19, 2016, we received the manuscript "Detection of Mycobacterium tuberculosis by an improved NWU-TB system potentially capable to differentiate between live and dead mycobacteria" by Isaac Mutingwende, Urban Vermeulen, Faans Steyn, Hendrik Viljoen, and Anne Grobler. The submission form indicates that this paper should be processed as a (n) Full-Length Text intended for publication in the section Mycobacteriology and Aerobic Actinomycetes.

The manuscript has been assigned the control number JCM00377-16. Take note of this number, and refer to it in any correspondence with the Journals Department or with the editor.

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Thank you for submitting your work to Journal of Clinical Microbiology.

Regards,
JCM staff

Detection of *Mycobacterium tuberculosis* by an improved NWU-TB system potentially capable to discriminate between live and dead mycobacteria

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Running title: Detection of *M. tuberculosis* by an improved NWU-TB

Key words: Tuberculosis; diagnosis; NWU-TB system; propidium monoazide; live/dead discrimination; sputum

Abstract (249 words)

Delays in tuberculosis diagnosis and initiation of appropriate treatment limit the success of global efforts to eradicate the disease. Inability of current molecular assays to discriminate between live and dead mycobacteria limit their deployment in endemic settings. In this study, the performance of the NWU-TB system under development was evaluated, in comparison to smear microscopy, Xpert MTB/Rif and MGIT culture. The effectiveness of propidium monoazide (PMA) to distinguish between live and dead mycobacteria was also evaluated. 176 randomly selected stored sputum samples were used to evaluate the NWU-TB system. Next, a random subset of 50 samples selected from the 176 samples was used for live/dead discrimination evaluation using PMA. The ΔC_T ($C_{T \text{ PMA-treated}} - C_{T \text{ PMA-untreated}}$) was calculated and samples were stratified by TB treatment status for analysis. Using MGIT culture as the gold standard, the sensitivities % (95% CI) of smear, Xpert MTB/Rif, and NWU-TB were 64.3 (55.4-72.6), 84 (76.2-90.1), and 94.6 (89.1-97.8) respectively, while the specificities % (95% CI) were 62.1 (42.3-79.3), 100 (88.1-100) and 86.2 (68.3-96.1) respectively. There was no statistically significant difference ($p=0.277$) between the ΔC_T s of treatment naive and on treatment groups. NWU-TB and Xpert MTB/Rif have similar overall diagnostic accuracies for detection of pulmonary TB. The potential of PMA to discriminate between live and dead mycobacteria has been demonstrated in clinical samples. This finding is important for TB endemic settings where it is critical for molecular tests to identify true reinfection cases in addition to being applicable to treatment outcomes monitoring.

1.0 Introduction

Despite being an ancient disease, *Mycobacterium tuberculosis* (MTB) is still one of the major infectious diseases in the 21st century. In 2013 alone, an estimated 9.0 million people developed TB and 1.5 million succumbed to the disease; 360,000 of whom were HIV-positive (WHO, 2014). Pulmonary TB is the most common form of the disease. South Africa has the highest TB incidence in the world, estimated to be 652/100,000 population in 2013. Prompt diagnosis and treatment are crucial to reduce the prevalence and incidence of the disease. Treatment initiation delay has been shown to vary from 30 to 90 days from onset of symptoms mainly due to diagnostic delay (1, 2). Modelling studies indicate that a sensitive, same-day and accessible TB diagnostic could reduce mortality by 20-35% as result of early treatment initiation (3). Rapid, accurate, affordable, and accessible TB diagnostics that allow same day diagnosis and treatment initiation are urgently needed if the TB fight is to be won in resource constrained settings.

Sputum smear microscopy (SSM) is the first and often the only TB diagnostic test used in resource limited settings and requires at least 2 specimens to be submitted on different days; it has a low and variable sensitivity, is tedious, and requires skilled microscopists (4). Mycobacteria Culture, the current global gold standard is highly sensitive and specific, but suffer from such problems as long turnaround time, need for expensive equipment, and it is prone to cross contamination especially in high prevalence settings (5-7). Both tests are thus associated with treatment initiation delays. Nucleic acid amplifications tests (NAATs) provide a viable option for a point-of-care TB (POC-TB) diagnostic. A number of in-house and commercial NAATs have been developed to identify MTB in clinical specimens rapidly and directly (8-15). Meta-analysis studies have reported sensitivity and specificity of in-house PCR assays to be 9.4%–100% and 5.6%–100% respectively (15) and of commercial assays to be 36–100% and 54–100% (9) respectively.

In general, improvement in accuracy is necessary if NAATs are to become more clinically relevant and beneficial to patients in low resource settings and areas with a high HIV prevalence. HIV infection reduces the bacillary load in sputum and consequently reduces sensitivity of molecular tests in HIV-infected individuals compared to HIV-uninfected individuals (16, 17). Therefore, new or improved NAATs should close this sensitivity gap compared to culture in individuals with paucibacillary disease. The inability of NAATs to discriminate between live and dead mycobacteria also contributes to their poor specificity when compared to culture. For example, a large proportion of patients presumed to have been successfully treated remain Xpert MTB/Rif - positive for up to 5 years (18). Detection of reinfection cases in endemic settings is another

challenge associated with all current molecular assays. In a study assessing the performance of Xpert MTB/Rif for monitoring treatment response, the proportion of positive Xpert MTB/Rif results was twice that of positive liquid cultures (84% vs. 42%) after 8 weeks of treatment and at the end of the 26 week monitoring period, 27% of samples were Xpert MTB/Rif positive compared to only 4% with MGIT culture (19). Therefore, new molecular assays should ideally also be suitable for diagnosing previously treated patients and treatment outcomes monitoring. Improving current molecular assays such as Xpert MTB/Rif is a viable option but the development of new systems also remains an attractive alternative. Current efforts with the Xpert MTB/RIF Ultra (Ultra) assay, which uses a new sample processing cartridge that doubles the amount of purified DNA delivered to the PCR reaction, are commendable as the new assay will have better a sensitivity than the original Xpert MTB/Rif assay (20, 21), but it still cannot distinguish between live and dead mycobacteria.

Pretreatment of clinical samples with a DNA-intercalating agent, propidium monoazide (PMA) prior to DNA isolation and amplification has been investigated as a possibility (22). The PMA dye can penetrate dead cells and covalently form a PCR-unamplifiable DNA complex upon light activation (22). However, PMA cannot penetrate live cells, and thus the DNA from live cells is PCR-amplifiable. After PMA processing, downstream processes remain unaltered, enabling coupling with any molecular assay. Current evidence on the effectiveness of the PMA approach in clinical specimens is limited (23, 24), therefore more evaluations are needed.

The aim of this study was to (i) evaluate the performance of an improved rapid molecular assay known as the NWU-TB (25) for detection of *M. tuberculosis* complex in clinical sputum samples and (ii) to evaluate whether PMA can be used in conjunction with the NWU-TB system for the selective amplification and detection of DNA from viable mycobacteria in patient sputum samples.

2.0 Methods

2.1 Study Design

From January 2013 to December 2014, a total 548 sputum samples were collected from tuberculosis presumptive miners visiting the Orkney West-Vaal hospital. The hospital is owned and privately operated by AngloGold Ashanti to cater for the healthcare needs of its miners. Upon submission of a fresh same day sputum sample, an aliquot was used to perform SSM, MGIT culture and Xpert MTB/Rif. The remainder of these fresh un-decontaminated samples was frozen at -20°C and stored at the hospital for an average of 2 weeks. The frozen samples were transferred

under appropriate conditions to the North-West University from Orkney-Westvaal hospital (a 52 km distance) and stored at -20 °C until used in evaluating the NWU-TB system. Samples were stored at North-West University for time periods ranging from 2 weeks to 1 year before testing.

Qualified laboratory scientists stationed at Orkney-Westvaal hospital performed the three reference tests (SSM, Xpert MTB/Rif, MGIT culture) as part of their routine work. MTB results for these 3 reference tests were kept blind to the North-West University (NWU) research team up until the data analysis stage. In this study, 176 stored random nonduplicated clinical sputum samples were used to evaluate the NWU-TB system at the North-West University and then results from the 3 reference tests for the tested samples were requested from the hospital for statistical analysis.

2.2 SSM, MGIT Culture and Xpert MTB/Rif

SSM and MGIT culture were performed by experienced and independent laboratory scientists working at Orkney-Westvaal hospital following appropriate standard protocols and manufacturers' instructions (26, 27). Briefly, equal volumes of sputum and N-acetyl-L-cysteine – sodium hydroxide solution were mixed. After 20 minutes, the mixture was neutralized with phosphate buffered saline and centrifuged. The sediment obtained was resuspended in 1 ml sterile phosphate buffer and used for smear preparation and inoculation in culture. Sputum smear grading of Ziehl-Neelsen stained smears was performed in accordance with the WHO criteria (26). For MGIT culture, MGIT tubes (Becton Dickinson, Franklin Lakes, NJ, USA) inoculated with 0.5ml of decontaminated sample were incubated at 37°C. Samples were considered negative when no growth was automatically detected by the MGIT instrument after 42 days. Mycobacterial species in positive cultures were identified using AccuProbe molecular probes (GenProbe, San Diego, CA, USA).

Xpert MTB/Rif was performed as per manufacturer's instructions (Cepheid, Sunnyvale, CA, USA). Briefly, 1 ml of sputum sample was mixed with 2 ml of Xpert sample reagent, vortexed for at least 10 seconds, and incubated at room temperature for 10 minutes, followed by another round of vortexing for 10 seconds and room temperature incubation for 5 minutes. A total of 2 ml of the mixture was transferred into the Xpert cartridge and loaded into the Xpert MTB/Rif instrument. The automatic detection and reporting procedure was then run and results automatically reported.

2.3 NWU-TB system

2.3.1 Sample preparation

Previously frozen sputum samples were thawed overnight at 4°C. A 250µl sputum aliquot was mixed with an equal volume of a lysis buffer followed by lysis on a lyser device for 7 minutes as previously described (25, 28). Initial experiments using 5µl of lysate without a DNA concentration step, using a real time instrument (as achieved with the endpoint PCR based system) (25) did not achieve optimal amplification even after intensive qPCR optimization with the aid of the Taguchi method (29). To address the challenge, MTB DNA was extracted and purified using a ZR Viral DNA/RNA Kit (Zymo Research, Irvine, CA), with modifications to concentrate DNA. To 500µL of the lysate, 400µL of genomic lysis buffer and 165µL isopropanol were added, and the mixture was then vortexed for 4-6s before incubation for 10 minutes at room temperature. The mixture was transferred into a Zymo-spin column and centrifuged at 10 000g for 80s. The collection tube was discarded, followed sequentially by addition of

- (i) 200µL DNA pre-wash buffer and centrifugation for 60s at 10 000g;
- (ii) 500µL of g-DNA wash buffer followed by 60s centrifugation at 10 000g;
- (iii) 60µL of elution buffer pre-equilibrated at 65°C was followed by centrifugation for 45s at 10 000g.

The eluted DNA was stored at -20°C until used in subsequent qPCR experiments. Five (5) microliters of each DNA sample was used as a template for amplification in real-time PCR. The Zymo kit was selected due to its quick (15 minutes) DNA recovery step after the lysis step, thus enabling the sample processing step to be carried out in less than 30 minutes.

2.3.2 Design and synthesis of primer and fluorogenic probe.

The IS6110 insertion sequence (Accession number: AJ242908), which is unique to the MTBC members [23, 24] was the target for PCR amplification. The primer and scorpion probe were designed using Beacon Designer software (Version 8.13, Premier Biosoft International). The sequence of the primer is: CGGCGGCTGGGCTCCC and of the scorpion probe:

[FAM]-GGTCACCGGTTGATGTGGTCGTAGTAGGTCGCGGTGACC-[BHQ1]-[HEG]-CGAGCTGGGTGTGCCGATC. The primer and scorpion probe were both synthesized by Sigma Aldrich (reference number: 8203265032).

2.3.3 Real-time PCR

The amplification reaction mixture contained 5 µl of template in a final volume of 20µl: 10µl KAPA Probe Fast qPCR Hotstart Mastermix 2x (Kapa Biosystems/Roche, Cape Town, South

Africa), 0.1µl (100nM final concentration) primer, 0.25µl (250nM final concentration) scorpion probe, and 4.65µl molecular grade water. PCR was carried out using a Bio-Rad CFX 96 (Biorad CFX Manager 3.1 software) with the following cycling protocol: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10s, 63°C for 15s, 72°C for 10s. All runs included a no-template control and a positive control. All samples were tested in triplicate. A result was considered negative when no amplification occurred or when the CT value was >37 cycles. The results were considered positive when CT values were <37 cycles for all three samples.

2.4 Live/dead discrimination

The next objective was to test the effectiveness of propidium monoazide (PMA) in distinguishing between live and dead mycobacteria in clinical sputum samples from TB treatment naive patients and patients at various stages of standard TB therapy. Of the 176 samples used to evaluate the performance of NWU-TB as described in the sections above, 50 sputum samples were randomly selected and used in the live/dead discrimination evaluation. Each of the 50 samples was split into 2 aliquots. The first aliquot was handled as per the standard NWU-TB protocol described above. The second aliquot was first treated with PMA according to the optimal conditions described elsewhere (23, 30, 31). Briefly, PMA (Biotium, Inc., Hayward, CA, USA) was dissolved in 20% of dimethylsulfoxide (DMSO) (Sigma) and aliquoted before storage in the dark at -20°C. PMA was added to 500µL of sample (buffer + sputum) to a final concentration of 100 µM and incubated in the dark for 10 minutes with occasional flipping to allow diffusion of the intercalating dyes into dead cells. Light exposure was performed for 5 minutes using a 650 watt halogen light source (GE, South Africa) placed at a distance of 20 centimeters from the samples. The samples were kept on ice in horizontal orientation to avoid excessive heating. The samples were then subjected to the same procedure described above for the NWU-TB system, from lysis to qPCR.

2.5 Ethics

This study was approved by the North-West University Research Ethics Committee under ethics number: NWU-00005-11-A5.

2.6 Statistical Analysis

Statistical analyses were performed using STATA 11 (StataCorp, College Station, TX, USA). A p-value of less than 0.05 was considered statistically significant. Sensitivity and specificity, positive predictive value (PPV), negative predictive value (NPV), and area under the curve (AUC) for smear microscopy, Xpert MTB/RIF, and NWU-TB were calculated using MGIT culture as

reference. The ΔC_T , *i.e.* is the difference between the C_T values of PMA-treated aliquot and the C_T value of the PMA-untreated aliquot was calculated for each sample. A C_T value > 37 was adjusted to 37 for ΔC_T calculations.

3.0 Results

3.1 Study population

Of the 176 samples tested, 158 patient sputum samples were included in the analysis to evaluate the NWU-TB system in comparison to SSM and Xpert MTB/Rif against MGIT culture as the gold standard. The other 18 samples had missing data from one of the 3 references tests and thus were not included in the analysis. The population predominantly consisted of 96.13% male patients that originated from a mining community. The mean age of patients was 44.49 years as shown in Table 1. Next, a subgroup of 50 randomly selected samples from the 176 samples was used to evaluate the effectiveness of the use of PMA for live/dead mycobacteria discrimination. The population characteristics of this subgroup are provided in Table 1 alongside those of the overall group.

3.2 Correlation of NWU-TB C_T values and smear grades

To determine the correlation between quantification of bacterial load by SSM and NWU-TB categorization, the C_T values were stratified according to smear grade using the World Health Organization grading system (26). The smear grading was performed by experienced laboratory staff at Orkney-Westvaal hospital. The correlation results between the smear grades and C_T values from the NWU-TB system are presented in Figure 1. A strong negative correlation between C_T values and smear grades was found (Spearman rank correlation -0.71 , $p < 0.001$).

Table 1: Study population characteristics

	NWU-TB evaluation group	PMA evaluation group
Age [% (95% CI)]	44.49 (43.01-45.97)	
Sex:		
Male n (%)	149 (96.13%)	47 (95.91%)
Female n (%)	6 (3.87%)	2 (4.08%)
HIV status where known:		
Positive n (%)	82 (78.85%)	28 (80.0%)
Negative n (%)	22 (21.15%)	7 (20.0%)
Current TB treatment:		
Yes n (%)	99 (63.87%)	19 (38.78%)
No n (%)	56 (36.13%)	30 (61.22%)

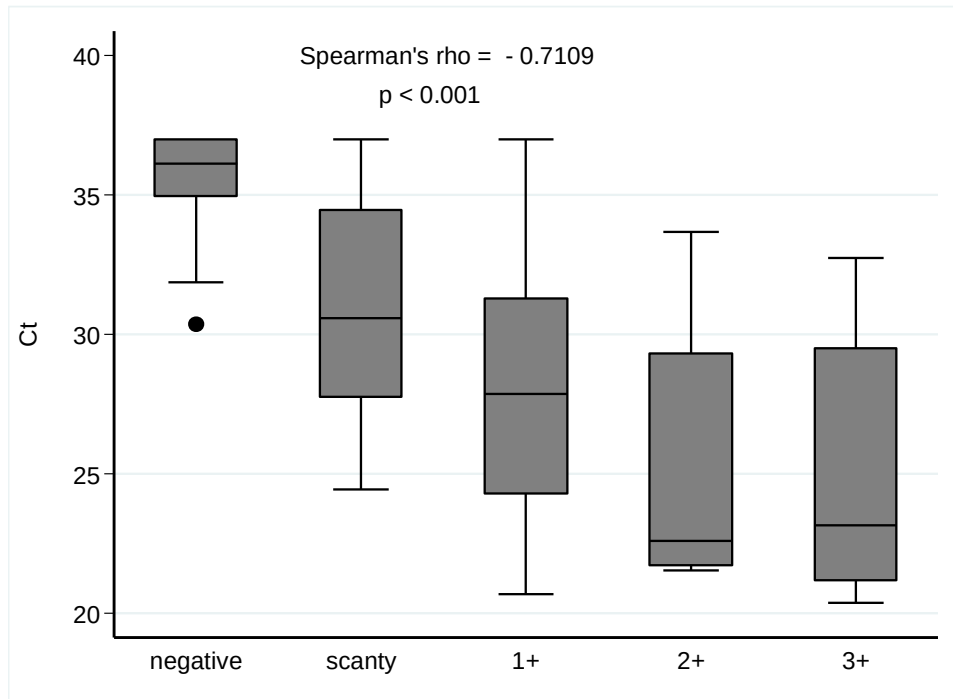


Figure 1: Correlation between NWU-TB CT values and bacteriology results. Boxes represent the 25th, 50th (median) and 75th quartiles with whiskers extending to the greatest and smallest values. SSM grading was done according to prescriptions of the World Health Organization (26).

3.3 Comparison of NWU-TB system and MGIT culture

NWU-TB had a higher sensitivity at 94.6% [95% CI: 89.1%; 97.8%) compared to Xpert MTB/Rif 84% (p=0.0025) and SSM 64.3% (p<0.001) as given in Table 2. However, Xpert MTB/Rif had a specificity of 100% [95% CI: 88.1%-100%), which was significantly higher than NWU-TB at 86.2% (p<0.001) and SSM at 62.1% (p<0.001). Overall, there was a strong correlation between Xpert MTB/Rif and NWU-TB (Mc Nemar's chisquare =11.64, p value = 0.0006).

Table 2: Performance of SSM, Xpert MTB/Rif and NWU-TB against MGIT culture as gold standard

	SSM	Xpert MTB/Rif	NWU-TB
Sensitivity% (95% CI)	64.3 (55.4-72.6)	84 (76.2-90.1)	94.6 (89.1-97.8)
Specificity% (95% CI)	62.1 (42.3-79.3)	100 (88.1-100)	86.2 (68.3-96.1)
PPV% (95%CI)	88.3 (80-94)	100 (96.4-100)	96.8 (92.1-99.1)
NPV% (95% CI)	28.1 (17.6-40.8)	60.4 (45.3-74.2)	78.1 (60-90.7)
*AUC	0.6255	0.9202	0.9016

*AUC – Area under the curve

Overall Diagnostic accuracy of NWU-TB in comparison to Xpert MTB/Rif and SSM using ROC curves

Receiver operator characteristic (ROC) analysis indicated that the area under the curve for SSM, GeneXpert and NWU-TB was 0.6255, 0.9202 and 0.9016 respectively. An assay with an area under the curve (AUC) greater than 0.9 is considered to have excellent performance, based on the ROC analysis both GeneXpert and NWU-TB showed excellent performance.

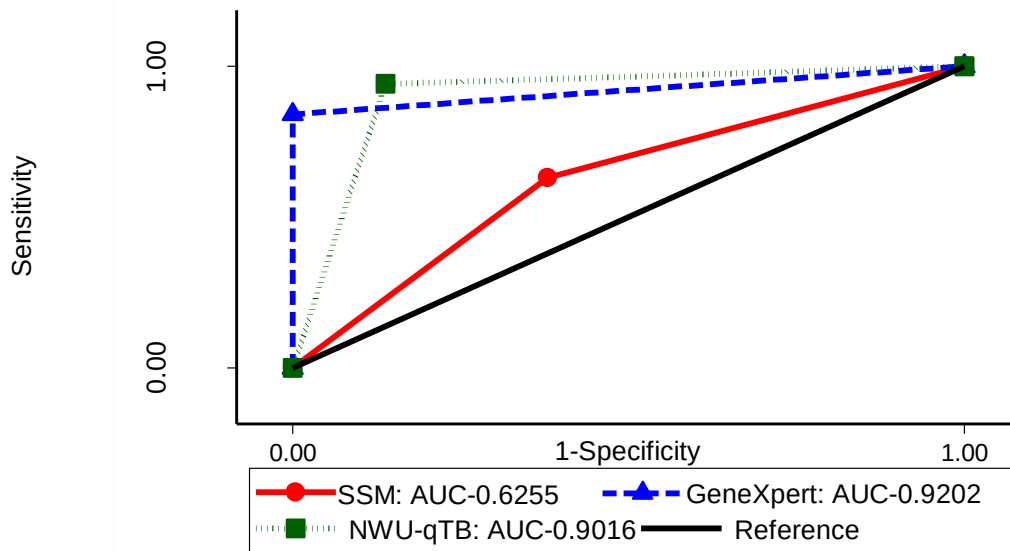


Figure 2: Receiver operator characteristic curves for NWU-TB, SSM and GeneXpert compared to MGIT culture as gold standard.

3.4 PMA treatment

The number of samples divided into 5 different smear grades (Negative, Scanty, 1+, 2+, and 3+) and the corresponding cultures results are given in Table 3. Of the 50 samples used in the sub-study most of the samples was classified as smear negative.

Table 3: Smear and culture results of 50 clinical sputum samples used in PMA testing

AFB grade*	Culture		Missing	Total
	Positive	Negative		
Negative	13	4	1	18
Scanty	9	3		12
1+	7	1		8
2+	5	0		5
3+	7	0		7
Total				50

*Smear grades categorized according to the World Health Organization guidelines for AFB smear positivity (26). Abbreviations: AFB, acid-fast bacilli; PMA, propidium monoazide.

All culture negative samples except one were undetectable by both and Xpert MTB/Rif and NWU-TB. The discrepant sample (Sample 12 in Table 4) tested positive with both Xpert MTB/Rif and NWU-TB without PMA treatment; however, when the sample was treated with PMA, followed by NWU-TB it tested negative giving a very large ΔC_T . In contrast, 8 culture positive samples tested positive on the NWU-TB system without PMA treatment, however after PMA treatment they tested negative. The C_T values without PMA treatment in these samples were above 30 cycles. In 3 patients who had NTM infection (*M. chelonae* and *M. Kansasii*), all the tests (Xpert MTB/Rif, NWU-TB and MGIT culture) reported negative results except SSM.

