Evaluation of a novel concentration method for the microscopic detection of *Mycobacterium tuberculosis* from induced sputum

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Abstract

Diagnosis of tuberculosis (TB) in developing countries relies on sputum smear microscopy, which has poor sensitivity compared to culture, particularly for immune-compromised people. Induction of sputum by inhalation of saline has the potential to increase case detection but centrifugation of the sample is necessary before microscopy. A systematic review was undertaken comparing the performance of microscopy and culture on induced sputum. A study was then undertaken in Zimbabwe on the use of magnetic beads (TB Beads) coated with chemical ligands to concentrate the mycobacteria from induced sputum, which if successful would negate the requirements for a biosafety cabinet and centrifuge, and therefore increase access to TB diagnosis in peripheral clinics. Adult TB suspects who were smear-negative on conventional microscopy underwent the induction procedure. The resulting sample was divided in two: half was processed with the TB Beads method with fluorescent and Ziehl-Neelsen microscopy; the other half was subjected to centrifugation followed by fluorescent smear microscopy, and culture on Löwenstein-Jensen medium. Of the 139 patients that were enrolled, 97% produced an induced sputum sample. Mild side-effects were experienced by 13% of patients. 26 smear-positive patients were found, of which 21 were positive on centrifugation, and 13 on TB Beads. Seven patients were culture-positive. One patient was smear-negative and culture-positive. The TB Beads elution buffer was altered by the manufacturer during enrollment, so results were analysed for both buffer types combined, as well as separately. For both buffers combined, the sensitivity and specificity of microscopy compared to culture were 43% and 95% respectively for ZN microscopy and 43% and 94% when using fluorescent microscopy. When

both ZN and fluorescent microscopy were combined the sensitivity was 57%. However, when centrifugation was used prior to fluorescent microscopy, sensitivity increased to 86%. In conclusion the TB Beads did not perform sufficiently well to replace centrifugation.

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Glossary

AFB	acid-fast bacilli
AIDS	acquired immune deficiency syndrome
BAL	bronchoalveolar lavage
BRTI	Biomedical Research and Training Institute
BSA	bovine serum albumin
CI	confidence interval
CLO	community liaison officer
DOTS	directly observed therapy (shortcourse)
DrPH	doctor of public health
DST	drug susceptibility testing
EBPHP	evidence-based public health policy
EPTB	extrapulmonary tuberculosis
ERB	ethics review board
FM	fluorescent microscopy
GDP	gross domestic product
HCW	health care worker
HIV	human immunodeficiency virus
LJ	Löwenstein-Jensen
LMPD	leadership, management and professional development
LSHTM	London School of Hygiene and Tropical Medicine
MDR	multi-drug resistant
MODS	microscopic-observation drug susceptibility
MoHCW	Ministry of Health and Child Welfare
MRCZ	Medical Research Council of Zimbabwe

MSc	Master of Science
MSF OCA	Médecins sans Frontières Operational Centre Amsterdam
NA	not applicable
NaDCC	sodium dichloroisocyanurate
NALC-NaOH	n-acetyl-l-cysteine sodium hydroxide
NPV	negative predictive value
NS	not stated
NTM	non-tuberculous mycobacteria
OPA	organisation and policy analysis
pDADMAC	poly diallyl dimethyl ammonium chloride
PI	principal investigator
PPV	positive predictive value
PTB	pulmonary tuberculosis
RDT	rapid diagnostic test
SAH	sociological approaches to health
SI	sputum induction
TB	tuberculosis
TDR	Tropical Disease Research
TLA	thin layer agar
TST	tuberculin skin test
USA	United States of America
WHO	World Health Organisation
XDR	extensive drug resistant
ZN	Ziehl-Neelsen

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At the LSHTM, thanks to Christian Bottomley for support with statistics. Mary Cooke kindly designed the Access database which was used.

Finally, one of the best decisions I have ever made was to ask Ruth McNerney to be my thesis supervisor. She provided me with excellent academic and emotional support throughout the whole degree, and was always available to brainstorm, troubleshoot, and console as required.

Chapter 1: Integrating statement

The Doctor of Public Health (DrPH) degree taught at the LSHTM aims to provide students with the skills required to transform them into effective leaders in public health organisations. The degree consists of three components: a taught component during which core modules are taken in combination with MSc modules that the student decides will be useful during the course of study; an Organisational and Policy analysis (OPA) project and report, utilising the information gained in the core modules; and a research project, in which the student carries out a piece of original research which is of shorter duration, but equal in intellectual rigour, as that of a traditional PhD degree.

I chose to undertake a DrPH degree as opposed to a PhD because while I wanted to improve my knowledge of undertaking and interpreting research, I also wanted to learn about specific topics, such as management and public health policy, in an interactive environment which would encourage debate with fellow students experienced with working in the public health arena. I also liked the fact the DrPH degree could be undertaken alongside part-time work. The degree components almost inevitably became closely-linked to my past experience and current work with Médecins Sans Frontières (MSF). MSF not only provided the funding for the school and research fees; also, 8 years of experience with the organisation has shaped my perspectives of global public health issues. It was therefore extremely helpful to use the components of the DrPH degree as a prism through which to see the procedures used and impact of the work that the organisation carries out.

The taught component consisted of two modules exclusive to the DrPH programme, the core modules: Evidence-based Public Health Policy (EBPHP) and Leadership, Management and Professional Development (LMPD). I also took three optional MSc modules. The EBPHP module introduced me to key aspects of public health such as the evaluation of evidence for policy-making, which culminated in the performance of a systematic review. This exercise not only helped me to learn how to assess the different components of available evidence, but set me up for the performance of a systematic review based on my thesis topic, which has subsequently been accepted for publication. The agenda-setting exercise underlined the importance of taking several different perspectives into account when attempting to influence policy: this exercise was useful almost straight-away, when it came to deciding on a topic for my research project which was immediately relevant to the patients in the field site in which it was to be performed, interesting to the staff at the field site, and beneficially in-line with the long-term strategy for the MSF headquarters advisors and directors who ultimately gave the go-ahead for the project to take place; in other words, getting the research project accepted was an exercise in working my own priorities into those of others.

The LMPD module was a pleasant surprise, as I had previously never considered that the field of organisational behaviour was of relevance in public health management. The components on change management and leadership skills were of particular interest to me, and I was able to use previous work experience in the prescribed assignment to investigate how poor leadership and lack of an adequate change management policy undermined policy change, even when the changes were in line with the staff's own objectives and ideals.

The module underlined the importance of good change management strategies to achieve plans in an effective and cohesive way.

Both of the core modules were quite new areas for me, and yet within a few months I felt I gained a solid grounding in both, which allowed me to undertake the OPA exercise confidently.

I took on the Design and Analysis of Epidemiological Studies module to ensure my skills in epidemiological research were up-to-date. This allowed for objective interpretation and presentation of data, useful for the research project and also for future evidence-based decision-making in a public health field. I updated my knowledge of molecular biology with the Molecular Biology of Infectious Diseases: Research Progress and Applications module. I took the opportunity to expand my knowledge of this topic as it is of relevance to my job as a laboratory specialist, therefore allowing me to keep abreast of advances which can have a significant impact on disease control and monitoring, such as the recent Xpert® MTB/RIF assay. I improved my qualitative/sociological knowledge by undertaking the Sociological Approaches to Health (SAH) module. This helped me to understand that public health management is not only about policies, numbers and costs, but also how these aspects are interpreted and accepted by individuals and societies.

I undertook the OPA project in the MSF in Ireland office, and it allowed me to put what I had learned in EBPHP, LMPD and SAH into practice. It helped me to move from my technical, paramedical understanding of how the organisation functioned, to appreciating its other vital components, e.g. its Fundraising and Communications activities. I gained an appreciation for how an organisation's structure leads to its culture, and how these then give an office its own

distinctive voice and strengths. The project report provided information on progress made to date regarding ambitions, and gave feedback on what worked well, and where improvements could be made. The OPA improved my understanding of how different motivational aspects influence the success with which an organisation achieves its priorities, and how the different components of a small office fit into the larger role of an international public health organisation such as MSF. The OPA taught me the skills to identify potential impediments to success, and to empower employees to achieve organisational objectives and personal potential.

The research project saw me back on the familiar territory of research in tuberculosis diagnosis. I chose to remain in this field in order to allow the research to be of use to my employer, and to ensure that I acquired research expertise in an area of interest to me, both in my current position and to enhance future career options. Although I had undertaken operational research before, this project required a different level of stringency and diligence. The project required me to think about the impact of evidence and its potential consequences for global public health, and not just answering an organisation's immediate needs. It required engagement with local research partners to determine the best implementation model for positive impact on the host population. Living and working with people living with HIV/AIDS in Zimbabwe emphasised the necessity of research to improve tuberculosis case-finding for this vulnerable population. The context also provided lessons on the consequences of the collapse of a previously-functional public health system, alongside a ravaging dual epidemic of HIV-TB which can challenge even functional public health systems. The research project evaluated a diagnostic

technique, but I had originally also planned to undertake semi-structured interviews to determine user acceptability of the technique under investigation, following on from the SAH module's focus on patient perceptions of public health interventions. This did not take place, following the advice of my Review panel; however, knowledge from the LMPD module was utilised for the management of the interdisciplinary study team.

The DrPH degree has given me a solid foundation in public health policy, leadership and management issues, enabling me to understand better the functioning and objectives of MSF and other public health organisations, and teaching me how to interact with external stakeholders. I now feel I can undertake high-quality public health-based research, or can commission research following careful analysis of the kind of research likely to prove useful in a given public health setting. With the skills acquired, the commissioned or undertaken research can then be critically interpreted and evaluated, with the end objective of improving the functioning of a public health organisation, or with influencing public health policy.

Chapter 2: Rationale and objectives

2.1) Background and justification

2.1.1) Tuberculosis

2.1.1.1) Tuberculosis: transmission and infection

Tuberculosis (TB) is an infectious disease most commonly caused by the bacterium *Mycobacterium tuberculosis*. The bacterium is usually transmitted through the inhalation of infectious micro-aerosols, which are expelled by infectious patients when coughing or sneezing. These droplet nuclei are inhaled into the alveoli, and the bacteria may then travel to other parts of the body to cause extra-pulmonary TB (EPTB). Infection progresses when the bacteria multiply within the lungs or other body sites (Enarson, Rieder et al. 2000). 10-20% of immune-competent people who are infected will develop TB disease over their lifetime, normally shortly after infection (Sutherland 1976; Vynnycky and Fine 1997), but disease can occur at any stage throughout their lives, through the renewed multiplication of dormant *M. tuberculosis*, i.e. endogenous reactivation (Vynnycky and Fine 1997). Not all people exposed to TB become infected, suggesting that the innate immune mechanisms of some can clear *M tuberculosis*, but this has not yet been proved (Walzl, Ronacher et al. 2011).

The immunopathology of TB infection in humans has yet to be fully elucidated, and animal models, including mice, have been used to study this. Once inhaled, *M. tuberculosis*-containing droplet nuclei are taken up by alveolar macrophages and immature dendritic cells, the latter of which present antigens to naive T cells and prime the immune response. Cytokine activity results in the recruitment of macrophages and neutrophils into the lungs, thus commencing

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the development of granulomas. Bacteria continue to multiply within the lung, resulting in interstitial pneumonitis up to 2 weeks after infection. In weeks 3 and 4 after infection, granuloma development takes place through influx of inflammatory cells and the formation of lesions in the lung, with macrophages and T cells predominating, resulting in an antigen-specific interferon-gamma producing T-cell response. Activated CD4 T cells produce interferon gamma, which cause the macrophages to suppress bacterial growth, eliminating 50-90% of *M. tuberculosis*. CD8 T cells release perforin and granulysin which kill the macrophage and the *M. tuberculosis* within. The bacterial load is not completely eliminated, and *M. tuberculosis* enters a quiescent phase and remain stable until a later phase of reactivation, at which point the host response is unable to suppress growth. The granulomas then increase in cellular structure and size, and lesions are present. The lesions enclose the infected macrophages and prevent bacterial dissemination. The chronic phase of granuloma development then begins, when fibrosis takes place around and throughout the granulomas. The final phase occurs when the granuloma is broken down, *M. tuberculosis* disseminates, and, without treatment, the host deteriorates. Human granulomas have a hypoxic, caseating or necrotic core, which is surrounded by layers of mixed T cells and macrophages. In over 90% of humans, granulomas are sufficient to contain infection and prevent transmission and disease development, resulting in a subclinical, or latent, state for the patient's life. While the factors influencing reactivation of infection are not yet fully understood, the depletion of CD4 T helper cells in human immune deficiency virus (HIV) infection has been linked with granuloma erosion and subsequent reactivation of disease. The continued enlargement of granulomas can also result in their erosion into air passages and blood vessels, thus allowing

dissemination into the blood stream and respiratory system (Kaufmann 2003; Kaufmann and Britton 2008). Current understanding of the immune response to TB infection is presented in Figure 1.

Figure 1: Major cells and immune mechanisms in TB. From Kaufmann, 2003: Immune response to tuberculosis: experimental animal models (Kaufmann 2003).
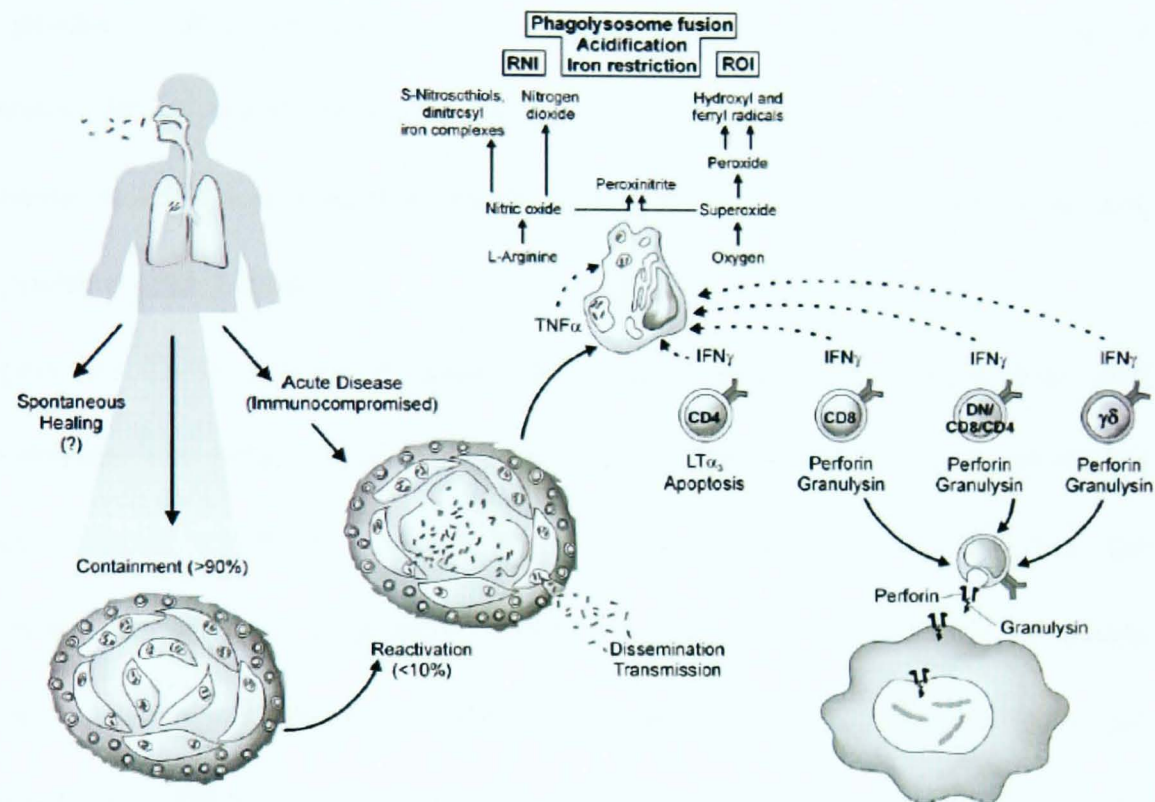
The immune system normally prevents infection from developing into TB. HIV-positive people have an impaired immune system, which allows new or dormant TB infections to develop into the active form of the disease, and these patients have an annual risk of 10% of developing post-primary TB infection (Selwyn, Hartel et al. 1989).

Pulmonary TB accounts for approximately 80% of the cases of TB (Kherad, Herrmann et al. 2009). EPTB can affect any body part, but most frequently affects the pleura and lymph nodes (Enarson, Rieder et al. 2000).

2.1.1.2) Clinical presentation

Primary disease occurs when a previously uninfected person is first infected with *M. tuberculosis*. Localised inflammation occurs in the lung, resulting in granuloma formation. The patient may have symptoms of upper respiratory tract infection, such as coughing and malaise. Post-primary TB is the most clinically significant form of the disease, and may result from: direct progression from primary TB; reactivation of a primary infection, following a period of dormancy; blood-borne or lymphatic spread to other parts of the lung; or exogenous re-infection, which is common in endemic areas, particularly among HIV-positive populations.

Post-primary TB is commonly associated with cough, fever, weight loss, and night sweats. The presence of the disease can be confirmed at this stage with a sputum culture which is positive for *M. tuberculosis*. This is currently the reference standard for diagnosis. Diagnosis can also be assisted by using sputum smear microscopy, tuberculin skin-testing, and chest x-ray. Complications which can arise from post-primary TB include infection of the larynx and pharynx, pericarditis, haemoptysis, airways obstruction, severe lung damage, carcinoma of the lung, and ultimately, death (Davies 2003). Death can result even in developed countries which provide effective drug treatment, with increased mortality being associated with advanced age (Abuaku, Tan et al. 2010). Mortality is much higher in developing countries, especially in areas of high HIV prevalence (Corbett, Watt et al. 2003). In 2010, an estimated 1.1 million deaths took place among HIV-negative TB cases, with an estimated 0.35 million deaths among HIV-positive TB cases. Mortality rates from TB appear to be falling globally, including in all the TB high-burden countries apart from Afghanistan (World Health Organisation 2011c).

2.1.1.3) Case definitions

Case definitions for TB infection are required to facilitate patient registration and case notification, to select appropriate treatment regimens, to standardise data collection, and for epidemiological purposes.

The World Health Organisation (WHO) provides the following case definitions to guide the management of TB, based on access to biological confirmation and the level of certainty of the diagnosis:

TB suspect: Any person who presents with symptoms or signs suggestive of TB. The most common symptom of pulmonary TB is a productive cough for more than 2 weeks, which may be accompanied by other respiratory symptoms (shortness of breath, chest pains, haemoptysis) and/or constitutional symptoms (loss of appetite, weight loss, fever, night sweats, and fatigue).

Case of TB: A definite case of TB (defined below) or one in which a health worker (clinician or other medical practitioner) has diagnosed TB and has decided to treat the patient with a full course of TB treatment.

Note: Any person given treatment for TB should be recorded as a case. Incomplete "trial" TB treatment should not be given as a method for diagnosis.

Definite case of TB: A patient with *M. tuberculosis* complex identified from a clinical specimen, either by culture or by a newer method such as molecular line probe assay. In countries that lack the laboratory capacity to routinely identify *M. tuberculosis*, a pulmonary case with one or more initial sputum smear examinations positive for acid-fast bacilli (AFB) is also considered to be a "definite" case, provided that there is a functional external quality assurance (EQA) system with blind rechecking.

Cases of TB are also classified according to the:

— anatomical site of disease;

— bacteriological results (including drug resistance);

— history of previous treatment;

— HIV status of the patient.

Defining the site of infection is important for monitoring purposes and to determine the infectiousness of the patient. The following definitions are provided for defining site of infection:

Pulmonary TB (PTB) refers to a case of TB (defined above) involving the lung parenchyma. Miliary TB is classified as pulmonary TB because there are lesions in the lungs. Tuberculous intrathoracic lymphadenopathy (mediastinal and/or hilar) or tuberculous pleural effusion, without radiographic abnormalities in the lungs, constitutes a case of EPTB. A patient with both pulmonary and EPTB should be classified as a case of pulmonary TB. **EPTB** refers to a case of TB (defined above) involving organs other than the lungs, e.g. pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, or meninges. Diagnosis should be based on at least one specimen with confirmed *M. tuberculosis* or histological or strong clinical evidence consistent with active EPTB, followed by a decision by a clinician to treat with a full course of TB chemotherapy. The case definition of an EPTB case with several sites affected depends on the site representing the most severe form of disease. Unless a case of EPTB is confirmed by culture as caused by *M. tuberculosis*, it cannot meet the "definite case" definition (World Health Organisation 2010e).

2.1.1.4) Case-finding

Different approaches can be used for the detection of TB cases. The WHO recommends passive case-finding, whereby symptomatic patients self-present at

clinical facilities for diagnosis, over active case-finding, whereby healthcare workers proactively investigate among populations by screening high-risk populations. This is in order to focus resources on those who are most likely to have the infection and who are most likely to transmit the infection to others (World Health Organisation 2006c). Active case-finding is recommended only for high-risk cases such as HIV-positive people (World Health Organisation 2008c).

2.1.1.5) Treatment

Post-primary TB should be treated according to the drug sensitivity profile of the organism causing the infection. However, these profiles are usually not available for most patients diagnosed with TB. Drug-susceptible TB is treated with a directly-observed, multi-drug regimen consisting of first-line drugs: isoniazid, rifampicin, ethambutol and pyrazinamide for 2 months. This is followed by 4 months of isoniazid plus rifampicin (if treatment is directly-observed), or 6 months of isoniazid plus ethambutol (if treatment if self-administered) (Rieder 2002).

M. tuberculosis can acquire resistance to the drugs used to treat it. Antituberculous drug resistance has been classified according to the following definitions:

- Mono-resistance: TB which is resistant *in vitro* to one first-line drug

- Poly-resistance: TB which is resistant *in vitro* to more than one first-line drug, other than both isoniazid plus rifampicin

- Multi-drug resistance (MDR): TB which is resistant *in vitro* to at least isoniazid and rifampicin (World Health Organisation 2006a).

In 2006, the World Health Organisation (WHO) described the emergence of extensive drug-resistant TB, or XDR TB. XDR TB is now defined as TB strains which are MDR, and resistant to any fluoroquinolone, and at least one of the three injectable second-line drugs (amikacin, kanamycin and capreomycin) (Centres for Disease Control and Prevention 2006; Jassal and Bishai 2009).

2.1.1.6) Epidemiology

It is estimated that 2 billion people, or 33% of the world's population, are infected with *M. tuberculosis* (World Health Organisation 2009a). The WHO reported the following TB statistics for 2010 (World Health Organisation 2011c):

- 8.8 million incident cases (range, 8.5-9.2 million)

- 1.1 million deaths in HIV-negative people (range, 0.9-1.2 million)

- 0.35 million deaths in HIV-positive people (range, 0.32-0.39 million).

Figure 2: Global estimated TB incidence in 2010 (World Health Organisation 2011c).

Estimated TB incidence rates, 2010
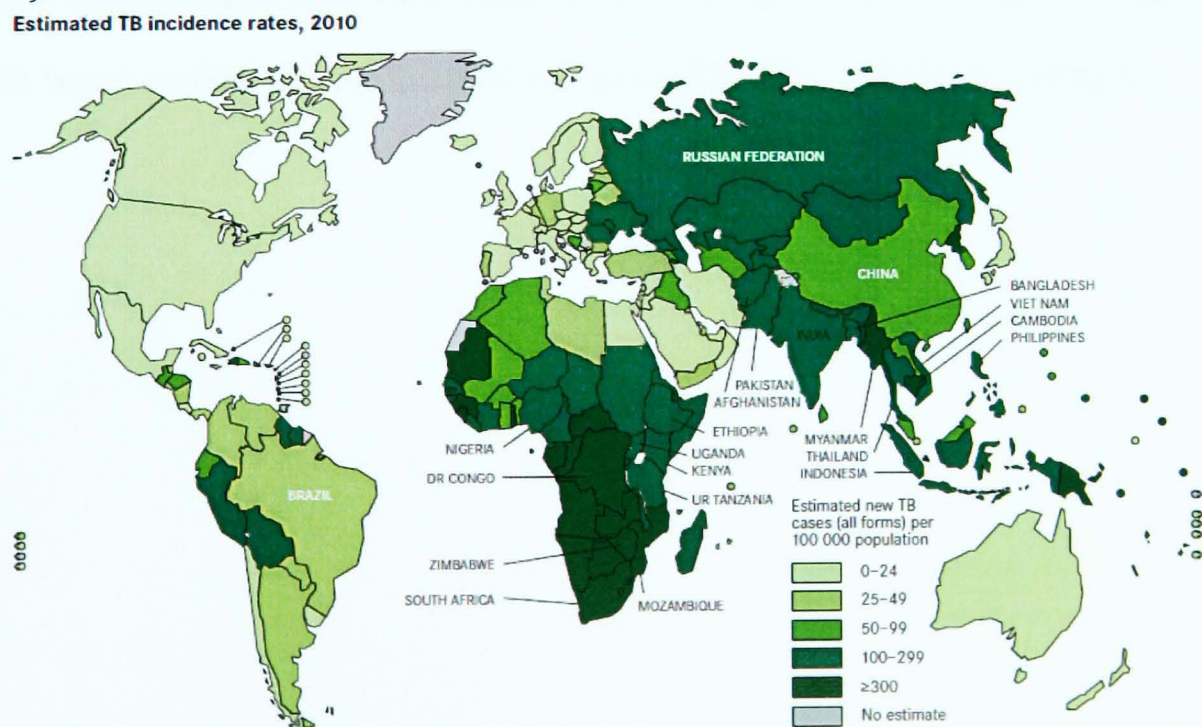
There were 5.7 million notified cases of disease in 2010. This is equivalent to an estimated 65% of all incident cases, which is 6% less than the target case detection rate of 71% defined by the Millennium Development Goal Target 6C (World Health Organisation 2010b). 87% of the patients in the 2009 smear-positive treatment cohort were successfully treated. Fewer than 5% of new and previously-treated TB suspects were tested for MDR-TB (World Health Organisation 2011c).

2.1 million TB patients knew their HIV status, with 46% of HIV-positive TB patients starting antiretroviral therapy, and 77% starting co-trimoxazole preventive treatment in 2010. There was an estimated prevalence of 650,000 cases of MDR-TB in 2010, with 46,000 patients enrolled in MDR-TB treatment, which is an estimated 16% of the people predicted to require treatment (World Health Organisation 2011b).

Twenty-two countries have been designated to be high-burden countries by the WHO, based on their epidemiological burden of TB. These are listed in table 1.