Table 4: Discrepant results from the four tests including PMA-treated results from NWU-TB

*Sample	SSM	Culture	Xpert MTB/Rif	NWU-TB PMA-untreated	C_T NWU-TB PMA-untreated	ΔC_T
1	Negative	Positive	MTB-low	Positive	34.93	2.07
2	Negative	Positive	Negative	Positive	35.55	1.45
3	Negative	Positive	MTB-Very low	Positive	36.73	0.27
4	Negative	Positive	Negative	Negative	37	0
5	Negative	Positive	Not done	Positive	34.99	2.01
6	Negative	Positive	Not done	Positive	35.78	1.22
7	Negative	Positive	Negative	Positive	36.13	0.87
8	Scanty 8	Positive	Negative	Positive	35.85	1.15
9	Scanty 5	Negative	MTB-Very low	Positive	27.71	9.29
10	Scanty 7	Positive	MTB-Very Low	Positive	30.57	6.43
11	1+	<i>M. chelonae</i>	Negative	Negative	37	0
12	Scanty 4	<i>M. kansasii</i>	Negative	Negative	37	0
13	Scanty 4	<i>M. kansasii</i>	Negative	Negative	37	0

Note: All C_T values for samples shown in this table were >37 after PMA-treatment, which means they all tested negative for live mycobacteria. $\Delta C_T = C_{T \text{ PMA-treated}} - C_{T \text{ PMA-Untreated}}$. The shaded cells indicated those samples where the results of Culture, Xpert MBT/Rif and NWU-TB differed.

* Re-coded numbering; Not the actual patient identification codes used in the study

PMA treatment did not yield statistically significant differences when data was stratified according to TB treatment status as shown in Figure 3. The nonparametric median equality test of the ΔC_T values of the treatment naive and treatment groups was 0.277. There were however observable differences with C_T values of PMA-treated samples higher than those of PMA-untreated samples in most of the cases. Since the sample size was too small to stratify the patient samples according

to TB treatment stage, sufficiently powered statistical analyses could not be performed to highlight the differences observed.

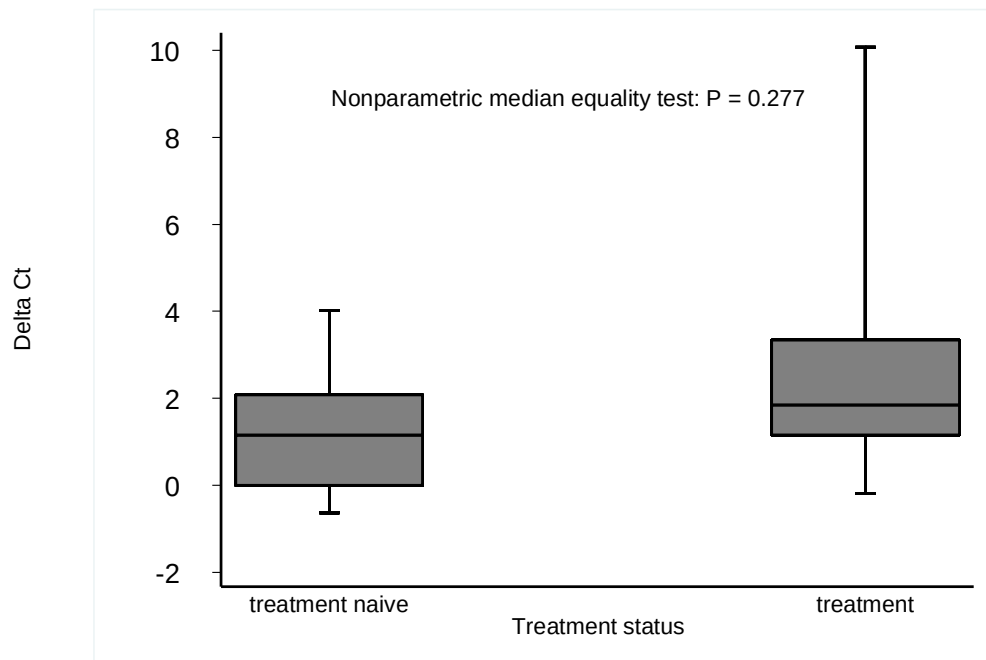


Figure 3: Comparison between the mean difference in threshold cycle (ΔCt) (propidium monoazide (PMA) treated minus PMA untreated) obtained from sputum samples collected from patients either receiving anti-tuberculosis therapy (treatment group) or not receiving treatment (treatment naive).

4.0 Discussion

Early detection of MTB and initiation of appropriate anti-tuberculosis therapy are critical steps in ensuring both better disease prognosis in patients and epidemic control efforts. Currently, SSM and culture are the most relied upon tests for the initial diagnosis and treatment response monitoring in most high burdened countries. Though relatively sensitive for both initial diagnosis and treatment monitoring, culture is largely handicapped by its long turnaround time. SSM can produce results within a day but it cannot determine the viability of mycobacteria. Recently, considerable focus has been placed on PCR-based assays due to their short turnaround times and good accuracy (9, 32-35). However, like SSM no current commercial PCR-based assay can distinguish between live and dead mycobacteria thereby making the assays unreliable, especially in high endemic areas where reinfections cases are common. In this study, the performance of a new qPCR based NWU-TB system in clinical sputum samples was evaluated, and then the effectiveness of using PMA to distinguish between live and dead mycobacteria in sputum from patients on either standard anti-tuberculosis therapy or treatment naive was also explored.

NWU-TB showed better sensitivity than both SSM and Xpert MTB/Rif. The sensitivity values for Xpert MTB/Rif and SSM are comparable to those reported in literature (4, 36), thus results from this study can be trusted but still some caution must be exercised when drawing conclusions. By extrapolation, this means that the sensitivity of NWU-TB is comparable to other commercial molecular systems and warrant is its further development.

On the other hand, the specificity of NWU-TB was significantly lower than that of Xpert MTB/Rif but comparable to that of other molecular assays reported elsewhere (37, 38). The lower specificity of NWU-TB may have been due to MTB-NTM coinfection though this was not investigated nor confirmed in this study. The high prevalence of NTMs in the cohort used as previously reported (25) strongly supports the MTB/NTM coinfection argument. Although speciation was done, the presence of a mixture of MTB and NTM in a patient sample may interfere with the performance of a molecular test depending on the relative concentrations of the MTB and NTM in that sample thereby reducing specificity (39).

Study results may be influenced by the reference standard used to compare test results. Although culture is the universally accepted gold standard, it is not 100% sensitive and can give false-negative results (40), thus presenting obstacles for evaluating new diagnostics especially in cohorts with a high HIV prevalence rate. However, in this study the effect of an imperfect reference standard on the reported accuracies of the tests is assumed to be minimal due to the experience of the technicians performing the tests at the hospital. The area under the curve (AUC) for both Xpert MTB/Rif and NWU-TB was above 0.9 indicating that they are both excellent tests according to a widely accepted guideline (41), while the AUC for SSM was 0.6255, confirming its widely reported lower accuracy.

The NWU-TB results reported in this paper demonstrate that current efforts to improve the system's sputum sample processing method as well as its usability in peripheral-level clinics on a real-time PCR platform have not reduced the accuracy of the system as the results are comparable to those previously reported (25). Strong correlation ($\rho = -0.7109$) between NWU-TB C_T values and the smear grades was demonstrated. This demonstrates the reproducibility and reliability of the NWU-TB sample processing step. Currently, the sample processing method is fully manual and efforts are underway to semi-automate the process and exclude the wash steps. Semi-automation will minimize variations due to skill levels and laboratory capacities after deployment. Although a Biorad CFX96 was used in this study, the final modular NWU-TB system will

incorporate a small fast thermal cycler currently being development by a collaborator. The new cycler will permit real-time amplification in less than 25 minutes. This study demonstrates that the NWU-TB sample processing method can be coupled to any real-time PCR platform, thus reducing installation costs in laboratories that already have existing real-time PCR instruments.

Currently, sputum culture conversion at 2 months is the only widely accepted marker of treatment efficacy and thus acceptable for monitoring therapy in patients on anti-tuberculosis treatment (42). Since MGIT culture can take 2 to 6 weeks for the results to be available, it is not suitable for rapid decision-making about the effectiveness of TB-therapy. The PMA method coupled to nucleic acid amplification tests has been used for rapid differentiation of live and dead cells in food and environmental microbiology (43, 44). Recently, attempts have been made to apply the PMA approach, linked to PCR, to differentiate between live and dead MTB. To our knowledge, very few studies have evaluated the effectiveness of PMA in clinical sputum samples. Some of the factors that influence the efficacy of PMA include: length of PMA incubation with the sample, concentration of PMA used and the duration of exposure to the halogen lamp. However, de Assuncao *et al.*, showed that at the optimal PMA concentration, different incubation (5 to 10 minutes) and halogen lamp exposure times (1 to 2 minutes) resulted in small differences in the efficacy of PMA (30). Therefore, shorter time intervals were adopted in this study. Previous studies with MTB used PMA concentrations ranging from 50 to 500 μ M (23, 30, 45). Since high PMA concentrations have been reported to penetrate viable cells and inhibit PCR (46), a moderate concentration of 100 μ M as employed in another study was selected (30). Some sample processing methods (sputasol and Cepheid sample reagent) has been reported to be incompatible with PMA (24), but there was no evidence that the lysis buffer used in this study was incompatible with PMA.

Noteworthy, one culture negative sample (sample 9 in Table 4) tested positive on both Xpert MTB/Rif and NWU-TB without PMA treatment. However, after PMA pretreatment in the NWU-TB system, a negative result was reported with a large ΔC_T . This is a classic case of a false positive obtained by NAAT systems, highlighting the weaknesses of current molecular assays in failing to distinguish between live and dead mycobacteria. Overall comparison of the ΔC_T values when patient data was stratified according to TB treatment status (treatment vs treatment naive), however, yielded no statistically significant differences although observable differences can be noted from the box plots in Figure 3. Specific cases are also reported in Table 4 where indeed PMA discriminated between live and dead mycobacteria. There were 8 culture-positive samples that tested positive without PMA treatment using the NWU-TB test but tested negative after PMA

treatment. This anomaly has also been reported in another study (47). Since in our case, stored samples as opposed to fresh samples were used, freezing and thawing could have affected the integrity of bacterial cell membranes, thus PMA could have penetrated the damaged membranes of MTB (47) leading to the false negative results after PMA pretreatment.

The study had some limitations. Thawed stored sputum samples were used for NWU-TB testing, which may not give the same results as fresh specimens. Limited availability of patient medical data such as duration of therapy and the small sample size limited the level of analyses that could be conducted. A large prospective longitudinal assessment will yield more information. For this pilot PMA evaluation, the sample size was not sufficiently powered to detect differences between the treatment naive patients and those on TB treatment. In conclusion, the NWU-TB sample processing method is simple, fast and permits quantitative grading correlating with TB disease burden. Use of PMA also shows potential for live/dead MTB discrimination, which is important for treatment monitoring and detection in endemic settings where reinfection is common.

Funding

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Conflict of Interest

The authors declare that they have no conflicts of interest. All rights in patents were assigned to North-West University.

Acknowledgements

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5.0 References

1. **Karim F, Islam MA, Chowdhury A, Johansson E, Diwan VK.** 2007. Gender differences in delays in diagnosis and treatment of tuberculosis. *Health Policy and Planning* **22**:329-334.
2. **Sreeramareddy CT, Qin ZZ, Satyanarayana S, Subbaraman R, Pai M.** 2014. Delays in diagnosis and treatment of pulmonary tuberculosis in India: a systematic review. *The International Journal of Tuberculosis and Lung Disease* **18**:255-266.
3. **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 Suppl 1**:49-57.
4. **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. *The Lancet Infectious Diseases* **6**:664-674.
5. **Diraa O, Fdany K, Boudouma M, Elmdaghri N, Benbachir M.** 2003. Assessment of the Mycobacteria Growth Indicator Tube for the bacteriological diagnosis of tuberculosis. *The International Journal of Tuberculosis and Lung Disease* **7**:1010-1012.
6. **Nijenbandring de Boer R, Baptista de Oliveira e Souza Filho J, Cobelens F, de Paula Ramalho D, Campino Miranda PF, de Logo K, Oliveira H, Mesquita E, Oliveira MM, Kritski A.** 2014. Delayed culture conversion due to cigarette smoking in active pulmonary tuberculosis patients. *Tuberculosis* **94**:87-91.
7. **Lee M-R, Chung K-P, Chen W-T, Huang Y-T, Lee L-N, Yu C-J, Teng L-J, Hsueh P-R, Yang P-C, Luh K-T.** 2012. Epidemiologic surveillance to detect false-positive *Mycobacterium tuberculosis* cultures. *Diagnostic Microbiology and Infectious Disease* **73**:343-349.
8. **Miyazaki Y, Koga H, Kohno S, Kaku M.** 1993. Nested polymerase chain reaction for detection of *Mycobacterium tuberculosis* in clinical samples. *Journal of Clinical Microbiology* **31**:2228 - 2232.

9. **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.
10. **Kox L, Rhienthong D, Miranda A, Udomsantisuk N, Ellis K, van Leeuwen J, van Heusden S, Kuijper S, Kolk A.** 1994. A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. *Journal of Clinical Microbiology* **32**:672 - 678.
11. **Kearns A, Freeman R, Steward M, Magee J.** 1998. A rapid polymerase chain reaction technique for detecting *M. tuberculosis* in a variety of clinical specimens. *Journal of Clinical Pathology* **51**:922 - 924.
12. **Ikonomopoulos J, Gorgoulis V, Zacharatos P, Manolis E, Kanavaros P, Rassidakis A, Kittas C.** 1999. Multiplex polymerase chain reaction for the detection of mycobacterial DNA in cases of tuberculosis and sarcoidosis. *Modern Pathology* **12**:854 - 862.
13. **Ikonomopoulos J, Gorgoulis V, Zacharatos P, Kotsinas A, Tsoli E, Karameris A, Panagou P, Kittas C.** 1998. Multiplex PCR assay for the detection of mycobacterial DNA sequences directly from sputum. *In Vivo* **12**:547 - 552.
14. **Gunisha P, Madhavan H, Jayanthi U, Therese K.** 2001. Polymerase chain reaction using IS6110 primer to detect *Mycobacterium tuberculosis* in clinical samples. *Indian Journal of Pathology and Microbiology* **44**:97 - 102.
15. **Flores L, Pai M, Colford J, Riley L.** 2005. In-house nucleic acid amplification tests for the detection of *Mycobacterium tuberculosis* in sputum specimens: meta-analysis and meta-regression. *BMC Microbiology* **5**:55.
16. **Scott LE, McCarthy K, Gous N, Nduna M, Van Rie A, Sanne I, Venter WF, Duse A, Stevens W.** 2011. Comparison of Xpert MTB/RIF with Other Nucleic Acid Technologies for Diagnosing Pulmonary Tuberculosis in a High HIV Prevalence Setting: A Prospective Study. *PLoS Medicine* **8**:e1001061.

17. **Adelman MW, Kurbatova E, Wang YF, Leonard MK, White N, McFarland DA, Blumberg HM.** 2014. Cost Analysis of a Nucleic Acid Amplification Test in the Diagnosis of Pulmonary Tuberculosis at an Urban Hospital with a High Prevalence of TB/HIV. *PLoS ONE* **9**:e100649.
18. **Boyles TH, Hughes J, Cox V, Burton R, Meintjes G, Mendelson M.** 2014. False-positive Xpert(R) MTB/RIF assays in previously treated patients: need for caution in interpreting results. *The International Journal of Tuberculosis and Lung Disease* **18**:876-878.
19. **Friedrich SO, Rachow A, Saathoff E, Singh K, Mangu CD, Dawson R, Phillips PPJ, Venter A, Bateson A, Boehme CC, Heinrich N, Hunt RD, Boeree MJ, Zumla A, McHugh TD, Gillespie SH, Diacon AH, Hoelscher M.** 2013. Assessment of the sensitivity and specificity of Xpert MTB/RIF assay as an early sputum biomarker of response to tuberculosis treatment. *The Lancet Respiratory Medicine* **1**:462-470.
20. **Harrington M, Benjamin W.** 2015. The Tuberculosis Diagnostics Pipeline. 2015 PIPELINE REPORT:153. URL:
<http://www.pipelinereport.org/sites/g/files/g575521/f/201507/TB%20Diagnostics.pdf>. Date of access: 16 November 2015
21. **Suzana S, Ninan MM, Gowri M, Venkatesh K, Rupali P, Michael JS.** 2015. Xpert MTB/Rif for the diagnosis of extrapulmonary tuberculosis-an experience from a tertiary care centre in South India. *Tropical Medicine & International Health* doi:10.1111/tmi.12655.
22. **Rogers GB, Stressmann FA, Koller G, Daniels T, Carroll MP, Bruce KD.** 2008. Assessing the diagnostic importance of nonviable bacterial cells in respiratory infections. *Diagnostic Microbiology and Infectious Disease* **62**:133-141.
23. **Kim YJ, Lee SM, Park BK, Kim SS, Yi J, Kim HH, Lee EY, Chang CL.** 2014. Evaluation of propidium monoazide real-time PCR for early detection of viable *Mycobacterium tuberculosis* in clinical respiratory specimens. *Annals of Laboratory Medicine* **34**:203-209.

24. **Nikolayevskyy V, Miotto P, Pimkina E, Balabanova Y, Kontsevaya I, Ignatyeva O, Ambrosi A, Skenders G, Ambrozaitis A, Kovalyov A, Sadykhova A, Simak T, Kritsky A, Mironova S, Tikhonova O, Dubrovskaya Y, Rodionova Y, Cirillo D, Drobniewski F.** Utility of propidium monoazide viability assay as a biomarker for a tuberculosis disease. *Tuberculosis* **95**:179-185.
25. **Mutingwende I, Vermeulen U, Steyn F, Viljoen H, Grobler A.** 2015. Development and evaluation of a rapid multiplex-PCR based system for *Mycobacterium tuberculosis* diagnosis using sputum samples. *Journal of Microbiological Methods* **116**:37-43.
26. **Kantor de Narvaiz I, Kim Jae S, Frieden T, Laszlo A, Luelmo F, Norval P-Y, Rieder H, Valenzuela P, Weyer K.** 1998. Laboratory services in tuberculosis control, part II: Microscopy. World Health Organization, Geneva, Switzerland. [http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_\(part2\).pdf](http://whqlibdoc.who.int/hq/1998/WHO_TB_98.258_(part2).pdf).
27. **Becton Dickinson and Company.** 2013. BBL™MGIT™Mycobacteria Growth Indicator Tube 7 mL With BACTEC™MGIT™960 Supplement Kit. [https://www.bd.com/ds/technicalCenter/inserts/L000180JAA\(201106\).pdf](https://www.bd.com/ds/technicalCenter/inserts/L000180JAA(201106).pdf). Accessed 16 June 2014.
28. **Grobler A, Levanets O, Whitney S, Booth C, Viljoen H.** 2012. Rapid cell lysis and DNA capture in a lysis microreactor. *Chemical Engineering Science* **81**:311-318.
29. **Thanakiatkrai P, Welch L.** 2011. Using the Taguchi method for rapid quantitative PCR optimization with SYBR Green I. *International Journal of Legal Medicine* **126**:161-165.
30. **de Assuncao TM, Batista EL, Jr., Deves C, Villela AD, Pagnussatti VE, de Oliveira Dias AC, Kritski A, Rodrigues-Junior V, Basso LA, Santos DS.** 2014. Real time PCR quantification of viable *Mycobacterium tuberculosis* from sputum samples treated with propidium monoazide. *Tuberculosis* **94**:421-427.
31. **Alvarez G, Gonzalez M, Isabal S, Blanc V, Leon R.** 2013. Method to quantify live and dead cells in multi-species oral biofilm by real-time PCR with propidium monoazide. *AMB Express* **3**:1.

32. **Müllerova M.** 1996. Gen-probe amplified MTD test in comparison with microscopy and culture. *Journal of Microbiological Methods* **27**:104.
33. **Nicol MP, Workman L, Isaacs W, Munro J, Black F, Eley B, Boehme CC, Zemanay W, Zar HJ.** 2011. Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: a descriptive study. *The Lancet Infectious Diseases* **11**:819-824.
34. **Park SH, Kim CK, Jeong HR, Son H, Kim SH, Park MS.** 2014. Evaluation and comparison of molecular and conventional diagnostic tests for detecting tuberculosis in Korea, 2013. *Osong Public Health and Research Perspectives* **5**:S3-7.
35. **Chen JH, She KK, Kwong TC, Wong OY, Siu GK, Leung CC, Chang KC, Tam CM, Ho PL, Cheng VC, Yuen KY, Yam WC.** 2015. Performance of the new automated Abbott RealTime MTB assay for rapid detection of *Mycobacterium tuberculosis* complex in respiratory specimens. *European Journal of Clinical Microbiology & Infectious Diseases* doi:10.1007/s10096-015-2419-5.
36. **Steingart KR, Sohn H, Schiller I, Kloda LA, Boehme CC, Pai M, Dendukuri N.** 2013. Xpert(R) MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. *Cochrane Database Syst Rev* **1**:CD009593.
37. **Nimesh M, Joon D, Pathak AK, Saluja D.** 2013. Comparative study of diagnostic accuracy of established PCR assays and in-house developed *sdaA* PCR method for detection of *Mycobacterium tuberculosis* in symptomatic patients with pulmonary tuberculosis. *Journal of Infection* **67**:399-407.
38. **Haldar S, Bose M, Chakrabarti P, Dagainawala HF, Harinath BC, Kashyap RS, Kulkarni S, Majumdar A, Prasad HK, Rodrigues C, Srivastava R, Taori GM, Varma-Basil M, Tyagi JS.** Improved laboratory diagnosis of tuberculosis – The Indian experience. *Tuberculosis* **91**:414-426.
39. **Cross LJ, Anscombe C, McHugh TD, Abubakar I, Shorten RJ, Thorne N, Arnold C.** 2015. A Rapid and Sensitive Diagnostic Screening Assay for Detection of *Mycobacteria* Including *Mycobacterium tuberculosis* Directly from Sputum without Extraction. *International Journal of Bacteriology* **2015**.

40. **Walter SD, Irwig L, Glasziou PP.** Meta-Analysis of Diagnostic Tests With Imperfect Reference Standards. *Journal of Clinical Epidemiology* **52**:943-951.
41. **Swets JA.** 1988. Measuring the accuracy of diagnostic systems. *Science* **240**:1285-1293.
42. **Mitchison DA.** 1993. Assessment of new sterilizing drugs for treating pulmonary tuberculosis by culture at 2 months. *The American Review of Respiratory Disease* **147**:1062-1063.
43. **Nocker A, Sossa-Fernandez P, Burr MD, Camper AK.** 2007. Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology. *Applied and Environmental Microbiology* **73**:5111-5117.
44. **Vesper S, McKinstry C, Hartmann C, Neace M, Yoder S, Vesper A.** 2008. Quantifying fungal viability in air and water samples using quantitative PCR after treatment with propidium monoazide (PMA). *Journal of Microbiological Methods* **72**:180-184.
45. **Miotto P, Bigoni S, Migliori GB, Matteelli A, Cirillo DM.** 2012. Early tuberculosis treatment monitoring by Xpert® MTB/RIF. *European Respiratory Journal* **39**:1269-1271.
46. **Pholwat S, Heysell S, Stroup S, Foongladda S, Houpt E.** 2011. Rapid First- and Second-Line Drug Susceptibility Assay for Mycobacterium tuberculosis Isolates by Use of Quantitative PCR. *Journal of Clinical Microbiology* **49**:69-75.
47. **Kim YJ, Lee SM, Park BK, Kim SS, Yi J, Kim HH, Lee EY, Chang CL.** 2014. Evaluation of Propidium Monoazide Real-Time PCR for Early Detection of Viable Mycobacterium tuberculosis in Clinical Respiratory Specimens. *Annals of Laboratory Medicine* **34**:203-209.

CHAPTER 6:

Cost-effectiveness of NWU-TB in South Africa

This chapter consists of a full length article to be submitted to *BMC Infectious Diseases* journal. The “instructions to authors” for the journal were followed in preparing the manuscript, except that for easy reading figures, schemes and tables are inserted at their logical places as they would appear in a printed version of the article and 1.5 line spacing was used to ensure consistency with the rest of the thesis. The complete author guidelines for the journal can be found in “Annexure E”. This chapter addresses the fourth objective in this study by estimating the cost per test of NWU-TB and its potential cost-effectiveness if adopted as a TB screening test for all miners in South Africa.

Estimating the cost per test and potential cost-effectiveness of the NWU-TB system for active tuberculosis screening in mining communities in South Africa

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Key words: Tuberculosis; screening; NWU-TB system; cost-effectiveness; DALY; ICER

Running title: Cost-effectiveness of NWU-TB in South Africa

Abstract

Background: mining communities act as reservoirs and drivers of new TB infections within South Africa and its neighboring countries; with a prevalence of tuberculosis estimated at 3,000/100,000 population in South African miners. As new diagnostic tests that are cheaper, portable, and more accurate continue to reach the market, active case finding (ACF) as opposed to passive case finding can help reduce disease burden in such hotspots. The objective of this study was to estimate the cost per test for NWU-TB and then evaluate the cost-effectiveness of using either NWU-TB or Xpert MTB/Rif or sputum smear microscopy (SSM) to screen all miners in South Africa for tuberculosis, relative to the current base scenario of passive case finding (PCF).

Methods: The “ingredients approach” was used to estimate the cost per test for NWU-TB from a laboratory perspective. A decision-analysis model was used to estimate the cost-effectiveness of screening all South African miners for TB, using NWU-TB, SSM and Xpert MTB/Rif relative to the current base scenario of PCF. We adopted the healthcare provider’s perspective. The primary outcome was the incremental cost-effectiveness (ICER) in US\$ cost per case detected. It should be emphasized that the modelling work described here is of a very preliminary nature as it sought to address a basic question of whether at the current costs and performance level, NWU-TB will be cost-effective for TB screening in a high prevalence setting.

Results: The total cost per test for NWU-TB was estimated at \$5.36, with material cost per test contributing the highest (75%) and equipment cost contributing the least (1.31%). The incremental cost effectiveness ratios (ICERs) for community based TB screening within South African mines using NWU-TB and Xpert MTB/Rif were US\$50.3 per case detected and \$181.52 per case detected respectively.

Conclusions: NWU-TB is cheaper than Xpert MTB/Rif but is almost double the cost of SSM. Active screening of all miners in South Africa for tuberculosis using NWU-TB was the most cost-effective strategy.

Introduction

The emergence of accurate, rapid and reliable TB diagnostic tests on the market provides hope to all stakeholders in low- and middle income countries involved in the fight against TB. Studies’ documenting the diagnostic accuracy (sensitivity and specificity) of these tests are widely available and it is an active research area [1-4]. However, accuracy alone does not directly translate into

better patient or population outcomes such as deaths averted, years of life gained and reduced transmissions [5]. Therefore, the ability to estimate the potential impact and cost-effectiveness of a promising diagnostic test before its deployment must be a high priority to avoid wasteful investments [6]. For example, the recently launched revolutionary Xpert MTB/Rif has well documented good accuracy [7-9], but evidence from recent studies indicates that in spite of this high accuracy, insignificant reductions in morbidity and mortality have been achieved [10, 11]. Due to the urgent need to deploy new TB diagnostics but within an environment of limited population-level data on impact of new diagnostic interventions, models provide a less costly alternative to translate available empirical data into estimates of impact and cost-effectiveness, thus enabling informed decision making [12].

Miners are an important reservoir of tuberculosis infection in South Africa and prevalence of TB in miners is more than three times that of a normal population [13]. Mines in South Africa employ many migrant workers from neighboring countries, which drives the spread of the epidemic in the region and the problem was well summarized by the South African Health Minister, (“If TB and HIV are a snake in Southern Africa, the head of the snake is here in South Africa. People come from all over the Southern African Development Community to work in our mines and they export TB and HIV, along with their earnings. If we want to kill a snake, we need to hit it on its head”) (Aaron Motsoaledi, June 2010). Active case finding (ACF) to identify and treat persons with active tuberculosis in hotspots like the mines is an attractive strategy that can interrupt future transmissions. However, for such programs to attract immediate funding there is need to highlight the direct economic benefits [14]. In this manuscript, the cost-effectiveness of adopting NWU-TB for community based screening of active tuberculosis in South African mines was determined.

To determine the cost-effectiveness, the cost per test from a laboratory perspective for NWU-TB was determined. The accuracy of NWU-TB was previously described [15, 16] for tuberculosis diagnosis in a hospital of a South African gold mine. Since the test is under final development information from this study will also assist in setting the appropriate target product profile (TPP) that informs final product (prototype) optimization to achieve optimal population-level impact that is at the same time cost-effective.

Methods

NWU-TB costing

To determine the total cost of NWU-TB from a health provider's perspective, we gathered cost information for the different activities and materials used. These included material costs per test, overhead and labour costs, equipment costs, and average number of tests performed per day. Statutory South African VAT (14%) was included in all the costs, and all costs are in U.S dollars [costs converted from South African rand to U.S\$ at 13.6940 R/\$US applicable on 23/09/2015 (<https://www.resbank.co.za/Research/Rates/Pages/CurrentMarketRates.aspx>)]. The South African rand has been declining since the time this analysis was conducted and this might have a significant impact on the outcomes and conclusions drawn from this study. In January 2016, the rand reached it all time low of 16.84 to the dollar which directly leads to high prices for all imported products. This shows how vulnerable developing countries are to first world technologies where the costs are pegged to major international currencies and the tests are not domestic-based.

Material costs per test

The unit cost per specimen tested from a provider's perspective for NWU-TB was estimated using the "ingredients" approach. This involved documenting all the materials used from the moment a patient sputum sample is received in the laboratory until patient results are dispatched.

Number of tests per day

To determine the labour cost per test and the equipment cost per test, it is necessary to determine the maximum number of NWU-TB tests that can be performed per day and assume that the system will operate at maximum capacity for the entire duration of its useful lifespan. The maximum number of tests that can be performed per day was determined as outlined below. The NWU-TB test can be divided into main 2 stages: sample processing stage and the amplification and detection of *M. tuberculosis* stage on a real-time platform. The real time instrument can run 48 specimens simultaneously, with each run lasting a maximum of 1 hour. The rate limiting step in the current NWU-TB workflow is the lysis step during the patient sample processing procedure [15]. In the current lyser device a maximum of 8 specimens can be processed per each run, with each run lasting a maximum of 20 minutes including the specimen processing and lysis. Assuming, a laboratory operates for 8 hours daily, a maximum of 128 specimens can be processed daily using a single lyser device.

Labour cost per test

NWU-TB can be performed by a qualified medical laboratory technologist with ease. In South Africa a medical laboratory technologist must possess a national diploma in biomedical technology and be registered with Health Professions Council of South Africa. In determining the labour costs, the assumption was that all the tests will be performed only by these technologists and therefore the salary of a technologist employed by the National Health Laboratory Services (NHLS) was used in calculating labour cost per test. Other labour costs such as sample collection, counseling and physician consultation, were not included in calculating the labour cost per test as these would apply to any TB test.

Equipment costs and daily equivalents

The cost of building a lyser device was taken as the purchase price, and this does not include normal costs associated with future commercial product. However, these incremental costs are likely to be offset by cost savings associated with economies of scale. The negotiated purchase price of the Streck real time thermal cycler is US\$ 8,000. The cost of building the lyser device came to US\$ 772.29, thus the total equipment of the NWU-TB system is US\$ 8,772.29. In order to estimate the daily equivalent costs, the following assumptions were made; (i) one year warranty provided by manufacturer, (ii) equipment comes with a \$1,000.00 insurance package, with the assumption that the client will purchase insurance annually after expiry of warranty, (iii) the equipment has a 5 year useful lifespan, and (iv) a discount rate of 5% is included.

Overhead cost per test

Overhead costs for NWU-TB were estimated to be equivalent to those calculated for sputum smear microscopy (SSM) reported elsewhere [17]. The assumption is premised on the fact that NWU-TB is designed to be stationed at a microscopy centre thus requiring the same level of infrastructure. The overhead cost was determined as \$0.74 with an uncertainty range of between \$0.18 and \$0.93.

Cost-effectiveness

The modelling work described here is of a very preliminary nature as it only seeks to inform further product development. A decision-analytic model was constructed to estimate the cost-effectiveness of community based screening of tuberculosis in South African mines using any of the three tests (SSM, Xpert MTB/Rif or NWU-TB) in comparison to the base scenario of passive case finding (PCF). The model structure is shown in Figure 1 and the main outcome measure is

the Incremental Cost Effectiveness Ratio (ICER which was expressed as US\$ cost per case detected). The decision tree and all cost-effectiveness analyses were performed by decision analysis using TreeAge Pro 2015 (TreeAge Pro Inc., Williamston, MA). The cost per test for NWU-TB used in cost-effectiveness analysis has been calculated in this manuscript, while costs for SSM, and Xpert MTB/Rif were obtained from literature [17]. Due to the lack of data on patient associated costs, the evaluation was conducted from a healthcare provider's perspective.

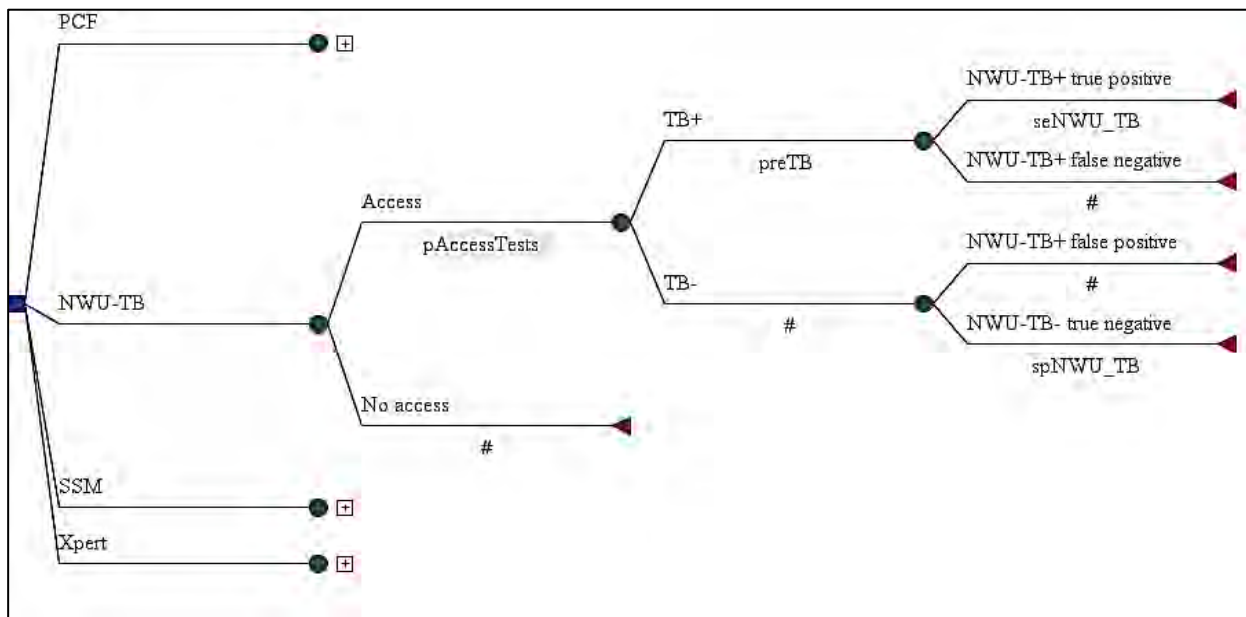


Figure 1: Simplified schematic of decision tree used in the study. The square represents a decision node, circles are chance nodes, and triangles are terminal nodes. Costs are calculated at each terminal node based on the parameters provided in Table 1.

Prevalence of tuberculosis, costs for SSM and Xpert, and other variables associated with different algorithms were based on literature estimates and are summarized in Table 1. Diagnostic accuracy data for SSM, Xpert MTB/Rif and NWU-TB was sourced elsewhere [15], conducted at a mining hospital using MGIT culture as gold standard. Sensitivity analyses were conducted for most of the parameters using ranges provided in Table 1. In spite of the high prevalence of HIV/AIDS in the study population; the costs and effectiveness of antiretroviral therapy were not modelled.

Table 1: Parameter values and ranges inputted in the model, and reference sources for the estimates used in the cost-effectiveness analysis

Parameter	Parameter value	Range (Min-Max)	Reference
Epidemiological parameters			
Prevalence of TB among miners	0.03	0.01-0.07	[13]
Characteristics of TB diagnosis			
Sensitivity SSM	0.621	0.553-0.684	[15]
Specificity SSM	0.561	0.447-0.67	[15]
Sensitivity NWU-TB	0.808	0.75-0.856	[15]
Specificity NWU-TB	0.756	0.649-0.844	[15]
Sensitivity Xpert	0.853	0.799-0.896	[15]
Specificity Xpert	0.732	0.622-0.824	[15]
Probability of being screened with a diagnostic test	1	0.95-1.0	Personal opinion
Probability of accessing care in PCF	0.57	0.25-1.00	[18]
pTruepos_combined_PCF	0.776	0.61-1.0	[18]
pTrueneg_combined_PCF	1.00	0.883-1.0	[18]
Laboratory costs			
SSM	\$2.25	1.40-3.24	[17]
NWU-TB*	\$5.36	4.56 - 10.64	This manuscript
Xpert	\$14.93	13.36-28.88	[17]
PCF	\$0.20	0.1 – 1	[19]

*Note: The cost per test for NWU-TB is calculated in this paper

Xpert, Xpert MTB/Rif; DALY, disability-adjusted life year; SSM, sputum smear microscopy, pTruepos_combined_PCF refers to the combined sensitivity of SSM and culture when confirming suspected TB cases, similarly pTrueneg_combined_PCF refers to the combined specificity of SSM and culture.

The following assumptions were made in the model in addition to those already mentioned elsewhere in this manuscript; a discount of 3%, the standard costs of TB treatment were not modelled, and benefits were assumed to only accrue over a one year static time period.

Results

NWU-TB costing

Material costs

The material cost per sample for NWU-TB is \$ 4.02 when the cost of propidium monoazide is not included as shown in Table . The cost of propidium monoazide per sample is \$5.28 which is 31.34 % above the cost of all other materials used in the NWU-TB system.

Table 2: Material costs per NWU-TB test

Item	US\$	% of total
Lysis buffer	0.11	2.74
Lysis microreactor (LMR)	0.80	19.90
ZR Viral DNA/RNA Kit	0.57	14.17
Scorpion primer	0.09	2.24
Scorpion probe	0.74	18.41
KAPA Probe qPCR Mastermix 2x	0.39	9.70
96-well plates	0.06	1.49
Micro seal adhesives for PCR plates	0.02	0.50
DNA extraction control- Internal control	0.92	22.89
PCR grade water pH 8.0	0.10	2.49
General consumables (Gloves, tips, detergents)	0.22	5.47
Total material costs per test	4.02	100.00

Labour costs

Determining the staff time per test is naturally the first step in determining labour cost per test and Table shows how the calculations were performed. The NWU-TB assay can be performed by any qualified Medical/Clinical Laboratory Technologist whose annual pay scale range is R 130,121.00 to R 291,807.00 according to the National Health Laboratory Services (NHLS)

(http://www.payscale.com/research/ZA/Employer=NHLS/Salary/by_Job). In this study a median salary of R 210,964.00 (equivalent to US\$ 15,405.58) was used. The actual work days per year are shown in Table .

Table 3: Medical Laboratory Technologist labour cost per NWU-TB test in South Africa

Item	Value
Total potential income per year	\$15,405.58
Potential working days per year	260 days
Annual leave	22 days
National holidays	12 days
Actual working days	226 days
Technologist salary per day	\$68.17
Working hours per day	8 hours
Number of tests per hour	16 tests
Labour cost per test	\$0.53

Equipment cost per test

Using standard procedures, the present value of equipment over its entire 5 year life span, was calculated to be \$12,318.24. Table shows how the equipment cost per test of US\$ 0.07 was calculated.

Table 4: Equipment cost per test for NWU-TB

	Value (units)
Present value of equipment with insurance	\$12, 318.24
monthly equivalent equipment cost (over 5 years)	\$205,304
Days equipment used per month	22 days
Daily equivalent equipment cost	\$9.33
Number of tests per day	128 tests
Equipment cost per test	\$0.07

Total cost per test

Using data from Table to Table , and the assumption made for the overhead cost per test, the total cost per test from the health provider's perspective was calculated as shown in Table . The estimated total cost per test was US\$ 5.36.

Table 5: Total cost per test for NWU-TB based on component costs

	Value (US\$)	% of total
Material cost per test	4.02	75%
Labour cost per test	0.53	9.89%
Equipment cost per test	0.07	1.31%
Overhead cost per test	0.74	13.80%
Total cost per test	5.36	100%

Cost-effectiveness

The incremental cost-effectiveness ratio (ICER) of NWU-TB was US\$ 50.3 per case detected, while both Xpert MTB/Rif and SSM were dominated as shown in Table . South Africa is a middle income country with a willingness-to-pay threshold of US 5,786.00 [20], thus ICER was compared to this threshold value. A test that is dominated by other alternatives is rejected from the onset as the dominated strategy has a greater cost with no greater benefits under all model conditions. Xpert MTB/Rif and SSM were dominated under the model conditions.

Table 6: Incremental cost-effectiveness ratios of the different diagnostic strategies

Strategy	Cost	Incr Cost	Eff	Incr Eff	C/E	Incr C/E
PCF	0.114	0	0.491	0	0.23	0
SSM	2.25	2.136	0.478	-0.013	4.71	“dominated”
NWU-TB	5.36	5.246	0.594	0.104	9.02	50.63
Xpert	14.93	14.816	0.572	0.082	26.08	\$ 181.52

All tests referencing common baseline. Incr Cost – incremental cost; Incr Eff – incremental effectiveness; Incr C/E – incremental cost-effectiveness;

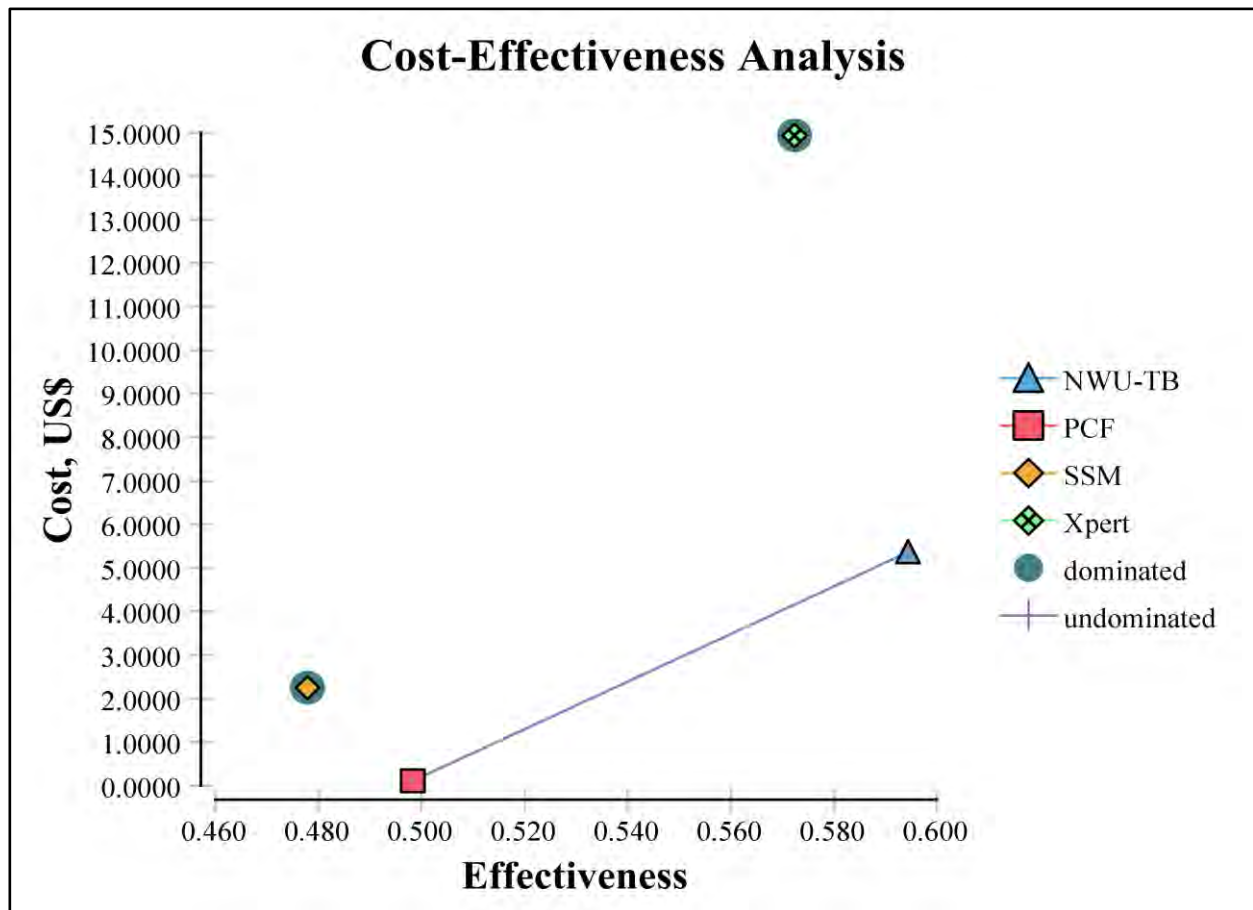


Figure 2: Cost-effectiveness analysis output graph

One-way sensitivity-analyses on all parameters were performed, and Figure 2 shows 8 parameters that had the highest effect on the model. The model was most sensitive to the specificities of NWU-TB and Xpert MTB/Rif, showing that increases in the specificities of the assays are associated with increased cost effectiveness. A decrease in the prevalence of tuberculosis in the target population will result in reduced ICERs.