Table 1: Estimated epidemiological burden of TB in 2010 (World Health Organisation 2011c)

Estimated epidemiological burden of TB, 2010. Rates per 100 000 population except where indicated

	POPULATION (THOUSANDS)	MORTALITY[a]			PREVALENCE			INCIDENCE			HIV PREVALENCE IN INCIDENT TB CASES (%)		
		BEST[b]	LOW	HIGH	BEST	LOW	HIGH	BEST	LOW	HIGH	BEST	LOW	HIGH
Afghanistan	31 412	38	27	50	352	161	578	189	155	226	–	–	–
Bangladesh	148 692	43	32	57	411	188	671	225	184	269	0.2	0.1	0.3
Brazil	194 946	2.6	1.6	4.3	47	17	80	43	36	51	23	23	23
Cambodia	14 138	61	44	82	660	296	1 070	437	373	506	6.6	6.3	6.8
China	1 341 335	4.1	3.9	4.2	108	93	123	78	68	88	1.7	1.0	2.8
DR Congo	65 966	54	41	69	535	250	850	327	281	376	8.2	6.0	11
Ethiopia	82 950	35	28	42	394	173	623	261	240	282	–	–	–
India[c]	1 224 614	26	17	39	256	161	373	185	167	205	5.0	3.3	7.1
Indonesia	239 871	27	18	38	289	123	484	189	155	226	4.0	2.3	6.4
Kenya	40 513	17	12	23	283	122	448	298	286	311	41	37	45
Mozambique	23 391	49	30	74	491	233	844	544	374	746	61	60	61
Myanmar	47 963	41	24	65	525	381	643	384	328	445	20	10	30
Nigeria	158 423	21	7.2	43	199	70	438	133	63	228	25	24	25
Pakistan	173 593	34	22	49	364	154	611	231	189	277	0.3	0.2	0.5
Philippines	93 261	33	22	46	502	438	566	275	226	329	0.4	0.2	0.7
Russiian Federation	142 958	18	11	29	136	49	233	106	90	124	5.3	5.2	5.4
South Africa	50 133	50	31	75	795	364	1 260	981	806	1 170	60	60	61
Thailand	69 122	16	10	23	182	80	300	137	112	163	16	16	17
Uganda	33 425	15	10	22	193	95	306	209	168	254	54	53	55
UR Tanzania	44 841	13	11	15	183	87	281	177	166	189	38	38	39
Viet Nam	87 848	34	21	49	334	147	576	199	152	253	4.3	2.9	6.2
Zimbabwe	12 571	27	17	40	402	185	639	633	486	799	75	75	76
High-burden countries	**4 321 967**	**20**	**17**	**23**	**231**	**196**	**268**	**166**	**158**	**174**	**12**	**11**	**14**
AFR	836 970	30	26	34	332	277	392	276	256	296	39	35	44
AMR	933 447	2.2	1.9	2.5	36	28	44	29	27	30	13	12	15
EMR	596 747	16	12	20	173	112	246	109	97	122	2.2	1.7	2.8
EUR	896 480	6.8	5.4	8.3	63	47	80	47	44	50	5.0	4.4	5.5
SEAR	1 807 594	27	21	35	278	206	360	193	179	207	5.4	4.1	6.9
WPR	1 798 335	7.5	6.6	8.5	139	124	156	93	85	102	2.1	1.5	2.7
Global	**6 869 573**	**15**	**13**	**18**	**178**	**156**	**201**	**128**	**123**	**133**	**13**	**12**	**14**

– indicates no estimate available.
[a] Mortality excludes deaths among HIV-positive TB cases. Deaths among HIV-positive TB cases are classified as HIV deaths according to ICD–10.
[b] Best, low and high indicate the point estimate and lower and upper bounds of the 95% uncertainty interval.
[c] Estimates for India have not yet been officially approved by the Ministry of Health & Family Welfare, Government of India and should therefore be considered provisional.

2.1.1.7) TB control

WHO launched the DOTS (Directly Observed Therapy Shortcourse) TB treatment strategy in 1995. This was based on five principle components: political commitment; case detection in self-reporting patients with prolonged cough, using sputum smear microscopy; standardised short-course chemotherapy, including directly-observed treatment; regular drug supply; and standardised data recording and reporting (World Health Organisation 1999b). The strategy was updated in 2006 as part of the Stop TB Strategy, and now includes the following six components:

1. Pursuing high-quality DOTS expansion and enhancement: making treatment available to all who require it, with a focus on the 22 high-burden countries which account for 80% of the world's TB cases

2. Addressing TB/HIV, MDR-TB and other challenges: recognition that this will require greater action than the DOTS approach

3. Contributing to health system strengthening: in order to advance financing, planning, management, and supply systems to facilitate systems scale-up

4. Engaging all care providers: including public, private, corporate and voluntary health care providers

5. Empowering people with TB, and communities: mobilising civil society to undertake TB control activities

6. Enabling and promoting research: developing new tools for diagnosis, treatment and prophylaxis (World Health Organisation 2011b).

In 2010, 5.7 million TB cases were notified through DOTS programmes, with 46 million people having been successfully treated and up to 6.8 million lives saved through DOTS and the Stop TB strategy (World Health Organisation 2011b).

The Stop TB Strategy is in collaboration with the Target 6C of the United Nation's Millennium Development Goals, which aims to 'have halted by 2015 and begun to reverse the incidence of malaria and other major diseases'. The target set for TB case detection was 71% (World Health Organisation 2010b).

2.1.1.8) HIV and TB

In 2009, approximately 33.3 million people were living with HIV/AIDS globally, with 1.8 million new infections occurring in this year (UNAIDS 2010).

Two-thirds of the world's infected people live in Sub-Saharan Africa. While the epidemic has begun to stabilise or decline in some countries, in other regions, infections are rising and disproportionately affecting injecting-drug users, sex workers and men who have sex with men (World Health Organisation 2009c). Mortality from HIV has been steadily decreasing, from a peak of an estimated 2.2 million in the mid-2000s, to an estimated 1.8 million in 2010, due to increased access to antiretroviral drugs in developing and middle-income countries (UNAIDS 2011).

TB is a leading cause of morbidity and mortality for people living with HIV/AIDS (PLWHA). There were an estimated 380,000 deaths from TB in HIV-infected people in 2010. HIV-TB co-infection in sub-Saharan Africa can be up to 80% in some countries (UNAIDS 2010). Of the 1.4 million TB-associated deaths in 2010, 350,000 occurred in PLWHA (World Health Organisation 2011b). It is essential to develop and maintain collaborative HIV/TB activities to ensure that patients who are co-infected with TB and HIV are identified and treated appropriately (World Health Organisation 2008b). The Three I's strategy aims to integrate prevention and treatment of TB in people living with HIV, thereby to decrease the impact of TB on HIV-positive people. The strategy focuses on isoniazid preventative treatment, intensified case-finding for active TB, and infection control to protect vulnerable patients, health care workers and the community from TB infection (World Health Organisation 2008c).

2.1.2) Diagnosis of TB

The diagnosis of TB is not straightforward. Various methods are available, ranging widely in technical complexity and cost. The cheaper, technically easier

methods in use in most high-burden countries have low sensitivity, particularly in paediatric and immune-compromised patients, and they cannot detect drug resistance. More sensitive methods are increasingly becoming available but due to cost and technical complexity tend to be limited to district or reference laboratory level.

2.1.2.1) Direct microscopy

The DOTS strategy relies on Ziehl-Neelsen-stained sputum smear microscopy to detect TB infection (World Health Organisation 2009b). In spite of innovations in molecular testing such as the Xpert® MTB/RIF assay (Cepheid, USA) (Boehme, Nabeta et al. 2010), and of preferential pricing for high-burden, low and middle-income countries (Foundation for Innovative New Diagnostics 2011), microscopy is likely to remain the cornerstone of TB diagnosis in most high-burden countries due to the Xpert® MTB/RIF assay's high costs, requirements for electricity and computer literacy (Trebucq, Enarson et al. 2011). HIV-TB co-infected patients are less likely to be found smear-microscopy positive than HIV-negative TB patients (Elliott, Halwiindi et al. 1995). Patients with smear-negative TB can have poor treatment outcomes (Hargreaves, Kadzakumanja et al. 2001). Smear microscopy has been found to have sensitivities ranging from 28% to 70% when compared to the culture reference-standard (Kanaujia, Lam et al. 2005; Matee, Mtei et al. 2008; Cattamanchi, Dowdy et al. 2009b; Lawn, Brooks et al. 2011a). Fluorescent staining and examination can improve the sensitivity of microscopy by 8%, but false positive results are more likely when compared with conventional microscopy (Cattamanchi, Davis et al. 2009a). Fluorescent microscopy (FM) has also been found to be more cost-efficient and give faster results than conventional

microscopy, particularly as less time is required to view the smear when using FM (Kivihya-Ndugga, van Cleeff et al. 2003). A multicentric cross-sectional study found FM to have greater sensitivity but lower specificity compared to Ziehl-Neelsen microscopy, and recommended that FM implementation be accompanied by thorough training and ongoing monitoring (Cuevas, Al-Sonboli et al. 2011).

The traditional sample collection strategy for microscopy is spot-morning-spot, i.e., the first sample is collected at the time of the patient's first visit to the health facility, with the second being expectorated at home the next morning, and the third being collected when the patient delivers the morning sample. In 2007, WHO recommended switching to a strategy whereby only two samples are collected and examined, in settings with a high microscopy workload and where quality control showed that smear microscopy quality was acceptable (World Health Organisation 2007b). The WHO went on to recommend front-loaded microscopy in 2010, whereby instead of a spot and a morning sample, two spot samples are collected on the day of the patient's initial visit to the health facility (World Health Organisation 2010d). This is based on evidence from a multi-country non-inferiority cluster randomised trial which showed that the sensitivity of the same-day collection approach was non-inferior to the spot-morning approach (Cuevas, Yassin et al. 2011).

Microscopy also provides information regarding infectiousness, with smear-positive patients considered to be more infectious. This information can be used for inpatient segregation and targeted contact tracing of patient contacts. The Xpert® MTB/RIF assay, if used as a first-line test as has been recommended by WHO for certain contexts (World Health Organisation 2011a), prevents this information from being obtained. The cycle threshold data which is generated

by the Xpert® MTB/RIF assay has been shown to offer a proxy indicator of smear-positivity, as the cycle threshold value is inversely proportional to smear-positivity – i.e., the cycle threshold decreases as smear-positivity increases. A cut-off of a cycle threshold of >31.8 is 80% sensitive as a rule-out for smear-positivity (Theron, Pinto et al. 2011).

2.1.2.2) Sputum concentration

Sputum processing and concentration can increase incremental yield and sensitivity of microscopy. A Kenyan study saw an increase of 18.6% in smear positivity when using overnight sedimentation following bleach homogenisation to concentrate sputum (Bonnet, Ramsay et al. 2008), and incremental yields or sensitivities, depending on study design, have been reported in the range of 7-253% (Angeby, Hoffner et al. 2004). Lack of consistency in methodology and results between studies means that this technique has not been adopted widely (Steingart, Ramsay et al. 2007). The use of overnight bleach sedimentation may improve infection control, as one study showed it was not possible to culture *M. tuberculosis* from positive samples after 15 minutes of exposure to bleach (Yassin, Cuevas et al. 2003) (Githui, Matu et al. 2007).

2.1.2.3) Concentration of mycobacteria using magnetic beads

Sputum samples are traditionally concentrated using centrifugation, and the optimal conditions for centrifugation have been described as being 3,200 x *g* for 22 minutes (den Hertog, Klatser et al. 2009). Most benchtop centrifuges which are routinely used in laboratories in resource-limited settings are not capable of

achieving this speed, and as such will not thoroughly sediment the mycobacteria present in the sample.

Due to the potential for generation of aerosols, it is recommended that samples which have been centrifuged are opened inside class I or II biosafety cabinets (Jensen, Lambert et al. 2005). Standard TB microscopy laboratories in resource-limited settings rely on natural ventilation for infection control, and as such do not routinely use biosafety cabinets (Rieder, Van Deun et al. 2007).

Paramagnetic beads, to which are attached synthetic ligands specific for mycobacteria, could be used in place of centrifugation for the concentration of mycobacteria. Polyvinyl-alcohol-based magnetic beads have been used in various nucleic acid separation applications, as a method to concentrate nucleic acid prior to amplification (Oster, Parker et al. 2001). The Nordiag Arrow BUGS n BEADS assay (Nordiag A.S.A., Oslo, Norway) uses magnetic bacteria-binding beads to extract a range of bacteria, including mycobacteria, from both direct clinical samples and primary culture (Nordiag 2011). The system can isolate both the bacteria and the bacterial DNA on the same magnetic particle surface. The system has been used on heat-inactivated sputum samples, and in a blind study of 58 samples, 100% were correctly determined and confirmed by a TB reference laboratory (Genpoint 2002). Another study using the system directly on clinical sputum and non-respiratory samples found that 46/61 (75.4%) of culture-positive samples were detected (Lysen, Hoidal et al. 2007).

Microsens Med:Tech has developed a magnetic bead-based system for use with sputum smear microscopy. Their TB Beads system uses poly diallyl dimethyl

ammonium chloride (pDADMAC), a chemical ligand which has a hydrophobic character with which to capture mycobacteria-like organisms by hydrophobic interaction, and a polar character, which allows the ligand to bind to an acidic surface. The ligand in this case is bound to a magnetic bead (Microsens Med:Tech 2008).

The procedure involves:

- thinning and decontaminating the sputum sample with sodium hydroxide

- extraction of the TB through incubation with TB Beads

- capture of the beads with a magnet

- elution of the mycobacteria from the beads

- preparation of the smears from the remaining supernatant (Microsens Med:Tech 2010; Microsens Med:Tech 2011).

The TB Beads system was evaluated on a blind panel of 130 sputum samples from the WHO sputum bank, and results were compared to microscopy results after concentration by centrifugation. The system showed a 96.1% correlation with centrifugation concentration, and a specificity of 96.7% (29/30). Sensitivity compared to culture was 89.4% (76/85) and the technique was 77.8% (77/99) sensitive compared to clinical diagnosis. Bead-concentrated samples had a higher microscopy score than those concentrated by centrifugation, making samples easier to read by microscopy (Wilson, Lane et al. 2010).

The TB Beads test was field-trialled in Uganda, where it was performed on spontaneously-expectorated sputum samples and compared with direct ZN microscopy, direct fluorescent microscopy, centrifugation fluorescent microscopy and culture on MGIT and LJ. Centrifugation fluorescent microscopy

had significantly higher sensitivity (74.8%) than TB Beads fluorescent microscopy (65.4%). The sensitivity of TB Beads fluorescent microscopy was significantly higher than that for direct ZN microscopy (51.4%), but not significantly higher than direct fluorescent microscopy (58.9%). The specificity of both TB Beads (88.6%) and centrifugation microscopy (94.3%) were lower than for direct ZN (98.9%). The authors concluded that the technique could be useful if the sensitivity were increased, but that in its current form, it was not yet suitable for implementation in resource-limited settings (Albert, Ademun et al. 2011).

2.1.2.4) Sample collection and quality

Microscopy centres on the production of 2 or 3 good sputum samples, but this can be problematic. An Indonesian survey (Sakundarno, Nurjazuli et al. 2009) of TB suspects who had undergone TB diagnosis found that, of those interviewed, 74.6% submitted 3 samples, 33.1% gave at least one good sample, and only 13.6% provided 3 good quality samples. 61.8% remembered that information on the reason for sputum examination had been provided, 24.3% recalled having been told how to produce good sputum, and 14.7% remembered being told characteristics of good-quality sputum. Results may have been influenced by recall bias, as up to a six-month gap between instruction and survey existed, and health care worker (HCW) behaviour may have been changed due to use of modified patient data forms. However, that only 13.6% provided 3 good-quality samples indicates that routine microscopy sensitivity can be impaired.

Sample quality has been analysed with regard to gender. In a Kenyan study (Ramsay, Bonnet et al. 2009), while there was no difference in the number of

samples submitted, significantly more men than women (63.9% vs. 53.7%) produced 3 good-quality sputum samples, and significantly more men were found to be smear-positive. It was found that lowering the threshold to include scanty positives resulted in significantly more female cases being detected.

A randomised controlled trial in Pakistan (Khan, Dar et al. 2007) found that improved patient instruction had a greater effect on sample quality on women vs. men, as well as leading to better sample quality and increased numbers returning with the 2nd sample (88% vs. 84%).

2.1.2.5) Sputum collection techniques

Sputum induction and gastric lavage have been used on children, who have been reported as having problems expectorating sputum. Both techniques are well-tolerated and improve case detection in children suspected of active TB (Iriso, Mudido et al. 2005; Zar, Hanslo et al. 2005). Sputum induction (SI) is also useful in adults who are smear-negative or have trouble expectorating sputum, and compares favourably with the more invasive bronchoalveolar lavage (BAL) technique: 97% of BAL and SI results for smear microscopy and culture were in agreement in a Brazilian study (Conde, Soares et al. 2000), and a second study in the same setting showed that culturing induced sputum had a sensitivity of 52% when compared to a range of diagnostic options, and performed similarly in HIV positive and negative suspects (Conde, Loivos et al. 2003).

2.1.2.5.1) Other applications for sputum induction

Sputum induction using hypertonic saline has been used to investigate airways inflammation in asthma and chronic obstructive pulmonary disease, by measuring sputum eosinophilia (Pin, Gibson et al. 1992; Brightling, Monteiro et

al. 2000; Green, Brightling et al. 2002). It has also been used for chronic cough investigation, specifically for the diagnosis of bronchitis (Brightling, Ward et al. 1999). Sputum induction has become a common diagnostic technique for *Pneumocystis jiroveci* pneumonia in HIV-positive patients (Metersky, Aslenzadeh et al. 1998), the diagnosis of lung cancer (Neumann, Meyer et al. 2009), and has also been used for the monitoring of cystic fibrosis (Henig, Tonelli et al. 2001).

2.1.2.6) Culture

In developed countries, it is standard practice to culture body fluids and tissues suspected of containing mycobacteria. Culture is the most sensitive and specific diagnostic technique in use, with an average sensitivity of 80-85%, and a specificity of 98% (ATS 2000). In 2007, WHO recommended the implementation of liquid culture in low and medium-income settings, provided correct infection control procedures were in place and adequate training had taken place (World Health Organisation 2007a). Novel techniques such as the Microscopic-Observation Drug-Susceptibility assay (MODS) and the Thin Layer Agar assay (TLA) combine microscopy with liquid and solid culture media, respectively, to speed up traditional culture techniques. A MODS assay study in Peru reported 97.8% sensitivity of detection compared to the reference standard of culture on Löwenstein-Jensen (LJ) medium (Moore, Evans et al. 2006). A TLA assay study in Colombia reported a sensitivity of 83% when compared with culture on LJ medium (Mejia, Castrillon et al. 1999). Both techniques rely on the microscopic identification of TB micro-colonies, which are invisible to the naked eye. TLA and MODS both have applications for concurrent diagnosis and drug sensitivity testing.

2.1.2.7) Phage-based techniques

Bacteriophage-based technology has been investigated as a means of indirectly diagnosing TB infection and rifampicin drug-resistance, with a reported sensitivity of 75.2% compared to culture, and a specificity of 98.7%, for TB diagnosis (Albert, Heydenrych et al. 2002). However, in another study using the Fastplaque Response test for detection of rifampicin resistance, high contamination rates meant only 70.6% percent of results were interpretable, compared with 86.3% of conventional DST results (Albert, Trollip et al. 2007).

2.1.2.8) Molecular techniques

TB can be diagnosed using nucleic acid amplification. Meta-analyses have found that this method is insufficiently accurate for routine diagnostic use (Sarmiento, Weigle et al. 2003), with sensitivity ranging from 9.4 to 100%, and specificity from 5.6 to 100% when using in-house tests (Flores, Pai et al. 2005). A commercial assay, the Becton Dickinson Probe Tec ET system, has been validated and sensitivities range from 56.7% to 97.1%, with specificities of 95.3% to 98.4% (Barrett, Magee et al. 2002; Wang, Lee et al. 2004; Wang, Lee et al. 2006). The Gen-Probe Amplified Mycobacterium Direct Test was evaluated in Zambia against FM, in-house PCR and culture, and was found to have the best sensitivity of all techniques when compared to a reference standard incorporating both microbiological and clinical data. However, the high cost of the technique limited its routine implementation in similar settings (Kambashi, Mbulo et al. 2001).

The Xpert® MTB/RIF assay (Cepheid, USA) is a fully-automated real-time PCR assay which allows for the simultaneous detection of *M. tuberculosis* complex and rifampicin resistance from a homogenised sputum sample (Helb, Jones et al.

2010). A multicentric trial found a single assay to have 98.2% sensitivity for *M. tuberculosis* complex detection in smear-positive, culture-positive samples. Sensitivity in smear-negative, culture-positive samples was 72.5%, which was increased by 12.6% and 5.1% by a second and third assay, respectively, to a total of 90.2% sensitivity based on 3 samples. Specificity was 99.2% on patients who did not have TB infection. The sensitivity for detection of rifampicin resistance was 97.6% (Boehme, Nabeta et al. 2010). A multicentric implementation study confirmed the findings, with 90.3% sensitivity for a single sample, and 76.9% sensitivity in smear-negative, culture-positive samples. Specificity was 99%. Performance was unaffected by HIV status, unlike microscopy. Sensitivity for detection of rifampicin resistance was 94.4%, with a specificity of 98.3% (Boehme, Nicol et al. 2011). However, while a South African study conducted on HIV-positive patients found a sensitivity for TB detection of 100% for smear- and culture-positive patients on a single test, the sensitivity was lower than expected in smear-negative patients, at 43.4% on one test, and 62.3% on two tests. False-positive results were also detected for rifampicin resistance, with a PPV of 57% (Lawn, Brooks et al. 2011a). Another South African study evaluated the assay using paediatric induced sputum samples, and recommended the processing of two induced sputum samples per patient to maximise sensitivity (Nicol, Workman et al. 2011).

The assay has also been evaluated for use with EP samples, with promising results for stool and urine, but lower sensitivity for tissue samples (Hillemann, Rusch-Gerdes et al. 2011).

2.1.2.9) Skin testing

Immunological tests have been used to diagnose latent and active TB. The tuberculin skin test (TST) was first reported in 1921 (Zinsser 1921) and is still recommended by the WHO for the diagnosis of paediatric TB (World Health Organisation 2006d). Commercially-available interferon-gamma release assays (IGRAs) can be used in place of TST, although they are more technically complex to perform, and have not been found to be more sensitive than the TST (Bamford, Crook et al. 2009). In 2011, the World Health Organisation issued a policy statement on IGRAs, which recommended that IGRAs should not replace TST in resource-limited settings, due to the lack of quality data on their performance in these settings, along with their cost and technical complexity (World Health Organisation 2011g).

2.1.2.10) Serology-based tests

While some antigen candidates have been identified for use in serology-based rapid diagnostic tests (RDT), none has been found to be sensitive enough to replace smear microscopy (Steingart, Dendukuri et al. 2009). The WHO Special Programme for Research and Training in Tropical Diseases (TDR) evaluated 19 commercially-available RDTs, and reported a sensitivity range from 0.9% to 59%, with a specificity range from 53% to 98.7%. The tests performed particularly poorly in HIV-positive people. While none of the RDTs performed well enough to replace sputum smear microscopy, used in combination with microscopy they could significantly increase the detection rate, but with an unacceptable rate of false positive results (World Health Organisation 2008a). In 2011, the World Health Organisation issued its first negative recommendation on these tests, with a policy statement strongly recommending that these tests

not be used for the diagnosis of pulmonary and EPTB (World Health Organisation 2011e).

2.1.2.11) Lipoarabinomannan detection

Lipoarabinomannan (LAM) is a component of the *M. tuberculosis* cell wall that can be detected in the urine of patients with pulmonary and EP TB. The antigen can be detected using an ELISA platform and has recently been developed as a lateral flow immunochromatographic test. This test was found to compare well with the ELISA version. The sensitivity was higher in HIV-positive patients with low CD4 counts. The assay was found to significantly increase the incremental yield when used in combination with smear microscopy, and has been recommended for use in this manner on HIV-positive patients with very low CD4 counts (Lawn, Kerkhoff et al. 2011b).

2.1.2.12) Smear-negative diagnosis

Smear-negative diagnosis, in the absence of culture or nucleic acid-testing facilities, is commonly managed using clinical algorithms. WHO updated their guidelines for the management of smear-negative pulmonary and - EPTB in adolescents and adults in 2006, with an emphasis on HIV-prevalent and resource-limited settings. Smear-negative TB is characterised as follows: at least two sputum samples negative for acid-fast bacilli, **and** radiological abnormalities consistent with active TB, **and** laboratory confirmation of HIV infection **or** strong clinical evidence of HIV infection, **and** decision by a clinician to treat with a full course of chemotherapy; **or** a patient with smear-negative sputum which is culture-positive for *M. tuberculosis* (World Health Organisation 2006b). In 2010, there were an estimated 2.0 million cases of smear-negative TB

among new cases, compared with 2.6 million cases of smear-positive TB and 0.8 million cases of EPTB. 57% of new pulmonary TB cases were smear-positive (World Health Organisation 2010c).

2.2) Study rationale

2.2.1) Introduction

This study aimed to investigate a method for increasing the sensitivity of sputum smear microscopy for the diagnosis of pulmonary TB in a population with a high prevalence of HIV. Combining sputum induction with a novel, simple technique for the concentration of mycobacteria (the TB Beads system) allowed the microscopic detection of TB in patients who were smear-negative by conventional smear microscopy. The simple concentration method does not require the use of a centrifuge or a biosafety cabinet, and is therefore appropriate for microscopy laboratories in district-level settings. This allows sputum induction to be combined with microscopy and may increase the sensitivity of TB diagnosis in the periphery.

2.2.2) Importance of improving TB diagnosis in resource-limited settings

The DOTS strategy is the most common TB control strategy in use, and has mainly been implemented in the 22 high-burden countries. Twenty of these countries are in Asia and Sub-Saharan Africa, the others being Brazil and the Russian Federation (World Health Organisation 2011d). Diagnosis in these settings relies on sputum smear microscopy, with the aim of identifying the most infectious TB patients, as being smear-positive implies that infectious *M. tuberculosis* are being expectorated and the coughing patient is therefore a public health risk (Rieder 2002). However, this approach, while working to reduce

incidence of infection, only diagnoses 61.8% to 70% of active pulmonary TB diseases when compared to sputum culture (Kanaujia, Lam et al. 2005; Matee, Mtei et al. 2008; Cattamanchi, Dowdy et al. 2009b).

Sputum culture, the reference standard for TB diagnosis, is not performed in a comprehensive manner across the high-burden countries due to the complexity and expense of implementation. Although WHO recommends the implementation of culture in these settings, it states that culture (particularly, liquid culture) should be implemented only after a thorough plan has been prepared which involves infrastructure requirements, training, infection control, and financing (World Health Organisation 2007a). While organisations such as the Global Laboratory Initiative provide support for capacity building in national TB control programmes (World Health Organisation 2010a), current TB culture services are still inadequate to support all TB suspects in the high-burden countries (World Health Organisation 2011c).

Smear microscopy relies on a good sputum sample and high bacillary load to be sensitive (Getahun, Harrington et al. 2007). TB patients with HIV co-infection tend to have a lower bacillary load and less sputum expectoration (Hargreaves, Kadzakumanja et al. 2001), therefore making smear microscopy insensitive. Therefore, relying on sputum smear microscopy disproportionately disadvantages HIV-positive patients.

Sputum induction (figure 3) has been shown to increase sensitivity of microscopy and culture in HIV-positive pulmonary TB patients (Conde, Soares et al. 2000; Conde, Loivos et al. 2003), but the induced sputum is usually processed prior to examination for maximum sensitivity. This is traditionally done by centrifugation at 3,000 x g for 20 minutes, although a recent study

suggests this should be increased to 3,200 x g for 22 minutes (den Hertog, Klatser et al. 2009). The centrifugation process generates infectious microaerosols, meaning centrifuged tubes must be manipulated inside a biosafety cabinet (Centres for Disease Control and Prevention 1999).
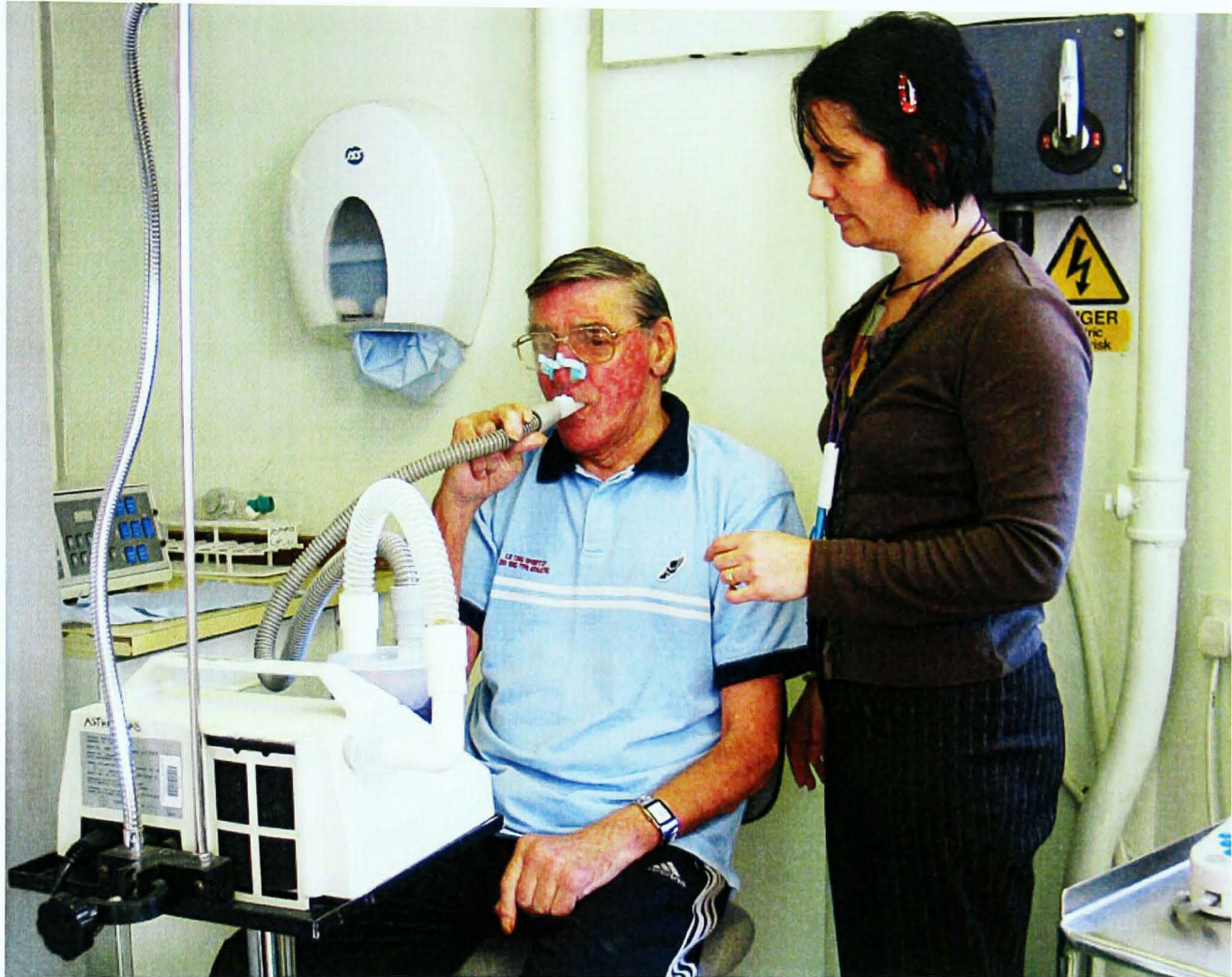
Figure 3: Patient undergoing sputum induction.