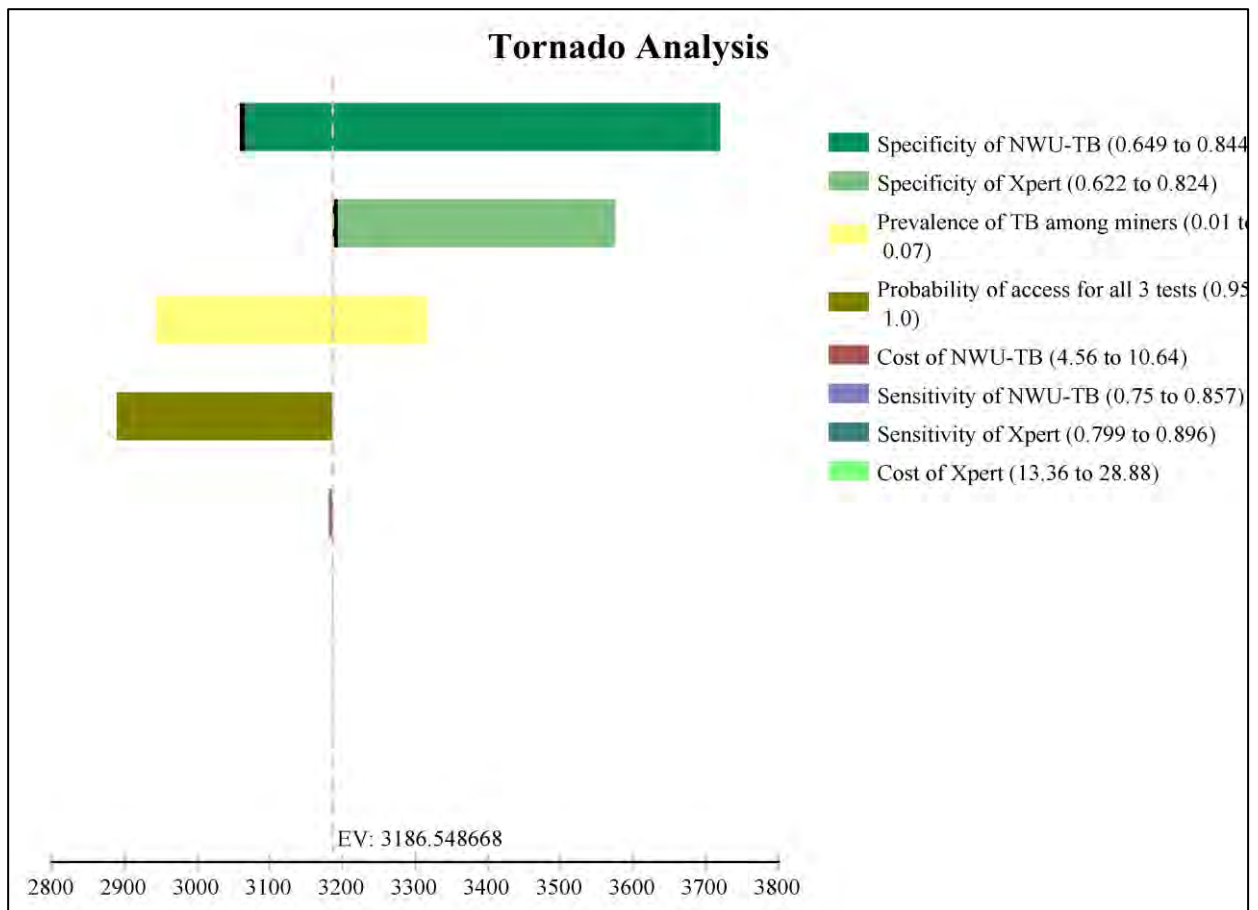


Figure 3: One-way sensitivity-analysis of key parameters used in the model. The vertical line represents the incremental cost-effectiveness ratio (ICER) of the baseline parameter values. Only the top 8 parameters are shown and the others are not shown. Black bars represent the low value of the sensitivity range and the coloured bars represent the high value. EV- expected value of ICER under baseline parameters; SSM- sputum smear microscopy; Xpert- Xpert MTB/Rif

Discussion

In South Africa, active tuberculosis cases are detected through passive case finding using mainly SSM to test presumptive patients presenting themselves at health care centres. MGIT culture, which is the current gold standard for tuberculosis diagnosis is slow and requires specialized facilities thereby making it unsuitable for community based screening of the disease [21]. In 1974, the World Health Organization abandoned its policy on ACF using mobile mass radiography, due to strong evidence against its effectiveness and inappropriateness [14]. However, screening of high risk groups was never condemned as long as it does not deter development of optimal tests. As better tools for TB diagnosis with lower risks than mobile mass radiography emerge on the market, there is need to predict their potential impact on future transmission, cost-effectiveness, and direct benefits for cases detected. We previously described a low cost and rapid TB diagnostic test with

the potential to be adapted for mobile ACF programs in high burden settings like mines in South Africa [15, 16].

In this study, the total cost per test for NWU-TB, which is a nucleic acid amplification test (NAAT) for rapid detection of *Mycobacterium Tuberculosis* (MTB) was first estimated. The cost of NWU-TB was found to be 64% lower than that of subsidized Xpert MTB/Rif [22, 17]. Consumables/materials accounted for 75% of the total cost per test, which is comparable to that reported for other molecular tests (Xpert MTB/Rif and MTBDRplus), whose consumable costs are described to be between 60% and 80% [17]. Reduction in consumable cost contribution for NWU-TB can be gained from economies of scale if the test is rolled out on a wider scale. Labour costs contributed 9.89%, which can increase if the number tests performed per day decreases. On the other hand, if less skilled cheaper labour can be trained to use the test or in low-income countries where salary costs are lower, the labour cost per test can also decrease further provided the number of tests per day remains unchanged. Equipment costs per test accounted for only 1.31%, highlighting the fact that simple technology can make a difference.

It was demonstrated that NWU-TB is the most cost-effective diagnostic test for community based TB screening among miners in South Africa. NWU-TB had an ICER of \$ 54.58 per case detected relative to PCF, while SSM and Xpert MTB/Rif were dominated under the model conditions. The results are comparable to those from an ACF program conducted in Cambodia which targeted TB contacts; the overall cost per case detected was US\$ 108 [23]. The prevalence of TB among household contacts is reported to be 3.1% (95% CI 2.1–4.5%) [24], and is similar to the TB prevalence [3%, sensitivity analysis range: 1% - 7%] used in the study, this study and the Cambodia study [23] highlight the cost-effectiveness of ACF in high TB prevalence settings. Different results may be obtained when ACF is conducted in low prevalence settings. One-way sensitivity analyses revealed that TB prevalence was of the top three factors whose uncertainty drives the largest impact. Therefore, current R&D effort to semi-automate and commercialize the NWU-TB test should focus on further improvements on the specificity of the test.

Although Xpert MTB/Rif was dominated it had an ICER of US\$ 181.52 per case detected, and can be considered cost-effective compared to results from large ACF multisite study which reported ICERs ranging from US\$ 60 to US\$ 1626 per case detected [25]. Therefore, policy makers must take into account its good performance characteristics and design features such as its low biohazard risk which makes it a suitable alternative for community-based screening. Caution must

still be exercised in recommending the most suitable alternative as policy makers do not make decisions in a vacuum. There are many programmes competing for limited funding and a seemingly “cost-effective strategy” may wipeout the entire health budget of the country if implemented. For example, it is reported that “In India, providing Xpert MTB/Rif to 15% of all TB suspects would consume the entire annual budget of the Revised National TB Control Program of US\$ 65million as of 2010”. There are about 500,000 mine workers in South Africa [26]. Assuming that a program was launched where all of them were screened at the stated costs for the TB tests given in Table , then screening all miners in South Africa for tuberculosis using either NWU-TB or Xpert MTB/Rif would cost US\$ 2,680,000 and US\$ 7,465,000 respectively in laboratory associated costs only.

Like any other modelling analyses, the study had some limitations. First, the model has a one year statistic time frame and future reductions in TB transmission that accrue from community-based screening in mining communities were not accounted for. It has been estimated that an individual with active TB can infect up to 12 persons per year [27, 28], leading to 0.9 future active TB cases [29]. Therefore, the model may have significantly underestimated the long-term cost-effectiveness of TB active case finding for all the tests relative to the no screening base scenario. Secondly, to a large degree, the cost-effectiveness analysis relied on literature data for parameter estimates. For example, effectiveness parameters were taken from a study conducted in a neighboring country [19], with a different socio-economic status to South Africa. In addition, costs per test for SSM and MGIT culture were obtained from literature and the methods used to calculate these costs are slightly different from the one adopted for determining NWU-TB cost per test as outlined in this study. Another assumption was that a negative test during screening will not influence clinical decision making.

In conclusion, a detailed costing calculation for estimating the cost per each NWU-TB test from a laboratory perspective is provided. The potential cost-effectiveness of community-based screening of all miners in South Africa for tuberculosis using either SSM or Xpert MTB/Rif or NWU-TB relative to the current base scenario of PCF was modelled. NWU-TB was found to be the most cost-effective strategy relative to PCF as base case scenario, while both Xpert MTB/Rif and SSM were dominated under the model conditions.

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Conflict of Interest

The authors declare that they have no conflicts of interest. All rights in patents were assigned to the North-West University.

References

1. Weekes K, Pearse M, Sievers A, Ross B, d'Apice A. The diagnostic use of the polymerase chain reaction for the detection of *Mycobacterium tuberculosis*. *Pathology*. 1994;26:482 - 6.
2. Weyer K, Mirzayev F, Migliori GB, Van Gemert W, D'Ambrosio L, Zignol M et al. Rapid molecular TB diagnosis: evidence, policy making and global implementation of Xpert MTB/RIF. *European Respiratory Journal*. 2013;42(1):252-71. doi:10.1183/09031936.00157212.
3. World Health Organization. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). Policy statement June. 2008.
4. Yoon K, Cho S, Lee T, Cheon S, Chang J, Kim S et al. Detection of *Mycobacterium tuberculosis* in clinical samples from patients with tuberculosis or other pulmonary diseases by polymerase chain reaction. *Yonsei Medical Journal*. 1992;33:209 - 16.
5. Mann G, Squire SB, Bissell K, Eliseev P, Du Toit E, Hesseling A et al. Beyond accuracy: creating a comprehensive evidence base for TB diagnostic tools. *International Journal of Tuberculosis and Lung Disease*. 2010;14(12):1518-24.
6. Dowdy DW, Cattamanchi A, Steingart KR, Pai M. Is Scale-Up Worth It? Challenges in Economic Analysis of Diagnostic Tests for Tuberculosis. *PLoS Medicine*. 2011;8(7):e1001063. doi:10.1371/journal.pmed.1001063.
7. Walusimbi S, Bwanga F, De Costa A, Haile M, Joloba M, Hoffner S. Meta-analysis to compare the accuracy of GeneXpert, MODS and the WHO 2007 algorithm for diagnosis of smear-negative pulmonary tuberculosis. *BMC Infectious Diseases*. 2013;13(1):507.

8. Steingart KR, Sohn H, Schiller I, Kloda LA, Boehme CC, Pai M et al. Xpert(R) MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. The Cochrane database of systematic reviews. 2013;1:CD009593. doi:10.1002/14651858.CD009593.pub2.
9. Park KS, Kim JY, Lee JW, Hwang YY, Jeon K, Koh WJ et al. Comparison of the Xpert MTB/RIF and Cobas TaqMan MTB assays for detection of Mycobacterium tuberculosis in respiratory specimens. Journal of Clinical Microbiology. 2013;51(10):3225-7. doi:10.1128/jcm.01335-13.
10. McNerney R, Zumla A. Impact of the Xpert MTB/RIF diagnostic test for tuberculosis in countries with a high burden of disease. Current Opinion in Pulmonary Medicine. 2015;21(3):304-8. doi:10.1097/mcp.0000000000000161.
11. Theron G, Zijenah L, Chanda D, Clowes P, Rachow A, Lesosky M et al. Feasibility, accuracy, and clinical effect of point-of-care Xpert MTB/RIF testing for tuberculosis in primary-care settings in Africa: a multicentre, randomised, controlled trial. The Lancet. 2014;383(9915):424-35. doi:[http://dx.doi.org/10.1016/S0140-6736\(13\)62073-5](http://dx.doi.org/10.1016/S0140-6736(13)62073-5).
12. Estill J, Egger M, Blaser N, Vizcaya LS, Garone D, Wood R et al. Cost-effectiveness of point-of-care viral load monitoring of ART in resource-limited settings: Mathematical modelling study. AIDS 2013;27(9):10.1097/QAD.0b013e328360a4e5. doi:10.1097/QAD.0b013e328360a4e5.
13. van Halsema CL, Fielding KL, Chihota VN, Lewis JJ, Churchyard GJ, Grant AD. Trends in drug-resistant tuberculosis in a gold-mining workforce in South Africa, 2002-2008. International Journal of Tuberculosis and Lung Disease. 2012;16(7):967-73. doi:10.5588/ijtld.11.0122.
14. Lönnroth K, Corbett E, Golub J, Godfrey-Faussett P, Uplekar M, Weil D et al. Systematic screening for active tuberculosis: rationale, definitions and key considerations [State of the art series. Active case finding/screening. Number 1 in the series]. The International Journal of Tuberculosis and Lung Disease. 2013;17(3):289-98. doi:10.5588/ijtld.12.0797.
15. Mutingwende I, Vermeulen U, Steyn F, Viljoen H, Grobler A. Development and evaluation of a rapid multiplex-PCR based system for Mycobacterium tuberculosis diagnosis using sputum

samples. *Journal of Microbiological Methods*. 2015;116(0):37-43. doi:<http://dx.doi.org/10.1016/j.mimet.2015.06.007>.

16. Grobler A, Levanets O, Whitney S, Booth C, Viljoen H. Rapid cell lysis and DNA capture in a lysis microreactor. *Chemical Engineering Science*. 2012;81(0):311-8. doi:<http://dx.doi.org/10.1016/j.ces.2012.07.009>.

17. Shah M, Chihota V, Coetzee G, Churchyard G, Dorman S. Comparison of laboratory costs of rapid molecular tests and conventional diagnostics for detection of tuberculosis and drug-resistant tuberculosis in South Africa. *BMC Infectious Diseases*. 2013;13(1):352.

18. Sekandi JN, Dobbin K, Oloya J, Okwera A, Whalen CC, Corso PS. Cost-Effectiveness Analysis of Community Active Case Finding and Household Contact Investigation for Tuberculosis Case Detection in Urban Africa. *PloS one*. 2015;10(2):e0117009. doi:10.1371/journal.pone.0117009.

19. Zwerling AA, Sahu M, Ngwira LG, Khundi M, Harawa T, Corbett EL et al. Screening for Tuberculosis Among Adults Newly Diagnosed With HIV in Sub-Saharan Africa: A Cost-Effectiveness Analysis. *Journal of Acquired Immune Deficiency Syndromes*. 2015;70(1):83-90. doi:10.1097/qai.0000000000000712.

20. Vassall A, van Kampen S, Sohn H, Michael JS, John K, den Boon S et al. Rapid diagnosis of tuberculosis with the Xpert MTB/RIF assay in high burden countries: a cost-effectiveness analysis. *PLoS Medicine*. 2011;8(11):e1001120.

21. Said HM, Ismail N, Osman A, Velsman C, Hoosen AA. Evaluation of TBc Identification Immunochromatographic Assay for Rapid Identification of Mycobacterium tuberculosis Complex in Samples from Broth Cultures. *Journal of Clinical Microbiology*. 2011;49(5):1939-42. doi:10.1128/JCM.01906-10.

22. Nikam C, Rodrigues C. Xpert MTB/RIF: The newly endorsed TB diagnostic. *Pediatric Infectious Disease*. 2014;6(2):75-8. doi:<http://dx.doi.org/10.1016/j.pid.2014.07.003>.

23. Eang MT, Satha P, Yadav RP, Morishita F, Nishikiori N, van-Maaren P et al. Early detection of tuberculosis through community-based active case finding in Cambodia. *BMC Public Health*. 2012;12(1):1-10. doi:10.1186/1471-2458-12-469.

24. Fox GJ, Barry SE, Britton WJ, Marks GB. Contact investigation for tuberculosis: a systematic review and meta-analysis. *European Respiratory Journal*. 2013;41(1):140-56. doi:10.1183/09031936.00070812.

25. Hinderaker SG, Rusen ID, Chiang CY, Yan L, Heldal E, Enarson DA. The FIDELIS initiative: innovative strategies for increased case finding. *The International Journal of Tuberculosis and Lung Disease*. 2011;15(1):71-6.

26. Gwatidzo T, Benhura M. Mining sector wages in South Africa. *Labour Market Intelligence Partnership*. 2013:2-8.

27. Keeler E, Perkins MD, Small P, Hanson C, Reed S, Cunningham J et al. Reducing the global burden of tuberculosis: the contribution of improved diagnostics. *Nature*. 2006;444 Suppl 1:49-57. doi:10.1038/nature05446.

28. Corbett EL, Charalambous S, Fielding K, Clayton T, Hayes RJ, De Cock KM et al. Stable Incidence Rates of Tuberculosis (TB) among Human Immunodeficiency Virus (HIV)–Negative South African Gold Miners during a Decade of Epidemic HIV-Associated TB. *Journal of Infectious Diseases*. 2003;188(8):1156-63. doi:10.1086/378519.

29. Salpeter EE, Salpeter SR. Mathematical Model for the Epidemiology of Tuberculosis, with Estimates of the Reproductive Number and Infection-Delay Function. *American Journal of Epidemiology*. 1998;147(4):398-406.

CHAPTER 7:

Summary & future prospects

This chapter summarizes all the preceding chapters and discusses recommendations for future research. The chapter starts with a recap of the objectives, followed by the main findings in each of the chapters, and finally highlights potential areas for future research. Thus, objectives 1, 2 and 3 were satisfactorily addressed.

1.0 Introduction

Annually, 3 million infectious cases of tuberculosis remain undiagnosed (World Health Organization, 2014). Evidence shows that a single TB diseased patient can infect as many as 15 more individuals per year if they remain untreated (Valadas & Antunes, 2005). TB is a curable disease but appropriate therapy cannot be initiated before a proper diagnosis is made. Sputum smear microscopy (SSM), the current mainstay test in low-income settings has poor accuracy, is labour intense, requires electricity and skilled microscopists and is thus inadequate to identify all infectious cases. If new diagnostic tests that result in dramatic increases in case detection in low-income settings where the disease burden is greatest do not see the light of the day, there is no way TB can be eradicated by 2050. Disturbingly, despite Sub-Saharan Africa being the highest bearer of the TB disease burden, its involvement in research and development of new TB diagnostic tests is minimal. Financial returns on investment to develop affordable point-of-care tests for low-income settings are narrow and therefore unattractive to international biotech firms (McNerney *et al.*, 2015). As an African student and together with my supervisors, we decided to be part of the solution and conceived this study.

The objectives of this study were as follows:

1. Develop a rapid and affordable nucleic acid amplification based test for the diagnosis of tuberculosis using sputum samples dedicated to be used for community-based screening in low-income settings.
2. To evaluate the performance of the developed test in clinical sputum samples collected between January 2013 and December 2015.
3. To be able to distinguish between live and dead mycobacteria, thereby preventing false positive results in a NAAT system and enabling the test to be used in treatment outcomes monitoring.
4. To determine the cost and potential cost-effectiveness if the system is adopted as replacement for SSM in South Africa.

2.0 Summary of study design, execution and results

Applying the principle of rapid cell lysis (Grobler *et al.*, 2012) that achieves mycobacterial cell lysis through chemical, mechanical and thermal means simultaneously, a 15 minute sputum sample processing method was developed. After the lysis step, 5µL of the lysate was directly used in a Multiplex PCR reaction using a Philisa fast thermal cycler (Streck Inc., Omaha, NE) which allows amplification for 40 cycles in 25 minutes. Biosafety risk of the lysis step was also assessed to avoid exposure of healthcare workers to bioaerosols during sample manipulation. The Total Assay Time (TAT) of the NWU-TB test is about 1 hour 30 minutes. Diagnostic accuracy in clinical sputum

samples using MGIT culture as gold standard showed that NWU-TB had better sensitivity [% (95% CI)] than both Xpert MTB/Rif and SSM in TB treatment naive individuals. In the initial evaluation described in chapter 4, the sensitivity of NWU-TB was [87.6% (79.8%–93.2%)] while sensitivities of SSM and Xpert MTB/Rif were [62.9% (52.9%–72.1%)], and [83.8% (75.3%–90.3%)], respectively. Specificity [% (95% CI)] for NWU-TB [88.9% (70.8%–97.6%)] was also better than for SSM [74.1% (53.7%–88.9%)] but lower than Xpert MTB/Rif [70.4% (49.8%–86.2%)]. In the problematic smear negative-culture positive group, mainly comprised of HIV infected patients, NWU-TB had better sensitivity of [76.1% (60.7%–88.9%)] than Xpert MTB/Rif [61.5% (44.6%–76.6%)]. Biosafety studies showed that there was complete mycobacterial inactivation during lysis and bioaerosols released were trapped by the filter fitted on the lysis microreactors. These results and features demonstrate that the new test (NWU-TB) can be suitable for community based TB screening in South Africa due to the short TAT, low cost, and good sensitivity especially in the smear negative-culture positive group.

However, the test still had some limitations that could prevent its widespread adoption. The first limitation of the test was the need to run agarose gels, and in the field or laboratory healthcare workers would not be interested in performing such a task. Secondly, the test could not distinguish between live and dead mycobacteria. DNA from dead mycobacteria has been reported to persist in sputum for up to 5 years (Boyles *et al.*, 2014). In endemic settings, exposed individuals and patients with a TB history are likely to have a false positive result. In order to address these two issues, a real-time PCR platform and a strategy using propidium monoazide (PMA) to distinguish between live and dead mycobacteria were adopted. PMA is a photoreactive DNA-binding dye that penetrates cell walls of dead cells and binds to DNA but cannot penetrate intact cell walls of living cells (Nocker *et al.*, 2006). Exposure to visible light from a 650W halogen lamp induces the photoreaction of PMA and dsDNA, forming a complex that is not amplifiable by PCR (Nocker *et al.*, 2007). The sensitivity of NWU-TB [94.6% (89.1%–97.8%)] was still higher than for both Xpert MTB/Rif [84% (76.2%–90.1%)] and SSM [64.3% (55.4%–72.6%)]. Specificity for NWU-TB [86.2% (68.3%–96.1%)] was higher than for SSM [62.1% (42.3%–79.3%)], but still remained lower than Xpert MTB/Rif [100% (88.1%–100%)]. Next, 50 paired PMA treated or PMA untreated sputum samples were randomly selected to evaluate the effectiveness of PMA to discriminate between live and dead mycobacteria. The Δ CT (PMA treated – PMA untreated) was calculated and samples stratified by TB treatment status for analysis. Though observable, Δ CT difference between patients receiving TB therapy and treatment naive were not statistically significant.

Proof-of-concept for the inclusion of PMA in the NWU-TB test was demonstrated in the study. In future, a large prospective study must be conducted.

The diagnostic accuracy (sensitivity and specificity) of any test is an important factor that is considered when selecting the most suitable test to adopt. Therefore, diagnostic accuracy studies reporting on the clinical evaluation of new tests must follow STARD guidelines for the results to be considered trustworthy and enable accurate comparison of results from different studies. STARD guidelines were followed in designing the three clinical evaluations of NWU-TB reported in this thesis. The first evaluation was performed at an independent site, the NICD, and blinded samples were used in the study. The other two studies were performed at North-West University by the team developing NWU-TB, however, the reference test (MGIT culture) together with Xpert MTB/Rif were performed by independent and experienced laboratory technicians stationed at Orkney-West Vaal hospital. The results from the comparative and reference tests were kept blind to the North-West University team until the data analysis stage. The current universally accepted “gold standard” for tuberculosis is MGIT culture and this was used in all the evaluations. As STARD guidelines were followed in designing the studies, the lower accuracy of Xpert MTB/Rif reported in chapter 4 when compared to results from other studies could not be dismissed on the basis that it is different from other studies. The diagnostic results reported in chapter 4 and chapter 5 indicate the actual performance of SSM, MGIT culture and Xpert MTB/Rif in the field and it can be observed that the field results may be different from results of evaluations done in controlled studies. This possibly explain why some tests with good performance indicators as reported in literature do not make huge impacts on morbidity and mortality once deployed in the field. It is recommended that future clinical evaluation studies of diagnostic tests be conducted in settings that closely mirror the sites at which they will be deployed and that they are performed by individuals with the same skills level of the healthcare workers anticipated to operate the instruments.