Source: http://www1.imperial.ac.uk/nhli/respiratory/airdisease/clinicalres/. Accessed 01.12.2011

Current guidelines state that routine sputum smear microscopy can be done without a biosafety cabinet, using natural ventilation (Rieder, Van Deun et al. 2007). Therefore routine TB microscopy laboratories cannot process induced sputum, as they lack not only the biosafety cabinets, but also the powerful centrifuges necessary to sediment *M. tuberculosis* from induced sputum. This implies that the sputum induction technique cannot be optimally utilised in

district-level settings, and therefore that patients who are unable to produce sputum spontaneously are being under-diagnosed for active TB. A technique which allowed induced sputum to be processed without requiring centrifugation and use of a biosafety cabinet would allow sputum induction to be used in the periphery and would potentially allow for the diagnosis of active pulmonary TB in those patients who would be missed by routine smear microscopy.

The TB Beads system was chosen because it is a platform which has been specifically developed for use in resource-limited settings. It has been designed for use outside of biosafety cabinets, and does not require electricity, or intensive training, to be performed, and as such is appropriate for use in TB microscopy laboratories in high-burden countries. The system is commercially available and was purchased by MSF. The manufacturer had no involvement in the study and was not involved in analysis or the dissemination of information following the study.

2.3) Background on setting

2.3.1) Médecins Sans Frontières in Zimbabwe
Médecins Sans Frontières (MSF) has been working in Zimbabwe since 2000, and has focused on comprehensive HIV care and response to emergencies, such as the 2008/9 cholera outbreak. HIV care includes provision of antiretroviral therapy, treatment of TB, and therapeutic feeding of malnourished HIV-positive children, with or without TB co-infection. MSF's main aim in Zimbabwe is to reduce the human suffering caused by the HIV/AIDS epidemic, through the model of decentralised treatment, in order to increase accessibility of patients to

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the health care infrastructure, as well as by advocating for other international actors to contribute to the reduction of suffering (Medecins Sans Frontieres 2009).

The study site was MSF Operational Centre Amsterdam's Epworth project, located on the outskirts of Harare, in Mashonaland East province. As of August 2011, MSF was providing comprehensive care in Epworth to 12,864 people living with HIV/AIDS, of which 9,306 were receiving anti-retroviral treatment at end June 2011. At this time the project was also providing TB treatment for 623 patients (Medecins Sans Frontieres 2011a).

The Zimbabwe location was chosen to be a study site for the following reasons:

- The mainly HIV-positive population of TB suspects produce poor-quality sputum samples, and efforts had been made to improve sample quality in the past

- It was estimated that the monthly number of suspects was sufficient to allow completion of data collection within 3 months: the laboratory tested on average 171 patients per month, with an average 18.2% positivity rate

- 88.8% of patients provide all samples requested (MSF OCA Zimbabwe 2010)

- The local staff is highly-educated and well-trained

- The country management team and headquarters-based advisors were willing to support operational research which would have a direct benefit for TB patients.

2.3.2) Zimbabwe

Zimbabwe is located in southern Africa, and borders Botswana to the south-west, Zambia to the north-west, Mozambique to the east, and South Africa to the south. The main languages spoken among its mainly Christian population of 13.5 million are English, Shona and Ndebele. Although it once had well-developed infrastructure and financial systems, the economy has declined severely due to poor management by the government, and Gross Domestic Product (GDP) has declined by 50% since 1998 (United Kingdom Foreign & Commonwealth Office 2011). However, the introduction of the US dollar has improved the GDP in recent years and the projected annual percentage change for 2011 is 4.5%, compared with -3.7% in 2006. No specific data is available on unemployment rate (International Monetary Fund 2010). However, unemployment is estimated at 94% (United Kingdom Foreign & Commonwealth Office 2011). Life expectancy in 2011 was 49.9 years for males and 43.3 years for women, which are among the lowest life expectancy rates in the world (IndexMundi 2011). The previously strong agricultural industry, which had dominated the economy, has been severely disrupted by land resettlement, with productivity falling by more than 50% since 1996 (United Kingdom Foreign & Commonwealth Office 2011). There is no up-to-date information on health expenditure as a proportion of GDP, with the latest data being from 2001, when total expenditure on health was 8.1% of GDP (World Health Organisation 2011f).

2.3.3) TB and HIV in Zimbabwe

Zimbabwe is ranked 17th in the list of 22 high-burden countries with regard to epidemiological burden of TB, and in 2010 had an estimated 80,000 incident

cases (range, 61,000 – 100,000), of which an estimated 60,000 cases occurred in HIV-infected patients (range, 47,000 – 76,000). The incidence per 100,000 was 633 cases (range, 486-799), with an estimated prevalence of 402 per 100,000 (range 185-639), and an estimated TB-related mortality (excluding HIV-infected) of 27 per 100,000 (range, 17-40). An estimated 1.9% (range, 1.0-3.3) of all new cases of TB are multi-drug resistant, as are an estimated 8.3% (range, 1.8-23) of retreatment cases (World Health Organisation 2011c).

Zimbabwe had an estimated 1.3 million people living with HIV and AIDS in 2008, with an estimated adult prevalence of 15.3% (UNAIDS 2008).

A study undertaken in Harare, Zimbabwe's capital, found that HIV positivity, male sex and overcrowding were risk factors for smear-positive TB, with an HIV prevalence of 21.1% among participants (Corbett, Bandason et al. 2009). An earlier study in the same location found that the incidence of culture-positive TB was significantly higher among HIV-positive people compared with HIV-negative people, with HIV-positive people having an incidence rate of 25.3 per 1,000 person-years follow-up, and HIV-negative having an incidence of 1.3 per 1,000 person-years follow-up (Corbett, Bandason et al. 2007). Among primary health care service attendees with chronic cough, Munyati *et al* found that TB was the most common diagnosis, with 46% of HIV-positive and 20% of HIV-negative people having probable or confirmed TB. HIV infection caused no adverse effect on the sensitivity of fluorescent microscopy in this setting, with only 3.7% of HIV-positive, and 2.2% of HIV-negative, patients being smear-negative/culture-positive. The explanation provided for this by the authors was the use of fluorescent, concentrated microscopy read by two microscopists, and the study protocol which required frequent sampling of sputum until TB

disease was confirmed or excluded (Munyati, Dhoba et al. 2005). Active case-finding in Harare using a mobile van approach was found to be more effective compared with a door-to-door case-finding approach (Corbett, Bandason et al. 2010a). Another study found that following up smear-negative TB suspects for one year led to 18.2% of them being found positive on subsequent investigation (Dimairo, MacPherson et al. 2010).

Chapter 3: Research hypothesis and objectives

<u>Study hypothesis</u>: Use of magnetic beads instead of centrifugation will minimise equipment and infrastructure requirements for the processing of induced sputum samples and will make the technique more appropriate for use with microscopy in peripheral settings.

<u>Key objective</u>: Comparison of 2 methods of induced sputum concentration:

a) routine decontamination and centrifugation, and

b) use of magnetic beads, specific for mycobacteria,

to demonstrate performance of the magnetic beads compared to conventional centrifugation.

<u>Secondary objective:</u>

Implementation of sputum induction for microscopy in a peripheral setting, without routine access to culture, to improve detection of pulmonary TB in HIV-positive TB suspects.

Chapter 4: Literature review

A critical review of the literature was undertaken to evaluate the performance of microscopy compared with culture on induced sputum. Where appropriate, other sample collection methods performed alongside sputum induction were also evaluated. This systematic review was published by the International Journal of Tuberculosis and Lung Disease in May 2012. It was published under the title: Microscopy compared to culture for the diagnosis of tuberculosis in induced sputum samples: a systematic review, with Pamela Hepple, Nathan Ford and Ruth McNerney as authors. The complete manuscript is available in annex 5.

Chapter 5: Design and methodology

5.1) Type of study

This prospective cross-sectional study hypothesised that use of magnetic beads instead of centrifugation would minimise equipment and infrastructure requirements for the processing of induced sputum samples and would make the technique more appropriate for use with microscopy in district-level settings. It compared 2 methods of sputum concentration: routine decontamination and centrifugation, and use of magnetic beads, specific for mycobacteria, to demonstrate non-inferiority of the magnetic beads compared to conventional centrifugation. Both techniques were compared against the reference standard for the diagnosis of pulmonary TB: sputum culture on Löwenstein-Jensen (LJ) medium.

A secondary objective was the implementation of sputum induction for microscopy in a district-level setting, without routine access to culture, to improve detection of pulmonary TB.

Sputum induction, induced sputum processing with magnetic beads and examination with fluorescent and light (Ziehl-Neelsen) microscopy were performed in the Epworth Polyclinic. Decontamination, sediment smear fluorescent microscopy and culture on LJ medium were performed at the Biomedical Research and Training Institute (BRTI) in Harare.

The study was set up by the student, who functioned as the primary investigator. The study took place in the Epworth Polyclinic, Mashonaland East, Zimbabwe. Enrolment started in February 2011 and took place over 5 months.

The study was funded entirely by Médecins Sans Frontières Operational Centre Amsterdam.

5.2) Study area

Epworth is a peri-urban settlement on the outskirts of Harare, and is populated by people from all over Zimbabwe, and surrounding countries. Epworth's population is estimated at 350,000, and, at >20%, the estimated HIV prevalence is higher than the national average of 15.3% (UNAIDS 2008). Médecins Sans Frontières has identified this population as vulnerable and provides free healthcare to those inhabitants who are TB and/or HIV-positive. The MSF programme operates within the Ministry of Health and Child Welfare (MoHCW) Epworth Polyclinic, and a close collaboration exists between the two organisations.

Figure 4: Diagnostic algorithm for TB suspects

TB suspect

Direct fluorescent smear microscopy x 2 samples

Negative

Positive

Smear microscopy positive

Ziehl-Neelsen smear microscopy

Smear microscopy negative

AFB confirmed

AFB not confirmed

Chest xray

Suggestive of TB infection

Not suggestive of TB

Treat for TB

Broad-spectrum antibiotics x 7 days

Reassess for TB treatment
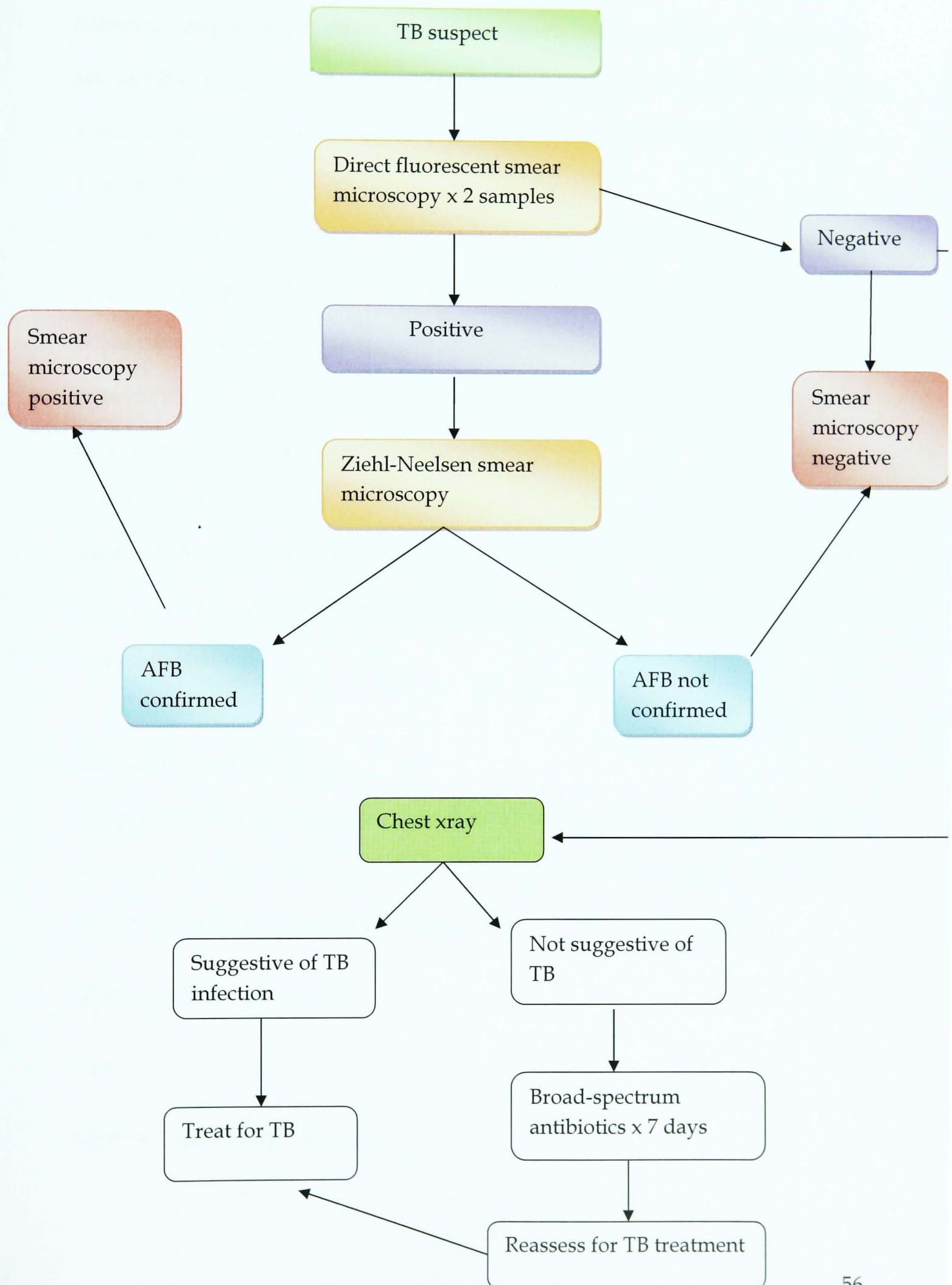
5.3) Patient selection and sample size

The study recruited adult patients attending the clinic who were smear-negative following diagnosis by direct fluorescent smear microscopy on unprocessed sputum (figure 7). At this point, patients were routinely either diagnosed with smear-negative TB, or were diagnosed as negative for pulmonary TB, according to standard algorithms.

To determine the required sample size it was necessary to estimate the proportion of culture positive subjects among the smear negatives recruited into the study. Given the sensitivity and specificity of smears relative to culture and the prevalence of smear negatives in the TB suspect population, this proportion could be calculated by an application of Bayes' theorem (Altman 1991):

$$(1-s)(q-t)/(q(1-s-t))$$

where s is the sensitivity and t the specificity of the direct smear test relative to the culture reference standard, and q is the prevalence of smear negatives in the TB suspect population.

This was derived as follows:

The proportion of interest is the proportion of culture positives among the smear negatives. This is equal to 1-NPV (NPV=Negative Predictive Value). NPV can be related to sensitivity and specificity using Bayes' theorem. The formula is

$$NPV=t(1-pr)/((1-s)pr+t(1-pr))$$

And

$$1-NPV=(1-s)pr/((1-s)pr+t(1-pr))$$

where pr is the prevalence of culture positives, s is sensitivity and t is specificity. Pr is unknown, so the formula is rewritten in terms of q, the prevalence of smear negatives. Pr can be written in terms of q, sensitivity and specificity by application of the law of total probability. Specifically,

$$pr=(t-q)/(s+t-1)$$

Replacing pr in the formula above with this expression for pr we get

$$1-NPV=(1-s)(q-t)/(q(1-s-t)).$$

Using the formula: $(1-s)(q-t)/(q(1-s-t))$,

if s= 0.4 (i.e. the sensitivity of direct microscopy compared to culture is 40% on average for an HIV-positive population),

t= 0.95 (i.e. the specificity of direct microscopy compared to culture is estimated to be 95%) and

q= 0.8 (i.e. 80% of TB suspects are smear-negative on direct microscopy in the clinic), then:

$$(1-0.4)(0.8-0.95)/(0.8*(1-0.4-0.95))=32\%.$$

I.e., 32% of the patients enrolled can be expected to be culture-positive.

Data collection was planned to take place over 3 months. Based on patient flow, proportion of smear-negatives, human and equipment resources, it was anticipated that 5 patients could be enrolled per day. With a 5-day working week, this would have allowed for 100 patients to be enrolled per month, or 300 in total. Of 300

patients, according to the formula above, 300 x 0.32= 96 could have been expected to be culture-positive.

Based on sputum induction studies in contexts similar to Zimbabwe (Parry, Kamoto et al. 1995; Wilson, Nachega et al. 2006; Souza Pinto and Bammann 2007; Morse, Kessler et al. 2008; Bell, Dacombe et al. 2009; Kranzer, Olson et al. 2011), the sensitivity range of centrifuged, induced sputum on microscopy compared to culture is 37.5-66.6%, mean 58%. The estimated sensitivity of beads compared to culture is 25-68%, mean 52%.

Assuming the sensitivity of centrifugation is 0.58 and that 96 culture positive patients had been recruited, the expected 95% confidence interval (CI) is [0.48, 0.68]. Similarly for the beads method, if we assume a sensitivity of 0.52 then the expected 95% CI is [0.42, 0.62]. The centrifugation method is not possible to conduct in most of the resource limited settings that the beads method is intended for, thus if the new method had a sensitivity within the estimated range it would have been considered a viable method to introduce.

5.4) Study procedures

5.4.1) Inclusion criteria
The inclusion criteria were the following:

- Suspected pulmonary TB (requiring sputum examination for diagnosis)

- Not having taken anti-TB drugs in the previous month, regardless of length of treatment

- Being smear-negative on direct fluorescent microscopy (FM)

- Aged 18 years or older.

The exclusion criteria were the following:

- Patient refused to provide consent

- Patient was too sick to undergo sputum induction following medical assessment, or to cooperate with the induction procedure as determined by the treating nurse/physician.

5.4.2) Enrolment procedure

Patients presenting at the clinic with suspected TB produced 2 spontaneous sputum samples, one on the spot and one at home the following morning. A direct (unconcentrated) smear was made from each sample, both smears were stained with Auramine-Rhodamine[1] and 70 fields were examined at x 400 for AFB with the PrimoStar iLED microscope (Carl Zeiss, Germany). Smears were graded following national guidelines (Ministry of Health and Child Welfare of Zimbabwe 2009), and all positive smears were re-stained using the Ziehl-Neelsen method[2] and the presence of AFB was confirmed with bright-field microscopy. If presence of AFB was confirmed, these patients were deemed smear-positive.

Patients who were smear-negative on 2 samples (no AFB seen on fluorescent or light microscopy) were assessed using the clinical diagnostic algorithm for infection with smear-negative pulmonary TB (World Health Organisation 2006b).

Patients who were smear-negative on 2 samples were also invited to participate in the study after receiving their direct microscopy results. A systematic record of refusal to participate was not maintained.

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[1] Merck TB-fluor phenol-free; 2012/07/31, batch 1.01597. 0001 (Merck KGaA, 64271 Darmstadt, Germany)

[2] Carbol fuchsin: Alere Healthcare, Bedfordview, UK. Lot – CCNDC, Ref – LG11, Exp – Oct 11
Decolouriser: Tb decolouriser, BD, Sparks, Maryland, USA, Lot – 0103474, Exp – 2011-04-30
Methylene blue solution 1% aqueous, Avonchem, Cheshire, UK (diluted with distilled water to 0.3%)
Batch no.-9248-2 Exp – August 2011

Those who were interested in participating were referred to the study nurse, who explained the details of the study and collected written informed consent (see annex 4). Those who consented to participate were assessed for clinical suitability for induction by a physician experienced in HIV-TB management. Patients were excluded based on a past history of asthma and/or bilateral wheezing; also excluded were patients who were deemed by the doctor to be unable to undergo the procedure safely due to severe acute cardiorespiratory failure with imminent risk of death. The enrolled patients were then taken to the outdoor sputum collection hut by the nurse and sputum induction was done, using an ultrasonic nebuliser[3] with 6% hypertonic saline[4]. Nursing staff wore protective goggles, gloves, a respirator mask, and a laboratory coat. The physician provided training in the induction procedure and patient monitoring. During routine induction he was available within 2 minutes in order to deal with any complications that could not be handled by nursing staff; in the event, this service was not required. The sputum induction protocol is described in annex 1. Patients expectorated into sterile 50 mL graduated Falcon tubes. No mechanical aspiration was used.

5.5) Laboratory methods

Samples were processed as outlined in figure 8.

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[3] Omron ultrasonic nebuliser, model NE-U17, Omron Matsusaka Co., Ltd., Japan
[4] Mucoclear 6%, Pari Pharma GmbH, Munchen, Germany. Lot: 007, exp: 04/13.

Figure 5: Algorithm for the processing of induced sputum

Induced sputum

Epworth laboratory

BRTI laboratory

TB Beads processing

Decontamination and centrifugation

ZN smear

Fluorescent smear

Fluorescent smear

LJ culture
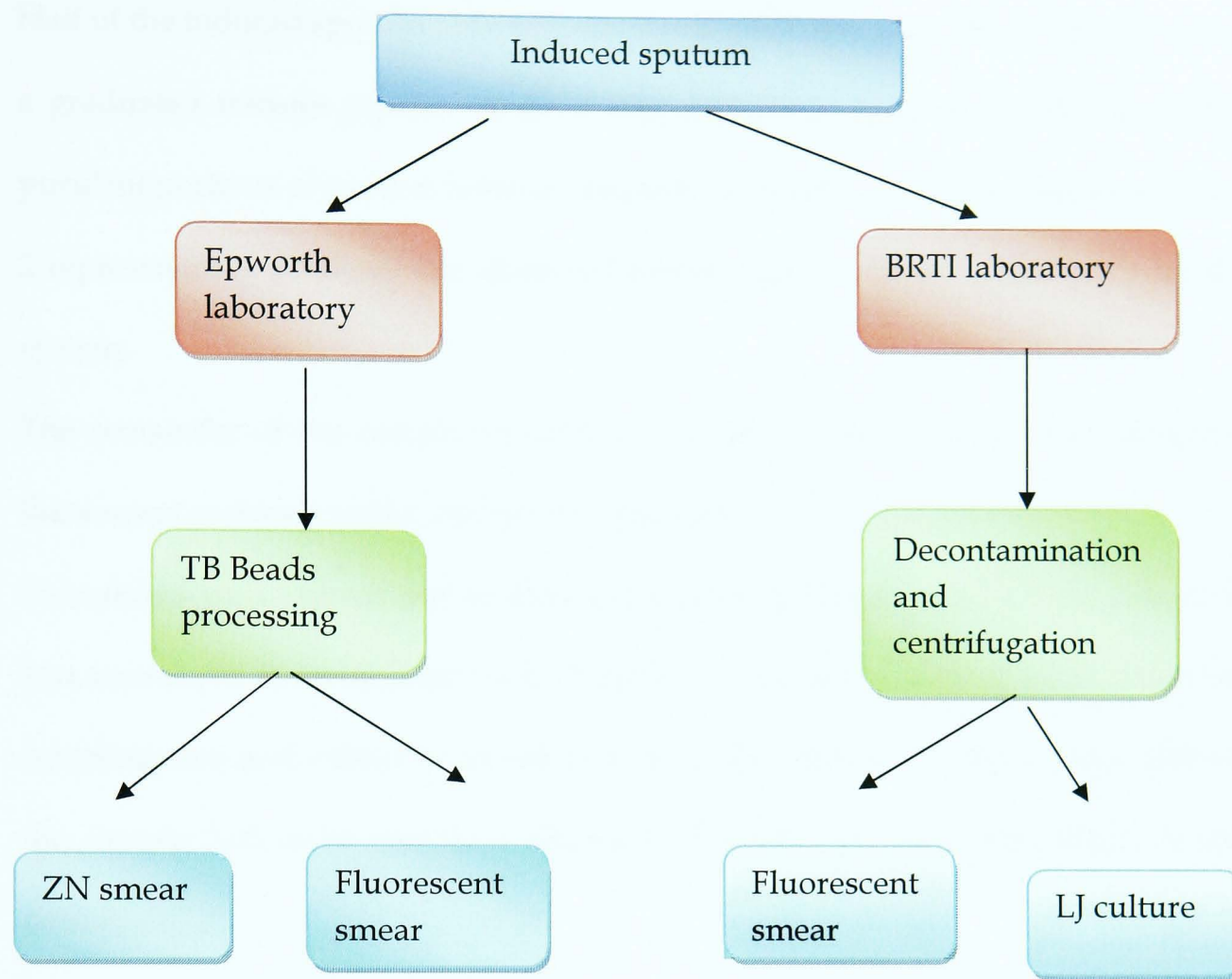
5.5.1) Aliquoting of induced sputum

Half of the induced sputum obtained was transferred into a sputum container using a graduated transfer pipette. While it was difficult at times to evenly distribute purulent portions of sputum between aliquots, we tried as much as possible to have 2 representative portions. The aliquoted sample was processed with the TB Beads system.

The remainder of the sample was sent to the BRTI, Harare, for decontamination, fluorescent sediment smear microscopy and culture on LJ medium.

Procedures were carried out in different laboratories because while the objective was to evaluate the performance of TB Beads in a peripheral setting, the facilities for centrifugation and culture were unavailable in this setting. For this reason, half of the sample had to be sent to a reference laboratory for these procedures to be performed.

5.5.2) Laboratory procedures

The Epworth laboratory standard operating procedures are described in annex 2.

5.5.2.1) Decontamination and centrifugation, fluorescent staining and examination, and culture on Löwenstein-Jensen medium

The remaining half of the induced sputum sample was taken to the BRTI laboratory in Harare. Samples were kept at 4 ^{0}C until they were transported in cold chain to the laboratory. Samples collected on Monday – Thursday were transported within 24 hours of collection, while those collected on Fridays were kept at 4 ^{0}C until the following Monday.

Samples were processed for centrifuged sediment smear fluorescent microscopy (Leica DM 1000, Leitz, Wetzlar, Germany), culture on Löwenstein-Jensen medium, and speciation with an MTP64 antigen test, according to the BRTI protocols in

annex 6. ZN microscopy was not conducted on samples in BRTI as this was not a routine test in BRTI. The principle data comparison was between TB Beads FM and centrifugation FM; therefore, data on TB Beads ZN was not compared with centrifugation ZN.

5.6) Quality control

5 positive Ziehl-Neelsen and 5 negative direct FM slides were randomly chosen and blindly cross-checked every month by a 2nd laboratory (Gweru General Hospital laboratory) as part of the clinic's routine quality control programme (Medecins Sans Frontieres 2008).

Additionally, 10% of sediment slides were re-examined every month, for each of the ZN and FM-stained slide cohorts. They were sent to the same laboratory as above for blind cross-checking.

The BRTI quality control procedures were followed as outlined in annex 3.

5.7) Follow-up of patient results

Patients were given appointments to collect their induction results the following day. Treatment was initiated, following clinical assessment, if bacilli were seen on TB Beads microscopy. Those who were TB Beads smear-negative were given the option to return after 4 weeks to receive their culture results. Patients who were negative on TB Beads smear-microscopy following induction, and on the clinical diagnostic algorithm, and had a centrifugation sediment smear-positive or culture-positive result were actively traced by the community liaison officers (CLO). They were placed on treatment when found, following clinical assessment. Use of CLOs is routine for patients who default from the diagnostic algorithm in the clinic, and tracing was done in a manner which minimised patient stigmatisation.

The ethical implications of using the results of the test under evaluation for guiding patient management were considered. It was believed that FM and ZN microscopy are sufficiently specific that a positive result could be relied upon to be a true positive. Most microscopy-positive patients had a negative culture result following a positive TB Beads or centrifugation sediment microscopy result. This outcome can also result from routine smear microscopy, as *M. tuberculosis* may be non-viable on culture. As the patients were usually already on treatment based on the microscopy result, they were reviewed by the clinician and follow-up decisions were made while taking the culture result into account.

5.8) Staffing

The PI had planned to be on-site for the duration of the field work and was ultimately responsible for the monitoring of the following: patient flow, enrolment, the informed consent procedure, referral of samples to the BRTI laboratory, and data management. The PI performed the TB Beads concentration technique in the clinic laboratory, with back-up from the Epworth laboratory scientists. Trained and experienced scientists covered 2 x 1 week PI absences, and continued the work after her departure. The PI's work permit expired before the sample size was reached and as such the tasks above were delegated to the clinical supervisor and the Epworth laboratory scientists for the final 2 months of enrolment.

The MSF clinical supervisor, who was a medical doctor, was responsible for training of nursing staff in the sputum induction procedure, including contra-indications, and monitoring during and post-induction. He assisted nursing staff in the monitoring of adverse reactions when required.

Three existing staff TB nurses were trained to function as study nurses (2 principal and 1 back-up), and they worked on a one week on/one week off basis. They received training in patient flow, data management, the informed consent procedure, and the induction procedure. S/he was responsible for enrolling patients, obtaining consent, performing the induction procedure, and data collection. S/he collated results and delivered them to the screening TB nurses for further action.

5.9) Specific objectives

The study obtained the following information:

1. Comparison of detection of bacilli on sediment smear microscopy following centrifugation and the TB Beads technique

2. Sensitivity of the TB Beads microscopy method compared to culture

3. Sensitivity of the centrifugation microscopy method compared to culture

4. Specificity of the TB Beads microscopy method compared to culture (i.e. what proportion of smear-positive induced sputum samples grew *M. tuberculosis* on culture)

5. Specificity of the centrifugation microscopy method compared to culture (i.e. what proportion of smear-positive induced sputum samples grew *M. tuberculosis* on culture)

6. Percentage of patients experiencing adverse reactions from the induction procedure, including details of symptoms.

5.10) Patient information

Every enrolled patient had a case report form, detailing the following:

- Age

- Sex

- HIV status (if known)

- Informed consent given

- Any adverse reactions during induction

- Any adverse reactions following induction

- Induction procedure completed

- Results of TB Beads microscopy

- Results of centrifugation microscopy

- Results of LJ culture (including speciation)

HIV status is routinely collected during the TB diagnosis procedure by the TB nurse, and this information was acquired from the patient records. If this information was not available in the patient records, the patient was asked to disclose his status, if known.

Enquiring about a person's HIV status can be difficult and this was done with context-specific sensitivity. However, as the polyclinic is a known HIV treatment centre, this was not usually problematic.

Enrolled patients were assigned unique codes. This code was the identification method for laboratory results. A register in which the patient name and code were linked (for feedback of results) was kept in the TB screening room, which was kept locked when a study nurse was not present. Only the study nurse and the PI had access to this information.

Once available, the study nurse added the results of the TB Beads microscopy to the patient's original TB microscopy request form, and this was returned to the screening TB nurse.

The TB request forms of the TB Beads-negative patients were retained until culture and centrifugation results were available, at which point these were added to the request form.

Data entry in the case report forms was done by the study nurse and the PI. The forms were kept in the TB screening room, under the conditions described above.

5.11) Statistical analysis

Data was double-entered using Microsoft ®Access® 2007 (©2006 Microsoft Corporation). The two databases were cross-checked to detect recording errors. Once the databases were deemed to be clean, they were locked and signed for by the PI. They were analysed using Microsoft® Excel® 2007 software (©2006 Microsoft Corporation) and STATA software (StataCorp. 2007. *Stata Statistical Software: Release 10*. College Station, TX: StataCorp LP).

5.12) Local engagement

Before the study commenced, ethical approval was granted by the Ethical Review Boards of the Medical Research Council of Zimbabwe, and the Biomedical Research and Training Institute, Harare. The study was discussed with the local medical team, and posters were prepared which explained the study to the patients. These were displayed prominently in the polyclinic.

After the study, plans were made for the findings to be shared in a meeting with local key health professionals, including the participating health care workers. This involves an open forum in which the findings are open for critique and a discussion held regarding the feasibility and relevance of the methods for implementation in Zimbabwe.

5.13) Ethics and informed consent

Ethical approval for this study was granted by the Ethical Review Boards of the Biomedical Research and Training Institute, Harare, the Medical Research Council of Zimbabwe, the London School of Hygiene and Tropical Medicine, and Médecins Sans Frontières.

Informed consent was acquired from all participants prior to the sputum induction procedure. Upon being found smear-negative by conventional microscopy, patients 18 years or older were invited to participate in the study by the study nurse, who had received training from the PI in obtaining informed consent, and who also participated in Ethics training provided by MRCZ. Patients had the principles of the study explained to them verbally in Shona or English. If they agreed to proceed, they were medically assessed for suitability to undergo sputum induction by the medical doctor. They were then invited to read or have read to them the patient informed consent form (annex 4), which was available in English and Shona. Any questions the patients had were answered by the study nurse. They then signed the informed consent form. Participants could withdraw from the study at any time. The consent forms were kept, in the TB screening room, accessible only to the PI and study nurse, for the duration of data collection. Once enrolment finished, the informed consent forms were transferred to the office of the MSF project coordinator, where they will be kept until 5 years after the study has been completed, in case of future medico-legal challenge.

Any patients refusing to participate in the study followed the routine diagnostic algorithm for the follow-up of smear-negative TB suspects.

It was originally decided not to provide reimbursement fees to patients, as it was believed that access to reference-standard testing in a population which would not normally benefit from this would be of sufficient benefit. This was accepted by 3 of our 4 ethics boards. MRCZ however indicated that a reimbursement fee of USD 10 should be given. This was introduced to the protocol once notification of amendment had been sent to the ERBs of MSF, BRTI and LSHTM. Amendment approval was granted by the MSF and LSHTM ERBs. BRTI was notified and confirmed receipt of the amendment.

While the provision of US$ 10 may be considered undue enticement in other sub-Saharan settings, this did not appear to be the case in Zimbabwe. This can be explained by the high cost of living and relatively high salaries. Several eligible patients refused to participate in the study, citing that they had other commitments, and this was seen as evidence that the reimbursement fee provided did not unduly influence people to participate in the study.

All members of the study team participated in ethics training, either provided by MRCZ, or online at the National Institutes of Health website (National Institutes of Health 2011).

Chapter 6: Results

6.1) Patient characteristics

One hundred and thirty nine patients were enrolled between February and June 2011. The original sample size of 300 was not obtained due to a number of factors; firstly, unexpected comparative results between microscopy and culture, in which there were several smear-positive, culture-negative patients. There was also a decrease in the enrolment rate, which would have prolonged the study duration beyond the planned implementation of the Xpert® MTB/RIF test (Cepheid, USA) (Boehme, Nabeta et al. 2010). Implementation of Xpert® MTB/RIF as a first-line test would have removed the direct smear-negative patient cohort from the programme. A decision was therefore taken to suspend enrolment prematurely.

One patient was erroneously included in the study prior to being diagnosed with pleural effusion during the pre-induction examination, and was therefore excluded from the study. Another patient's sample was misplaced, and this patient was thus excluded from further analysis. Of the 138 patients who underwent the induction procedure (including the patient whose sample was misplaced), 134 (97%) were able to produce an induced sputum sample. This resulted in 133 patient samples for microscopy and culture analysis (figure 6). Seventy-five (54%) of patients were female. The mean age was 38.8 years, with a median age of 37 years (IQR 30, 45).

Ninety-eight of 138 patients (71%) were HIV-positive. Twenty-eight of 138 (20%) were HIV-negative, and the status of 12 patients (9%) was unknown.

Results for all microscopy- and/or culture-positive patients are presented in table 2. These results include two culture-negative patients who subsequently became culture-positive when their sediment was re-cultured.

Figure 6: Flow chart outlining patient enrolment
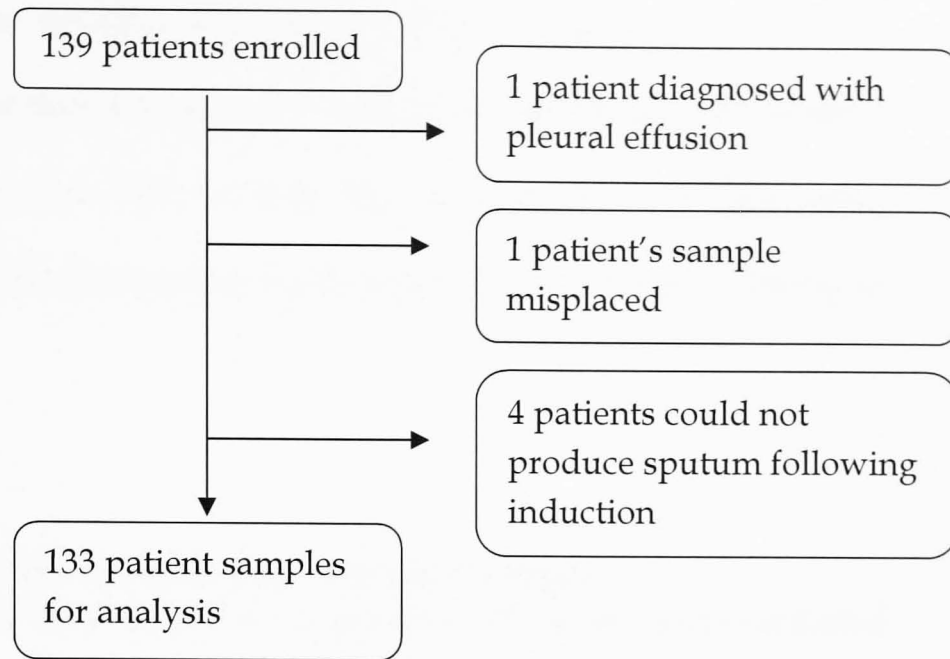
Table 2: Results per microscopy- and/or culture-positive patient

Patient number	TB Beads	Centrifugation	Culture
003	-	+	-
005	-	+	-
007	+	-	-
016	-	+	+
017	-	+	Contaminated
026	+	+	-
027	+	+	-
028	-	+	Contaminated
029	-	+	+
030	+	+	+
032	+	-	-
038	+	-	-
041	+	+	-
042	+	+	-
043	-	+	-
049	-	+	-
052	-	+	-
053	-	+	-
054	-	+	-
057	+	+	+
058	-	+	-
078	-	+	Contaminated
083	+	+	+
099	+	-	-
105	+	-	-
110	-	-	+
139	+	+	+

6.2) Microscopy

After 43 patients had been enrolled, the manufacturers announced that they had changed the formulation of their elution buffer, and advised us to move to version 2 of their test. All patients from 044 onwards had their samples analysed using version 2 of the test. Data analysis has been done separately for each test version, as well as combined.

6.2.1) TB beads used with the Ziehl-Neelsen staining technique

Nine patients of 133 (7%) were found to be positive on TB Beads-concentrated Ziehl-Neelsen (ZN) smears, all of which were scanty-positive[5]. Six of these patients (67%) were HIV-positive, 2 (22%) were HIV-negative, and one (11%) was of unknown HIV status. Five of 9 (56%) smear-positive patients were male.

For test version 1 alone, 5 patients of 41 (12%) were scanty-positive on TB Beads ZN smears. Three of these patients (60%) were HIV-positive, one (20%) was HIV-negative, and 1 (20%) was of unknown HIV status. 3 of these patients (60%) were female.

For test version 2 alone, 4 patients of 92 (4%) were scanty-positive, of which 3 (75%) were HIV-positive and one (25%) was HIV-negative. Three of four (75%) of these patients were male.

Most (124/133, 93%) patients were smear-negative, of which 87 (70%) were HIV-positive, 26 (21%) were HIV-negative, and 11 (9%) were of unknown HIV status. Fifty-four of 70 (77%) smear-negative patients were male.

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[5] Scanty positive on ZN microscopy = 1-9 AFB/100 high power fields. Scanty positive on fluorescent microscopy = 1-19 AFB/40 high power fields.

For test version 1 alone, thirty-six patients (86%) were smear-negative, of which 24 (67%) were HIV-positive, 10 (28%) were HIV-negative, and 2 (6%) were of unknown status. Twenty of 36 (56%) of smear-negative patients were female.

For test version 2 alone, eighty-eight patients (96%) were smear-negative, of which 63 (72%) were HIV-positive, 16 (18%) were HIV-negative, and 9 (10%) were of unknown HIV status. Fifty of 88 (57%) of these patients were female.

Results are presented in table 3.

6.2.2) TB beads used with Auramine-Rhodamine fluorescent staining technique

Ten patients of 133 (8%) were found to be positive on TB Beads-concentrated Auramine-Rhodamine fluorescent smears. Nine of these (90%) were scanty-positive, and one (10%) was 1+. Eight of these patients (80%) were HIV-positive, and 2 (20%) were HIV-negative. Six of 10 (60%) positive patients were female.

For test version 1 alone, 6 patients of 41 (15%) were scanty-positive on TB Beads concentrated Auramine-Rhodamine fluorescent smears. Four of these patients (67%) were HIV-positive, and 2 (33%) were HIV-negative. Five of 6 (83%) of these patients were female.

For test version 2 alone, 4 patients of 92 (4%) were scanty-positive, all of which were HIV-positive. Three of 4 of these patients (75%) were female.

One hundred and twenty three of 133 patients (93%) were smear-negative, of which 85 (69%) were HIV-positive, 26 (21%) were HIV-negative, and 12 (10%) were of unknown HIV status. Sixty-eight of 123 (55%) negative patients were female.

For test version 1 alone, thirty-five patients of 41 (85%) were smear-negative, of which 23 (66%) were HIV-positive, 9 (26%) were HIV-negative, and 3 (9%) were of unknown status. Eighteen of 35 (51%) of these patients were female.

For test version 2 alone, eighty-eight patients (96%) were smear-negative, of which 62 (71%) were HIV-positive, 17 (19%) were HIV-negative, and 9 (10%) were of unknown HIV status. Fifty of 88 (57%) of these patients were female.

Results are presented in table 3.

6.2.3) Centrifugation used with Auramine-O staining technique

Twenty-one patients of 133 (16%) were found to be positive on centrifugation Auramine-O fluorescent smears. Four of these (19%) were scanty-positive, 13 (62%) were 1+, 2 (10%) were 2+, and 2 (10%) were 3+. Thirteen of these patients (62%) were HIV-positive, 7 (33%) were HIV-negative, and the HIV status of one patient (5%) was unknown. Fifteen of 21 (71%) positive patients were male.

Of those samples processed with TB Beads test version 1 alone, 12 of 41 patients were smear-positive (29%). 1 patient of 12 (8 %) was scanty-positive, 8 patients of 12 (67%) were 1+, 1 patient of 12 (8%) was 2+, and 2 patients of 12 (17%) were 3+. Of the 12 positive patients, 5 were HIV-positive, 6 were HIV-negative, and 1 was of unknown HIV status. Seven of 12 (58%) positive patients were male.

Of those samples processed with TB Beads test version 2 alone, 9 patients of 92 (10%) were positive, of which 3 (33%) were scanty-positive, 5 (56%) were 1+, and 1 (11%) was 2+. 8 of 9 (89%) were HIV-positive, with 1 (11%) being HIV-negative. Eight (89%) of these patients were male.

One hundred and twelve of 133 patients (84%) were smear-negative, of which 80 (71%) were HIV-positive, 21 (19%) were HIV-negative, and 11 (10%) were of unknown HIV status. Sixty-eight of 112 (61%) negative patients were female.

Of those samples processed with TB Beads test version 1 alone, twenty-nine patients of 41 (71%) were smear-negative, of which 22 (76%) were HIV-positive, 5 (17%) were HIV-negative, and 2 (7%) were of unknown status. Eighteen of 29 (62%) of these patients were female.

Of those samples processed with TB Beads test version 2 alone, eighty-three patients (90%) were smear-negative, of which 58 (70%) were HIV-positive, 16 (19%) were HIV-negative, and 9 (11%) were of unknown HIV status. Fifty of 83 (60%) of these patients were female.

Results are presented in table 3.

6.3) Culture on Löwenstein-Jensen medium

Seven of 133 (5%) patients had positive culture results on Löwenstein-Jensen medium, of which 4 (57%) were HIV-positive, and 3 (43%) were HIV-negative. Six of 7 positive patients (86%) were male. All species isolated were *M. tuberculosis* complex.

For test version 1 alone, 3 of 41 patients (7%) had positive culture results, of which 1 (33%) was HIV-positive, and 2 (67%) were HIV-negative. All 3 culture-positive patients were male.

For test version 2 alone, 4 of 92 patients (4%) had positive culture results, of which 3 (75%) were HIV-positive, and 1 (25%) was HIV-negative. Three (75%) culture-positive patients were male.

One hundred and twenty-six of 133 patients (95%) were culture-negative. Eighty-nine (71%) were HIV-positive, 25 (20%) were HIV-negative, and 12 (10%) were of unknown HIV status. Seventy-three of 126 culture-negative patients (58%) were female.

For test version 1 alone, thirty-eight of 41 (93%) of patients were culture-negative, of which 26 (68%) were HIV-positive, 9 (24%) were HIV-negative, and 3 (8%) were of unknown HIV status. Twenty-three of 38 culture-negative patients (61%) were female.

For test version 2 alone, eighty-eight of 92 (96%) patients were culture-negative, of which 63 (72%) were HIV-positive, 16 (18%) were HIV-negative, and 9 (10%) were of unknown HIV status. Fifty of 88 culture-negative patients (57%) were female. Results are presented in table 3.

Table 3: Results per TB Beads test version and technique

TB Beads ZN	Test version 1	Test version 2	Combined test versions
# Samples	41	92	133
# Positive samples	5 (12%)	4 (4%)	9 (7%)
# HIV+ patients	27 (66%)	66 (72%)	93 (70%)
Gender ratio	18:23 (0.78)	41:51 (0.80)	59:74 (0.80)
TB Beads FM			
# Samples	41	92	133
# Positive samples	6 (14.3%)	4 (4.3%)	10 (8%)
# HIV+ patients	27 (65.9%)	66 (71.7%)	93 (70%)
Gender ratio	18:23 (0.78)	39:53 (0.74)	57:76 (0.75)
Centrifugation FM			
# samples	41	92	133
# positive samples	12 (29.3%)	9 (9.8%)	21 (16%)
# HIV+ patients	27 (65.9%)	66 (71.7%)	93 (70%)
Gender ratio	18:23 (0.78)	41:51 (0.80)	59:74 (0.80)
Culture on LJ			
# samples	41	92	133
# positive samples	3 (7.3%)	4 (4.3%)	7 (5%)
# HIV+ patients	27 (65.9%)	66 (71.7%)	93 (70%)
Gender ratio	18:23 (0.78)	41:51 (0.80)	59:74 (0.80)

6.4) Comparison of TB Beads and centrifugation

6.4.1) Comparison of Ziehl-Neelsen and/or Auramine-Rhodamine TB Beads microscopy vs. Auramine-O centrifugation microscopy

TB Beads results for ZN and Auramine-Rhodamine staining were combined and compared with Auramine-O centrifugation microscopy.

Eight of 133 patients (6%) were smear-positive on both concentration techniques, with 107 of 133 patients (81%) being negative on both techniques. Five patients (4%) were positive on the TB Beads concentration technique only; while 13 patients (10%) were positive on centrifugation microscopy but negative on TB Beads microscopy. Results are presented in table 4.

For version 1 of the test alone, 5 patients of 41 (12%) were positive on both concentration techniques, while 26 patients of 41 (63%) were negative on both techniques. Three patients of 41 (7%) were positive on the TB Beads concentration technique only; while 7 of 41 patients (17%) were positive on centrifugation microscopy only.

For version 2 of the test alone, 3 patients of 92 (3%) were positive on both concentration techniques, with 81 patients (88%) being negative on both concentration techniques. Two patients of 92 (2%) were positive on the TB Beads concentration technique only, while 6 patients (7%) were positive on centrifugation microscopy only.

Table 4: Comparison of TB Beads microscopy (combined staining techniques) with Auramine-O centrifugation microscopy

Centrifugation microscopy	TB Beads combined microscopy				Total
	Test version 1		Test version 2		Total
	+	-	+	-	
+	5	7	3	6	21
-	3	26	2	81	112
Total	8	33	5	87	133

6.4.2) Comparison of Auramine-Rhodamine TB Beads microscopy vs. Auramine-O centrifugation microscopy

Results for TB Beads Auramine-Rhodamine microscopy were compared with Auramine-O centrifugation microscopy.

Six patients of 133 (5%) were positive on both concentration techniques, with 108 patients (81%) negative on both techniques. Four patients (3%) were positive on Auramine-Rhodamine TB Beads microscopy only, while 15 patients (11%) were positive on Auramine-O centrifugation microscopy only. Overall results are presented in table 5, and results based on smear grade are shown in table 6.

For test version 1, 3 of 41 patients (7%) were found positive on both concentration techniques, with 26 patients (63%) negative on both techniques. Three patients (7%) were positive on TB Beads microscopy only, while 9 patients (22%) were positive on centrifugation microscopy only.

For test version 2, 3 patients of 92 (3%) were found positive on both concentration techniques, with 82 patients (89%) negative on both techniques. 1 patient (1%) was positive on TB Beads Auramine-Rhodamine microscopy only, while 6 patients (7%) were positive on centrifugation microscopy only.

Table 5: Comparison of Auramine-Rhodamine TB Beads microscopy with Auramine-O centrifugation microscopy

Centrifugation microscopy	TB Beads Auramine-Rhodamine microscopy				Total
	Test version 1		Test version 2		Total
	+	-	+	-	
+	3	9	3	6	21
-	3	26	1	82	112
Total	6	35	4	88	133

Table 6: Comparison of TB Beads Auramine-Rhodamine microscopy with centrifugation Auramine-O microscopy, according to smear grade

TB Beads Auramine-Rhodamine microscopy	Centrifugation Auramine-O microscopy					
		Scanty	1+	2+	3+	Negative	Total
	Scanty	1	4	1	0	4	10
	1+	0	0	0	0	0	0
	2+	0	0	0	0	0	0
	3+	0	0	0	0	0	0
	Negative	3	9	1	2	108	123
	Total	4	13	2	2	112	133

6.5) Comparison of TB Beads microscopy vs. culture on Löwenstein-Jensen medium

Four patients of 133 (3%) were positive on both TB Beads microscopy (ZN and/or Auramine-Rhodamine) and culture on Löwenstein-Jensen medium, while 117 (88%) were negative on both tests. Nine patients (7%) were positive on TB Beads microscopy only, while 3 patients (2%) were positive on LJ culture only. Combined results are presented in table 7, and results based on smear grade are shown in tables 8 and 9.

For test version 1, one patient of 41 (2%) was positive on both TB Beads microscopy and culture, while 31 patients (76%) were negative on both tests. Seven patients (17%) were positive on TB Beads microscopy only, while 2 patients (5%) were positive on LJ culture only.

For test version 2, three patients of 92 (3%) were positive on both TB Beads microscopy and culture, while 86 patients (94%) were negative on both tests. Two patients (2%) were positive on TB Beads microscopy and negative on LJ culture, while one patient (1%) was positive on LJ culture only.

Table 7: Comparison of TB Beads microscopy (combined techniques) with culture on Löwenstein-Jensen medium

TB Beads combined microscopy					
Culture on Löwenstein-Jensen medium	Test version 1		Test version 2		Total
	+	-	+	-	
+	1	5	3	2	11
-	4	31	1	86	122
Total	5	36	4	88	133

Table 8: Comparison of TB Beads ZN microscopy with culture on Löwenstein-Jensen medium, according to smear grade

TB Beads ZN microscopy	Culture on Löwenstein-Jensen medium			
		Positive	Negative	Total
	Scanty	3	6	9
	Negative	4	120	124
	Total	7	126	133

Table 9: Comparison of TB Beads Auramine-Rhodamine microscopy with culture on Löwenstein-Jensen medium, according to smear grade

TB Beads Auramine-Rhodamine microscopy	Culture on Löwenstein-Jensen medium			
		Positive	Negative	Total
	Scanty	2	7	9
	1+	1	0	1
	Negative	4	119	123
	Total	7	126	133

6.6) Comparison of centrifugation microscopy vs. culture on Löwenstein-Jensen medium

Six patients of 133 (5%) were positive on both centrifugation microscopy and culture on Löwenstein-Jensen medium, while 111 patients (84%) were negative on both tests. Fifteen patients (11%) were positive on centrifugation microscopy and negative on LJ culture, and 1 patient (1%) was positive on LJ culture and negative on centrifugation microscopy. Overall results are presented in table 10, and centrifugation FM results based on smear grade are shown in table 11.

Table 10: Comparison of centrifugation Auramine-O microscopy with culture on Löwenstein-Jensen medium

	Centrifugation microscopy	Auramine-O	
Culture	+	-	Total
+	6	1	7
-	15	111	126
Total	21	112	133

Table 11: Comparison of centrifugation Auramine-O microscopy with culture on Löwenstein-Jensen medium, according to smear grade

	Culture on Löwenstein-Jensen medium		
Centrifugation		**Positive**	**Negative**	**Total**
Auramine-O	**Scanty**	1	3	4
microscopy	**1+**	2	11	13
	2+	2	0	2
	3+	1	1	2
	Negative	1	111	112
	Total	7	126	133

6.7) Sensitivity and specificity of the techniques compared to culture on Löwenstein-Jensen medium

6.7.1) Sensitivity, specificity and predictive values for TB Beads on Ziehl-Neelsen microscopy

The sensitivity for results of test versions 1 and 2 combined, using the ZN staining technique, was 43% (95% CI 10-82). The specificity was 95% (95% CI 90-98). The

positive predictive value was 33% (95% CI 7-70), and the negative predictive value was 97% (95% CI 92-99).

For test version 1, the sensitivity was 33% (95% CI 1-91). The specificity was 90% (95% CI 75-97). The positive predictive value was 20% (95% CI 1-72), and the negative predictive value was 94% (95% CI 81-99).

For test version 2, the sensitivity was 50% (95% CI 7-93), and the specificity was 98% (95% CI 92-100). The positive predictive value was 50% (95% CI 7-93), and the negative predictive value was 98% (95% CI 92-100).

6.7.2) Sensitivity, specificity and predictive values for TB Beads on Auramine-Rhodamine microscopy

The sensitivity for results of test versions 1 and 2 combined, using the Auramine-Rhodamine staining technique, was 43% (95% CI 10-82). The specificity was 94% (95% CI 89-98). The positive predictive value was 30% (95% CI 7-65), and the negative predictive value was 97% (95% CI 92-99).

For test version 1, the sensitivity was 0% (95% CI 0, 71), while the specificity was 84% (95% CI 69-94). The positive predictive value was 0% (95% CI 0-46) and the negative predictive value was 91% (95% CI 77-98).

For test version 2, the sensitivity was 75% (95% CI 19-99), and the specificity was 99% (95% CI 94-100). The positive predictive value was 75% (95% CI 19-99), and the negative predictive value was 99% (95% CI 94-100).