In addition to diagnostic accuracy, it is necessary to estimate the cost-effectiveness of adopting the new strategy as tests with good accuracy may not be affordable in the target market. This is particularly critical for a disease like tuberculosis that mainly affects the poor as they stay in overcrowded and poorly ventilated houses, therefore a good test which is unaffordable will not make a big impact. The cost of NWU-TB and its cost-effectiveness for community-based screening of tuberculosis in all South African miners was evaluated. Costs for NWU-TB were estimated using the “ingredients approach” and were estimated to be \$5.36 without PMA treatment

and \$10.64 with PMA treatment. NWU-TB was also found to be cost-effective for tuberculosis screening in all South African miners with an ICER of \$50.3 per case detected. Xpert MTB/Rif and SSM were both dominated under the model conditions. Although Xpert MTB/Rif was dominated its ICER was \$181.52 per case detected which is comparable to the ICERs of other ACF studies reported elsewhere (Hinderaker, 2011; Eang *et al.*, 2012)

Table 4 of Chapter 2 provided a list of the proposed minimum specifications for the design of any new TB POC diagnostic test that was drafted by a TB diagnostics working group (http://www.msfaccess.org/TB_POC_Parismmeeting/). A comparison of NWU-TB to other tests following those points is provided in Table 1 below to indicate how NWU-TB fares when compared to other tests. NWU-TB cost per test is lower than that of Xpert MTB/Rif and MGIT culture, but is more expensive compared to SSM. It also shows better sensitivity than Xpert MTB/Rif in the problematic smear-negative and culture-positive group, which is associated with paucibacillary disease. However, NWU-TB still has some weaknesses: it is still manual test making it susceptible to operator variability and it still lacks an internal amplification control which may result in false negatives due to presence of PCR inhibitors.

Table 1: Comparison of Performance and features of NWU-TB to commonly used TB diagnostic tests in South Africa

	SSM	MGIT culture	Xpert MTB/Rif	NWU-TB system
Cost	\$2.25	\$23.46	\$14.93	\$5.36
Equipment cost	\$3,409.00	\$38,950.00	\$17,000.00	\$ 8,772.29
Sensitivity : smear ⁻ -Culture ⁺	N/A	N/A	61.5%	76.1%
Number of samples	3	1	1	1
Time to Result	2-3 days	10-21 days	< 2 hours	< 2 hours
Number of samples per run	1 (20/day)	960	1 (64/day)	8 (128/day)
Sample processing	BSL 1	BSL 3	BSL 1	BSL 1
Level of training	High	High	Low	High
Results read out	Complex	simple	Simple	simple
Battery powered	Yes	No	No	Yes
Where sited (laboratory)	primary level	reference level	district level	Primary level/mobile
Live/dead discrimination	No	Yes	No	Yes
Major limitations	MTB vs. MOTT	Slow	expensive	manual

3.0 Discussion

This section aims to provide a proper perspective on some of the outcomes reported on in this thesis and firstly to put the specific results on the use of PMA to distinguish between live and dead bacteria into context and secondly to discuss the low specificities of SSM and Xpert MTB/Rif reported here since these are discordant with results from several other publications.

3.1 PMA use in live/dead discrimination

Molecular methods promise to change the tuberculosis diagnostic landscape due to their quick turnaround times, and high accuracy. A number of such tests are already on the market. In spite of the advantages of the molecular tests, these methods have not managed to completely replace culture methods with its long turnaround times of 2-6 weeks. One of the main reasons for this is that molecular methods are not able to distinguish between live and dead mycobacterium, which makes them unsuitable for treatment outcomes monitoring and also prone to false positives in previously treated individuals. The use of propidium monoazide has been explored in a few studies (Miotto *et al.*, 2012; de Assuncao *et al.*, 2014; Kim *et al.*, 2014) in an attempt to address this inherent shortcoming of all molecular methods. The PMA approach is attractive in that if properly optimised it can be coupled to any molecular method. Since few studies have evaluated this approach particularly using clinical sputum samples, a pilot study was conceived and executed as described in one of the manuscripts (chapter 5) in this thesis. The aim of the pilot was to optimize the PMA assay using the light exposure instrument (Figure 1) that was assembled specifically for this study. Only 50 frozen sputum samples were used in the evaluation in which each sample was split into two aliquots. To enable a direct comparison, one aliquot was treated with PMA and the other was not. The samples were further processed in exactly the same manner following the NWU-TB standard protocol.

The results of this pilot study show that PMA treatment alters C_T values of the samples (denoted by ΔC_T), with larger ΔC_T values occurring in samples from patients who were receiving anti-tuberculosis therapy. This indicates that PMA was able to penetrate dead cells and cross-link with DNA to form a complex that is non-amplifiable by PCR using the conditions described in the manuscript. The protocol developed in the study will be used in a large prospective cohort study where fresh samples will be used.



Figure 1: Light exposure apparatus used in the PMA assay

It is hypothesized that freezing and thawing of samples damage the integrity of the bacterial cell wall of viable cells, which may allow PMA to penetrate the cells (Kim *et al.*, 2014). In addition, propidium monoazide is currently relatively expensive and this deters its wide scale use if the cost is not significantly reduced by for instance decreasing the sample volume. The cost was of this study was one of the reasons for analyzing only 50 samples in our study.

3.2 Specificity of SSM, Xpert MTB/Rif and NWU-TB

There may be concerns with reference to the low specificity results for Xpert MTB/Rif and SSM that were presented in this thesis. These results appear to be discordant with results from several other publications that show that both Xpert MTB/Rif and SSM have high specificities (Steingart *et al.*, 2006; Steingart *et al.*, 2014). The following facts may go some way in explaining the results: First, samples used in all studies reported in this thesis were collected from the same site (Orkney-Westvaal hospital) therefore any factors unique to this site would be replicated throughout all the studies. It should be noted that the laboratory where the reference tests (MGIT culture, SSM, and Xpert MTB/Rif) were performed is certified by the South African National Accreditation System (SANAS). As described in our study design the reference tests were carried out by registered medical scientists at the site as part of their daily routine work, with no interference from the NWU. In addition, the studies were blinded, with unblinding at the data analysis stage only. These results reinforce the known fact that all the current TB diagnostic tests have weaknesses when deployed in the field, especially in high prevalence settings. The reference used in all the analysis is MGIT culture, referred to as the “gold standard” in the literature; it is currently the universally acceptable microbiological reference standard.

4.0 Future research focus

In the present study; a rapid and affordable point-of-care TB diagnostic test suitable to be used as a community-based TB screening tool has been developed and evaluated. However, some R&D activities are still outstanding in order for the system to become commercially available.

The areas for further research include the following:

1. Designing and optimizing an internal amplification control. An internal amplification control needs to be introduced to each sample at the beginning of sample preparation to monitor the success of the process rather than the diagnosis of the sample, without losing sensitivity. As internal control a DNA sequence that is unrelated to the *M. tuberculosis* target sequences must be used. This unrelated DNA sequence is simultaneously amplified by PCR. A negative internal control result with a negative patient result is regarded as inconclusive and the test is repeated. This helps reduce the number of false negative that may be caused by the presence of PCR inhibitors in the sample.
2. Conduct a full prospective clinical evaluation on the effectiveness of PMA to discriminate between live and dead mycobacteria. A proof-of-concept study described in chapter 5 demonstrated that PMA is potentially effective strategy when coupled to NAATs for differentiating between live and dead mycobacteria in clinical samples. The strategy can enable NAATs to be used in treatment outcomes monitoring, which currently relies on the slow culture method. In the future study, during the first 2 months weekly sputum samples must be obtained from patients commenced on therapy and then monthly thereafter. This will ensure that there is a sufficient number of time points for statistical analysis which was not possible in the proof-of-concept study.
3. Screening of miners in South Africa to determine whether NWU-TB is actually a cost-effective strategy for community based TB screening. The final prototype of the NWU-TB system incorporating the improvements suggested above needs to be evaluated in the South African mines in order to validate the cost-effectiveness results reported in chapter 6. Other clinical evaluations studies can also be conducted during this phase in order to build a sufficient dossier for presentation to the WHO prequalification programme.
4. Incorporating drug resistance detection capabilities in the NWU-TB assay algorithm. Approximately 95% of rifampin resistance is associated with mutations in an 81-bp region

(corresponding to codons 511 to 533) of the *rpoB* gene known as the rifampicin resistance determining region (RRDR) (Htike Min *et al.*, 2014). Efforts to use sloppy molecular beacons (SMBs) targeting the entire 81bp RRDR are already underway using three SMBs (Chakravorty *et al.*, 2012) and their sequences are provided in Table 5. SMBs enable rapid and high-throughput identification of drug resistance based on accurate “probe-target hybrid” melting temperature (T_m) values (Roh *et al.*, 2015). The primers and SMBs were synthesized by Biomers.net (GmbH, Germany).

Table 5: Sequences of primers and sloppy molecular beacons for RMP drug resistance detection

Name	Sequence	Fragment length
<i>RpoB-F</i> <i>RpoB-R</i>	5'-agacgttgatcaacatccg-3' 5'-acctccagcccggcacgctcacgt-3'	172bp
<i>rpo1</i>	5'-FAM-cgaccgCccatgaattggctcagctggctggtgAcggtcg-BHQ1-3'	
<i>rpo2</i>	5'-HEX-ggcgcgaaccAcgacagcgggtgttctggtccatgaacgcgcc-BHQ1-3'	
<i>rpo3</i>	5' –Cy5-cgcgcgcaTcAccAacagtcggTgcttgtgggtcaaccgcgcg-BHQ2-3'	

The envisaged algorithm involves first detecting the presence of *M.tuberculosis* in a patient sample and in positive samples the presence of any mutations in the RRDR that are associated with RMP resistance is then probed. The rationale behind this approach is to minimize costs associated with drug resistance detection as the SMB probes are expensive. Two of the SMB probes (*rpo1* and *rpo2*) have been successfully optimised and optimization of the third probe (*rpo3*) is still ongoing. The melt peak of *rpo1* that corresponds to a wild type genotype is 80.0°C and that of *rpo2* is 83.2 °C. Further validation using a large panel of wild type clinical isolates will be needed to determine the range within which all possible wild type genotypes will fall. Future work must also focus on optimization of the *rpo3* probe. Thereafter the concordance between the SMB approach will be evaluated against Sanger sequencing as the “gold standard” in patient sputum samples.

5.0 References

- Boyles, T.H., Hughes, J., Cox, V., Burton, R., Meintjes, G. & Mendelson, M. 2014. False-positive Xpert(R) MTB/RIF assays in previously treated patients: need for caution in interpreting results. *The International Journal of Tuberculosis and Lung Disease*, 18(7):876-878.
- Chakravorty, S., Kothari, H., Aladegbami, B., Cho, E.J., Lee, J.S., Roh, S.S., Kim, H., Kwak, H., Lee, E.G., Hwang, S.H., Banada, P.P., Safi, H., Via, L.E., Cho, S.-N., Barry, C.E. & Alland, D. 2012. Rapid, High-Throughput Detection of Rifampin Resistance and Heteroresistance in Mycobacterium tuberculosis by Use of Sloppy Molecular Beacon Melting Temperature Coding. *Journal of Clinical Microbiology*, 50(7):2194-2202.
- de Assuncao, T.M., Batista, E.L., Jr., Deves, C., Villela, A.D., Pagnussatti, V.E., de Oliveira Dias, A.C., Kritski, A., Rodrigues-Junior, V., Basso, L.A. & Santos, D.S. 2014. Real time PCR quantification of viable Mycobacterium tuberculosis from sputum samples treated with propidium monoazide. *Tuberculosis* 94(4):421-427.
- Eang, M.T., Satha, P., Yadav, R.P., Morishita, F., Nishikiori, N., van-Maaren, P. & Weezenbeek, C.L.-v. 2012. Early detection of tuberculosis through community-based active case finding in Cambodia. *BMC Public Health*, 12(1):1-10.
- Grobler, A., Levanets, O., Whitney, S., Booth, C. & Viljoen, H. 2012. Rapid cell lysis and DNA capture in a lysis microreactor. *Chemical Engineering Science*, 81(0):311-318.
- Hinderaker, S.G. 2011. The FIDELIS initiative: innovative strategies for increased case finding. *The International Journal of Tuberculosis and Lung Disease*, 15.
- Htike Min, P.K., Pitaksajjakul, P., Tipkrua, N., Wongwit, W., Jintaridh, P. & Ramasoota, P. 2014. Novel mutation detection IN rpoB OF rifampicin-resistant Mycobacterium tuberculosis using pyrosequencing. *Southeast Asian J Trop Med Public Health*, 45(4):843-852.
- Kim, Y.J., Lee, S.M., Park, B.K., Kim, S.S., Yi, J., Kim, H.H., Lee, E.Y. & Chang, C.L. 2014. Evaluation of Propidium Monoazide Real-Time PCR for Early Detection of Viable Mycobacterium tuberculosis in Clinical Respiratory Specimens. *Annals of Laboratory Medicine*, 34(3):203-209.

- McNerney, R., Cunningham, J., Hepple, P. & Zumla, A. 2015. New tuberculosis diagnostics and rollout. *The International Journal of Tuberculosis and Lung Disease*, 32:81-86.
- Miotto, P., Bigoni, S., Migliori, G.B., Matteelli, A. & Cirillo, D.M. 2012. Early tuberculosis treatment monitoring by Xpert® MTB/RIF. *European Respiratory Journal*, 39(5):1269-1271.
- Nocker, A., Cheung, C.Y. & Camper, A.K. 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *Journal of Microbiological Methods*, 67(2):310-320.
- Nocker, A., Sossa-Fernandez, P., Burr, M.D. & Camper, A.K. 2007. Use of Propidium Monoazide for Live/Dead Distinction in Microbial Ecology. *Applied and Environmental Microbiology*, 73(16):5111-5117.
- Roh, S.S., Smith, L.E., Lee, J.S., Via, L.E., Barry, C.E., III, Alland, D. & Chakravorty, S. 2015. Comparative Evaluation of Sloppy Molecular Beacon and Dual-Labeled Probe Melting Temperature Assays to Identify Mutations in *Mycobacterium tuberculosis* Resulting in Rifampin, Fluoroquinolone and Aminoglycoside Resistance. *PLoS ONE*, 10(5):e0126257.
- Steingart, K.R., Henry, M., Ng, V., Hopewell, P.C., Ramsay, A., Cunningham, J., Urbanczik, R., Perkins, M., Aziz, M.A. & Pai, M. 2006. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *The Lancet Infectious Diseases*, 6(9):570-581.
- Steingart, K.R., Schiller, I., Horne, D.J., Pai, M., Boehme, C.C. & Dendukuri, N. 2014. Xpert® Mtb/Rif assay for pulmonary tuberculosis and rifampicin resistance in adults. *The Cochrane Database of Systematic Reviews*(1):1-166.
- Valadas, E. & Antunes, F. 2005. Tuberculosis, a re-emergent disease. *European Journal of Radiology*, 55(2):154-157.
- World Health Organization. 2014. Global tuberculosis report 2014. Geneva Switzerland. URL: http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf. Date of access: 16 August 2015

Annexure A: Independent evaluation report



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24 March 2014

Faculty of Health Sciences
DST/NWU Preclinical Drug Development Platform (PCDDP)
North West University
Potchefstroom, South Africa

RE: S²D² National TB Reference Laboratory evaluation

The S²D² is a South African developed rapid diagnostic PCR based platform designed for Tuberculosis detection. The system uses a heat-spin lysis DNA extraction, conventional fast PCR with three targets on the TB genome and the result is read by transferring amplicons to a fast gel where amplified product are identified using a size marker. Currently the design is an open system comprising of three separate instruments, a DNA extraction system, conventional rapid thermocycler and a flash gel.

Evaluation at the Centre for Tuberculosis – National TB Reference Laboratory

The instrument was placed at the Centre by the PCDDP and validated; an application specialist from the PCDDP was onsite for the evaluation and performed all system related procedures. The Centre had supplied a first set of 109 raw sputum samples blinded for evaluation, where a sample was classified as positive/negative based on the culture result (the current gold standard). The samples had a distribution of 36 positives and 73 negatives.

Initial evaluation was halted due to a high number of positive samples generated from the sample set and unblinding was done to resolve the issue. It was discovered by the application specialist that the primer set used in the mastermix was incorrectly composed and needed to be replaced.

The new mastermix was then evaluated and the performance of the S²D² in comparison with culture as the gold standard showed a **sensitivity of 67% and a specificity of 88%**. Smear microscopy in comparison showed a sensitivity of 40% and specificity 100% in the same set.

The system does show better performance than smear, however, in comparison to the currently available commercial products for TB, both sensitivity and specificity are not in the region of expectation i.e. **sensitivity 75 - 91% and specificity >95%**.



We strongly recommend an improvement in the platform; we are unable to determine the weakness of the platform since we have not evaluated the extraction system and PCR methodology independently. We would recommend such an evaluation initially be carried out to determine the component that requires strengthening. The lack of an internal control is concerning and we would recommend inclusion as the system is further developed. The handling of amplicons for detection also poses a risk for cross-contamination particularly in a high burden routine laboratory environment as South Africa.

Kind Regards,

Dr NA Ismail (Head of Department)



Annexure B: Journal of Microbiological Methods information pack



JOURNAL OF MICROBIOLOGICAL METHODS

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

• Description	p.1
• Audience	p.2
• Impact Factor	p.2
• Abstracting and Indexing	p.2
• Editorial Board	p.2
• Guide for Authors	p.4



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Annexure C: Supplementary information, paper 1

The Lyser device

The current system:

Rapid spin-heat-lysis of the mycobacterial organisms from biological samples are performed in an inexpensive lysis micro reactor (LMR). The Lyser device that has been built can achieve efficient mycobacterial lysis of 8 samples within 7 minutes. The applications of the Lyser device are not restricted only to our current work but can be used for other applications in biological and pharmaceutical fields. It is 7 minute efficient and safe sputum sample processing method that can potentially be coupled to any PCR based Mycobacterium tuberculosis detection system. System cost is **US\$ 776.29**



Figure 1: The Lyser Device

Key features of the Lyser device

- 8 samples lysed in 7 minutes
- Stirring range: 60-10 000 rpm (± 50 rpm variance)
- Stirring current operation: 3600 rpm
- Good torque to handle various biological samples
- Sample volumes: 250 – 3000 μ l
- Portable
- Built in control systems
- 25 °C to 100 °C temperature range (± 0.2 °C variance)
- Completely lysed and safe TB sputum sample: 3600 rpm, 95 °C, 7 min with lysis buffer.

Advances

- A smaller, more compact and refined unit
- Unit running from a battery. Solar panel charging as an option.

The Lysis Micro Reactor (LMR) –Consumable

The lysis micro reactor was developed to be cost effective, safe and easy to work with, simple to manufacture, lower the risk of contamination and to be disposable.

The unit can be handled in one hand and the lid can be opened and closed by the same hand. The lid seals tightly with smoke and pressure tests done up to 0.60 kPa. The unit has an added filter to release buildup of pressure during processing and to limit the risk of contamination. Specialized chrome-coated magnets are used that deliver optimum shearing performance up to speeds of 10 000 rpm to handle various biological sample compositions. The unit is fully disposable. Current unit cost is **US\$ 1.26**, can easily be lowered to **US\$ 0.80**.



Figure 2: The lysis micro reactor (LMR)

Key features of the lysis micro reactor (LMR)

- Capacity: 10 000 μ l
- LMR material: HTPE plastic
- Filter: Polypropylene-reinforced, 0.2 μ m pore size
- Stir bar: Rare earth magnet, 3 mm x 6 mm
- Burst pressure: 60 kPa/ 8.71 PSI
- Leakage tests: No leakages during smoke test, < 60 kPa

Lysis buffer-Consumable

Reagent	Final concentration
TE Buffer, 100 x	20
TCEP, 0.25 M	20 mM
Tween 20	1.5 %

Reagent cost for one sample is **US\$ 0.11**

Cost for Lysis step (Only consumables)

LMR + Lysis buffer = **US\$ 0.91**

Annexure D: JCM author guidelines

zjm-update/zjm-ITA-final takkarv S-138 12/22/15 7:48 ArtID: 1912-10

Journal of
Clinical Microbiology

January 2016, Instructions to Authors, pages 1–22

INSTRUCTIONS TO AUTHORS

SCOPE

The *Journal of Clinical Microbiology* (JCM) is devoted to the dissemination of new knowledge concerning the laboratory diagnosis of human and animal infections. In addition, JCM is an appropriate forum for the publication of information related to the role of the laboratory in both the management of infectious diseases and the elucidation of the epidemiology of infections. Manuscripts which present the results of original scientific investigations are encouraged. The three principal attributes that we require of papers published in JCM are timeliness, relevance to the practice of clinical microbiology, and quality science. Manuscripts that present information that is largely only of relevance to a restricted geographic area are discouraged.

JCM welcomes submission of manuscripts that present the results of investigations pertaining to new technologies in clinical microbiology when they address new applications, substantially extend our understanding of the role of the technology in the clinical microbiology laboratory, or provide clinical or laboratory outcome data.

JCM will consider manuscripts which describe truly novel molecular methods for use in the diagnosis or elucidation of infection. However, we discourage submission of manuscripts predicated on the application of previously well-described methods (e.g., RT-PCR, RAPD analysis, PFGE, and real-time PCR, etc.), even though the application may be new. Papers employing established molecular methods will be considered only when the application is examined in comparison to some other existing diagnostic method(s). Such comparative studies should include information regarding assay sensitivity, specificity, and diagnostic accuracy.

Studies with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) as a primary focus must offer something that is truly novel in order to merit consideration for publication in JCM, e.g., application of MALDI-TOF MS to previously unstudied organisms or organism groups, applications that extend beyond organism identification, clinical outcome studies, comparisons of different MALDI-TOF instruments, broad comparisons with conventional methods, or descriptions of modifications of existing MALDI-TOF MS methods. JCM will not consider manuscripts that describe routine applications of MALDI-TOF MS.

Papers submitted to JCM with whole-genome sequencing or the microbiome as a focus will be considered only when the studies performed are scientifically sound, the observations timely and novel, and the information presented of relevance to the practice of clinical microbiology. Similarly, papers that convey the results of meta-analysis studies will be considered when the data presented are extensive and when the observations made in the study are broadly applicable to the practice of clinical microbiology.

Case Reports are no longer being accepted for consideration in JCM.

The use of guidelines for the reporting of research studies can improve the accuracy and completeness of manuscripts.

Authors of papers about the diagnostic accuracy of clinical assays should refer to the Standards for the Reporting of Diagnostic Accuracy (STARD) guidelines and checklist at <http://stard-statement.org/>. Authors of papers about molecular epidemiology should refer to the Strengthening the Reporting of Molecular Epidemiology for Infectious Diseases (STROME-ID) guidelines and checklist at [http://www.thelancet.com/journals/laninf/article/PIIS1473-3099\(13\)70324-4/abstract](http://www.thelancet.com/journals/laninf/article/PIIS1473-3099(13)70324-4/abstract).

ASM publishes a number of different journals covering various aspects of the field of microbiology. Each journal has a prescribed scope which must be considered in determining the most appropriate journal for each manuscript.

(i) With respect to antimicrobial agents, JCM will consider clinically relevant manuscripts (a) that pertain to *in vitro* susceptibility test methods; (b) that are concerned with quality control procedures related to antimicrobial susceptibility tests; (c) that deal with investigations of test methods aimed at measuring levels of antimicrobial agents in clinical specimens; or (d) that describe the use of antimicrobial agents as tools in the isolation, identification, or epidemiologic assessment of microorganisms associated with disease. Manuscripts pertaining to other aspects of antimicrobial agents, such as their basic mechanisms of action, the elucidation of resistance determinants, pharmacokinetics and pharmacodynamics, and the development of new agents, will be considered for publication in *Antimicrobial Agents and Chemotherapy*.

(ii) Manuscripts that present the results of investigations with a primary focus on the basic mechanisms of pathogenesis of microorganisms or the pathophysiology of infections should be directed to *Infection and Immunity* (for bacteria, parasites, and fungi) or the *Journal of Virology* (for viruses).

(iii) Reports of clinical microbiology investigations or studies of the hospital population and the environment as they relate to nosocomial infections should be submitted to JCM. Manuscripts dealing with ecology or environmental studies or with the application of microorganisms to agricultural or industrial processes are more appropriate for *Applied and Environmental Microbiology*.