6.7.3) Sensitivity, specificity and predictive values for TB Beads on Ziehl-Neelsen and Auramine-Rhodamine microscopy (staining techniques combined)

The sensitivity for results for test versions 1 and 2 combined, using results for both ZN and Auramine-Rhodamine staining techniques on TB Beads microscopy, was 57% (95% CI 18-90). The specificity was 93% (95% CI 87-97). The positive predictive value was 31% (95% CI 9-61), and the negative predictive value was 98% (95% CI 93-100).

For test version 1, the sensitivity was 33% (95% CI 1-91), and the specificity was 82% (95% CI 66-92). The positive predictive value was 13% (95% CI 1-53), and the negative predictive value was 94% (95% CI 80-99).

For test version 2, the sensitivity was 75% (95% CI 19-99), and the specificity was 98% (92-100). The positive predictive value was 60% (15-95), and the negative predictive value was 99% (94-100).

6.7.4) Sensitivity, specificity and predictive values for centrifugation on Auramine-O microscopy

The sensitivity for centrifugation concentration on Auramine-O microscopy was 86% (95% CI 42-100). The specificity was 88% (95% CI 81-93). The positive predictive value was 29% (11-52), and the negative predictive value was 99% (95-100).

Results for all microscopy procedures are presented in figure 7.

Figure 7: Sensitivity, specificity, PPV and NPV of the different microscopy procedures
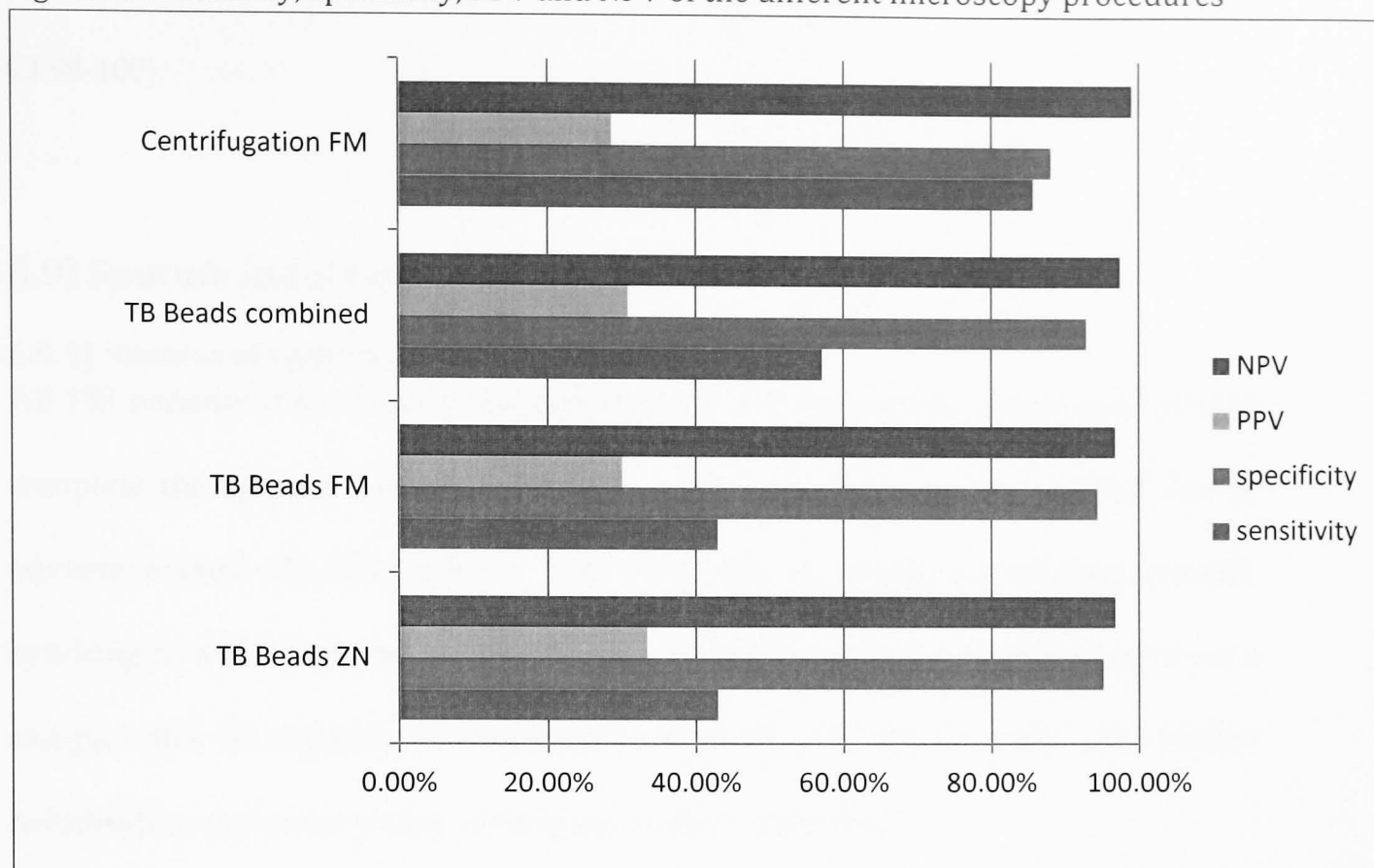
6.8) Performance of TB Beads test version 1 compared to test version 2

TB Beads elution buffer version 2 did appear to perform better than version 1, in line with the manufacturer's claims. However, due to the small sample size, it is not possible to verify this conclusively.

For ZN microscopy, test version 1 had a sensitivity of 33% (95% CI 1-91), a specificity of 90% (95% CI 75-97), a positive predictive value of 20% (95% CI 1-72), and a negative predictive value of 94% (95% CI 81-99); while test version 2 had a sensitivity of 50% (95% CI 7-93), a specificity of 98% (92-100), a positivity predictive value of 50% (95% CI 7-93), and a negative predictive value of 98% (95% CI 92-99).

For fluorescent microscopy, test version 1 had a sensitivity of 0% (95% CI 0-71), a specificity of 84% (95% CI 69-94), a positive predictive value of 0% (95% CI 0-46), and a negative predictive value of 91% (95% CI 77-98); while test version 2 had a sensitivity of 75% (95% CI 19-99), a specificity of 99% (95% CI 94-100), a positive

predictive value of 75% (95% CI 19-99), and a negative predictive value of 99% (95% CI 94-100).

6.9) Sputum induction

6.9.1) Success of sputum induction procedure

All 138 patients who commenced nebulisation with hypertonic saline were able to complete the sputum induction procedure. No procedure was suspended due to adverse events. Of 138 patients, 134 were able to produce a sputum sample, resulting in a 97.1% induction success rate. Four patients were unable to produce a sample after 20 minutes of nebulisation with 6% hypertonic saline. No further nebulisation procedures were attempted on these patients.

6.9.2) Tolerance of the sputum induction procedure

One hundred and twenty of 138 patients (87%) experienced no adverse reactions during the induction procedure. Of these 120 patients, 103 (86%) were under the age of 50, with 17 (14%) being over 50 years of age. Female patients comprised 52% of the under-50s, and 59% of the over-50s.

Of the 18 patients (13%) who experienced adverse reactions during the induction procedure, most patients (83%) had coughing spells. Of fifteen patients with coughing spells, 12 (80%) were under 50 years of age, and of these, 8 (67%) were female. Among those over 50 years of age, one was female (33%) and 2 were male (67%). One male patient aged over 50 years experienced cough and nausea. Three patients experienced nausea alone, one being a female patient aged under 50 years, one a female patient aged over 50 years, and the third being a male patient over 50 years of age.

Two patients of 138 (1%) experienced coughing spells up to 30 minutes after the induction procedure. Both were female, and one (50%) was under 50 years of age, with the other being over 50 years of age.

6.10) Results based on HIV status

6% (6/97) of HIV-positive patients were positive on TB Beads concentration with ZN staining, with 7% (2/28) of HIV-negative being positive on this technique, and no patients of unknown HIV status. The difference was not statistically significant (p=0.778).

8% (8/97) of HIV-positive patients were positive on TB Beads concentration with Auramine-O staining, with 7% (2/28) of HIV-negative patients being positive on this technique, and no patients of unknown HIV status. The difference was not statistically significant (p=0.581).

13% (13/97) of HIV-positive patients tested were positive on centrifugation, with 25% (7/28) of HIV-negative patients being positive on this technique, and 8% (1/12) patients of unknown HIV status. The difference was not statistically significant (p=0.369).

Among 97 HIV-positive patients, 4 (4%) were positive on culture, with 11% (3/28) of HIV-negative patients negative on this technique, and no patients of unknown HIV status were positive on culture. The difference was not statistically significant (p=0.370).

All 4 patients who could not provide a sample after induction were HIV-positive. 11% (11/97) of HIV-positive patients has side-effects during the induction procedure, with 11% (3/28) of HIV-negative patients having side-effects, and 25% (3/12) of patients with unknown HIV status. The two patients who had side-effects after induction were both HIV-positive.

6.11) Results based on gender

Slightly more female patients (54%) were enrolled in the study than male patients. Positivity patterns for TB Beads were not biased toward either gender, with more men (56%) being positive on ZN microscopy, and more women (60%) being positive for fluorescent microscopy. Significantly more men than women were positive on centrifugation microscopy: 25% of all men were positive on centrifugation, with only 8% of women being positive (p=0.007). This also applied to culture, where 10% of all men were positive, compared to 1% of all women (p=0.024).

Side-effects during induction were reported for 7% of men and 15% of women; this difference was not statistically significant (p= 0.255). After induction, side-effects were reported for 2% of men and 2% of women; again, the difference was not statistically significant (p=0.847).

Chapter 7: Discussion

7.1) Patient demographic data

The mean age of 38.8 years was consistent with the mean ages reported in sputum induction studies performed on adults. Results were analysed in cohorts of less than or equal to 50 years, and over 50 years. Life expectancy in 2011 in Zimbabwe was 49.93 years for men, and 43.34 years for women (IndexMundi 2011); we therefore describe those over 50 years of age as being 'elderly' for the purposes of this data analysis.

Slightly more (54%) female than male patients were enrolled. This could be due to the practice of screening all HIV-positive counselling and testing clients for TB as standard. As all pregnant women are screened for HIV during their ante-natal visits, this could result in more women than men being found HIV-positive, and therefore in the disproportionate amount of women being screened for TB. Zimbabwe's gender ratio for 2011 was 0.83 male in the 15-64 years age range, and 0.75 male in those over 65 years of age (IndexMundi 2011); this probably accounts for having enrolled slightly more women than men. This gender imbalance is seen across the medical activities in the polyclinic.

However, it has been found that women are more likely to be found to be smear-negative on direct smear microscopy than men, due to the submission of poorer-quality sputum specimens (Khan, Dar et al. 2007). This phenomenon may also apply to this setting.

Another possible explanation is that women had more free time than men, which allowed them to come to collect their smear-negative results, and to participate in the study when requested. It is possible that men were more likely to be employed

and therefore unable to stay in the clinic for induction to be performed, or to return the following day for their results.

7.2) Gender

While gender did not have an impact on TB Beads results, significantly more men than women were positive on centrifugation (25% of men c.f. 8% of women) and culture (10% of men c.f. 1% of women). The lack of impact in TB Beads results could be due to lower numbers of positives and lower sensitivity compared with centrifugation. WHO's 2010 data indicates that smear-positive notification rates in Zimbabwe had a male/female ratio of 1.1 in 2005 and 2010, therefore while it may be expected that slightly more men than women have a biological confirmation of TB infection, the numbers should not differ so much between the genders (World Health Organisation 2011c). However, other studies performed in Harare have also found male gender to be linked with increased risk of biologically-confirmed TB. In one study, business employees with a cough of three weeks or greater duration had sputum collected for culture, and male sex was found, on multivariate analysis, to be significantly associated with culture-positive TB, with an incidence rate ratio of 4.4 (95% CI 1.0-19.8) (Corbett, Bandason et al. 2007). A further community-based survey in Harare, investigating TB in people regardless of symptoms, found significant association of male sex with smear-positive TB, with an adjusted odds ratio of 3.1 (95% CI 1.5-6.4) (Corbett, Bandason et al. 2009).

Side-effects during and following induction were not significantly different between the genders.

7.3) HIV status

Seventy-one percent of enrolled patients were HIV-positive, with 20% being HIV-negative. The status of 9% of patients was unknown. A patient's HIV status was only recorded as being positive or negative if the patient knew the results of a recent HIV test. The proportion of HIV-positive people was high due to the clinic being a centre for comprehensive HIV care, to which people were referred for treatment and testing for opportunistic infections. Part of the clinic functioned as a polyclinic for routine health care, and patients with symptoms suggestive of TB were referred for screening, which explains why some people screened were either HIV-negative or of unknown status.

These proportions of HIV positivity and negativity were broadly maintained in the positivity rates for microscopy and culture: 66.6% of those patients positive on TB Beads ZN microscopy, 80% of TB Beads FM, and 62% of FM centrifugation were HIV-positive, with 57% of culture-positive patients being HIV-positive.

HIV status did not significantly affect the results on any technique. However, the small number of smear- and culture-positive patients means results should be interpreted with caution.

The only patients who could not provide a sample after induction were all HIV-positive, and more HIV-positive than HIV-negative patients had side-effects during the procedure, but small numbers make this hard to interpret.

7.4) Performance of TB Beads on Ziehl-Neelsen microscopy vs. Auramine-Rhodamine microscopy

TB Beads fluorescent microscopy did not have a statistically-significant higher yield (p-0.812) vs. Ziehl-Neelsen microscopy, with 9 patients of 133 (7%) being positive on

ZN microscopy, and 10 (8%) positive on Auramine-Rhodamine microscopy. The same was seen when the test version 1 cohort was analysed separately, with 5 patients (12%) positive on ZN microscopy, and 6 patients (15%) positive on FM. Again, this difference was not statistically significant (p=0.746). Four patients (4%) were found positive for both microscopy techniques in the test version 2 patient cohort.

7.5) Performance of fluorescent microscopy on TB Beads concentration vs. centrifugation concentration

Ten patients in total were found to be positive on fluorescent microscopy with TB Beads concentration, with 21 patients being positive on fluorescent microscopy with centrifugation concentration. TB Beads fluorescent microscopy had a sensitivity of 43% (95% CI 10-82), with centrifugation fluorescent microscopy having a sensitivity of 86% (95% CI 42-100). The point estimates indicate that the performance of TB Beads may be inferior to that of centrifugation concentration with regard to sensitivity, and that the TB Beads technique cannot in this version be considered as a substitute for centrifugation concentration. The specificity for the TB Beads technique and centrifugation concentration was 94% (95% CI 89-98) and 88% (95% CI 81-93) respectively.

Negative predictive values for both concentration techniques were high, at 97% (95% CI 92-99) for the TB Beads technique, and 99% (95% CI 95-100) for centrifugation concentration – this is because most patients were negative on all techniques. Positive predictive values were considerably lower for both techniques, with 30% (95% CI 7-65) for TB Beads concentration, and 29% (95% CI 11-52) for centrifugation concentration.

Test version 2 performed better than test version 1, with a sensitivity of 75% (95% CI 19-99) and a specificity of 99% (95% CI 94-100). While this might imply that the sensitivity of the new version is closer to that of centrifugation, the number of positive samples is too small to allow any meaningful conclusions to be drawn.

These findings follow the same trends as those found in a recent study describing the performance of the TB Beads technique (version 0) in Uganda (Albert, Ademun et al. 2011). In this study, fluorescent microscopy with TB Beads concentration was found to have a sensitivity of 65.4% (95% CI 55.6-74.4), compared with a sensitivity of 74.8% (95% CI 65.4-82.7) for fluorescent microscopy with centrifugation concentration. The authors concluded that the TB Beads technique was not a viable alternative to centrifugation at its current sensitivity performance. This study found that both TB Beads and centrifugation concentration methods had lower than expected specificity, with 88.6% (95% CI 82.9-92.9) and 94.3% (95% CI 89.7-97.2) specificity reported respectively.

The higher sensitivity of TB Beads FM relative to centrifugation in the Uganda study, compared with our findings for both test versions combined, may have been because version 0 of the test did not use an elution buffer. Because the bacteria remained attached to the beads, and the beads remained on the slide, there may have been less likelihood of bacteria floating off the slide during the staining technique.

7.6) Performance of microscopy vs. culture

The results generated in the study represent paired data. This was disregarded in the analysis due to the small sample size and low positivity rates found in the techniques used.

Twenty-seven patients of 133 (20%) were found positive for TB on microscopy and/or culture. Of these, 26 (96%) were positive on microscopy, with only one patient being microscopy-negative and culture-positive.

The average sensitivity of smear microscopy on induced sputum compared to culture was found in the literature review to be 44.6%, ranging from 0-100%. This must be interpreted with caution, due to the variety of microscopy and culture techniques used, each of which has an impact on sensitivity. The characteristics of the patient groups enrolled would also have had an impact on the probability of positivity for TB. This estimated sensitivity is within the range (28-70%) of sensitivity expected from direct smear microscopy (Yassin, Cuevas et al. 2003; Kanaujia, Lam et al. 2005; Matee, Mtei et al. 2008; Cattamanchi, Dowdy et al. 2009b). However, reduced sensitivity might be explained by the fact that many studies reviewed were performed on people who had been found negative on direct microscopy, or children. Both of these groups can be expected to be less likely to be smear-positive than the average TB suspect undergoing direct sputum smear microscopy. The sensitivity of microscopy on induced sputum from patients who are direct smear-negative cannot be accurately compared with that of smear microscopy on unselected patients with chronic cough.

The estimated sensitivity of 44.6% would imply that we should have found at least twice as many culture-positive patients as smear-positive patients. We found only 1 extra patient through culture, and 17 of the smear-positive patients were culture-negative (with 3 extra patient samples being contaminated on culture).

These results have two possible implications. Either the smear-positive, culture-negative results were false positives, as the use of culture on Löwenstein-Jensen medium as a reference standard would lead us to believe. This is possible, but unlikely, as slides were read by experienced microscopists in two separate

laboratory facilities, and each positive (and negative) smear was confirmed by at least one other microscopist. The positive fluorescent slides found in BRTI were confirmed by re-staining with the ZN technique, and all were found to contain AFB using this technique as well. It is possible that the AFB seen were non-tuberculous mycobacteria (NTM) which could not be grown on LJ medium; alternatively, the bacteria may have been *Nocardia* spp., which has been found in HIV-positive TB suspects in Sudan (Alnaum, Elhassan et al. 2011) and Ethiopia (Rasheed and Belay 2008). It has also been previously isolated in patients in Zimbabwe (Baily, Neill et al. 1988; Freland, Fur et al. 1995; Bhagat, Ibrahim et al. 1999). *Nocardia* spp. are a genera of the Corynebacterineae family, which are weakly acid-fast and can be confused with mycobacteria in microscopy (Scharfen, Buncek et al. 2010). *Nocardia* spp. can be overlooked in sputum cultures as it can be mistaken for non-pathogenic organisms, such as diphtheroids, and discarded (McNeil and Brown 1994). However, TB is a far more prevalent infection in the study population than nocardiosis, and it is far more likely that the organisms found were mycobacteria. Definitive confirmation of the species would be possible by performing genotyping on the bacteria present on the positive slides (Van Der Zanden, Te Koppele-Vije et al. 2003), but this was outside the remit of this study.

The second, and more likely, implication is that LJ culture in this case did not function as a true reference standard. It is believed that the smear microscopy results were true positives, but it is impossible to make conclusions on sensitivity and specificity based on these results. There are several potential explanations for the failure of culture as a reference standard in this study. Instead of the routine n-acetyl-l-cysteine sodium hydroxide (NALC-NaOH) decontamination method (Siddiqi and Ruesch-Gerdes 2006), which results in a final sodium hydroxide concentration of 1%, the harsher modified Petroff decontamination method was

used (Petroff 1915), in which no NALC is used, and the final sodium hydroxide concentration is 2% (see annex 6: BRTI protocols TB microscopy and culture). The samples were predominantly paucibacillary (scanty or 1+ on centrifugation), which has also been described elsewhere as being associated with induced sputum (Hatherill, Hawkridge et al. 2009). Induced sputum is thinner than spontaneously expectorated sputum, and so it could be hypothesised that less contact time for homogenisation may be required than when compared with directly expectorated sputum.

Once results trends became evident, BRTI was requested to re-culture stored sediment of those samples which had been centrifugation FM-positive but culture-negative. This was performed on 11 samples, of which 2 became positive, and 9 remained negative.

This implies that the decontamination method used may have been too harsh for these samples, and suggests that further research is required to ascertain ideal decontamination procedures for induced sputum. None of the studies reviewed for this thesis states that a non-standard or modified decontamination procedure was used on the induced sputum samples analysed. Where stated, either modified Petroff or NALC-NaOH decontamination was performed, and only two papers state the concentration of sodium hydroxide used: one used 2% NALC-NaOH, resulting in a final concentration of 1% (Al Zahrani, Al Jahdali et al. 2000), and another used NaOH with a final concentration of 2%, as was done in our study (Brown, Varia et al. 2007).

Four other sputum induction studies describe having found smear-positive, culture-negative samples (Shata, Coulter et al. 1996; Zar, Hanslo et al. 2005; Hatherill, Hawkridge et al. 2009; Moore, Apolles et al. 2011). One of these studies (Moore, Apolles et al. 2011) states that there was an average delay of 42 hours between

sample collection and reception at the laboratory, and offers this as an explanation for the high smear-positive, culture-negative rates.

The study also found higher TB yields on microscopy than culture, and with no smear-positives being culture-positive, and vice versa. This study was carried out on children, in a setting similar the one used in this study, a peripheral clinic in South Africa. The study used liquid culture (the sputum decontamination method used was not described), which could be hypothesised to be more sensitive than solid culture, and yet similar issues were experienced, which implies that this phenomenon requires further investigation.

The samples in this study had a delay of on average 24 hours between collection and delivery to the laboratory, but as samples were kept and transported in cold chain after having been aliquoted, this delay is not perceived to have had an impact on *M. tuberculosis* viability. The longest delays occurred when samples were collected on Fridays, and then transported to the BRTI laboratory on Mondays, but again, being kept in cold chain should have maintained *M. tuberculosis* viability and minimised contamination rates, as this has been shown to keep mycobacteria viable for 8 days or more (Bhat, Selvakumar et al. 2011).

It has been shown in South Africa that paediatric samples have a higher yield when lower a NaOH concentration (1%) is used, compared with the concentration used for adult samples (1.5%) (Whitelaw, Mentoor et al. 2010). The largest rise in recovery rates was seen among nasopharyngeal aspirate samples, which, like induced sputum, tend to be less purulent than spontaneously-expectorated sputum. Paediatric samples are traditionally thin and paucibacillary (Hopewell, Pai et al. 2006), and so this finding could be extrapolated to induced sputum. The results do not state whether induced or spontaneous sputum was analysed in this case.

Use of solid Löwenstein-Jensen medium instead of liquid medium may have reduced *M. tuberculosis* culture yield. Culture on liquid medium is known to have greater sensitivity compared with solid culture, particularly for NTM (Chihota, Grant et al. 2010). Therefore, it is possible that some smear-microscopy positive samples were in fact NTM which did not grow on the solid medium. Culture of induced sputum has been found to recover significant rates of NTM (Hatherill, Hawkridge et al. 2006), but while this study was designed to investigate this phenomenon, due to low culture sensitivity, this could not be confirmed.

7.7) Use of Auramine-Rhodamine compared with Auramine-O

Auramine-O is the standard stain used for fluorescent TB microscopy. Rhodamine B is sometimes added to enhance differentiation of *M. tuberculosis* from artifacts (Rieder, Van Deun et al. 2007). The fact that Auramine-Rhodamine was used with the TB Beads-concentrated samples, and Auramine-O alone with the centrifuged samples, may explain the higher yields found in centrifuged samples, as it may be possible that artifacts in centrifuged samples were confused with AFB. However, this is unlikely for two reasons: Auramine-O FM-positive smears were confirmed by two experienced microscopists; also, all Auramine-O fluorescent-positive smears were re-stained with the ZN staining technique, and the presence of AFB was verified in all FM-positive samples.

7.8) Smear adherence

Induced sputum samples are less purulent than spontaneously-expectorated samples. This may be due to their being mixed with saline as they are expectorated – up to 20 mL of saline was inhaled by patients over 20 minutes. Being less purulent, this implies that a smear prepared from an induced sputum sample adheres less well to a microscope slide, compared with directly-expectorated sputum, in which the protein content is higher. This problem is further exacerbated by the digestion of the induced sputum with 4% sodium hydroxide.

Because of this, initial smears made from TB Beads-processed sputum were very thin and sometimes washed off during staining. This was in spite of heat-fixation, and much care being taken during the staining procedure. The staff was familiar with working with digested, sediment smears, having previously performed a bleach concentration study in Epworth. Use of 100 µL of the sample instead of 50µL improved adherence of sample to slides, as did leaving slides to dry for at least one hour.

It was beyond the scope of this study to investigate other methods of fixation to improve smear adherence, but some suggestions for further research can be proposed. The addition of a proteinaceous substance such as bovine serum albumin (BSA) to the elution buffer or sediment may improve adherence. BSA is used in the Borstel Supranational Reference Mycobacteriology Laboratory, Germany, for smears made from positive MGIT tubes, which suffer from the same adherence problems. This may however make decolourisation more difficult, as the BSA retains carbol fuchsin more strongly than sputum (Hepple, Nguele et al. 2010). Use

of slides coated with poly-l-lysine may also have improved adherence (Leif, Ingram et al. 1977).

It is also possible that small quantities of sediment (e.g. 50 μL at a time) could be layered, with time allowed for drying between layers. However, this is not feasible for routine laboratory operations.

While the study was in progress, the manufacturer changed the smearing procedure, as well as the elution buffer, to enhance smear adherence, based on feedback from the PI and others (Microsens, personal communication). The new smearing procedure, whereby a smear is first made with the undigested sample, and then covered with the concentrated sample, may have improved adherence. However, this new version (version 3) was not implemented as the new buffer and instructions were supplied one week before the PI was due to leave the study site, and over half of the samples had already been processed when this change to the procedure was announced.

The procedure may have worked better on version 0 of test, where the beads were retained on the slide and therefore the mycobacteria were much less likely to be removed during the staining procedure. It should be noted that the research used to choose this technique for investigation, and which therefore led us to believe that the technique could be non-inferior to centrifugation, was performed on version 0. The same data was used in the package insert of versions 1 and 2 (Microsens Med:Tech 2010; Microsens Med:Tech 2011), and the manufacturers did not specify which specific amendments had been made to the elution buffer.

7.9) Quality control

Approximately 10% of TB Beads slides were randomly selected on a monthly basis and sent to the MSF-supported laboratory in Gweru Provincial Hospital, where

they were examined blindly by an experienced TB microscopist. Percentage agreement was good at 96%. A single ZN slide was found to be a false negative, with 4 AFB found on cross-check. This can be classified as a low false negative error and can arise due to chance alone (Medecins Sans Frontieres 2008). Known positive samples were also concentrated with TB Beads to act as daily positive controls. The fluorescent and ZN stains were tested daily with known positive and negative smears to ensure correct performance.

BRTI undertook its regular quality control procedures, outlined in annex 6.

7.10) Success and tolerance of the sputum induction procedure

All patients on whom the nebulisation procedure was commenced were able to complete it. No procedure was terminated due to side-effects. The success rate of the induction procedure, defined as production of a sample of sufficient volume so as to be capable of analysis with all the procedures being investigated, was 97.1%. Sample volume ranged from 3-10 mL. Only four patients were incapable of producing a sample after 20 minutes of nebulisation. All four were men less than or equal to 50 years of age, all were HIV positive, and none experienced adverse reactions during or following the procedure. No further clinical data is available to describe which treatment decisions were made in these cases. Our literature review identified induction success rates ranging from 76.4% to 100%, implying our success rate can be described as very high.

Only 13.1% of patients experienced side-effects. All of these were mild and could be dealt with on-site and without specific medical intervention. The most common adverse event was a coughing spell, experienced by 15 patients (11%) during the procedure, and by 2 patients (1%) up to 30 minutes after the procedure. There were

no specific trends observed with regard to age, gender or HIV status. Since numbers were small, it would not be possible to ascribe significance to any associations which may have been found.

Three patients experienced nausea, two of which were aged over 50 years. The remaining patient was a woman in the early stage of pregnancy, so the nausea could have been partially attributed to this state. Two of these patients, including the pregnant woman, were HIV-positive, with the status of the third patient being unknown. A fourth, HIV-positive male patient over 50 years of age experienced both cough and nausea. This implies that increased age may be associated with higher risk of nausea, but the sample size is too small for this result to be considered significant.

The literature review we performed listed possible adverse events such as cough, nausea, vomiting, epistaxis, and wheezing. Studies listing coughing as an adverse event describe a range of 0% to 26.8% of patients being affected, but cough could be seen as a somewhat subjective indicator, particularly as the induction procedure is designed to provoke coughing in the first place. Studies describing nausea or vomiting state ranges of incidence of <1% to 14.4%, with nausea being more frequent than vomiting. Our findings are therefore in line with what has been previously described in other studies.

The study proved that sputum induction by ultrasonic nebulisation of 6% saline could be performed safely in a polyclinic setting, on ambulant HIV positive and negative patients, with only minor side-effects and with minimal training of health care staff, as also seen in South Africa (Moore, Apolles et al. 2011). The most

complicated and limiting piece of equipment was the nebuliser itself, which required a power source. In this study, mains power was available, but a battery and inverter system could also be used if electrical supply was limited.

The Lung Flute has also been proposed as an electricity-free method of promoting sputum production (Fujita, Murata et al. 2009). A human-powered nebuliser has been evaluated in South Africa and has been found to yield good-quality sputum in comparison with an ultrasonic nebuliser (Kranzer, Olson et al. 2011). A human-powered centrifuge, used in combination with chlorine-based disinfection of reusable nebuliser parts, and an outdoor collection booth, would allow for sputum induction to be performed in settings with no power supply. If TB Beads were to be used, then the concentration procedure prior to microscopy could also be done without electricity; however, the sensitivity of the version(s) evaluated would need to be improved before this could be done.

7.11) Patient and staff perceptions of the sputum induction procedure

Little data exists on patient and staff perceptions of the sputum induction procedure. It was originally planned to perform exit interviews, using structured questionnaires, with patients to determine their perceptions of the induction procedure, as well as end-of-study interviews with the nurses who had been performing the induction procedure. This qualitative arm of the study was removed on the advice of the review panel, and so no formal perception data is available.

However, anecdotal evidence showed that the nurses were excited to be trained in the procedure, and enjoyed performing it. A team from BRTI came to visit the site in order to gather facts for a sputum induction study that they were planning to implement, and by the end of their tour, which was conducted by the nurses, they

had the impression that the procedure was easy to perform and well-tolerated by patients. The study site was audited by the MRCZ, and again the study group (in the absence of the PI, who was abroad at the time) conveyed a lot of enthusiasm regarding the procedure, especially regarding the fact of being able to get patients to expectorate larger volumes of sample than had been possible spontaneously.

The PI underwent the induction procedure during the training session on use of the nebuliser. As the procedure is designed to stimulate coughing by irritating the mucous membranes, it cannot be described as pleasant. However, other than a profound thirst, no other negative aspects were found. As a result of this experience, patients were provided with filtered water once they had undergone the procedure. This side-effect was not mentioned in any of the literature reviewed, and while it has few clinical consequences, being aware of it made the induction procedure and its aftermath more comfortable for the patients.

7.12) Nebuliser maintenance

Staff were thoroughly trained in the functioning and maintenance of the ultrasonic nebuliser used. The nebuliser was composed of several parts which required disinfection between patients: the medication cup, medication cup cover, inhalation hose and face mask. It was decided, to decrease the turn-around time, to use chlorine disinfection instead of autoclaving, as was originally planned[6]. Use of chlorine was also deemed safer and less technically-demanding, as there was no need for a heat source or training in the operation of a pressurised device.

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[6] Following the nebuliser manufacturer's instructions, the items listed above were soaked in a 0.1% NaDCC (sodium dichloroisocyanurate) solution, prepared by dissolving one NaDCC tablet (chlorine content 1.9g/tablet; final free chlorine: 1g/tablet) per litre of filtered water. Contact time was 15 minutes, after which the nebuliser manufacturer stated that disinfection would be complete (Omron Matsusaka Co. Ltd Omron ultrasonic nebuliser, model NE-U17, instruction manual. Japan.)

Use of chlorine disinfection requires no power source or special training, making it appropriate for resource-limited settings.

7.13) Sample size

The sample size was derived based on several assumptions:

- 40% sensitivity of direct fluorescent smear microscopy c.f. culture

- specificity of direct smear microscopy of 95% (with culture on LJ medium as a reference-standard)

- 80% negativity rate on direct fluorescent smear microscopy among TB suspects.

Based on the above, 32% of enrolled patients were expected to be culture-positive, of which around half (52% for TB Beads, 58% for centrifugation) were expected to be microscopy-positive. In the study, 7% of patients were positive on ZN TB Beads microscopy, 8% on fluorescent TB Beads microscopy, and 16% on fluorescent centrifugation microscopy. However, the culture positivity rate was 5%, which was lower than the microscopy positivity rates found, instead of double these rates, as was expected.

The actual sensitivity for TB beads (combined staining techniques) was 57% (95% CI 18-90). This was within the expected range of the confidence interval based on 52% sensitivity (42-62). However, the actual sensitivity for centrifugation was higher than expected, at 86% (95% CI 42-100). This was a result of culture not functioning as a reference standard, implying that the false-negative rate among culture samples

was high. This means that the true sensitivity of centrifugation and TB Beads concentration is unknown.

There is limited evidence, based on our findings, to suggest that TB Beads are not sensitive enough to function as a replacement for centrifugation, based on the disparity between the observed sensitivity of TB Beads (57%, 95% CI 18-90) and centrifugation (86%, 95% CI 42-100).

It is not believed that any biases existed which would have resulted in a higher positivity rate for centrifugation c.f. microscopy. If the induction process had introduced NTM into the samples, these would have been expected to have had equal chance of detection by both concentration techniques. Any problems encountered with the functioning of the reference standard were independent of the performance of microscopy. While the centrifuged sediment was the sample inoculated onto LJ medium, the performance of the centrifugation microscopy technique is expected to be independent of the concentration of sodium hydroxide used. The above leads us to believe that the sensitivity findings based on comparison of concentration techniques are valid.

The sample size of 300 patients wasn't achieved. Reasons for this are multi-factorial. Enrolment was lower than anticipated from the start: instead of the expected 5 per day, enrolment stabilised at 2-4 patients per day while the PI was on-site. The expected enrolment rate was based on laboratory direct smear-microscopy data, i.e. the number of patients who had direct smear-negative results issued per month. Following this, at least 6 smear-negative patients were expected to collect their results every weekday and would subsequently be invited to participate in the study. In practice, not all suspects returned to collect results, and some of those who

did claimed to be feeling better, and therefore did not want to undergo further investigation.

Enrolment decreased after the departure of the PI, whose work permit expired before study completion due to delays in acquiring ethical and research approval, and due to the lower than expected enrolment rate. Another reason given by the local study team included the temporary suspension of two Voluntary Counselling and Testing clinics, which had been feeding HIV-positive patients to the clinic for TB screening.

While at first patients readily agreed to participate in the study, there were rumours that, after the departure of the PI, some patients were discouraging others from participation in a study being undertaken in a clinic run by foreigners. This coincided with political events during which anti-Western sentiments were being expressed.

An interim analysis of the data undertaken in May 2011 showed that the rates of smear-positive, culture-negative results were unexpectedly high, and that the true performance characteristics of the test were unlikely to be clearly defined.

The decision had already been taken, in line with WHO recommendations (World Health Organisation 2011a), for the project to implement the Xpert® MTB/RIF assay (Cepheid, USA) as a microscopy replacement. This would have therefore made the inclusion criteria (direct smear-microscopy negative patients) redundant. For the reasons stated above, it was decided, in consultation with the MSF OCA Medical Director, the study team and the PI's doctoral supervisor, to terminate the study and analyse the data already collected.

Patients were informed of this decision via Shona and English-language posters placed prominently in the clinic. The Biomedical Research and Training Institute, and the Medical Research Council of Zimbabwe, were also informed.

7.14) Exclusion from enrolment

Fewer than 10 patients who consented to participate in the study were excluded on clinical grounds. Severe asthma, respiratory distress and bleeding were used as exclusion criteria, following the MSF sputum induction guidelines (Medecins Sans Frontieres 2010). Patients who were found to have pleural effusion were also excluded, and were directly treated on the basis of this finding.

7.15) Change of TB Beads test versions

The study planned to test a version of TB Beads ('version 0') in which the captured bacteria remained attached to the magnetic beads. A smear was then to be made of the beads, stained and examined for mycobacteria (Wilson, Lane et al. 2010).

After the protocol had been submitted for ethical approval, the manufacturer informed us that they had changed their test to allow for the captured bacteria to be eluted from the magnetic beads. They recommended that we evaluate this new version ('version 1') as they planned to withdraw version 0 from the market. As the manufacturer cited the same evidence for version 1's performance characteristics, it was assumed that the ligand properties remained unchanged (Microsens Med:Tech 2010). Therefore it was decided to evaluate version 1, and to adapt the protocol appropriately, following approval from the MSF OCA Medical Director and the doctoral supervisor.

In February 2011, when 43 patients had already been enrolled, the manufacturer informed us that they had changed the formulation of their elution buffer, and

advised us to change to the new version ('version 2') (Microsens Med:Tech 2011). The decision was made to process all subsequent samples using version 2 of the test buffer, as this was believed to be the product which would be commercially available. The patient cohorts would then be analysed separately, and also combined, on the assumption that the intrinsic performance characteristics were similar.

Version 2 of the elution buffer was incorrectly labelled as being '10x', and so was diluted 10-fold, as had been done for the version 1 buffer (Microsens Med:Tech 2010). This was done for samples 044-076. Closer examination of the revised protocol (Microsens Med:Tech 2011) made it clear that the buffer should be used in an undiluted form, and communication with the manufacturer confirmed this. Therefore, samples 044-076 were processed with an erroneously-diluted elution buffer, which may have had an impact on sensitivity.

In April 2011, the manufacturer informed us that they had changed their elution buffer once again ('version 3'), along with the sample processing procedure. The procedure now involved making a preliminary smear from the undigested sputum, which would form a proteinaceous base for the concentrated, eluted bacteria. This new version was not implemented, as it was felt that 3 different test cohorts would have been too complicated for data analysis; also, at this point it was clear that the test, far from being a commercial product, was in fact still under development. It is possible that version 3 may have been more sensitive than version 2. Version 2 of the test is user-friendly and simple to perform, but version 3, although it was never implemented, seemed to be less so. The step introduced to improve adherence (pre-smearing of the slide with undigested sputum) was cumbersome, open to administrative and procedural error, and it is unknown how practical this would be for everyday use.

The PI had to leave Zimbabwe due to work permit expiration a week after this announcement, so there was no time for a brief comparison to be performed, before the routine handling of samples was handed over to the Epworth laboratory scientists.

7.16) Human resources

A dedicated study nurse was planned and budgeted for. However, due to existing excess workload capacity in the polyclinic, it was decided to use two existing TB nurses instead, working in rotations of one week each, with a third nurse as back-up in case the first two were unavailable. All three nurses underwent training in all relevant study-specific issues, including ethics training. Advantages were that the nurses were firmly established in the clinic and so understood patient flow and clinic processes. They were also used to working together closely, which facilitated patient referral from the screening nurse to the acting study nurse. As they were also the ones attending to TB suspects and distributing results, on alternating weeks when they were not performing study duties, they could give preliminary information about study participation during routine consultations, and were alert to potential study participants during their routine work. Disadvantages were that competing priorities sometimes arose, at which point routine clinic work was ascribed priority over study work. This resulted in some potential study participants not being recruited due to occasional staff shortages.

According to Zimbabwean law, a nurse was unable to perform the clinical assessment of people undergoing induction; this had to be done by a medical doctor. Patient assessment was performed by a medical doctor, once the patient had been identified by the study nurse and general consent/interest had been obtained

(detailed informed consent was sought once the doctor had deemed the patient to be eligible to participate). The assessment procedure took less than ten minutes per patient, and with an enrolment rate of 2-4 patients per day, it made no sense to hire a doctor specifically for this purpose; therefore it was decided that a regular programme physician (of which three were available) would provide this service. This proved to be a bottleneck to enrolment on occasion, as the doctors had to be located in the clinic when necessary, and the study nurses had to wait until the doctor had finished his current task before he was able to assess the potential study patient. Assessment procedures consisted of checking for fasting over the previous two hours, severe respiratory distress, visible signs of bleeding, reduced levels of consciousness, and history of significant asthma and/or allergies. Patients also had salbutamol administered 5 minutes prior to the induction being performed, to minimise the chance of adverse effects. This assessment was straightforward and could have been performed by nursing staff, along with the induction procedure itself, if the law had permitted. In settings where nurses are given greater authority, it should be possible for nurses to complete all induction-related procedures, as in the recent study performed in a South African primary care clinic (Moore, Apolles et al. 2011).

7.17) Delays to study initiation

It took longer than expected to get ethical approval and registration: the procedure for acquiring ethical approval was not clearly explained by the MRCZ at the outset, and the next step in the process was usually discovered only on completion of the previous step. Apart from ethical approval, the laboratory had to be registered as a research facility and the PI as a foreign researcher, also as a research scientist with the laboratory council of Zimbabwe. Applications were submitted in September

2010, but consideration of most coincided with the Christmas period, further delaying approval. Enrolment began the day after the final registration was granted. This left the PI with just under three months of the original six-month temporary employment permit, which was not renewable. The enrolment rate was envisaged to be 6 patients per day, but as stated elsewhere, in fact it was lower than this. This meant that the original timeframe of three months for enrolment of 300 patients was not realistic. For this reason, the PI had to hand over the field work to the Epworth study team, and was unable to process all samples and remain on-site until the study end, as originally planned.

7.18) Patient positivity trends

Most of the patients who were found positive either on microscopy or culture were identified in the first 2 months of the study. The final 3 months of the study identified only 3 positive patients: one positive on microscopy only, one on culture only, and one on both microscopy and culture.

An explanation for this may have been a steady improvement in the performance of fluorescent microscopy for direct smear microscopy. Fluorescent microscopy was introduced to the Epworth laboratory in late November 2010, and so had been in place for 2 months when the study began. Quality control results for direct microscopy demonstrate that percentage agreement increased over time. January's results showed 80% agreement, while February's results showed 100%, and in March, the laboratory had 90% agreement. Results from April-June were 100% in agreement (Medecins Sans Frontieres 2011b). This may imply that true direct smear-positives were missed in February and March 2011, which were then detected when the patients were retested with TB Beads, centrifugation and culture. More accurate

testing in April-June would then have resulted in fewer of these direct microscopy false-negative patients from being enrolled.

It is also possible that the daily presence of the PI resulted in improved performance of routine testing, thereby contributing to the increased accuracy of fluorescence microscopy, along with the improvement that would have resulted from experience with the technique.

7.19) Bias

The TB Beads and centrifugation procedures were performed in different laboratories by different personnel. TB Beads processing and slide reading was done in the Epworth polyclinic laboratory by the PI and the Epworth laboratory scientists; while centrifugation and culture was done in the BRTI laboratory. Samples were aliquoted and sent to BRTI without any TB Beads results, and so BRTI was blinded to the TB Beads results. However, the Epworth laboratory, and the PI, was aware of incoming results from BRTI. TB Beads processing was completed 2-3 days before the BRTI results were available, and TB Beads results were not changed based on discordance with Laboratory 2.

However, this could potentially have introduced bias. The study was set up this way to facilitate patient follow-up: TB Beads-negative, centrifugation-positive patients had to be evaluated and put on treatment as appropriate, and the dissemination of these results was coordinated by the PI. It would have been preferable for someone unconnected to the TB Beads processing to have followed these results up.

7.20) Benefits and risks to patients

The study had the following benefits for patients:

- Next-day diagnosis of patients with smear-positive pulmonary TB following induction and processing with TB Beads, who were smear-negative on conventional microscopy, who could then be commenced on treatment immediately, instead of the 1-2 week delay when following the standard WHO algorithms which would otherwise require an antibiotic trial and follow up smears prior to diagnosis

- Diagnosis of culture-positive, smear-negative and algorithm-negative TB patients who would otherwise have been missed with microscopy – treatment could commence as soon as cultures were found to be positive.

This study detected patients who were smear-negative on conventional microscopy and smear-positive on microscopy following sputum induction. One patient who was smear-negative and culture-positive was also detected. These patients would otherwise have had to follow diagnostic algorithms including repeat direct microscopy and antibiotic therapy prior to diagnosis.

Sputum induction was performed on the same day as results for conventional microscopy were received. Patients had to return the following day to receive these results, and so incurred travel and incidental costs for this extra appointment. MSF provided reimbursement of US$10 to cover travel expenses and time lost.

While normally well-tolerated, sputum induction can lead to side-effects such as nausea, vomiting, epistaxis and wheezing, usually in less than 10% of patients. Patients were monitored by nursing staff for 30 minutes following the induction procedure in order to effectively deal with any side-effects. Plans were made to terminate the induction procedure if it was deemed that the side-effects were too severe.

If the TB Beads technique had been found to be comparable to centrifugation on induced sputum (a previous study found 96% sensitivity compared to centrifugation (Wilson, Lane et al. 2010)), then this would have allowed sputum induction to be rolled out to district-level clinics in Zimbabwe and other TB high burden countries, for use with smear microscopy where centrifugation and biosafety cabinet facilities do not exist. MSF clinics in Zimbabwe and elsewhere in resource-limited settings would have implemented the technique routinely, and would have supported and encouraged implementation by Ministries of Health. The TB Beads system has a cost of approximately US$1.50 per sample processed (based on the price quoted by Microsens for 800 tests and associated equipment). The price for routine diagnostic culture on Löwenstein-Jensen medium in Harare was US$30 (BRTI laboratory, Harare). Given the much higher cost for culture compared to processing with TB Beads, it was believed to be cost-effective to implement this technique routinely compared to culture.

Chapter 8: Conclusion

The objective of the study was to determine whether TB Beads, performed with fluorescent microscopy alone or in combination with fluorescent and ZN microscopy, performed well enough for induced sputum concentration to substitute for centrifugation in resource-limited settings. This could therefore have contributed to improved case-finding of TB among vulnerable populations in resource-limited settings. Our findings show that the TB Beads concentration technique did not perform as well as centrifugation fluorescent microscopy and so cannot in its current form replace centrifugation. Version 2 of the TB Beads test had a performance which was closer to that of centrifugation microscopy, but the sample size was small and further research is required to determine true performance when compared to a functional reference standard.

Microscopy results did not differ based on HIV status. Men and women were equally likely to be TB Beads positive, but men were more likely to be positive on centrifugation microscopy and culture. These findings were in line with those from other studies which have been conducted in the population. There was no significant gender-based incidence of side-effects.

Centrifugation with fluorescent microscopy recovered more bacteria than culture in this study, most probably due to over-decontamination of induced sputum samples, which were mostly non-purulent and paucibacillary in nature when found positive on microscopy. Culture on liquid media may have been more sensitive and may have led to the recovery of some species of NTM.

Poor smear adherence may have adversely influenced TB Beads positivity rates. Methods which improve smear adherence during the staining procedure should be investigated for use in conjunction with this technique.

The sputum induction procedure, with 6% hypertonic saline disseminated by an ultrasonic nebuliser, resulted in 97% of patients being able to produce a sputum sample. All 4 patients who were unable to produce a sample were HIV-positive, but small sample size means this finding is not significant. The procedure worked well on ambulant population in which 71% were HIV-positive. Adverse reactions were observed in 13.1% of patients. Reactions during the induction procedure mainly comprised of coughing spells, along with some nausea. Only 2 patients had a coughing spell after induction. This implies that people who are unable to expectorate spontaneously, or who are smear-negative on spontaneous sputum smear microscopy, can safely undergo sputum induction and increase their chances of obtaining a biological confirmation of TB infection, therefore being placed on treatment and decreasing chances of transmission to others. Interpretation of results was limited by the sample size not having been reached.

Future research on the TB Beads technique should include improvement of smear adherence during the staining procedure. Research in processing of induced sputum should consider evaluation of the optimal decontamination procedure which can preserve viable *M. tuberculosis* in paucibacillary, non-purulent samples, and a research project has been planned in conjunction with MSF to further develop this on paediatric induced sputum samples. It would also be interesting to undertake research on patient and staff perceptions of the induction procedure, in order to make the procedure more comfortable and acceptable. It may also be

relevant to combine TB Beads with emerging technology such as the Xpert®
MTB/RIF assay (Cepheid, USA), in order to improve the sensitivity of the assay.

From a public health perspective, this research indicates that TB Beads should not
be implemented as a substitute for centrifugation. However, the study added to the
growing body of evidence that sputum induction can be performed safely on
ambulant HIV-positive TB suspects in a peripheral setting. Induced sputum can be
used in combination with emerging molecular technologies to enhance case
detection and to rapidly identify drug resistance, thus decreasing transmission and
incidence of drug-sensitive and drug-resistant TB.

Chapter 9: References

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Annex 1: Sputum induction protocol

Sputum induction was performed according to the standard MSF protocol. The procedures below were extracted from the 'MSF Sputum induction – background paper' (Medecins Sans Frontieres 2010).

Pre requisite Factors

If any of these factors are not possible, the procedure should not be done

- Only trained staff must conduct the procedure, preferably a nurse

- An appropriate site **must** be available. The minimum required is:

  A small room or temporary structure with good ventilation

  if possible with

  Negative pressure *and/or*

  UV light *and/or*

  Extractor fan

- Staff must use respirators, eye protection and non sterile gloves.

Material required

General:

- Mask (respirator) for the operator and carer (if present)

- Eye protection and non sterile gloves for operator

- Oxygen (on standby in case of emergency)

- Pulse oximetre

- Request form

Preparation Pre - nebulization:

- Spacer device (holding chamber) and mask

- Salbutamol metered dose inhaler

Nebulization:

- Mask, chamber and tubing

- Antibacterial filter

- Ultrasonic nebulizer (if possible to obtain) [5,9]

- Sterile solution of 3-6% sodium chloride – refrigerated if possible (more irritant)

Aspiration:

- Suction catheter (7 or 8F)

- Ideally obtain a mechanical suction device & mucus trap. If not available, obtain a 50ml syringe

- Sputum collection container

- Sterile solution of 0.9% sodium chloride to add to the specimen

IMPORTANT: *Infection control measures*

Management of materials

- Spacer devices (holding chambers) – Either sterilize after each patient (preferred) or disinfect after each patient by soaking in hexanios for at

least 15 minutes then rinse, then soak again in a new bath of hexanios for 15 minutes. <u>Rinse well</u> and then wipe dry

- All masks, tubing, suction catheters and syringes should be disinfected with 2% chlorine and then discarded

- Antibacterial filters should be fitted and changed for each patient to protect the nebulizer, oxygen cylinder (if used), and any aspiration device (if used)

Management of the environment

- The room must be left unused with the windows open, or extraction fan on for a least 30mins after the procedure to allow adequate replacement of air in the room. A fan may be necessary to facilitate this. No one should enter this room during the period without a respirator. A sign should be placed on the door saying 'No Entry Without a Respirator' until the minimum period has passed

Annex 2: Laboratory procedures

2.1) Magnetic beads concentration (Microsens Med:Tech 2010; Microsens Med:Tech 2011)

The induced sputum will be concentrated using the TB Beads method[7], prior to examination by Ziehl-Neelsen and fluorescent microscopy.

1) The sputum is thinned and decontaminated using incubation with 4% NaOH for 15-20 minutes

2) The TB Beads solution is added and the mixture is left to incubate at room temperature for 2 minutes

3) The tubes are placed in a magnetic holder and the magnetic beads are collected at the bottom of the container

4) The supernatant is removed, wash solution is added and the beads are resuspended

5) Step 4 is repeated and the beads are washed again

6) The wash solution is removed and elution buffer is added

7) The beads are collected magnetically, and the bead-free supernatant is used to make smears for microscopy

8) Smears are air-dried, heat-fixed and stained as appropriate.

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[7] Version 1: Lot no. TBGM09.02, exp. 2011 - 09
Version 2: Lot no. TBMP0103.1, exp. 2011 - 12

2.2) ZN staining and examination

The concentrated sputum smear will be fixed and stained according to the Ziehl-Neelsen technique[8], followed by examination by bright-field microscopy:

1) The smear is allowed to dry, then passed through a flame 2-3 times for fixation to occur

2) The slide is placed on a staining rack and covered completely with 1% carbol fuchsin stain (Alere, UK)

3) The slide is heated until steam rises, then left to stain for 5 minutes

4) The slide is washed with water and covered completely with 3% acid-alcohol decolouriser (Becton Dickinson, USA) for 2 minutes

5) The slide is washed with water and covered completely with 0.3% methylene blue counterstain (Avonchem, UK) for 1 minute

6) The slide is washed with water, the back of the slide is wiped with gauze, and the slide is left to dry on a dying rack

7) Once dry, the smear is examined at x1000 for AFB, using immersion oil, and graded according to the WHO-IUATLD scale (Rieder, Van Deun et al. 2007).

8) All slides (positive and negative) are read by 2 laboratory scientists (the PI plus one other scientist)

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[8] Carbol fuchsin: Alere Healthcare, Bedfordview, UK. Lot – CCNDC, Ref – LG11, Exp – Oct 11
Decolouriser: Tb decolouriser, BD, Sparks, Maryland, USA, Lot – 0103474, Exp – 2011-04-30
Methylene blue solution 1% aqueous, Avonchem, Cheshire, UK (diluted with distilled water to 0.3%)
Batch no.-9248-2 Exp – August 2011

2.3) Fluorescent staining and examination

The concentrated sputum smear will be fixed and stained according to the Auramine-Rhodamine technique (TB Fluor phenol-free kit, Merck, Germany)[9], followed by examination by fluorescent microscopy:

1) The smear is allowed to dry, then passed through a flame 2-3 times for fixation to occur

2) The slide is placed on a staining rack and covered completely with Auramine-Rhodamine solution for 15 minutes

3) The slide is rinsed with water

4) The slide is covered completely with decolourising solution for 1 minute

5) The slide is washed with water

6) The slide is covered completely with potassium permanganate counterstain solution for 5 minutes

7) The slide is washed with water, the back of the slide is wiped with gauze, and the slide is left to dry on a dying rack

8) Once dry, the smear is examined at x400 for fluorescent rods, and graded according to the WHO-IUATLD scale (Rieder, Van Deun et al. 2007)

9) All slides (positive and negative) are read by 2 laboratory scientists (the PI plus one other scientist)

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[9] Merck TB-fluor phenol-free; 2012/07/31, batch 1.01597. 0001 (Merck KGaA, 64271 Darmstadt, Germany)

Annex 3: Case report form

Sputum induction study case report form

Date:_____

Patient number:_____

Age:_____

Sex: Male   Female

HIV status: Positive   Negative   Unknown

Informed consent given: Yes   No

Adverse reactions DURING induction (circle as appropriate):

None   Cough   Nausea   Vomiting   Nosebleed   Wheezing

Adverse reactions up to one hour AFTER induction (circle as appropriate):

None   Cough   Nausea   Vomiting   Nosebleed   Wheezing

Induction procedure completed: Yes   No

TB Beads microscopy result:

Ziehl-Neelsen_____

Auramine_____

Centrifugation microscopy result:

Auramine_____

Löwenstein-Jensen culture result:_____

Species:_____

Annex 4: Patient informed consent form

Version 09.11.2010

MEDECINS SANS FRONTIERES

Evaluation of a novel concentration method for the microscopic detection of *Mycobacterium tuberculosis* from induced sputum

Principal Investigator: Pamela Hepple, MSc

Phone number: 0773 235 297

What you should know about this research study:

- We give you this consent form so that you may read about the purpose, risks, and benefits of this research study.

- Routine care is based upon the best known treatment and is provided with the main goal of helping the individual patient. The main goal of research studies is to gain knowledge that may help future patients.

- We cannot promise that this research will benefit you. Just like regular care, this research can have side effects that can be serious or minor.

- You have the right to refuse to take part, or agree to take part now and change your mind later.

- Whatever you decide, it will not affect your regular care.

- Please review this consent form carefully. Ask any questions before you make a decision.

- Your participation is voluntary. You will be given USD10 as reimbursement for your time and for travel expenses.