(iv) JCM considers papers involving immunologic assays for use in the diagnosis of infection. Manuscripts that pertain to studies that evaluate immune responses and elucidate immune mechanisms associated with infection, all studies that pertain to vaccines, and papers that address the assessment and laboratory diagnosis of immunologic diseases (e.g., autoimmune diseases and primary immunodeficiencies) are considered outside the purview of JCM and should be submitted to *Clinical and Vaccine Immunology* (formerly *Clinical and Diagnostic Laboratory Immunology*).

Questions about these guidelines may be directed to the editor in chief of the journal being considered.

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ASM recognizes that there are valid concerns regarding the publication of information in scientific journals that could be put to inappropriate use as described in the CPC resolution mentioned above. Members of the ASM Journals Board will evaluate the rare manuscript that might raise such issues during the review process. However, as indicated elsewhere in these Instructions, research articles must contain sufficient detail, and material/information must be made available, to permit the work to be repeated by others. Supply of materials should be in accordance with laws and regulations governing the shipment, transfer, possession, and use of biological materials and must be for legitimate, bona fide research needs. We ask that authors pay particular attention to the NSAR Select Agents and Toxins list on the CDC website <http://www.selectagents.gov/index.html> and the U.S. Government Policy for Oversight of Life Sciences Dual Use Research of Concern (March 2012; <http://www.phe.gov/s3/dualuse/Documents/us-policy-durc-032812.pdf>).

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ASM requirements for submitted manuscripts are consistent with the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals, as last updated by the International Committee of Medical Journal Editors in August 2013 (<http://www.icmje.org/>).

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Informed consent is not needed if the patient cannot be identified from any material in a manuscript. In the absence of informed consent, identifying details, such as patient initials, specific dates, specific geographic exposures, or other identifying features (including body features in figures), should be omitted, but this must not alter the scientific meaning. Important information that is relevant to the scientific meaning should be stated so that the patient cannot be identified, e.g., by stating a season instead of a date or a region instead of a city. If a patient can be identified from the material in a manuscript, all efforts should be made to obtain informed consent to publish from patients or parents/legal guardians of minors. Informed consent requires that the patient have the opportunity to see the manuscript prior to submission. The written consent must state either that the patient has seen the complete manuscript or that the patient declines to do so. Patient consent should be archived with the authors and be available upon request. A statement attesting the receipt and archiving of written patient consent should be included in the published article.

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Authors of manuscripts which contain quantitative real-time PCR applications are encouraged to consult the article concerning minimum information for publication of quantitative real-time PCR experiments (the MIQE guidelines) by Bustin et al. (*Clin Chem* 55:611–622, 2009) for guidance as to what information should be considered for inclusion in their submission.

Newly determined nucleotide and/or amino acid sequence data must be deposited and GenBank/ENA/DDJB accession numbers must be included in the manuscript no later than the modification stage of the review process. It is expected that the sequence data will be released to the public no later than the publication (online posting) date of the accepted manuscript. The accession numbers should be included in a separate paragraph at the end of the Materials and Methods section for full-length papers or at the end of the text for Short-Form papers. If conclusions in a manuscript are based on the analysis of sequences and a GenBank/ENA/DDJB accession number is not provided at the time of the review, authors should provide the annotated sequence data as supplemental material not for publication.

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Analyses should specify the database, and the date of each analysis should be indicated as, e.g., January 2016. If relevant, the version of the software used should be specified.

See "Presentation of Nucleic Acid Sequences" for nucleic acid sequence formatting instructions.

The URLs of the databases mentioned above are as follows: DNA Data Bank of Japan (DDBJ), <http://www.ddbj.nig.ac.jp/>; European Nucleotide Archive (ENA), <http://www.ebi.ac.uk/ena/>; and GenBank, National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/nucleotide>.

Proper Use of Locus Tags as Systematic Identifiers for Genes

To comply with recommendations from the International Nucleotide Sequence Database (INSD) Collaborators and to avoid conflicts in gene identification, researchers should implement the following two fundamental guidelines as standards for utilization of locus tags in genome analysis, annotation, submission, reporting, and publication. (i) Locus tag prefixes are systematic gene identifiers for all of the replicons of a genome and as such should be associated with a single genome project submission. (ii) New genome projects must be registered with the INSD, and new locus tag prefixes must be assigned in cooperation with the INSD to ensure that they conform to the agreed-upon criteria.

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2. Falagas ME, Kasiakou SK. 2006. Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. *Antimicrob Agents Chemother* 50:2274–2275. (Letter.) {"Letter" or "Letter to the editor" is allowed but not required at the end of such an entry.}
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5. da Costa MS, Nobre MF, Rainey FA. 2001. Genus I. *Thermus* Brock and Freeze 1969, 295^{AL} emend. Nobre, Trüper and da Costa 1996b, 605, p 404–414. In Boone DR, Castenholz RW, Garrity GM (ed), *Bergey's manual of systematic bacteriology*, 2nd ed, vol 1. Springer, New York, NY.
6. Fitzgerald G, Shaw D. In Waters AE (ed), *Clinical microbiology*, in press. EFH Publishing Co, Boston, MA.* {Chapter title is optional.}
7. Green PN, Hood D, Dow CS. 1984. Taxonomic status of some methylotrophic bacteria, p 251–254. In Crawford RL, Hanson RS (ed), *Microbial growth on C₁ compounds*. Proceedings of the 4th International Symposium. American Society for Microbiology, Washington, DC.
8. Rotimi VO, Salako NO, Mohaddas EM, Philip LP. 2005. Abstr 45th Intersci Conf Antimicrob Agents Chemother, abstr D-1658. {Abstract title is optional.}
9. Smith D, Johnson C, Maier M, Maurer JJ. 2005. Distribution of fimbrial, phage and plasmid associated virulence genes among poultry *Salmonella enterica* serovars, abstr P-038, p 445. Abstr 105th Gen Meet Am Soc Microbiol. American Society for Microbiology, Washington, DC. {Abstract title is optional.}
10. Garcia CO, Pairs S, Burgos R, Molina J, Molina JF, Calvo C, Vega L, Jara LJ, Garcia-Kutzbach A, Cuellar ML, Espinoza LR. 1996. Detection of *Salmonella* DNA in synovial membrane and synovial fluid from Latin American patients using the polymerase chain reaction. *Arthritis Rheum* 39(Suppl 9):S185. {Meeting abstract published in journal supplement.}
11. O'Malley DR. 1998. Ph.D. thesis. University of California, Los Angeles, CA. {Title is optional.}
12. Stratagene. 2006. Yeast DNA isolation system: instruction manual. Stratagene, La Jolla, CA. {Use the company name as the author if none is provided for a company publication.}
13. Odell JC. April 1970. Process for batch culturing. US patent 484,363,770. {Include the name of the patented item/process if possible; the patent number is mandatory.}

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2. Winnick S, Lucas DO, Hartman AL, Toll D. 2005. How do you improve compliance? *Pediatrics* 115:e718–e724. doi:10.1542/peds.2004-1133.
3. Dionne MS, Schneider DS. 2002. Screening the fruitfly immune system. *Genome Biol* 3:reviews1010-reviews1010.2. doi:10.1186/gb-2002-3-4-reviews1010.
4. Magalon A, Mendel RR. 15 June 2015, posting date. Biosynthesis and insertion of the molybdenum cofactor. *EcoSal Plus* 2015 doi:10.1128/ecosalplus.ESP-0006-2013.
5. Johnson J, Robinson VR. 2016. Cleavage of JPS-1 in cells infected with human rhinovirus. *mSystems* 1(1):e00001-15. doi:10.1128/mSystems.00001-15.

Note: a posting or accession date is required for any online reference that is periodically updated or changed.

Citations of ASM Accepts manuscripts should look like the following example.

Wang GG, Pasillas MP, Kamps MP. 15 May 2006. Persistent transactivation by Meis1 replaces Hox function in myeloid leukemogenesis models: evidence for co-occupancy of Meis1-Pbx and Hox-Pbx complexes on promoters of leukemia-associated genes. *Mol Cell Biol* doi:10.1128/MCB.00586-06.

Other journals may use different styles for their publish-ahead-of-print manuscripts, but citation entries must include the following information: author name(s), posting date, title, journal title, and volume and page numbers and/or DOI. The following is an example:

Zhou FX, Merianos HJ, Brunger AT, Engelman DM. 13 February 2001. Polar residues drive association of polyleucine transmembrane helices. *Proc Natl Acad Sci U S A* doi:10.1073/pnas.041593698.

(ii) **References cited in the text.** References that should be cited in the text include

- Unpublished data
- Manuscripts submitted for publication
- Unpublished conference presentations (e.g., a report or poster that has not appeared in published conference proceedings)
- Personal communications
- Patent applications and patents pending
- Computer software, databases, and websites

These references should be made parenthetically in the text as follows:

... similar results (R. B. Layton and C. C. Weathers, unpublished data).
 ... system was used (J. L. McInerney, A. F. Holden, and P. N. Brighton, submitted for publication).
 ... as described previously (M. G. Gordon and F. L. Ratter, presented at the Fourth Symposium on Food Microbiology, Overton, IL, 13 to 15 June 1989). {For non-published abstracts and posters, etc.}
 ... this new process (V. R. Smoll, 20 June 1999, Australian Patent Office). {For non-U.S. patent applications, give the date of publication of the application.}
 ... available in the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>).
 ... using ABC software (version 2.2; Department of Microbiology, State University [<http://www.state.micro.edu>]).

URLs for companies that produce any of the products mentioned in your study or for products being sold may not be included in the article. However, company URLs that permit access to scientific data related to the study or to shareware used in the study are permitted.

(iii) **Citations in abstracts.** Because the abstract must be able to stand apart from the article, references cited in it should be clear without recourse to the References section. Use an abbreviated form of citation, omitting the article title, as follows.

(P. S. Satheshkumar, A. S. Weisberg, and B. Moss, *J Virol* 87:10700–10709, 2013, doi:10.1128/JVI.01258-13)
 (J. H. Coggin, Jr., p. 93–114, in D. O. Fleming and D. L. Hunt, ed., *Biological Safety: Principles and Practices*, 4th ed., 2006)
 "... in a recent report by D. A. Hopwood [*mBio* 4(5): e00612-13, 2013, doi:10.1128/mBio00612-13] ..."

This style should also be used for Addenda in Proof.

(iv) **References related to supplemental material.** If references must be cited in the supplemental material, list them in a separate References section within the supplemental material and cite them by those numbers; do not simply include citations of numbers from the reference list of the associated article. If the same reference(s) is to be cited in both the article itself and the supplemental material, then that reference would be listed in both References sections.

Short-Form Papers

The Short-Form format is intended for the presentation of brief observations that do not warrant full-length papers. However, Short-Form papers should contain firm data; observations alone are not acceptable. Submit Short-Form papers in the same way as full-length papers. They receive the same review, they are not published more rapidly than full-length papers, and they are not considered preliminary communications.

The title, running title (not to exceed 54 characters and spaces), byline, and correspondent footnote should be pre-

pared as for a full-length paper. Each Short-Form paper must have an abstract of no more than 50 words. Do not use section headings in the body of the Short Form; combine methods, results, and discussion in a single section. Paragraph lead-ins are permissible. The text should be kept to a minimum and if possible should not exceed 1,000 words; the number of figures and tables should also be kept to a minimum. Materials and methods should be described in the text, not in figure legends or table footnotes. Present acknowledgments as in full-length papers. The References section is identical to that of full-length papers.

Minireviews

Minireviews are expected to be focused discussions of defined topics relevant to clinical microbiologists. In general, they are to be submitted only following invitation by the editor in chief or the Minireview section editor of JCM. Suggestions for Minireview topics may be sent to the editor in chief by e-mail.

Minireviews are not expected to be comprehensive reviews of the literature but rather focused discussions and relevant updates of specific topics. A standard title page should be provided. This is followed by an abstract of 100 words or less and then the text of the Minireview, which should not exceed 12 double-spaced manuscript pages in length, exclusive of tables, figures, photographs, and references. Up to three tables, figures, or photographs, total, may be included. References should be limited to no more than 30. Minireviews will be sent for peer review.

Author bio. A short biographical sketch and photograph of each author of the Minireview should be submitted along with the initial version of the manuscript. These will be published at the end of the article.

- The text limit is 150 words for each author and should include WHO you are (your name), WHERE you received your education, WHAT positions you have held and at WHICH institutions, WHERE you are now (your current institution), WHY you have this interest, and HOW LONG you have been in this area, as well as a brief review of your scholarly interests and record of publication. In addition, please list pertinent significant awards you have received.
- The photo should be a recent black-and-white head shot of passport size. It will be reduced to approximately 1.125 inches wide by 1.375 inches high. The photo must meet the production criteria for regular figures and should be checked for production quality by using Rapid Inspector, provided at the following URL: <http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp>.
- To submit, upload the text and photo with your manuscript in the submission and review system. Include the biographical text immediately after the References section of your manuscript, in the same file. It should be labeled with the heading "Biosketch." Upload the head shot photograph in the submission system as a "Minireview Bio Photo"; include the author's name or

Instructions to Authors

enough of it for identification in the photo's file name.

Contact the [scientific editor](#) if you have questions about what to write. Contact the [production editor](#) if you have questions about submitting your files.

Commentaries

Commentaries are invited communications concerning topics relevant to the readership of JCM and are intended to engender discussion. Reviews of the literature, methods and other how-to papers, and responses targeted at a specific published paper are not appropriate. Commentaries are subject to review.

A standard title page should be provided. This is followed by an abstract of 75 words or less and then the text of the Commentary, which should not exceed 10 double-spaced manuscript pages in length, exclusive of tables, figures, photographs, and references. Up to two tables, figures, or photographs, total, may be included. References should be limited to no more than 30.

Point-Counterpoint

Point-Counterpoint is a feature of JCM in which two experts present opposing views on a contemporary issue in the laboratory diagnosis of infectious diseases. This feature will be the lead article in the issue of JCM in which it appears. Participation as an author of a Point-Counterpoint feature is by invitation only.

A JCM editor will write a brief introductory piece of approximately 200 words outlining why a specific issue is important and then present the issue in the form of a question. The two experts will then each write a commentary, no more than 1,000 words in length, in which they present evidence in support of either the pro or con view. One table or one figure may be included. Since these discussions will be evidence based, authors may also cite up to 10 references. Unpublished or in-press data which reflect the current practice in their laboratory may be used but should not be the sole basis for their position.

Authors should send commentaries directly back to the JCM editor within 30 days of receipt of the introductory statement. Following receipt of both the pro and con commentaries, the editor will review the submissions and may return them to the author(s) with comments and/or suggested revisions. If revisions are required, the author(s) will have 14 days to craft a revised commentary, which will be sent directly back to the editor. Upon receipt of final commentaries, the JCM editor will write a brief summary consisting of no more than six one-sentence bullet points, outlining where the experts agree (no more than three points) and disagree (no more than three points). The JCM editor will then upload the introduction, both commentaries, and the summary in eJP.

Photo Quiz

A Photo Quiz submission should present the findings of some relevant, interesting, and new observation pertinent to

the practice of clinical microbiology in which a photograph is particularly useful in conveying important information and where the observation can serve as the basis for both a question and an answer. The photograph may be of a micrograph, some other laboratory material, a clinical lesion, or the results of an imaging study.

A Photo Quiz consists of two parts: (i) a case presentation featuring a photograph depicting some unusual and/or informative finding in clinical microbiology and (ii) an answer to the quiz. The case presentation and the answer must be submitted as two separate articles. Note that authors and affiliations are listed below the title.

Photo Quiz case presentation. The text in the Photo Quiz case presentation should be limited to 200 to 300 words. The header for the case presentation should read "Photo Quiz."

Please include a photograph about 39 picas (6.5 inches) wide and 28 picas (4.625 inches) high. Since photos appearing with published Photo Quizzes appear on the cover of the journal, a high-resolution TIFF or EPS file is preferred. A short legend for the photo must be provided, and the photo must be cited in the case presentation. Refer to a recently published Photo Quiz for correct formatting.

Answer to Photo Quiz. The text of the answer to the Photo Quiz should also be limited to 200 to 300 words. The header to the answer should read "Answer to Photo Quiz." Four to six references may be cited at the end of the Photo Quiz answer.

Submission. The Photo Quiz case presentation should be submitted in the "Photo Quiz" manuscript category. The Photo Quiz answer should be submitted in the "Photo Quiz Answer" manuscript category.

Letters to the Editor

Two types of Letters to the Editor may be submitted. The first type (Comment Letter) is intended for comments on final, typeset articles published in the journal (not on accepted manuscripts posted online) and must cite published references to support the writer's argument. The second type (New-Data Letter) may report new, concise findings that are not appropriate for publication as full-length papers or Short-Form papers.

Letters may be no more than 500 words long and must be typed double spaced. Refer to a recently published Letter for correct formatting. Note that authors and affiliations are listed below the title.

All Letters to the Editor must be submitted electronically, and the type of Letter (New Data or Comment) must be selected from the drop-down list in the submission form. For Letters commenting on published articles, the cover letter should state the volume and issue in which the article was published, the title of the article, and the last name of the first author. In the Abstract section of the submission form, put "Not Applicable." Letters to the Editor do not have abstracts. Both types of Letter must have a title, which must appear on the manuscript and on the submission form. Figures and tables should be kept to a minimum.

If the Letter is related to a published article, it will be sent to the editor who handled the article in question. If the editor believes that publication is warranted, he/she will solicit a reply from the corresponding author of the article and give approval for publication.

New-Data Letters will be assigned to an editor according to subject matter and will be reviewed by that editor and/or a reviewer.

Please note that some indexing/abstracting services do not include Letters to the Editor in their databases.

Errata

The Erratum section provides a means of correcting errors that occurred during the writing, typing, editing, or publication (e.g., a misspelling, a dropped word or line, or mislabeling in a figure) of a published article. Submit Errata via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Erratum as a Microsoft Word file. Please see a recent issue for correct formatting.

Author Corrections

The Author Correction section provides a means of correcting errors of omission (e.g., author names or citations) and errors of a scientific nature that do not alter the overall basic results or conclusions of a published article (e.g., an incorrect unit of measurement or order of magnitude used throughout, contamination of one of numerous cultures, or misidentification of a mutant strain, causing erroneous data for only a [non-critical] portion of the study). Note that the addition of new data is not permitted.

For corrections of a scientific nature or issues involving authorship, including contributions and use or ownership of data and/or materials, all disputing parties must agree, in writing, to publication of the Correction. For omission of an author's name, letters must be signed by the authors of the article and the author whose name was omitted. The editor who handled the article will be consulted if necessary.

Submit an Author Correction via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). Select Author Correction as the manuscript type. In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Author Correction as a Microsoft Word file. Please see a recent issue for correct formatting. Signed letters of agreement must be supplied as supplemental material not for publication (scanned PDF files).

Retractions

Retractions are reserved for major errors or breaches of ethics that, for example, may call into question the source of the data or the validity of the results and conclusions of an article. Submit Retractions via the eJP online manuscript submission and peer review system (see "Submission, Review, and Publication Processes"). In the Abstract section of the submission form (a required field), put "Not Applicable." Upload the text of your Retraction as a Microsoft Word file. Letters of agreement signed by all of the authors must be supplied as supplemental material not for publication (scanned PDF files). The Retraction will be assigned to the editor in chief of the journal,

and the editor who handled the paper and the chairperson of the ASM Journals Board will be consulted. If all parties agree to the publication and content of the Retraction, it will be sent to the Journals Department for publication.

CrossMark

ASM has implemented CrossMark. CrossMark is a multi-publisher initiative to provide a standard way for readers to locate the current version of an article. Clicking on the CrossMark logo will indicate whether an article is current or whether updates have been published. Additional information about CrossMark can be found on CrossMark's [website](#) and on ASM's CrossMark [policy page](#).

ILLUSTRATIONS AND TABLES

Illustrations

Image manipulation. Digital images submitted for publication may be inspected by ASM production specialists for any manipulations or electronic enhancements that may be considered to be the result of scientific misconduct based on the guidelines provided below. Any images/data found to contain manipulations of concern will be referred to the editor in chief, and authors may then be requested to provide their primary data for comparison with the submitted image file. Investigation of the concerns may delay publication and may result in revocation of acceptance and/or additional action by ASM.

Linear adjustments to contrast, brightness, and/or color are generally acceptable, as long as the measures taken are necessary to view elements that are already present in the data and the adjustments are applied to the entire image and not just specific areas. Unacceptable adjustments to images include, but are not limited to, the removal or deletion, concealment, duplication (copying and pasting), addition, selective enhancement, or repositioning of elements within the image.

Nonlinear adjustments made to images, such as changes to gamma settings, should be fully disclosed in the figure legends at the time of submission. In addition, images created by compiling multiple files, including noncontiguous portions of the same image, should clearly distinguish that these multiple files are not a single image. This can be done by "tooling," or **inserting thin lines**, between the individual images.

File types and formats. Illustrations may be continuous-tone images, line drawings, or composites. Color graphics may be submitted. Suggestions about how to ensure accurate color reproduction are given below.

On initial submission, figures may be uploaded as individual PDF files or combined and uploaded as a single PDF file. Place each legend in the text file, as well as on the same page with the figure to assist review. At the modification stage, production-quality digital files must be provided. The legends will be copy-edited and typeset for final publication and should not be included as part of the figure itself at this stage. All graphics submitted with modified manuscripts must be bitmap, grayscale, or in the RGB (preferred) or CMYK color mode. See "[Color illustrations](#)." Halftone images (those with various

Instructions to Authors

densities or shades) must be grayscale, not bitmap. JCM accepts TIFF or EPS files but discourages PowerPoint for either black-and-white or color images.

For instructions on creating acceptable EPS and TIFF files, refer to the Cadmus digital art website, <http://art.cadmus.com/da/index.jsp>. PowerPoint requires users to pay close attention to the fonts used in their images (see the section on fonts below). If instructions for fonts are not followed exactly, images prepared for publication are subject to missing characters, improperly converted characters, or shifting/obscuring of elements or text in the figure. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp. Note that, due to page composition system requirements, you must verify that your PowerPoint files can be converted to PDF without any errors.

We strongly recommend that before returning their modified manuscripts, authors check the acceptability of their digital images for production by running their files through Rapid Inspector, a tool provided at the following URL: <http://rapidinspector.cadmus.com/RapidInspector/zmw/index.jsp>. Rapid Inspector is an easy-to-use, Web-based application that identifies file characteristics that may render the image unusable for production. Please note when using Rapid Inspector to check PowerPoint files that there is a known bug in the application that can occasionally fail PowerPoint Presentation (.pptx) files, even though the files meet all required production criteria. If you experience this bug, the issue can be corrected by saving the PowerPoint files as an older version, PowerPoint 97-2004 Presentation (.ppt), during the Save As process (use the drop-down format menu and select this format). Once you save your files as .ppt, they will pass Rapid Inspector if all required production criteria have been met.

If you have additional questions about using the Rapid Inspector preflighting tool, please send an e-mail inquiry to helpdesk.digitalartsupport@cenveo.com.

Minimum resolution. It is extremely important that a high enough resolution is used. All separate images that you import into a figure file must be at the correct resolution before they are placed. (For instance, placing a 72-dpi image in a 300-dpi EPS file will not result in the placed image meeting the minimum requirements for file resolution.) Note, however, that the higher the resolution, the larger the file and the longer the upload time. Publication quality will not be improved by using a resolution higher than the minimum. Minimum resolutions are as follows:

- 300 dpi for grayscale and color
- 600 dpi for combination art (lettering and images)
- 1,200 dpi for line art

Size. All graphics should be submitted at their intended publication size; that is, the image uploaded should be 100% of its print dimensions so that no reduction or enlargement is necessary. Resolution must be at the required level at the submitted size. Include only the significant portion of an illustration. White space must be cropped from the image, and excess space between panel labels and the image must be eliminated.