PURPOSE

You are being asked to participate in a research study to improve testing for tuberculosis. The purpose of the study is to see if a new way of collecting and testing sputum makes it easier to find out if someone is positive for tuberculosis. You were selected as a possible participant in this study because our normal TB tests said that you do not have TB, but those tests cannot pick up all infections. We think that it is possible that although you were not found to have tuberculosis with normal testing, this new sputum collection and testing procedure could find that you are positive for tuberculosis. If you are found to be positive, we will provide you with the treatment. If you are found to be negative, you can seek

further care from your local clinic if you still feel sick. We will be asking approximately 300 people in Epworth polyclinic to participate in this study.

PROCEDURES AND DURATION

If you decide to participate, you will undergo a sputum collection procedure called sputum induction. This is a standard procedure used in many countries, and is not experimental.

Sputum induction means that you will be asked to breathe in some salty water which has been made into a mist. This will make you cough up sputum, which you will spit into a container.

We will then take this sputum into the laboratory and examine it, using a new testing procedure, for tuberculosis bacteria. If we see the bacteria, we will tell your nurses that you have TB and need treatment. The medical team will then decide how to treat you.

We will also send your sputum to a laboratory in Harare for another, standard, test. This test takes a few weeks to do. We will find you and tell you if you are positive on this test when we have the results. Please make sure we have your correct address so that we can find you easily. We can make an appointment for you to come and discuss your results in one month's time. Please tell us if you would like to do this.

The induction procedure takes about 15 minutes, and we will ask you to stay in the clinic for another 30 minutes to make sure that you are OK afterwards. The whole procedure should take around 1 hour.

We will then ask you to return tomorrow to get the results of the new testing procedure.

We will use all of the sputum in our tests, and none will be left over. The microscope slides we make from your sputum will be kept for 5 years in case we need to check them again but they will not be used for any other tests or studies. The bacteria that we may grow from your sputum will be destroyed as soon as we have written down the results. Your sputum will be marked with a number, not your name, and only 2 people (your nurse and the study investigator) will be able to connect your name to your number.

RISKS AND DISCOMFORTS

The sputum induction procedure can give you some unpleasant side-effects. Out of 100 people, we think that 5 or 6 people will have nausea, or vomiting, or nosebleeds, or wheezing. This is why we want you to stay for 30 minutes after the procedure has finished. If you have any of these side-effects, we will look after you in the Epworth polyclinic.

If you decide not to continue with the induction procedure, you will follow the same procedures as for those patients not enrolled in the study – you will be checked for tuberculosis based on your symptoms and put on medication if the doctor thinks you need it.

BENEFITS AND/OR COMPENSATION

Taking part in this study can mean that we find that you have a tuberculosis infection. If you are infected with tuberculosis, we will treat you for this infection, free of charge. We cannot and do not guarantee or promise that you will receive any benefits from this study.

CONFIDENTIALITY

If you indicate your willingness to participate in this study by signing this document, we plan to disclose if you are positive or negative for tuberculosis on the 3 different tests that we will perform. We will also write down your HIV status, if you know it. It is OK if you don't want to give us this information. This information will be used by MSF researchers who are also working with the London School of Hygiene and Tropical Medicine, in England.

This information will not contain your name. You will be given a special study number. Only 2 people will know which number relates to you, and this information will not leave the Epworth polyclinic.

Under some circumstances, the Medical Research Council of Zimbabwe may need to review patient records for compliance audits.

ADDITIONAL COSTS

We will give you USD10 for participating in the study, to cover travel costs and the time you spend here. Tuberculosis treatment will be provided for free by MSF, if you are found to have tuberculosis.

IN THE EVENT OF INJURY

In the event of any side effects resulting from your participation in this study, treatment can be obtained at Epworth Polyclinic. You should understand that the costs of such treatment will be our responsibility. Financial compensation is not available.

If you need further information, please contact the study investigator.

VOLUNTARY PARTICIPATION

Participation in this study is voluntary. If you decide not to participate in this study, your decision will not affect your future relations with the Epworth polyclinic, its personnel, and MSF. If you decide to participate, you are free to withdraw your consent and to discontinue participation at any time without any consequences for your care.

OFFER TO ANSWER QUESTIONS

Before you sign this form, please ask any questions on any aspect of this study that is unclear to you. You may take as much time as necessary to think it over.

AUTHORIZATION

You are making a decision whether or not to participate in this study. Your signature indicates that you have read and understood the information provided above, have had all your questions answered, and have decided to participate.

The date you sign this document to enrol in this study, that is, today's date, MUST fall between the dates indicated on the approval stamp affixed to each page. These dates indicate that this form is valid when you enrol in the study but do not reflect how long you may participate in the study. Each page of this Informed Consent Form is stamped to indicate the form's validity as approved by the Medical Research Council of Zimbabwe.

_____   _____

Name of Research Participant (please print)   Date

_____   _____

                                                                    AM

Signature of Participant or legally authorized representative   Time

                    PM

_____

_____   _____

Signature of Witness   Signature of Staff Obtaining

Consent

(*Optional*)

YOU WILL BE GIVEN A COPY OF THIS CONSENT FORM TO KEEP.

If you have any questions concerning this study or consent form beyond those answered by the investigator, including questions about the research, your rights as a research subject or research-related injuries; or if you feel that you have been treated unfairly and would like to talk to someone other than a member of the research team, please feel free to contact the Medical Research Council of Zimbabwe on telephone 791792 or 791193.

Annex 5: Systematic review

Microscopy compared to culture for the diagnosis of tuberculosis in induced sputum samples: a systematic review

P. Hepple,*† N. Ford,‡§ R. McNerney†

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_____ S U M M A R Y

BACKGROUND: Resource-limited settings rely on sputum examination using microscopy to diagnose tuberculosis (TB); however, the sensitivity of the test is poor and case detection rates are low. Sputum induction is proposed as a way to improve sample collection and enhance test sensitivity.

OBJECTIVE: To undertake a systematic review of studies comparing microscopy and culture sensitivity in induced sputum samples.

METHODS: We ran duplicate searches of databases (up to August 2011) and searchable websites of major human immunodeficiency virus (HIV) and TB conferences (up to November 2010) to identify studies comparing the performance of microscopy compared to culture on induced sputum samples, with culture as the reference standard.

RESULTS: A total of 23 studies met our inclusion criteria. The overall success of the induction was high, ranging from 76.4% (95%CI 68.5–83.2) to 100% (95%CI 98.5–100), while adverse events associated with sputum induction were infrequent and mild. The sensitivity of microscopy compared to culture ranged from 0% to 100%; only eight studies reported on the species of mycobacterium isolated in culture. Yield was generally higher for sputum induction compared to nasopharyngeal aspiration and gastric lavage, and compared equally well to bronchoalveolar lavage and physiotherapy.

DISCUSSION: Sputum induction increases TB case detection and is useful for people who are negative on spontaneous smear microscopy or unable to expectorate spontaneously. It is well-tolerated by children and adults, irrespective of HIV status, and can be used where culture is not available. The use of induced sputum samples with molecular tests, such as Xpert® MTB/RIF, warrants further investigation.

KEY WORDS: sputum induction; tuberculosis; microscopy; culture; sensitivity

IN 2010, there were an estimated 12 million prevalent tuberculosis (TB) cases worldwide and 8.8 million incident cases, resulting in 1.45 million deaths, including 0.35 million in persons co-infected with the human immunodeficiency virus (HIV). Case detection rates remain poor, particularly in the World Health Organization (WHO) Africa Region where only 60% of estimated incident cases were detected and notified during 2010.[1] Globally, 6.2 million TB cases were notified during 2010, of which 75% (4.6 million) were new pulmonary cases. Delayed diagnosis is detrimental to patient outcomes,[2] and untreated infectious pulmonary disease leads to further disease transmission.[3]

Pulmonary TB is commonly diagnosed by microscopic examination of spontaneously expectorated sputum. It is not a sensitive technique, and only 57% of notified new pulmonary cases in 2010 were smear-positive.[1] In the absence of alternative diagnostic tests, smear-negative cases may remain undetected and unreported, contributing to the burden of untreated infectious disease.

Smear microscopy has reported sensitivities ranging from 61.8% to 70% when compared to culture.[4–6] However, the HIV epidemic has led to a substantial increase in the frequency of smear-negative pulmonary TB,[7] and sensitivities of below 30% are reported in parts of Africa where HIV prevalence is high.[8] Microscopy relies on the production of purulent sputum samples, but quality varies: one study, from Indonesia, found that only a third of TB suspects who had undergone TB diagnosis provided at least one good sample, and less than one in seven were able to provide three good quality samples;[9] this may vary depending on the patient characteristics.[10] Samples that contain mainly saliva rather than bronchial expectoration rarely contain mycobacteria, and are of reduced value for TB diagnosis.[11]

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Sputum induction is frequently proposed as a technique to improve sample collection, and has been found to be relatively easy to perform and generally well tolerated.[12–15] The technique involves using sterile water or hypertonic saline to irritate the airways, which promotes coughing and production of a specimen. While sputum induction does not require high levels of technology or training, its utility at the peripheral level is limited by the fact that culture is usually only available at national reference laboratories. If microscopy could be used instead of culture this would improve the utility of sputum induction in such settings. However, the usefulness of sputum induction for TB diagnosis is unclear: studies reporting similar results have concluded that sputum induction had no added value over spontaneous expectoration,[16] or that induction was a useful and cost-effective intervention.[17]

We undertook a systematic review of studies assessing the performance of microscopy compared to culture in induced sputum samples. Culture of induced sputum was taken as the reference standard.

METHODS

Search strategy

We developed a search strategy combining key terms that may indicate sputum induction (sputum induction, induced sputum, sputum expect* and sputum sampl*) with TB. The following databases were searched from inception up to August 2011: PubMed, Embase, the Web of Science and Google Scholar. We also searched abstracts of the following conferences: all electronic abstract books of the Union World Conferences on Lung Health conferences (up to Berlin, November 2010), the Society of General Microbiology (up to Nottingham, September 2010), the American Society of Tropical Medicine (up to Washington 2009) and all International AIDS Society conferences (up to Vienna, July 2010) and all Conferences on Retroviruses and Opportunistic Infections (up to San Francisco, February 2010). Our search was complemented by reviewing the bibliographies of relevant papers. No language restriction was applied. Where needed, authors of original studies were contacted for additional information.

Study selection and data extraction

One of the authors (PH) scanned all articles by title and abstract for initial inclusion according to pre-defined inclusion criteria. We included any studies investigating the use of sputum induction for the diagnosis of pulmonary TB, regardless of age, HIV status or presence or absence of symptoms. Studies were excluded if they did not provide separate results for microscopy and culture for comparison. Final inclusion of potentially eligible articles was assessed in dupli-

cate (PH, NF). Data extraction was done in duplicate (PH, NF) using pre-defined extraction tables to collect information about study characteristics. We extracted data on culture and microscopy results for all sputum collection methods. We defined a positive result as one or more positive smears or culture, irrespective of how many samples were processed per patient. The methodological quality of studies was assessed using a framework adapted from the Cochrane handbook for the systematic review of diagnostic test accuracy.[18]

Data analysis

We assessed inter-rater reliability on inclusion of articles by calculating the φ statistic. We calculated point estimates and 95% confidence intervals (CIs) for the sensitivity of microscopy compared to culture. All P values were two-sided, and $P < 0.05$ was considered significant. All analyses were conducted using Stata version 11 (Stata Corp, College Station, TX, USA). NF and PH conducted all statistical analyses.

RESULTS

Our initial search identified 668 abstracts (Figure 1): 42 studies met the inclusion criteria for assessment of full articles, and 23 studies (3127 participants) were included for analysis. Agreement on final inclusion was high ($\varphi = 0.92$). Nineteen studies were excluded
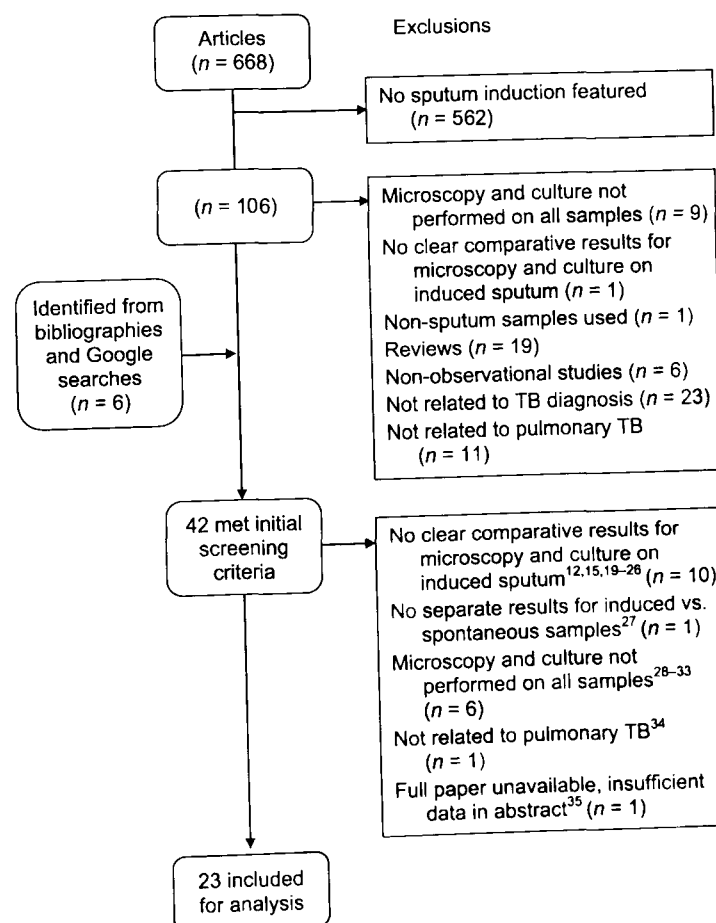
Figure 1 Flow diagram of study selection process.

for the following reasons: 10 had no clear comparative results for microscopy and culture on induced sputum;[12,15,19–26] 1 had no separate results for induced vs. spontaneous samples;[27] for 6 studies, microscopy and culture were not performed on all samples;[28–33] 1 study was not related to pulmonary TB,[34] and for 1 study, the full paper was unavailable, in spite of attempts to contact the authors.[35]

Heterogeneity among studies

Study characteristics are summarised in the Table. Ten studies were carried out in Africa,[36–45] 5 in Asia,[13,46–49] 4 in the Americas[16,50–52] and 4 in Europe.[14,17,53,54] The majority of the studies included HIV-positive patients: 13 had mixed HIV-positive and -negative populations,[14,16,36,37,39–42,44,45,50,51,53] two included only HIV-positive patients,[43,52] one included only HIV-negative patients,[48] and the remainder did not report HIV status.[13,17,38,46,47,49,54] Fifteen studies were conducted exclusively among adults,[13,14,37,41–43,45,47–54] six exclusively among children,[36,38–40,42,44,46] one included both children and adults,[17] and one did not state age.[16]

Most studies (20/23) selected patients based on clinical suspicion of TB infection.[13,14,17,36–39,41–50,52–54] One also included HIV-positive patients, regardless of symptoms.[45] Seven studies used radiology to confirm suspicion,[13,14,42,46–48,54] 10 recruited patients who were unable to expectorate spontaneously,[14,17,37,42,43,47–50,53] and 7 recruited patients who had had smear-negative microscopy results following spontaneous expectoration.[37,41–43,48,50,53] Sixteen studies were prospective cohort studies,[13,14,17,37,40–46,48,50–53] 4 were cross-sectional studies,[36,38,47,49] 2 were prospective multicentric cohorts,[39,54] and 1 was a retrospective review.[16]

Ultrasonic nebulisers were used in 15 studies,[14,17,37,38,41–43,45,47–51,53,54] 4 used jet nebulisers,[16,39,40,45] 1 used a Venturi-type face mask nebuliser,[17] 3 used other techniques,[13,46,52] and 2 did not state type of nebuliser.[36,44] Sputum induction was the only sampling method investigated in 12 studies.[13,16,17,36–38,44,45,47,50,51,53] Six studies also investigated gastric lavage,[14,39–41,46,49] 2 investigated fibreoptic bronchoscopy,[48,54] 1 investigated nasopharyngeal aspiration,[46] 2 investigated bronchoalveolar lavage (BAL),[14,41] and 2 investigated physiotherapy (Table 1).[41,52] One study also investigated the string test and blood culture,[42] while another study performed culture of extra-pulmonary samples as appropriate.[43]

Of 23 studies, 3 used direct Ziehl-Neelsen (ZN) microscopy,[13,37,46] 1 used concentrated ZN microscopy,[41] 1 used direct fluorescent microscopy,[38] 7 used concentrated fluorescent microscopy,[14,39,40,42,44,45,50] 8 used ZN or Kinyoun microscopy where use of sputum concentration was not stated,[16,36,38,47–49,52,54] and 4 used fluorescent microscopy where use of sputum concentration was not stated.[17,43,51,53] Thirteen studies exclusively used solid media (Löwenstein-Jensen, Middlebrook, Ogawa or a combination),[13,17,36–38,41,42,46–49,51,52] 5 ex-

clusively used liquid media (BACTEC™, BacT/ALERT® or Mycobacteria Growth Indicator Tube),[14,39,40,44,45] and 3 used a combination of liquid and solid media.[43,50,54] The remainder did not state the culture media used.[16,53] Half (11/23) collected one sample,[13,17,36,38,40–42,47,49,52,53] and the remainder collected multiple samples.[14,16,37,39,43,44–46,50,51,54] Two studies collected 1 sample per type of nebuliser assessed,[17,45] and 1 study did not clearly state the number of samples collected.[48]

Study outcomes

The success of the induction procedure could be calculated for all but one study.[16] Overall, success was high, ranging from 76.4% (95%CI 68.5–83.2) to 100% (95%CI 98.5–100). Adverse events associated with sputum induction were infrequent and mild, and included cough,[13,39,40] nausea,[13,17] vomiting,[42] epistaxis, coughing, wheezing and vomiting,[39,40] and nose bleeds, vomiting, increased cough and wheezing.[44] Four studies reported that there were no adverse events.[37,38,45,48] Eleven studies did not report adverse events (Appendix).[16,36,42,43,46,47,50–54]*

Positivity rates ranged from 0% (95%CI 0–13.7) to 41.8% (95%CI 28.7–55.9) for microscopy smears, and from 2.4% (95%CI 1.1–4.5) to 100% (95%CI 85.8–100) for cultures. These estimates are primarily dependent on background TB prevalence rather than method of assessment. Among those patients who had previously been found negative on spontaneous smear microscopy, or who had not been able to expectorate, microscopy positivity rates ranged from 2% to 41.8%.[37,41–43,48–50,53]

The sensitivity of microscopy compared to culture ranged from 0% (95%CI 0–26.5)[47] to 100%.[44] These results are summarised in Figure 2. (Note that for one study, more patients were found to be positive on microscopy than culture; as confidence intervals could not be generated, this study is not represented).[44] Three studies reported smear-positive, culture-negative samples: one study reported that one sample that was positive on microscopy was negative on culture,[38] another stated that sputum samples from four children were microscopy-positive and culture-negative,[39] and a third reported that no smear-positive samples were culture-positive, and no culture-positive samples were smear-positive.[44] Eight of 23 studies investigated the species of mycobacteria discovered. Two studies reported that 100% of species found were *Mycobacterium tuberculosis* complex,[37,44] and two more that speciation had been performed, and did not describe the isolation of non-tuberculous mycobacteria (NTM).[43,45] One study isolated NTM, but excluded these patients from the study.[47] Another

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* The Appendix is available in the online version of this article.

Study	Country	Year	Study type	Age	HIV status n/N	Selection criteria	Sample size*	Samples per patient	Type of nebuliser	Comparison with other sampling methods
Al-Aghbari[46]	Yemen	2003–2005	Prospective cohort	Children; median: 5 years	NS	Clinical suspicion of PTB, including radiological suspicion	88	1–3	Salbutamol via metered dose inhaler and oxygen	Nasopharyngeal aspiration and gastric aspiration
Al Zahrani[50]	Canada	1995–1998	Prospective cohort	Adults; mean: 44 years†	2/60 HIV+ (3.3%)	Clinical suspicion of PTB; spontaneous smear-negative or unable to expectorate spontaneously	503	>1	Ultrasonic	None
Atiq-ur-Rehman[13]	Pakistan	2006	Prospective cohort	Adults; mean: 34.3 years	NS	Strong clinical and radiological suspicion of PTB	164	1	Compressor	None
Bell[41]	Malawi	2005–2006	Prospective cohort	Adults; mean: 36 years	75/111 HIV+ (89%)	Clinical diagnosis of smear-negative PTB	111	1	Ultrasonic	Physiotherapy, gastric washing and BAL
Breen[53]	United Kingdom	2005–2006	Prospective cohort	Adults; median: 32 years	16/42 HIV+ (38%)	Clinical suspicion of TB; spontaneous smear negative or unable to expectorate spontaneously	42	1	Ultrasonic	None
Brown[14]	United Kingdom	2004–2006	Prospective cohort	Adults; median: 28 years	3/84 HIV+ (3.6%)	Clinical suspicion of TB; unable to expectorate spontaneously, radiography suggestive of TB	127	5	Ultrasonic	Gastric washing/aspiration and BAL
Iriso[36]	Uganda	NS	Cross-sectional	Children; mean: 25.5 months	62/126 HIV+ (49%)	Clinical suspicion of TB	101	1	NS	None
Kawada[49]	Japan	1994–1996	Cross-sectional	Adults; mean: 39 years	NS	Clinical suspicion of TB; unable to expectorate spontaneously	22	1	Ultrasonic	Gastric aspiration
Kawada[47]	Urban hospital, Japan	1996–1997	Cross-sectional	Adults; mean: 37 years	NS	Clinical suspicion of PTB, including radiological suspicion, unable to expectorate spontaneously	27	1	Ultrasonic	None
Klein[51]	Urban Teaching Hospital, USA	1990–1992	Prospective cohort	Adults; age not stated	251/373 HIV+ (67%)	Suspected *Pneumocystis carinii* pneumonia	373†	1 or more	Ultrasonic	None
Kranzer[45]	South Africa	2010	Prospective cohort	Adults; median: 39 years	66/123 HIV+ (53.7%)	HIV+ or symptoms suggestive of TB infection	109	1 per nebuliser type	Ultrasonic and human-powered (jet)	None
Merrick[16]	Urban Teaching Hospital, USA	1989-1994	Retrospective review	NS	17/24 HIV+ (70.8%)	Patients with culture-positive TB	24	3	Jet	None
Moore[44]	Primary health care clinic, South Africa	2007–2009	Prospective cohort	Children; median: 38 months	48/270 HIV+ (18%)	Clinical suspicion of TB, adult household contact, or newly HIV-diagnosed with respiratory symptoms	270	2	NS	None

Morse[42]	Botswana	2006–2007	Prospective cohort	Adults; male range: 20–80 years; female range: 21–69 years	114/140 HIV+ (81.4%)	Clinical suspicion of TB, including radiological suspicion, unable to expectorate spontaneously or smear-negative on spontaneous expectoration	140	1	Ultrasonic	Urine culture, string test culture and blood culture
Parry[37]	Urban hospital, Malawi	NS	Prospective cohort	Adults; mean: 35 years	37/82 HIV+ (45.1%)	Clinical suspicion of TB, unable to expectorate spontaneously or smear-negative on spontaneous smear	82	1 to 3	Ultrasonic	None
Saglam[48]	Turkey	2001–2003	Prospective cohort	Adults; mean: 35.8 years	100% HIV−	Clinical suspicion of TB, including radiological suspicion; smear-negative on spontaneous expectoration or unable to expectorate spontaneously	55	Unclear	Ultrasonic	Fibreoptic bronchoscopy
Schoch[54]	Switzerland	2003–2005	Prospective cohort	Adults; mean: 38 years	NS	Asylum seekers with radiology suggestive of TB infection	91	2 (results presented are for second sample only)	Ultrasonic	Bronchoscopy
Shata[38]	Malawi	NS	Cross-sectional	Children; range: 3–15 years	NS	Clinical suspicion of TB infection	30	1	Ultrasonic	None
Souza Pinto[52]	Brazil	2004–2005	Prospective cohort	Adults; age not stated	100% HIV+	Adult clinical TB suspects, HIV+	132	1	Regular-flow oxygen	Chest physiotherapy
Toubes[17]	Spain	1997-2000	Prospective cohort	Adults and children; range: 7–90 years	NS	Active TB suspects, unable to expectorate spontaneously	90	1 sample per induction technique (results of both techniques combined)	Ultrasonic + Venturi-type face mask	None
Wilson[43]	South Africa	2002	Prospective cohort	Adults; age not stated	100% HIV+	Smear-negative TB suspects, unable to produce sputum or 2× spontaneous smear-negative	147	1–2	Ultrasonic	Culture of blood, urine and extra-pulmonary aspirates where appropriate
Zar[40]	South Africa	1998	Prospective cohort	Children; median: 9 months	100/142 HIV+ (70.4%)	Primary diagnosis of pneumonia; HIV+ or suspected positive; or admitted to ICU	149	1	Jet	Gastric lavage
Zar[39]	South Africa	2000–2002	Prospective cohort	Children; median: 13 months	95/250 HIV+ (38%)	Suspected pulmonary tuberculosis	250	3	Jet	Gastric lavage

* Sample size reported for cohort attempting sputum induction.
† Calculated.
‡ Final sample size (initial sample on which induction was attempted is unclear).
HIV = human immunodeficiency virus; NS = not stated; PTB = pulmonary TB; + = positive; TB = tuberculosis; − = negative; BAL = bronchoalveolar lavage; ICU = intensive care unit.

Study	Year	Sensitivity (95% CI)	Microscopy +ve	Culture +ve
Children				
Al-Aghbari	2009	46.20 (19.20–74.90)	6	13
Iriso	2005	40.00 (22.70–59.40)	12	30
Shata	1996	57.10 (18.40–90.10)	4	7
Zar	2000	20.00 (4.30–48.10)	3	15
Zar	2005	49.00 (34.80–63.40)	25	51
Adults				
Al Zahrani	2000	22.70 (11.50–37.80)	10	44
Atiq-ur-Rehman	2009	77.80 (60.80–89.90)	28	36
Bell	2009	44.40 (13.70–78.80)	4	9
Breen	2007	30.80 (9.10–61.40)	4	13
Brown	2007	30.90 (17.60–47.10)	13	42
Kawada	1996	29.40 (10.30–60.00)	5	17
Kawada	1998	0.00 (0.00–26.50)	0	12
Klein	1993	66.70 (29.90–92.50)	6	9
Kranzer	2011	42.90 (9.90–81.60)	3	7
Morse	2008	37.50 (24.00–52.30)	18	48
Parry	1995	60.00 (40.60–77.30)	18	30
Saglam	2005	74.20 (55.40–88.10)	23	31
Schoch	2007	47.10 (23.00–72.20)	8	17
Souza Pinto	2007	66.60 (41.00–86.70)	12	18
Wilson	2006	38.40 (28.00–49.50)	33	86
Mixed/unknown age				
Merrick	1997	25.00 (9.80–46.70)	6	24
Toubes	2005	20.50 (9.30–36.50)	8	39

0 20 40 60 80 100
Percentage

Figure 2 Forest plot summarising the sensitivity of microscopy compared to culture for induced sputum.

study reported that two microscopy smears (0.54%) were positive for *M. avium* complex and 64 cultures (17.2%) were positive for NTM.[51] A further two studies reported NTM cultures, but it was unclear if these were from induced sputum,[52] or included in the main analysis.[17]

Comparison with other sputum collection techniques
Eleven of 23 studies compared sputum induction with other sputum collection techniques. Six studies compared sputum induction with gastric aspiration: in three of these studies, the positivity rate for microscopy was the same for both techniques, while the sensitivity of microscopy compared to culture was higher for gastric aspiration,[14,40,46] and in three studies the microscopy positivity rate was higher for induced sputum, while sensitivity compared to culture was higher for sputum induction.[39,41,49] One study investigated use of nasopharyngeal aspiration, and the yield for both microscopy and culture was higher with nasopharyngeal aspiration, with the technique also showing a higher sensitivity for microscopy compared to culture.[46] BAL and fibreoptic bronchoscopy were compared with sputum induction in four studies. For two studies, the yield using culture and microscopy, as well as the sensitivity of microscopy in relation to culture, was higher on induced sputum.[14,54] One study showed higher yields from in-

duced sputum for both microscopy and culture, yet the sensitivity for microscopy compared to culture was higher for BAL,[41] and another study had higher positivity rates on bronchoscopy for both microscopy and culture, as well as higher sensitivity for microscopy than culture.[48] Physiotherapy was investigated in two studies. In one study, positivity rates for microscopy, as well as sensitivity compared to culture, were higher from the chest physiotherapy sample, but positivity rates for culture were the same;[52] the second study had higher positivity rates on both microscopy and culture, as well as higher sensitivity for microscopy compared with culture, for chest physiotherapy samples.[41] Figure 3 summarises the sensitivity of microscopy compared to culture for those studies in which multiple sample collection techniques were assessed. Results from two studies reported sensitivities of over 100% (for lymph node biopsy,[43] and physiotherapy;[52] these studies are not represented in Figure 3).