- Maximum width for a 1-column figure: 20.6 picas (ca. 8.7 cm)
- Maximum width for a 2-column figure: 42 picas (ca. 17.8 cm)
- Minimum width for a 2-column figure: 26 picas (11.1 cm)
- Maximum height for a standard figure: 54.7 picas (ca. 23.2 cm)
- Maximum height for an oversized figure (no running title): 57.4 picas (ca. 24.3 cm)

Contrast. Illustrations must contain sufficient contrast to be viewed easily on a monitor or on the printed page.

Labeling and assembly. All final lettering and labeling must be incorporated into the figures. On initial submission, illustrations should be provided as PDF files, with the legends in the text file and with a legend beneath each image to assist review. At the modification stage, production-quality digital figure files (without legends) must be provided. Put the figure number well outside the boundaries of the image itself. (Numbering may need to be changed at the copyediting stage.) Each figure must be uploaded as a separate file, and any multipanel figures must be assembled into one file; i.e., rather than uploading a separate file for each panel in a figure, assemble all panels in one piece and supply them as one file.

Fonts. To avoid font problems, set all type in one of the following fonts: Arial, Helvetica, Times Roman, European PI, Mathematical PI, or Symbol. Courier may be used but should be limited to nucleotide or amino acid sequences, where a non-proportional (monospace) font is required. All fonts other than these must be converted to paths (or outlines) in the application with which they were created. For proper font use in PowerPoint images, refer to the Cadmus digital art website, http://art.cadmus.com/da/instructions/ppt_disclaimer.jsp.

Color illustrations. All figures submitted in color will be processed as color. Adherence to the following guidelines will help to ensure color reproduction that is as accurate as possible.

The final online version is considered the version of record for JCM and all other ASM journals. To maximize online reproduction, color illustrations should be supplied in the RGB color mode as either (i) RGB TIFF images with a resolution of at least 300 pixels per inch (raster files, consisting of pixels) or (ii) Illustrator-compatible EPS files with RGB color elements (vector files, consisting of lines, fonts, fills, and images). CMYK files are also accepted. Other than in color space, CMYK files must meet the same production criteria as RGB files. The RGB color space is the native color space of computer monitors and of most of the equipment and software used to capture scientific data, and it can display a wider range of colors (especially bright fluorescent hues) than the CMYK (cyan, magenta, yellow, black) color space used by print devices that put ink (or toner) on paper. For the print version (and reprints), ASM's print provider will automatically create CMYK versions of color illustrations from the supplied RGB versions. Color in the print journal may not match that in the online journal of record because of the smaller range of colors capable of being

reproduced by CMYK inks on a printing press. For additional information on RGB versus CMYK color, refer to the Cadmus digital art site, http://art.cadmus.com/da/guidelines_rgb.jsp.

Drawings

Submit graphs, charts, complicated chemical or mathematical formulas, diagrams, and other drawings as finished products not requiring additional artwork or typesetting. All elements, including letters, numbers, and symbols, must be easily readable, and both axes of a graph must be labeled. Keep in mind that the journal is published both in print and online and that the same electronic files submitted by the authors are used to produce both.

When creating line art, please use the following guidelines:

(i) **All art must be submitted at its intended publication size.** For acceptable dimensions, see "Size," above.

(ii) **Avoid using screens (i.e., shading) in line art.** It can be difficult and time-consuming to reproduce these images without moiré patterns. Various pattern backgrounds are preferable to screens as long as the patterns are not imported from another application. If you must use images containing screens,

(a) Generate the image at line screens of 85 lines per inch or less.

(b) When applying multiple shades of gray, differentiate the gray levels by at least 20%.

(c) Never use levels of gray below 5% or above 95%, as they are likely to fade out or become totally black when output.

(iii) Use thick, solid lines that are no finer than 1 point in thickness.

(iv) No type should be smaller than 6 points at the final publication size.

(v) Avoid layering type directly over shaded or textured areas.

(vi) Avoid the use of reversed type (white lettering on a black background).

(vii) Avoid heavy letters, which tend to close up, and unusual symbols, which the printer may not be able to reproduce in the legend.

(viii) If colors are used, avoid using similar shades of the same color and avoid very light colors.

In figure ordinate and abscissa scales (as well as table column headings), avoid the ambiguous use of numbers with exponents. Usually, it is preferable to use the appropriate *Système International d'Unités* (SI) symbols (μ for 10^{-6} , m for 10^{-3} , k for 10^3 , and M for 10^6 , etc.). Thus, a representation of 20,000

cpm on a figure ordinate should be made by the number 20 accompanied by the label kcpm. A complete listing of SI symbols can be found in the International Union of Pure and Applied Chemistry (IUPAC) publication *Quantities, Units and Symbols in Physical Chemistry*, 3rd ed. (RSC Publishing, Cambridge, United Kingdom, 2011); an abbreviated list is available at <http://old.iupac.org/reports/1993/homann/index.html>.

When powers of 10 must be used, the journal requires that the exponent power be associated with the number shown. In representing 20,000 cells per ml, the numeral of the ordinate should be "2" and the label should be " 10^4 cells per ml" (not "cells per ml $\times 10^{-4}$ "). Likewise, an enzyme activity of 0.06 U/ml might be shown as 6 accompanied by the label 10^{-2} U/ml. The preferred designation is 60 mU/ml (milliunits per milliliter).

Presentation of Nucleic Acid Sequences

Long nucleic acid sequences must be presented as figures in the following format to conserve space. Print the sequence in lines of approximately 100 to 120 nucleotides in a nonproportional (monospace) font that is easily legible when published with a line length of 6 inches (ca. 15.2 cm). If possible, lines of nucleic acid sequence should be further subdivided into blocks of 10 or 20 nucleotides by spaces within the sequence or by marks above it. Uppercase and lowercase letters may be used to designate the exon-intron structure or transcribed regions, etc., if the lowercase letters remain legible at a 6-inch (ca. 15.2-cm) line length. Number the sequence line by line; place numerals representing the first base of each line to the left of the lines. Minimize spacing between lines of sequence, leaving room only for annotation of the sequence. Annotation may include boldface, underlining, brackets, and boxes, etc. Encoded amino acid sequences may be presented, if necessary, immediately above or below the first nucleotide of each codon, by using the single-letter amino acid symbols. Comparisons of multiple nucleic acid sequences should conform as nearly as possible to the same format.

Figure Legends

On initial submission, each legend should be placed in the text file and be incorporated into the image file beneath the figure to assist review.

Legends should provide enough information so that the figure is understandable without frequent reference to the text. However, detailed experimental methods must be described in the Materials and Methods section, not in a figure legend. A method that is unique to one of several experiments may be reported in a legend only if the discussion is very brief (one or two sentences). Define all symbols used in the figure and define all abbreviations that are not used in the text.

Tables

Tables that contain artwork, chemical structures, or shading must be submitted as illustrations in an acceptable format at the modification stage. The preferred format for regular tables is Microsoft Word; however, WordPerfect and Acrobat PDF

Instructions to Authors

TABLE 1 Distribution of protein and ATPase in fractions of dialyzed membranes^a

Membrane	Fraction	ATPase	
		U/mg of protein	Total U
Control	Depleted membrane	0.036	2.3
	Concentrated supernatant	0.134	4.82
E1 treated	Depleted membrane	0.034	1.98
	Concentrated supernatant	0.11	4.6

^a Specific activities of ATPase of nondepleted membranes from control and treated bacteria were 0.21 and 0.20, respectively.

are also acceptable. Note that a straight Excel file is not currently an acceptable format. Excel files must be either embedded in a Word or WordPerfect document or converted to PDF before being uploaded.

Tables should be formatted as follows. Arrange the data so that columns of like material read down, not across. The headings should be sufficiently clear so that the meaning of the data is understandable without reference to the text. See the "Abbreviations" section of these Instructions for those that should be used in tables. Explanatory footnotes are acceptable, but more-extensive table "legends" are not. Footnotes should not include detailed descriptions of the experiment. Tables must include enough information to warrant table format; those with fewer than six pieces of data will be incorporated into the text by the copy editor. Table 1 is an example of a well-constructed table.

NOMENCLATURE

Chemical and Biochemical Nomenclature

The recognized authority for the names of chemical compounds is *Chemical Abstracts* (CAS; <http://www.cas.org/>) and its indexes. *The Merck Index*, 15th ed. (RSC Books, Cambridge, UK, 2013), is also an excellent source. For biochemical terminology, including abbreviations and symbols, consult *Biochemical Nomenclature and Related Documents* (Portland Press, London, United Kingdom, 1992) available at <http://www.chem.qmul.ac.uk/iupac/bibliog/white.html>, and the instructions to authors of the *Journal of Biological Chemistry* and the *Archives of Biochemistry and Biophysics*.

Do not express molecular weight in daltons; molecular weight is a unitless ratio. Molecular mass is expressed in daltons.

For enzymes, use the recommended (trivial) name assigned by the Nomenclature Committee of the International Union of Biochemistry (IUB) as described in *Enzyme Nomenclature* (Academic Press, Inc., New York, NY, 1992) and its supplements and at <http://www.chem.qmul.ac.uk/iubmb/enzyme/>. If a nonrecommended name is used, place the proper (trivial) name in parentheses at first use in the abstract and text. Use the EC number when one has been assigned. Authors of papers describing enzymological studies should review the standards of the STREND Commission for information required for adequate description of experimental conditions and for report-

ing enzyme activity data (<http://www.beilstein-institut.de/en/projects/strenda/guidelines>).

For nomenclature of restriction enzymes, DNA methyltransferases, homing endonucleases, and their genes, refer to the article by Roberts et al. (*Nucleic Acids Res* 31:1805–1812, 2003).

Drugs

Whenever possible, use generic names of drugs; the use of trade names is not permitted.

Nomenclature of Microorganisms

Binary names, consisting of a generic name and a specific epithet (e.g., *Escherichia coli*), must be used for all microorganisms. Names of categories at or above the genus level may be used alone, but specific and subspecific epithets may not. A specific epithet must be preceded by a generic name, written out in full the first time it is used in a paper. Thereafter, the generic name should be abbreviated to the initial capital letter (e.g., *E. coli*), provided there can be no confusion with other genera used in the paper. Names of all taxa (kingdoms, phyla, classes, orders, families, genera, species, and subspecies) are printed in italics and should be italicized in the manuscript; strain designations and numbers are not. Vernacular (common) names should be in lowercase roman type (e.g., streptococcus, brucella). For *Salmonella*, genus, species, and subspecies names should be rendered in standard form: *Salmonella enterica* at first use, *S. enterica* thereafter; *Salmonella enterica* subsp. *arizonae* at first use, *S. enterica* subsp. *arizonae* thereafter. Names of serovars should be in roman type with the first letter capitalized: *Salmonella enterica* serovar Typhimurium. After the first use, the serovar may also be given without a species name: *Salmonella* Typhimurium, *S. Typhimurium*, or *Salmonella* serovar Typhimurium. For other information regarding serovar designations, see *Antigenic Formulae of the Salmonella Serovars*, 9th ed. (P. A. D. Grimont and F.-X. Weill, WHO Collaborating Centre for Reference and Research on Salmonella, Institut Pasteur, Paris, France, 2007; see <http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089>). For a summary of the current standards for *Salmonella* nomenclature and the Kaufmann-White criteria, see the article by Brenner et al. (*J Clin Microbiol* 38:2465–2467, 2000), the opinion of the Judicial Commission of the International Committee on Systematics of Prokaryotes (*Int J Syst Evol Microbiol* 55:519–520, 2005), and the article by Tindall et al. (*Int J Syst Evol Microbiol* 55:521–524, 2005).

The spelling of bacterial names should follow the *Approved Lists of Bacterial Names (Amended) & Index of the Bacterial and Yeast Nomenclatural Changes* (V. B. D. Skerman et al., ed., American Society for Microbiology, Washington, DC, 1989) and the validation lists and notification lists published in the *International Journal of Systematic and Evolutionary Microbiology* (formerly the *International Journal of Systematic Bacteriology*) since January 1989. In addition, two sites on the World Wide Web list current approved bacterial names: Prokaryotic Nomenclature Up-to-Date (<http://www.dsmz.de/bacterial-diversity/prokaryotic-nomenclature-up-to-date.html>) and List of Prokaryotic Names with Standing in Nomenclature (<http://www>

.bacterio.net/). If there is reason to use a name that does not have standing in nomenclature, the name should be enclosed in quotation marks in the title and at its first use in the abstract and the text and an appropriate statement concerning the nomenclatural status of the name should be made in the text. "*Candidatus*" species should always be set in quotation marks.

For guidelines regarding new names and descriptions of new genera and species, see the articles by Tindall (Int J Syst Bacteriol 49:1309–1312, 1999) and Stackebrandt et al. (Int J Syst Evol Microbiol 52:1043–1047, 2002). To validate new names and/or combinations, authors must submit three copies of their published article to the *International Journal of Systematic and Evolutionary Microbiology*.

It is recommended that a strain be deposited in at least two recognized culture collections in different countries when that strain is necessary for the description of a new taxon (Int J Syst Evol Microbiol 50:2239–2244, 2000).

Since the classification of fungi is not complete, it is the responsibility of the author to determine the accepted binomial for a given organism. Sources for these names include *The Yeasts: a Taxonomic Study*, 5th ed. (C. P. Kurtzman, J. W. Fell, and T. Boekhout, ed., Elsevier Science, Amsterdam, Netherlands, 2011), and *Dictionary of the Fungi*, 10th ed. (P. M. Kirk, P. F. Cannon, D. W. Minter, and J. A. Stalpers, ed., CABI International, Wallingford, Oxfordshire, United Kingdom, 2008); see also <http://www.speciesfungorum.org/Names/Fundic.asp>.

Names used for viruses should be those approved by the International Committee on Taxonomy of Viruses (ICTV) and reported on the ICTV Virus Taxonomy website (<http://www.ictvonline.org/index.asp>). In addition, the recommendations of the ICTV regarding the use of species names should generally be followed: when the entire species is discussed as a taxonomic entity, the species name, as with other taxa, is italic and has the first letter and any proper nouns capitalized (e.g., *Tobacco mosaic virus*, *Murray Valley encephalitis virus*). When the behavior or manipulation of individual viruses is discussed, the vernacular (e.g., tobacco mosaic virus, Murray Valley encephalitis virus) should be used. If desired, synonyms may be added parenthetically when the name is first mentioned. Approved generic (or group) and family names may also be used.

Microorganisms, viruses, and plasmids should be given designations consisting of letters and serial numbers. It is generally advisable to include a worker's initials or a descriptive symbol of locale or laboratory, etc., in the designation. Each new strain, mutant, isolate, or derivative should be given a new (serial) designation. This designation should be distinct from those of the genotype and phenotype, and italicized genotypic and phenotypic symbols should not be included. Plasmids are named with a lowercase "p" followed by the designation in uppercase letters and numbers. To avoid the use of the same designation as that of a widely used strain or plasmid, check the designation against a publication database such as Medline.

Genetic Nomenclature

To facilitate accurate communication, it is important that standard genetic nomenclature be used whenever possible and

that deviations or proposals for new naming systems be endorsed by an appropriate authoritative body. Review and/or publication of submitted manuscripts that contain new or nonstandard nomenclature may be delayed by the editor or the Journals Department so that they may be reviewed.

Bacteria. The genetic properties of bacteria are described in terms of phenotypes and genotypes. The phenotype describes the observable properties of an organism. The genotype refers to the genetic constitution of an organism, usually in reference to some standard wild type. Use the recommendations of Demerec et al. (Genetics 54:61–64, 1966) as a guide to the use of these terms. If your manuscript contains information including genetic nomenclature, please refer to the Instructions to Authors of the *Journal of Bacteriology*.

"Mutant" versus "mutation." Keep in mind the distinction between a mutation (an alteration of the primary sequence of the genetic material) and a mutant (a strain carrying one or more mutations). One may speak about the mapping of a mutation, but one cannot map a mutant. Likewise, a mutant has no genetic locus, only a phenotype.

"Homology" versus "similarity." For use of terms that describe relationships between genes, consult the articles by Theissen (Nature 415:741, 2002) and Fitch (Trends Genet 16:227–231, 2000). "Homology" implies a relationship between genes that have a common evolutionary origin; partial homology is not recognized. When sequence comparisons are discussed, it is more appropriate to use the term "percent sequence similarity" or "percent sequence identity," as appropriate.

Tetracycline resistance determinants. The nomenclature for tetracycline resistance determinants is based on the proposal of Levy et al. (Antimicrob Agents Chemother 43:1523–1524, 1999). The style for such determinants is, e.g., Tet B; the space helps distinguish the determinant designation from that for phenotypes and proteins (TetB). The above-referenced article also gives the correct format for genes, proteins, and determinants in this family.

Locus tags. Locus tags are systematic, unique identifiers that are assigned to each gene in GenBank. All genes mentioned in a manuscript should be traceable to their sequences by the reader, and locus tags may be used for this purpose in manuscripts to identify uncharacterized genes. In addition, authors should check GenBank to make sure that they are using the correct, up-to-date format for locus tags (e.g., uppercase versus lowercase letters and the presence or absence of an underscore, etc.). Locus tag formats vary between different organisms and also may be updated for a given organism, so it is important to check GenBank at the time of manuscript preparation.

Viruses. The genetic nomenclature for viruses differs from that for bacteria. In most instances, viruses have no phenotype, since they have no metabolism outside host cells. Therefore,

Instructions to Authors

distinctions between phenotype and genotype cannot be made. Superscripts are used to indicate hybrid genomes. Genetic symbols may be one, two, or three letters.

Eukaryotes. FlyBase (<http://flybase.org/>) is the genetic nomenclature authority for *Drosophila melanogaster*. WormBase (<http://www.wormbase.org/#01-23-6>) is the genetic nomenclature authority for *Caenorhabditis elegans*. When naming genes for *Aspergillus* species, the nomenclature guidelines posted at <http://www.aspergillusgenome.org/Nomenclature.shtml> should be followed, and the *Aspergillus* Genome Database (<http://www.aspgd.org/>) should be searched to ensure that any new name is not already in use. The *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>) and the *Candida* Genome Database (<http://www.candidagenome.org/>) are authorities for *Saccharomyces cerevisiae* and *Candida albicans* genetic nomenclature, respectively. For more information about the genetic nomenclature of eukaryotes, see the Instructions to Authors for *Molecular and Cellular Biology*.

ABBREVIATIONS AND CONVENTIONS

Verb Tense

ASM strongly recommends that for clarity you use the **past** tense to narrate particular events in the past, including the procedures, observations, and data of the study that you are reporting. Use the present tense for your own general conclusions, the conclusions of previous researchers, and generally accepted facts. Thus, most of the abstract, Materials and Methods, and Results will be in the past tense, and most of the introduction and some of the Discussion will be in the present tense.

Be aware that it may be necessary to vary the tense in a single sentence. For example, it is correct to say "White (30) demonstrated that XYZ cells grow at pH 6.8," "Figure 2 shows that ABC cells failed to grow at room temperature," and "Air was removed from the chamber and the mice died, which proves that mice require air." In reporting statistics and calculations, it is correct to say "The values for the ABC cells are statistically significant, indicating that the drug inhibited . . ."

For an in-depth discussion of tense in scientific writing, see *How To Write and Publish a Scientific Paper*, 7th ed.

Abbreviations

General. Abbreviations should be used as an aid to the reader, rather than as a convenience for the author, and therefore their use should be limited. Abbreviations other than those recommended by the IUPAC-IUB (*Biochemical Nomenclature and Related Documents*, 1992) should be used only when a case can be made for necessity, such as in tables and figures.

It is often possible to use pronouns or to paraphrase a long word after its first use (e.g., "the drug" or "the substrate"). Standard chemical symbols and trivial names or their symbols (folate, Ala, and Leu, etc.) may also be used.

Define each abbreviation and introduce it in parentheses the first time it is used; e.g., "Cultures were grown in Eagle minimal

essential medium (MEM)." Generally, eliminate abbreviations that are not used at least three times in the text (including tables and figure legends).

Not requiring introduction. In addition to abbreviations for Système International d'Unités (SI) units of measurement, other common units (e.g., bp, kb, and Da), and chemical symbols for the elements, the following should be used without definition in the title, abstract, text, figure legends, and tables:

DNA (deoxyribonucleic acid)	reduced)
cDNA (complementary DNA)	NADP ⁺ (nicotinamide adenine
RNA (ribonucleic acid)	dimucleotide phosphate,
cRNA (complementary RNA)	oxidized)
RNAse (ribonuclease)	poly(A) and poly(dT), etc.
DNase (deoxyribonuclease)	(polyadenylic acid and
rRNA (ribosomal RNA)	polydeoxythymidylic acid,
mRNA (messenger RNA)	etc.)
tRNA (transfer RNA)	oligo(dT), etc. (oligodeoxy-
AMP, ADP, ATP, dAMP,	thymidylic acid, etc.)
ddATP, and GTP, etc. (for the	UV (ultraviolet)
respective 5' phosphates	PFU (plaque-forming units)
of adenosine and other	CFU (colony-forming units)
nucleosides) (add 2',	MIC (minimal inhibitory
3', or 5'- when needed for	concentration)
contrast)	Tris (tris(hydroxymethyl)
ATPase and dGTPase, etc.	aminomethane)
(adenosine triphosphatase	DEAE (diethylaminoethyl)
and deoxyguanosine	EDTA (ethylenediamine-
triphosphatase, etc.)	tetraacetic acid)
NAD (nicotinamide adenine	EGTA (ethylene glycol-bis[β-
dimucleotide)	aminoethyl ether]-N,N',N''-
NAD ⁺ (nicotinamide adenine	tetraacetic acid)
dimucleotide, oxidized)	HEPES (N-2-hydroxyethyl-
NADH (nicotinamide adenine	piperazine-N'-2-
dimucleotide, reduced)	ethanesulfonic acid)
NADP (nicotinamide adenine	PCR (polymerase chain reaction)
dimucleotide phosphate)	AIDS (acquired immuno-
NADPH (nicotinamide adenine	deficiency syndrome)
dimucleotide phosphate,	

Abbreviations for cell lines (e.g., HeLa) also need not be defined.

The following abbreviations should be used without definition in tables:

amt (amount)	SD (standard deviation)
approx (approximately)	SE (standard error)
avg (average)	SEM (standard error of the
concn (concentration)	mean)
diam (diameter)	sp act (specific activity)
expt (experiment)	sp gr (specific gravity)
exptl (experimental)	temp (temperature)
ht (height)	vol (volume)
mo (month)	vs (versus)
mol wt (molecular weight)	wk (week)
no. (number)	wt (weight)
prepn (preparation)	yr (year)

Drugs. Should an author decide to abbreviate the names of antimicrobial agents in a manuscript, the following standard abbreviations are strongly recommended.

Antibacterial agents. Use the indicated abbreviations for the following antibacterial agents.

amikacin (AMK)	erythromycin (ERY)
amoxicillin (AMX)	fleroxacin (FLE)
amoxicillin-clavulanic acid (AMC)	fosfomycin (FOF)
ampicillin (AMP)	gatifloxacin (GAT)
ampicillin-sulbactam (SAM)	gentamicin (GEN)
azithromycin (AZM)	grepafloxacin (GRX)
azlocillin (AZL)	imipenem (IPM)
aztreonam (ATM)	kanamycin (KAN)
carbenicillin (CAR)	levofloxacin (LVX)
cefactor (CEC)	linezolid (LZD)
cefadroxil (CFR)	lomefloxacin (LOM)
cefamandole (FAM)	loracarbef (LOR)
cefazolin (CFZ)	meropenem (MEM)
cefdimir (CDR)	methicillin (MET)
cefditoren (CDN)	mezlocillin (MEZ)
cefepime (FEP)	minocycline (MIN)
cefetamet (FET)	moxalactam (MOX)
cefixime (CFM)	moxifloxacin (MXF)
cefmetazole (CMZ)	nafcillin (NAF)
cefonicid (CID)	nalidixic acid (NAL)
cefoperazone (CFP)	netilmicin (NET)
cefotaxime (CTX)	nitrofurantoin (NTT)
cefotetan (CTT)	norfloxacin (NOR)
cefotetan (CTT)	ofloxacin (OFX)
cefotetan (CTT)	oxacillin (OXA)
cefprozil (CPR)	penicillin (PEN)
ceftazidime (CAZ)	piperacillin (PIP)
ceftazidime (CAZ)	piperacillin-tazobactam (TZP)
ceftazidime (CAZ)	quinupristin-dalfopristin (Synercid) (Q-D)
ceftazidime (CAZ)	rifabutin (RFB)
ceftazidime (CAZ)	rifampin (RIF)
ceftazidime (CAZ)	rifapentine (RFP)
ceftazidime (CAZ)	sparfloxacin (SPX)
ceftazidime (CAZ)	spectinomycin (SPT)
ceftazidime (CAZ)	streptomycin (STR)
ceftazidime (CAZ)	teicoplanin (TEC)
ceftazidime (CAZ)	telithromycin (TEL)
ceftazidime (CAZ)	tetracycline (TET)
ceftazidime (CAZ)	ticarcillin (TIC)
ceftazidime (CAZ)	ticarcillin-clavulanic acid (TIM)
ceftazidime (CAZ)	tolramycin (TOB)
ceftazidime (CAZ)	trimethoprim (TMP)
ceftazidime (CAZ)	trimethoprim-sulfamethoxazole (SXT)
ceftazidime (CAZ)	trovafloxacin (TVA)
ceftazidime (CAZ)	vancomycin (VAN)

β -Lactamase inhibitors. Use the indicated abbreviations for the following β -lactamase inhibitors.

clavulanic acid (CLA)	tazobactam (TZB)
sulbactam (SUL)	

Antifungal agents. Use the indicated abbreviations for the following antifungal agents.

amphotericin B (AMB)	ketoconazole (KTC)
clotrimazole (CLT)	nystatin (NYT)
flucytosine (5FC)	terbinafine (TRB)
fluconazole (FLC)	voriconazole (VRC)
itraconazole (ITC)	

Antiviral agents. Use the indicated abbreviations for the following antiviral agents.

acyclovir (ACV)	ganciclovir (GCV)
cidofovir (CDV)	peniciclovir (PCV)
famciclovir (FCV)	valacyclovir (VCV)
foscarnet (FOS)	zidovudine (AZT)

Reporting Numerical Data

Standard metric units are used for reporting length, weight, and volume. For these units and for molarity, use the prefixes m, μ , n, and p for 10^{-3} , 10^{-6} , 10^{-9} , and 10^{-12} , respectively. Likewise, use the prefix k for 10^3 . Avoid compound prefixes such as m μ or $\mu\mu$. Use $\mu\text{g/ml}$ or $\mu\text{g/g}$ in place of the ambiguous ppm. Units of temperature are presented as follows: 37°C or 324 K .

When fractions are used to express units such as enzymatic activities, it is preferable to use whole units, such as "g" or "min," in the denominator instead of fractional or multiple units, such as μg or 10 min . For example, "pmol/min" is preferable to "nmol/10 min," and " $\mu\text{mol/g}$ " is preferable to "nmol/ μg ." It is also preferable that an unambiguous form, such as exponential notation, be used; for example, " $\mu\text{mol g}^{-1}\text{ min}^{-1}$ " is preferable to " $\mu\text{mol/g min}$." Always report numerical data in the appropriate SI units.

Representation of data as accurate to more than two significant figures must be justified by presentation of appropriate statistical analyses.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the articles by Olsen (*Infect Immun* 71:6689–6692, 2003; *Infect Immun* 82:916–920, 2014).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (*J Virol* 79:669–676, 2005).

Statistics

Statistical analysis of data is a crucial component of scientific publication. Authors who are unsure of proper statistical analysis should have their manuscripts checked by a qualified statistician.

The following is a list of important items that must be considered before manuscript submission. Deficiencies in any of these areas may delay review and/or publication.

(i) Statistical analyses were performed on all quantitative data regardless of how significant the differences look in the tables or figures.

(ii) Data were appropriately analyzed as parametric (normally distributed) or nonparametric data.

(iii) Parametric and nonparametric data are presented appropriately. Means and standard deviations or standard errors are appropriate means of presenting data analyzed by parametric analyses (i.e., *t* test and analysis of variance [ANOVA]), but only medians and surrounding levels (quartiles, quintiles, and 10th and 90th percentiles, etc.) are appropriate for nonparametric statistics (Mann-Whitney test and Kruskal-Wallis test, etc.). Means have no meaning in nonparametric analyses.

(iv) For any data in which there are more than two comparisons (i.e., between one control and more than one experimental group), an analysis must be done for multigroup comparisons. Such an analysis would usually be an ANOVA for parametric data or a Kruskal-Wallis test for nonpara-

Instructions to Authors

metric data. *t* tests cannot be used when more than two groups are being compared (except as indicated below). Failure to use multigroup tests generates type I errors: concluding that two data sets within the overall data set being compared are different when in fact they are not. Exception: some statisticians argue that two-group comparisons can be used on multigroup data if the expected outcomes are appropriately anticipated before the experiment. For example, data generated by individually testing two unrelated factors for their effects on a target with only a single, untreated target as a control could be appropriately analyzed by *t* tests instead of ANOVA.

(v) For all appropriate multigroup comparisons, two *P* values must be generated and provided in the manuscript. The main *P* value applies to the overall data set and indicates that within that data set at least two groups differ from each other. The overall *P* value does not indicate which two groups are different. The main *P* value and the overall *P* value should be computed by using a *post hoc* test. For ANOVA, these *post hoc* tests are usually Dunnett's test (used to compare multiple experimental groups to a single control), the Fisher protected least significant difference (PLSD) test, the Tukey-Kramer test, and the Games-Howell test. Others may be used. Note that each *post hoc* test has certain underlying assumptions that may not be applicable to the data under analysis. For a Kruskal-Wallis nonparametric ANOVA, the Dunn procedure is appropriate to generate *P* values for two-group comparisons.

(vi) Data presented as endpoints (i.e., LD₅₀ and ID₅₀, etc.) contain both the calculated value and a confidence interval with a statistical significance associated with it (95%, 99%, or similar confidence interval), calculated by logit or probit analysis. Simple LD₅₀ values, such as Reed-Muench calculations, may not be used alone.

(vii) When samples are taken multiple times from one experimental entity (i.e., multiple serum samples from one animal, gross pathology scores measured for the same animal over time or growth curves, etc.), one cannot use analyses such as *t* tests, ANOVA, or the Mann-Whitney test,

etc., because these tests assume that each measure is independent. An entity with a high score on day 1 is more likely to have a high score on day 2 than is an entity with a low score. It is likely that some expert statistical help will be needed for these situations, usually involving regression analysis or survival analysis, etc.

(viii) Statistical significance and biological significance are not the same. There is nothing magical about a *P* value of 0.05. When results from large sample sizes are compared, a *P* value of <0.05 will often be obtained, as *P* value is a function of both sample size and effect size. If sample sizes are large, then more-rigorous (i.e., smaller) *P* values may be desirable. If sample sizes are small, *P* values of >0.05 may still be important. There should be both statistical and biological significance to the results and conclusions in the manuscript.

For a review of some common errors associated with statistical analyses and reports, plus guidelines on how to avoid them, see the articles by Olsen (Infect Immun 71:6689–6692, 2003; Infect Immun 82:916–920, 2014).

For a review of basic statistical considerations for virology experiments, see the article by Richardson and Overbaugh (J Virol 79:669–676, 2005).

Isotopically Labeled Compounds

For simple molecules, labeling is indicated in the chemical formula (e.g., ¹⁴C₂, ³H₂O, and H₂³⁵SO₄). Brackets are not used when the isotopic symbol is attached to the name of a compound that in its natural state does not contain the element (e.g., ³²S-ATP) or to a word that is not a specific chemical name (e.g., ¹³¹I-labeled protein, ¹⁴C-amino acids, and ³H-ligands).

For specific chemicals, the symbol for the isotope introduced is placed in square brackets directly preceding the part of the name that describes the labeled entity. Note that configuration symbols and modifiers precede the isotopic symbol. The following examples illustrate correct usage:

[¹⁴ C]urea	UDP-[U- ¹⁴ C]glucose
L-[methyl- ¹⁴ C]methionine	<i>E. coli</i> [³² P]DNA
[2,3- ³ H]serine	fructose 1,6-[1- ³² P]bisphosphate
[α- ¹⁴ C]lysine	[γ- ³² P]ATP

Annexure E: BMC author guidelines

Appendix B



Instructions for preparation of manuscripts for publication in supplements to BioMed Central journals

[General information](#) | [Style and language](#) | [Manuscript preparation](#) | [Figures](#) | [Tables](#) | [Additional files](#) | [Author presubmission checklist](#)

General information

Publication

Manuscripts accepted by the Editor-in-Chief will be published online in a BioMed Central journal in fully browseable web forms and formatted PDF files. Articles will be available through BioMed Central websites and submitted for inclusion in PubMed.

Editorial policies

Submission of a manuscript to a BioMed Central journal implies that all authors have read and agreed to its content, and that readily reproducible materials described in the manuscript will be freely available to any scientist wishing to use them for non-commercial purposes. Nucleic acid sequences, protein sequences, and atomic coordinates should be deposited in an appropriate database in time for the accession number to be included in the article. Where appropriate, authors are encouraged to adhere to the standards proposed by the Microarray Gene Expression Data Society (<http://www.mged.org>) and to deposit microarray data in one of the public repositories.

Authors are required to ensure that no material submitted as part of a manuscript infringes existing copyrights, or the rights of a third party. The article should not already have been published in another journal and should not be under consideration by any other journal, but it can already have been deposited on a preprint server.

Copying text from previously published work without acknowledging the source is plagiarism and such misconduct may be reported to the authors' institution(s). Any quotations must be clearly indicated by quote marks and the source must be cited.

Ethics and consent

Submission of a manuscript reporting experimental research on humans or animals implies that authors have obtained approval for their research from the appropriate ethics committee, or government body regulating animal or human research. A statement to this effect must be included in the Methods section of your manuscript. Experimental methods must be in compliance with the Helsinki Declaration (www.wma.net/en/30publications/10policies/b3/). Where appropriate, a statement to this effect must be included in the Methods section of the manuscript.

For all articles that include information or clinical photographs relating to individual patients, written and signed consent from each patient to publish must also be submitted to BioMed Central. The manuscript should include a statement to this effect in the Acknowledgements section, as follows: "Written consent was obtained from the patient or their relative for publication of this study".

Competing interests

Authors of manuscripts submitted for publication in supplements to BioMed Central journals must complete a declaration of competing interests (see ["Competing interests"](#)). Where an author has no competing interests, they should state this. See <http://www.nlm.nih.gov/pubs/factsheets/supplements.html>.

Special requirements – RCT registration and reporting checklists

BioMed Central supports prospective registration and numbering of randomized controlled trials (RCTs). Authors of reports of controlled trials of health care interventions must register their trial in a suitable publicly accessible registry prior to submission. The trial registers that currently meet all of the ICMJE guidelines can be found at <http://www.icmje.org/faq.html>.

BioMed Central also supports initiatives aimed at improving the reporting of biomedical research. We recommend authors refer to the [EQUATOR](#) network website for further information on the available reporting guidelines for health research, and the [MIBBI](#) Portal for prescriptive checklists for reporting biological and biomedical research

E&OE March 2012

1

Appendix B

where applicable. Authors are requested to make use of these when drafting their manuscript and peer reviewers will also be asked to refer to these checklists when evaluating these studies. Checklists are available for a number of study designs, including randomized controlled trials ([CONSORT](#)), systematic reviews ([PRISMA](#)), observational studies ([STROBE](#)), meta-analyses of observational studies ([MOOSE](#)), diagnostic accuracy studies ([STARD](#)) and qualitative studies ([RATS](#)). For authors of systematic reviews, a supplementary file, linked from the Methods section, should reproduce all details concerning the search strategy. For an example of how a search strategy should be presented, see the [Cochrane Reviewers' Handbook](#).

Authors from pharmaceutical companies, or other commercial organizations that sponsor clinical trials, should adhere to the Good Publication Practice guidelines for pharmaceutical companies (<http://www.gpp-guidelines.org/>), which are designed to ensure that publications are produced in a responsible and ethical manner. The guidelines also apply to any companies or individuals that work on industry-sponsored publications, such as freelance writers, contract research organizations and communications companies.

The involvement of medical writers or anyone else who assisted with the preparation of the manuscript content should be acknowledged, along with their source of funding, as described in the European Medical Writers Association (EMWA) guidelines on the role of medical writers in developing peer-reviewed publications (<http://www.emwa.org/Mum/EMWAguidelines.pdf>). If medical writers are not listed among the authors, it is important that their role be acknowledged explicitly. We suggest working such as "We thank Jane Doe who provided medical writing services on behalf of XYZ Pharmaceuticals Ltd".

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Style and language**General**

Currently, BioMed Central can only accept manuscripts written in English. Spelling should be US English or British English, but not a mixture. Full stops rather than commas should be used to indicate decimal points.

BioMed Central will not edit submitted manuscripts for style or language, so please ensure that inconsistencies in style, and incorrect grammar, spelling and punctuation have been addressed prior to submission.

Gene names should be in italic, but protein products should be in plain type.

Generic drug names should generally be used. When proprietary brands are used, include the brand names in parentheses.

There is no explicit limit on the length of articles submitted for publication in a supplement, unless otherwise instructed by the supplement organizers, but authors are encouraged to be concise. There is also no restriction on the number of figures or tables, but note that these should be limited to a reasonable number for the length of the article. Authors should include all relevant supporting data with each article.

Typography

- Please use double line spacing.
- Type the text unjustified, without hyphenating words at line breaks.
- Use hard returns only to end headings and paragraphs, not to rearrange lines.
- Capitalise only the first word and proper nouns in the title and headings.
- All pages should be numbered.
- Use the BioMed Central reference format (see [References](#) section in [Manuscript preparation](#)).
- Greek and other special characters may be included. If you are unable to reproduce a particular special character, please type out the name of the symbol in full. Please ensure that all special characters used are embedded in the text, otherwise they may be lost during conversion to PDF.

Units

SI units should be used throughout (litre and molar are permitted, however).

Manuscript preparation: text and sections

Preparing text

File formats

The following file formats are acceptable for the main text document:

- Microsoft Word (version 2 and above) – see below for template
- Rich text format (RTF)
- Portable document format (PDF)

TeX/LaTeX - please use BioMed Central's TeX template: <http://www.biomedcentral.com/fora/tex> and submit your file as a PDF file.

Microsoft Word template

We have designed a MS Word template that can be used to generate a standard style and format for your article. It can be used if you have not yet started to write your article, or if it is already written and needs to be put into BioMed Central journal style.

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- If there are sections that you do not need, delete them (but check the rest of the Instructions for Authors to see which sections are compulsory).
- Please include a [Competing interests](#) section, as these are compulsory for all Supplement articles.
- If you need an additional copy of a heading (e.g. for additional figure legends), just copy and paste.
- Please note you can put the Methods section after the Conclusion if you prefer.
- Enter the references using the reference style given, see [References](#) section.

Sections of the manuscript

All manuscripts must be structured and include a title page and abstract. Manuscripts should be divided into sections as appropriate to the article - please do not number the sections or any subheadings. The following sections are required:

Title page

Abstract

Background or Introduction

Main body of text

- Research articles must include **Methods**, **Results** and **Conclusion** sections in the main text, with an optional **Discussion** section

- Review articles – please include appropriate headings and subheadings

Conclusions

List of abbreviations used (if any)

Competing interests

Authors' contributions (research articles only)

Acknowledgements

References

Figure legends (if any)

Tables and captions (if any)

Description of additional data files (if any)

Please note: footnotes and text boxes are not allowed

Appendix B

Title page

This should include the title of the article, the full author names (with no titles or qualifications), institutional addresses (Department, Institute, City, Post/Zip code, Country), and email addresses for all authors. Authors and affiliations must be linked using superscript numerals. The corresponding author should also be indicated. The title should be in bold, sentence case with no full stop at the end and no underlining.

Abstract

This should not exceed 350 words and, for research articles, should be structured into separate sections headed Background, Methods (if applicable), Results and Conclusions. (Background: the context and purpose of the study; Methods: how the study was performed and statistical tests used; Results: the main findings; Conclusions: brief summary and potential implications.) Please do not cite references, figures or tables in the abstract, and minimise the use of abbreviations.

Background or Introduction

The Background section should be written in a way that is accessible to researchers without specialist knowledge in that area and must clearly state – and, if helpful, illustrate – the background to the research and its aims. Reports of clinical research should, where appropriate, include a summary of a search of the literature to indicate why this study was necessary and what it aimed to contribute to the field. The section should end with a brief statement of what is being reported in the article.

Main body of text

The main text should be arranged under appropriate subheadings *typed in sentence case*. Research articles should include the following sections: ('Methods' may be placed before 'Results' or after 'Conclusions'):

Methods

The Methods section should include the design of the study, the setting, the type of participants or materials involved, a clear description of all interventions and comparisons, and the type of analysis used, including a power calculation if appropriate. Generic drug names should generally be used. When proprietary brands are used in research, include the brand names in parentheses. The section should be divided into subsections if several methods are described.

The accession numbers of any nucleic acid sequences, protein sequences or atomic coordinates cited in the manuscript should be provided in square brackets with the corresponding database name; for example, [EMBL:AB028295, EMBL:AC137000, DDBJ:AE000812, GenBank:U49845, PDB:1BFM, Swiss-Prot:Q96KQ7, PIR:S86116].

The databases for which we can provide direct links are: EMBL Nucleotide Sequence Database (EMBL, <http://www.ebi.ac.uk/embl/>), DNA Data Bank of Japan (DDBJ, <http://www.ddbj.nig.ac.jp/>), GenBank at the NCBI (GenBank, <http://www.ncbi.nlm.nih.gov/genbank/>), Protein Data Bank (PDB, <http://www.pdb.org/pdb/home/home.do>), Protein Information Resource (PIR, <http://pir.georgetown.edu>) and the Swiss-Prot Protein Database (Swiss-Prot, <http://us.expasy.org/sprot/>).

Results

The Results and Discussion may be combined into a single section or presented separately. Results of statistical analysis should include, where appropriate, relative and absolute risks or risk reductions, and confidence intervals. The Results and Discussion sections may also be broken into subsections with short, informative headings, typed in sentence case.

Conclusions

This should state clearly the main conclusions and give a clear explanation of their importance and relevance. Summary illustrations may be included.

List of abbreviations used

If abbreviations are used in the text they should be defined in the text where first used and a list of abbreviations provided.

Competing interests

A competing interest exists when your interpretation of data or presentation of information may be influenced by your personal or financial relationship with other people or organizations. Authors should disclose any financial competing

Appendix B

interests but also any non-financial competing interests that may cause them embarrassment were they to become public after the publication of the article.

All authors are required to complete a declaration of competing interests. All competing interests that are declared will be listed at the end of published articles. Where author(s) have no competing interests, please state 'The author(s) [or certain named authors] declare that they have no competing interests'.

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- Do you hold any stocks or shares in an organization that may in any way gain or lose financially from the publication of this manuscript, either now or in the future? If so, please specify.
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Appendix B

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Article within a journal

1. Koonin EV, Altschul SF, Bork P: **BRCA1 protein products: functional motifs**. *Nat Genet* 1996, 13:266-267.

Article within a journal supplement

2. Orengo CA, Bray JE, Hubbard T, LoConte L, Sillitoe I: **Analysis and assessment of ab initio three-dimensional prediction, secondary structure, and contacts prediction**. *Proteins* 1999, 43(Suppl 3):149-170.

In press article

3. Kharitonov SA, Barnes PJ: **Clinical aspects of exhaled nitric oxide**. *Eur Respir J*, In press.

Published abstract

4. Zvaifler NJ, Burger JA, Marinova-Mutatchieva L, Taylor P, Maini RN: **Mesenchymal cells, stromal derived factor-1 and rheumatoid arthritis [abstract]**. *Arthritis Rheum* 1999, 42:s250.

Article within conference proceedings

5. Jones X: **Zooflora and synthetic mechanisms**. In *Proceedings of the First National Conference on Porous Sieves: 27-30 June 1996; Baltimore*. Edited by Smith Y. Stoneham: Butterworth-Heinemann; 1996:16-27.

Book chapter, or article within a book

6. Schnepf E: **From prey via endosymbiont to plastids: comparative studies in dinoflagellates**. In *Origins of Plastids. Volume 2*. 2nd edition. Edited by Lewin RA. New York: Chapman and Hall; 1993:53-76.

Whole issue of journal

7. Ponder B, Johnston S, Chodosh L (Eds): **Innovative oncology**. In *Breast Cancer Res* 1998, 10:1-72.

Whole conference proceedings

8. Smith Y (Ed): **Proceedings of the First National Conference on Porous Sieves: 27-30 June 1996; Baltimore**. Stoneham: Butterworth-Heinemann; 1996.

Complete book

9. Margulis L: **Origin of Eukaryotic Cells**. New Haven: Yale University Press; 1970.

Monograph or book in a series

10. Hunninghake GW, Gadek JE: **The alveolar macrophage**. In *Cultured Human Cells and Tissues*. Edited by Harris TJR. New York: Academic Press; 1995:54-56. [Stoner G (Series Editor): *Methods and Perspectives in Cell Biology*, vol 1.]

Book with institutional author

11. Advisory Committee on Genetic Modification: **Annual Report**. London; 1999.

Appendix B

PhD thesis

12. Kohavi R: **Wrappers for performance enhancement and oblivious decision graphs**. PhD thesis. Stanford University, Computer Science Department; 1995.

Link / URL

13. **Mouse Tumor Biology Database** [http://tumor.informatics.jax.org/cancer_links.html]

Dataset with persistent Identifier

Zheng, L-Y; Guo, X-S; He, B; Sun, L-J; Peng, Y; Dong, S-S; Liu, T-F; Jlang, S; Ramachandran, S; Liu, C-M; Jing, H-C (2011): Genome data from sweet and grain sorghum (*Sorghum bicolor*). *GigaScience*. <http://dx.doi.org/10.5524/100012>

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Figures may be submitted in colour. Each figure should be closely cropped to minimize the amount of white space surrounding the illustration.

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Illustrations should be designed such that all information is legible when scaled to a horizontal width of 800 pixels, since this is the default size for a BioMed Central journal (and PubMed Central) illustration on the web. Therefore, image files should be submitted at a resolution of 300 dpi or greater. Note that lower resolution files might not reproduce clearly.

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Small tables [less than one side of A4 (210mm x 297mm)] that are integral to the manuscript should be pasted into the end of the document text file in portrait form, and will be displayed in the final published form of the article. Tables may be subdivided into labelled parts, but each table should form a single unit. Such tables should be formatted using the 'Table object' in a word processing program to ensure that columns of data are kept aligned – do not use tabs to separate the columns. The title and legend should not be inside table cells, but appear outside the table.

Tables should not include shading or colour as this won't be reproduced in the online version. Tables should not include vertical rules, and horizontal rules should only be used within the body of the table if essential for clarity (horizontal rules under main headings/subheadings and at the end of the table should be included). Full stops rather than commas should be used to indicate decimal points.

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Appendix B

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E&OE March 2012

10