Assessment of methodological quality
We assessed all full articles for methodological quality. All but one[51] studied a sample of patients that could be considered to be representative of the population who would undergo sputum induction for TB diagnosis in practice, and all but three[17,46,54] studies accounted for withdrawals; 9 studies provided a clear

Study	Year	Sensitivity (95% CI)	Microscopy +ve	Culture +ve
Sputum induction				
Al-Aghbari	2009	46.20 (19.20–74.90)	6	13
Bell	2009	44.40 (13.70–78.80)	4	9
Brown	2007	30.90 (17.60–47.10)	13	42
Kawada	1996	29.40 (10.30–60.00)	5	17
Saglam	2005	74.20 (55.40–88.10)	23	31
Schoch	2007	47.10 (23.00–72.20)	8	17
Souza Pinto	2007	66.60 (41.00–86.70)	12	18
Wilson	2006	38.40 (28.00–49.50)	33	86
Zar	2000	20.00 (4.30–48.10)	3	15
Zar	2005	49.00 (34.80–63.40)	25	51
Nasoph. aspirate				
Al-Aghbari	2009	71.40 (41.90–91.60)	10	14
Gastric aspirate				
Al-Aghbari	2009	52.60 (28.90–75.60)	10	19
Bell	2009	30.00 (6.70–65.20)	3	10
Brown	2007	37.50 (21.10–56.30)	12	32
Kawada	1996	14.30 (0.40–57.90)	1	7
Zar	2000	33.30 (7.50–70.10)	3	9
Zar	2005	44.70 (28.60–61.70)	17	38
Physiotherapy				
Bell	2009	50.00 (18.70–81.30)	5	10
Bronch. lavage				
Bell	2009	50.00 (11.80–88.20)	3	6
Brown	2007	23.80 (8.20–47.20)	5	21
Saglam	2005	78.80 (61.10–91.00)	26	33
Schoch	2007	26.70 (7.80–55.10)	4	15
Urine				
Wilson	2006	16.30 (6.80–30.70)	7	43
Cold abscess aspirate				
Wilson	2006	50.00 (18.70–81.30)	5	10

0 20 40 60 80 100
Percentage

Figure 3 Forest plot summarising the sensitivity of microscopy compared to culture for studies comparing different sputum collection methods.

definition of what was considered to be a positive result.[16,17,39,40,42–44,50,53] Overall, we rated the methodological quality of the studies as adequate.

DISCUSSION

Improved detection of pulmonary TB is considered a priority by the World Health Organization and the Stop TB Partnership. The current frontline diagnostic test, smear microscopy, lacks sensitivity. Case finding might be augmented through the implementation of sputum induction to improve the quality of samples examined. Our systematic review of the published data found that the success of sputum induction was high across a range of study settings and patient groups.

Studies used both ZN and fluorescent microscopy. Fluorescent microscopy has been found to be more sensitive than ZN microscopy, but it can also be prone to lower specificity due to the appearance of artifacts.[55] Both solid and liquid culture was performed. Liquid culture is known to be more sensitive than solid, but it is also more prone to contamination and to grow NTM.[56] Due to the slow growth of

M. tuberculosis and the sophisticated laboratory facilities required, culture is rarely available as a diagnostic option in high-burden countries.

The overall success rate for induction was high, with no important difference between patient groups of study settings, implying that the procedure is worth implementing in diverse settings where patients find spontaneous expectoration difficult. Sputum induction is potentially highly beneficial for HIV-positive patients, as they commonly have problems expectorating spontaneously and sputum smear microscopy is known to be less sensitive in this patient population. Results were similar for studies that included children, another group for whom sputum expectoration may be difficult. Good results were found among people who were spontaneous smear-negative or who were unable to expectorate. The procedure was well tolerated, with most studies reporting only mild and infrequent side-effects. Epistaxis, reported by two paediatric studies, should be carefully considered as it poses an infection risk to health care workers, particularly in high HIV prevalence settings. Because sputum induction induces coughing, which generates micro-aerosols, it has been recommended that it

should only be performed when spontaneous expectoration has failed, and using exhaust ventilation devices, with health care workers using respiratory protection.[57] Guidelines for resource-limited settings recommend optimising natural ventilation in places where sputum induction is performed.[58]

The sensitivity of microscopy on induced sputum compared to culture varied considerably across studies, with no clear influence of study setting or patient population. Although sensitivity was low, it allows for a more accurate diagnosis compared to the use of non-specific clinical diagnostic algorithms that are often the only alternative at peripheral health centres in resource-limited settings where culture facilities are not available or difficult to access. The sensitivity reported by some studies for microscopy compared to culture is similar to sensitivity for direct smear microscopy, and thus implies that it is worth implementing when only microscopy can be performed. Patients with negative results can be reassessed following a diagnostic algorithm. On-site testing also reduces loss to follow-up that can result when samples are referred to a central laboratory for further investigation.

We did not find any important difference in yield when comparing studies in which single or multiple sampling was done, which is an important consideration for resource-limited settings, as single sampling reduces processing time and cost. This would also facilitate same-day diagnosis, in line with the proposed move to same-day sample collection and microscopy to replace sample collection on consecutive days.[59]

The other sampling techniques evaluated in these studies mostly resulted in lower positivity rates than sputum induction. Notably, physiotherapy resulted in the same yield as sputum induction in one study; this merits further investigation—physiotherapy is an intervention that may be of particular use in resource-limited settings as it does not require expensive equipment or intensive training. However, the overall performance of these different techniques could not be assessed due to insufficient reporting of specificity data.

Strengths of this review include an extensive search strategy that identified 23 studies reporting our primary outcome, and analytical approaches to assess differences between study and patient characteristics. We described potential sources of heterogeneity, but were unable to provide pooled estimates, as very few studies reported specificity, which limited our ability to account for the trade-off between sensitivity and specificity required for more robust assessments of diagnostic accuracy.[60] The sensitivity of spontaneous smear microscopy can be affected by numerous factors, including sputum quality, smear preparation, staining procedures, examination time and the amount of training received in accurate smear examination, but too few studies reported this information for it to be formally assessed. Finally, all systematic reviews

are subject to potential publication bias. However, the determinants and extent of publication bias for diagnostic studies is unclear, and because statistical tests are not generally applicable,[18] this was not formally assessed.

A potential limitation is the use of strict exclusion criteria, which led to the exclusion of some large-scale studies.[15,19,20,28] The question being researched by this review involved the comparison of microscopy yields on induced sputum compared with culture yields. As such, studies had to demonstrate clear and comparable data for yields on microscopy and culture to be included.

Our systematic review highlights several areas for future research. In particular, future research should report the proportion of microscopy-positive and culture-negative samples, as well as the species of mycobacteria isolated so that conclusions on the accuracy of induced sputum can be made. The performance of sputum induction compared to other techniques merits further assessment. Further research is also warranted to determine the optimal combination of nebuliser and saline concentrations, as this varies considerably between studies. Finally, the use of induced sputum in newer tests such as the Xpert® MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) should be vaildated.[61,62]

In conclusion, sputum induction can be useful when implemented for use with smear microscopy for people who are negative on spontaneous smear microscopy or who are unable to expectorate spontaneously, and is well-tolerated by children and adults alike. Induction procedures have a high success rate. As it is a cough-generating procedure, biosafety issues must be carefully considered prior to implementation. The technique requires less infrastructure than that required for culture facilities, and as such can potentially be implemented for use with microscopy in peripheral areas. However, most studies were not performed in peripheral settings, and further research is required to determine the applicability of sputum induction to such settings, although initial evidence of implementation in resource-limited settings is promising.[44]

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Appendix Study outcomes

Study	Microscopy method	Culture method	Success of sputum induction		Adverse events	Positivity rate		Sensitivity (microscopy vs. culture)		Specificity of sputum induction microscopy vs. culture
			Successfully induced n/N, % (95%CI)	Reasons for failure		Microscopy n/N, % (95%CI)	Culture n/N, % (95%CI)	Sputum induction n/N, % (95%CI)	Other methods n/N, % (95%CI)	
Al-Aghbari[46]	Direct ZN	Solid (Ogawa)	82/88, 93.1 (85.7–97.4)	NS	NS	6/82, 7.3 (2.7–15.2)	13/82, 15.9 (8.7–25.6)	6/13, 46.2 (19.2–74.9)	Nasopharyngeal aspirate 10/14, 71.4 (41.9–91.6) Gastric aspirate 10/19, 52.6 (28.9–75.6)	NS
Al Zahrani[50]	Concentrated fluorescent	Liquid (BACTEC™ 460) + solid (LJ)	500/503, 99.4 (98.2–99.9)	NS	NS	10/500, 2 (1.0–3.6)	44/500, 8.8 (6.5–11.6)	10/44, 22.7 (11.5–37.8)	NA	NS
Atiq-ur-Rehman[13]	Direct ZN	Solid (LJ)	132/164, 80.5 (73.6–86.3)	NS	Cough (21; 5 led to bronchospasm) Nausea (19)	28/132, 21.2 (14.6–29.2)	36/132, 27.3 (19.9–35.7)	28/36, 77.8 (60.8–89.9)	NA	NS
Bell[41]	Concentrated ZN	Solid (LJ)	111/111, 100 (96.7–100)	—	NS	4/111, 3.6 (1.0–9.0)	9/111, 8.1 (3.8–14.8)	4/9, 44.4 (13.7–78.8)	Physiotherapy 5/10, 50 (18.7–81.3) Gastric washing 3/10, 30 (6.7–65.2) BAL 3/6, 50 (11.8–88.2)	NS
Breen[53]	Fluorescent, unclear if concentrated	Unclear	42/42, 100 (91.6–100)	—	NS	4/42, 9.5 (2.7–22.6)	13/42, 40.0 (17.6–47.1)	4/13, 30.8 (9.1–61.4)	NA	NS
Brown[14]	Concentrated fluorescent	Liquid (BacT/ALERT®)	107/140, 76.4 (68.5–83.2)	NS (excluded if fewer than 5 samples provided)	Intolerance (1/126)*	13/107, 12.1 (6.6–19.9)	42/107, 39.2 (30.0–49.2)	13/42, 30.9 (17.6–47.1)	Gastric aspirate 12/32, 37.5 (21.1–56.3) BAL 5/21, 23.8 (8.2–47.2)	NS
Iriso[36]	ZN, unclear if concentrated	Solid (LJ)	101/101, 100 (96.4–100)	—	NS	12/101, 11.9 (6.3–19.8)	30/101, 29.7 (21.0–39.6)	12/30, 40 (22.7–59.4)	NA	NS
Kawada[49]	ZN, unclear if concentrated	Solid (Ogawa)	22/22, 100 (84.6–100)	—	NS	5/22, 22.7 (7.8–45.4)	17/22, 77.3 (54.6–92.2)	5/17, 29.4 (10.3–60.0)	Gastric aspirate 1/7, 14.3 (0.4–57.9)	NS
Kawada[47]	ZN, unclear if concentrated	Solid (Ogawa)	25/27, 92.6 (75.7–99.1)	NS	NS	0/25, 0.0 (0–13.7)	12/25, 48 (27.8–68.7)	0/12, 0.0 (0–26.5)	NA	NTM isolated from 4 patients; these were excluded from the study
Klein[51]	Fluorescent, unclear if concentrated	Solid (LJ + Middlebrook 7H10)	519/563, 92.2 (89.7–94.3)‡	Inadequate for staining or smear	NS	6/373, 1.6 (0.1–3.5)	9/373, 2.4 (1.1–4.5%)	6/9, 66.7 (29.9–92.5)§	NA	Microscopy: 2/373 (0.54%) = M. avium complex Culture: 64/373 (17.2%) = NTM
Kranzer[45]	Fluorescent, concentrated	Liquid (MGIT)	109/114, 95.6 (90.0–98.6)	NS	None	3/109, 2.7 (0.6–7.8)	7/109, 6.4 (2.6–12.8)	3/7, 42.9 (9.9–81.6)	NA	Speciation performed, no NTM reported
Merrick[48]	Kinyoun, unclear if concentrated	NS	24 (denominator unclear)	NS	NS	6/24, 25 (9.8–46.7)	24/24, 100 (85.8–100)	6/24, 25 (9.8–46.7)	NA	NS

Study	Staining	Culture media	Sensitivity (95% CI)	Inability to produce sputum	Adverse events				Other specimens	Notes
Moore[44]	Fluorescent, concentrated	Liquid (MGIT)	268/270, 99.3 (97.3–99.9)	NS	Nose bleed 75/496, 15.1 (12.1–18.6) Vomiting 11/496, 2 (1.1–3.9) Increased cough 30/496, 6 (4.1–8.5) Wheeze 3/496, 0.6 (0.1–1.8)	17/270, 6.3 (3.7–9.9)	12/270, 4.4 (2.3–7.6)	17/12, 100	NA	All culture-positive samples M. tuberculosis
Morse[42]	Fluorescent, concentrated	Solid (LJ)	139/140, 99.3 (96.1–100.0)	Failure to produce sputum	Vomiting 2/140, 1.4 (0.2–5.1)	18/139, 12.9 (7.9–20.0)	48/139, 34.5 (26.7–43.1)	18/48, 37.5 (24.0–52.3)	Microscopy not performed on other methods	NS
Parry[37]	Direct ZN or fluorescent	Solid (LJ)	73/82, 89.0 (80.2–94.9)	NS	0/73	18/73, 24.7 (15.3–36.1)	30/73, 41.1 (29.7–53.2)	18/30, 60 (40.6–77.3)	NA	All culture-positive samples M. tuberculosis complex
Saglam[48]	ZN, unclear if concentrated	Solid (LJ)	55/55, 100 (93.5–100)	NA	0/55	23/55, 41.8 (28.7–55.9)	31/55, 56.4 (42.3–69.7)	23/31, 74.2 (55.4–88.1)	BAL 26/33, 78.8 (61.1–91.0)	NS
Schoch[54]	ZN, unclear if concentrated	Solid and liquid - media not stated	91/91, 100 (96.0–100)	NA	NS	8/91, 8.8 (3.9–16.6)	17/91, 18.7 (11.3–28.2)	8/17, 47.1 (23.0–72.2)	BAL 4/15, 26.7 (7.8–55.1)	NS
Shata[38]	ZN, unclear if concentrated	Solid (LJ)	29/30 96.6 (82.8–99.1)	NS	0/30	4/29, 13.8 (3.9–31.6)	7/29, 24.1 (10.3–43.5)	4/7, 57.1 (18.4–90.1)	NA	1 sample microscopy-positive and culture-negative
Souza Pinto[52]	ZN, unclear if concentrated	Solid (LJ)	132/132, 100 (97.2–100)	NA	NS	12/132, 9.1 (4.8–15.3)	18/132, 13.6 (8.2–20.7)	12/18, 66.6 (41.0–86.7)	Physiotherapy 21/18 (116.6)	9/34 (26.5%) of all strains cultured NTM; number occurring in induced sputum samples unclear
Toubes[17]	Fluorescent, unclear if concentrated	Solid (LJ + Middlebrook)	89/94, 94.7 (88.0–98.3)	Nausea	Nausea 1/94	8/89, 9.0 (4.0–16.9)	39/89, 43.8 (33.3–54.7)	8/39, 20.5 (9.3–36.5)	NA	2 samples culture-positive for NTM; unclear if these samples were included in analysis
Wilson[43]	Fluorescent, unclear if concentrated	Liquid (MGIT) and solid (LJ)	136/147, 92.5 (87.0–96.2)	NS	NS	33/130, 25.8 (18.2–33.8)	86/130, 66.2 (57.3–74.2)	33/86, 38.4 (28.0–49.5)	Urine 7/43, 16.3 (6.8–30.7) Blood NA/36 Lymph node biopsy 21/17, 123.5 Pleural/ascetic exudate NA/14 Cold abscess aspirate 5/10, 50 (18.7–81.3)	Speciation performed: no NTM described
Zar[40]	Concentrated fluorescent	Liquid (BACTEC™ 12B)	142/149, 95.3 (90.6–98.1)	Too ill; increasing tachypnoea or cough	Mild epistaxis (6/142) Coughing (8/142) Wheezing (3/142)	13/142, 9.1 (5.0–15.1)	15/142, 10.6 (6.0–16.8)	3/15, 20 (4.3–48.1)	Gastric aspirate 3/9, 33.3 (7.5–70.1)	Not stated
Zar[39]	Concentrated fluorescent	Liquid (BACTEC™ 12B)	250/250, 100 (98.5–100)	NA	Coughing¶ (293/721) Epistaxis (55/721) Vomiting (3/721) Wheezing 2/721	25/250, 10.0 (6.6–14.4)	51/250, 20.4 (15.6–25.9)	25/51, 49.0 (34.8–63.4)	Gastric aspirate 17/38, 44.7 (28.6–61.7)	Sputum samples from 4 children (6%) microscopy-positive, culture-negative

* Denominator includes patients who underwent at least one induction.
† 2/22 could only expectorate when saline concentration was increased from 3% to 5%.
‡ Study reports number of adequate sputum samples collected rather than number of patients.
§ Excludes NTM.
¶ Data refer to number of inductions.
ZN = Ziehl-Neelsen; NS = not stated; LJ = Löwenstein-Jensen; NA = not applicable; BAL = bronchioalveolar lavage; NTM = non-tuberculosis mycobacteria; MGIT = Mycobacteria Growth Indicator Tube.

_____ R É S U M É

CONTEXTE : Dans les contextes à ressources limitées, on utilise l'examen microscopique des crachats pour le diagnostic de la tuberculose (TB), mais la sensibilité de ce test est médiocre et les taux de détection des cas sont faibles. On a proposé l'expectoration provoquée comme moyen d'améliorer le recueil des échantillons et de renforcer la sensibilité du test.

OBJECTIF : Entreprendre une revue systématique des études comparant la sensibilité de la bacilloscopie et de la culture dans les échantillons d'expectorations provoquées.

MÉTHODES : Nous avons mené en double des recherches des bases de données jusqu'à août 2011 et des sites accessibles concernant les principales conférences sur le VIH et la TB (jusqu'à novembre 2010) afin d'identifier les études comparant les performances de la bacilloscopie par comparaison avec la culture dans les échantillons d'expectorations provoquées en utilisant comme standard de référence le résultat de la culture.

RÉSULTATS : Nos critères d'inclusion ont été rencontrés dans 23 études. Les taux globaux de succès de l'induction ont été élevés, allant de 76,4% (IC95% 68,5–83,2) à 100% (IC95% 98,5–100), alors que les effets indésirables associés à l'induction des expectorations étaient rares et peu graves. La sensibilité de l'examen microscopique par comparaison avec la culture s'est étalée entre 0% et 100% ; huit études seulement ont signalé l'espèce de mycobactéries isolée par la culture. Le rendement est généralement plus élevé pour l'induction des crachats par comparaison avec l'aspiration naso-pharyngienne et le tubage gastrique et se compare à égalité avec le lavage broncho-alvéolaire et la physiothérapie.

DISCUSSION : L'induction des crachats augmente le taux de détection des cas de TB et est utile chez les sujets dont l'examen direct des frottis d'expectoration spontanée est négatif ou qui sont incapables d'expectorer spontanément. Cette technique est bien tolérée chez les enfants et les adultes, quel que soit leur statut VIH, et peut être utilisée lorsque la culture n'est pas disponible. L'utilisation d'échantillons de crachats provoqués pour les tests moléculaires tels que Xpert® MTB/RIF mérite des investigations complémentaires.

_____ R E S U M E N

MARCO DE REFERENCIA: En los entornos con recursos limitados el diagnóstico de la tuberculosis (TB) se basa en el examen microscópico del esputo, pero la sensibilidad de esta prueba es baja y se logran tasas bajas de detección de casos.

OBJETIVO: Utilizar la inducción del esputo como un método que permite mejorar la calidad de las muestras recogidas y aumentar la sensibilidad de la prueba. Se llevó a cabo un análisis sistemático de los estudios publicados que comparan la sensibilidad de la baciloscopia y el cultivo en las muestras obtenidas mediante inducción del esputo.

MÉTODOS: Se llevaron a cabo búsquedas sistemáticas en duplicado en las bases de datos (hasta agosto del 2011) y en los sitios web consultables de las principales conferencias sobre la infección por el virus de la inmunodeficiencia humana (VIH) y la TB (hasta noviembre del 2010), con el fin de escoger los estudios que comparaban el rendimiento diagnóstico de la baciloscopia y el cultivo en muestras de esputo inducido, tomando el cultivo como norma de referencia.

RESULTADOS: Veintitrés estudios cumplieron con los criterios de inclusión. La eficacia global de la inducción fue alta, entre 76,4% (95%IC 68,5–83,2) y 100% (IC95% 98,5–100) y los efectos adversos relacionados con la inducción del esputo fueron infrecuentes y leves. La sensibilidad del examen microscópico comparado con el cultivo osciló entre 0% y 100%; solo en ocho estudios se comunicó la especie de micobacteria aislada en el cultivo. El rendimiento diagnóstico fue generalmente superior con las muestras obtenidas mediante la inducción del esputo que con la aspiración nasofaríngea o el lavado gástrico y fue equivalente al rendimiento de las muestras tomadas durante el lavado broncoalveolar o con la asistencia de la fisioterapia.

CONCLUSIÓN: La inducción del esputo aumenta la detección de casos de TB y es útil en las personas con una baciloscopia negativa en muestras recogidas de manera natural o en quienes no consiguen expectorar espontáneamente. La inducción es bien tolerada por los niños y los adultos, independientemente de su estado frente al VIH y se puede realizar en medios donde no se cuenta con el cultivo. Se debe ampliar la investigación con las muestras de esputo inducido al uso de las pruebas moleculares como el Xpert® MTB/RIF.

Annex 6: BRTI protocols

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1.0 Purpose and scope:

This procedure describes how to perform *Mycobacterium tuberculosis* (TB) microscopy and culture of Sputum, Pleural fluids and Pus aspirate   samples

2.0 Responsibility:

The Departmental head maintains this procedure and ensures its implementation

3.0 Safety considerations

HAZARD	WHEN,HOW AND WHY COULD CONTACT OCCUR	CONTROL SYSTEM
Biological -Bacterial Cultures -Blood and body fluids	-Entry through skin contact -Hand to mouth entry -Aerolisation	-Strict methods of personal hygiene must be observed. -Wearing of personal protective equipment -Prevention of aerolisation -Disposal of biological waste in biological waste bags. Disposal of chemicals as stipulated by manufacturer - Use of Safety Cabinets Class II always
Physical -Bunsen burner -Glass slides, coverslips and capillary tubes	-Possible burns -Sharps injury	-Awareness of naked flame -Disposal of glass in sharps container

4.0 Introduction

Microscopic examination of appropriately stained sputum specimens for tubercle bacilli by either auramine or Ziehl-Neelsen (ZN) staining is the cornerstone of Laboratory diagnosis of tuberculosis.

Culture provides the definitive diagnosis of tuberculosis because it is virtually impossible to distinguish different mycobacterium species by microscopy. Also, not all positive tuberculosis patients harbour large enough numbers of organisms to be detected by microscopy (5000-10000 tubercle bacilli/ml) but culture picks up these few organisms that are missed by microscopy. It is therefore necessary to carry out bacteriological cultures in parallel with microscopic examinations in a research setting.

5.0 SPECIMEN COLLECTION AND HANDLING
Refer to Primary sample collection SOP

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6.0   Criteria for specimen acceptance

6.1   The specimen label should include:
   a) Patients/ participant ID number or study ID.
   b) Collector's initials
   c) Type of specimen

6.2   The requisition on request form should include:
   a) Patients first and last name/ Participants ID number
   b) Collectors initials
   c) Indication whether patient is on TB treatment or not
   d) Date of collection
   e) Time of collection (where applicable)

Note: All specimens are accepted regardless of volume and appearance. However, with reference to Policy manual clause 5.4 subsection 5.4.8 if information on the specimen does not match the information on the request form, the sample is processed and comments will be included in the final report.

7.0   Specimen receipt

7.1   Materials

   a) Gloves
   b) Specimen Accession Book (Work Book)
   c) 5% Phenol disinfectant

7.2   Procedure

7.2.1   Specimen reception

   a) Wear gloves before handling specimens.
   b) Take specimens from the delivery counter into the laboratory   check regularly for specimen arrival).
   c) Separate specimens from the request forms, checking that the   sample labels correspond to the information on the request forms.
   d) If a sample has leaked, wash with 5% Phenol disinfectant before removing from the plastic bag and dry with paper towel soaked in 5% Phenol.
   e) Criteria for specimen acceptance: See note on section 6, subsection 6.2

7.2.2   Specimen sorting and recording

   a) Sort specimens according to the project phase specific working instructions
   b) Assign a laboratory number to the request form and the sample as follows;

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c) Give a code, which denotes the sample type as illustrated below:

    i.   Type of specimen   Code

    ii.   Prevalence Survey specimen   PL
    iii.   Prevalence Symptomatic specimen   PD
    iv.   Household Diagnostic specimen   TD
    v.   Household Contact specimen   TC
    vi.   Two monthly Survey specimen   TS
    vii.   Fluid specimen   TK

d) Give a number starting from one up to the end of the study.
e) Give a letter from a choice of a 17- letter alphabet, starting with letter A and ending with letter Y before the lettering starts with A again. (See illustration below).

```
1  2  3  4  5  6  7 8 9 10 11 12 13 14 15 16 17  18 19 20 21
A  B  C  F  G  H  J K M  P  Q  R  T  U  W  X  Y  A  B  C  F etc..........
```

f) Make sure that each number with the letter Y is a multiple of 17 and always check this to ensure correct numbering.
g) Record all samples received in an Accession book, (see page 20 of 34 Appendix I), using information from the request form.

8.0   PROCESSING METHODS

8.1   Microscopy

8.1.1   Smear preparation

8.1.1.1 Materials and equipment:

a) Microscope slides
b) Slide drier
c) Slide Storage Boxes
d) Pipettes

8.1.1.2 Procedure:

(a)   **Sputum**

    i.   Label a new, clean and unscratched slide with the client's ID number and the Laboratory number.
    ii.   Place slides on a slide drier.
    iii.   Select and suck the most dense particles of sputa (blood stained, purulent or cheesy mucus particles) using a Pasteur pipette.

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iv. Spread specimen over an area approximately 1x 2cm in circular motion making the smear thin enough to be able to read through it.
v.   Note the appearance of the sputa on the request form.
vi. Allow slides to dry.

b) Pleural fluids smear preparation

i.   Write the description of the effusion on the request form.
ii.   Crush any clots, if present, with a Pasteur pipette.
iii. Prepare smear by spreading a drop of fluid on a clean, labeled slide.
iv.   Centrifuge the rest of the fluid at 2800rpm for 10 minutes.
v.   Decant the supernatant and re-suspend sediment in residual fluid then spread on a clean, labeled slide.
vi.   Allow slides to dry.

c) Thick aspirate smear preparation

i.   Prepare a thin direct smear from the aspirate by spreading a drop of the aspirate on a clean, labeled slide.
ii. Allow slides to dry.

8.1.2 Staining procedures

8.1.2.1 Auramine O Staining Procedure

a) Principle of test:

When stained with Auramine, tubercle bacilli will take up the yellow fluorescence colour of the stain. Upon decolourisation with acid alcohol, the bacilli will remain with the colour whereas everything else in the smear will loose the colour and then take up the colour of the counter-stain. Tubercle bacilli will therefore appear as rod shaped, emitting a bright yellow fluorescence against a dark background of either Potassium permanganate or Acridine Orange counter-stain, under x40 objective.

b) Reagents
i.
ii.   Auramine
iii. Acid alcohol
iv.   Potassium permanganate
v.   Distilled water

c) Procedure
i.   Fix smears to the slides by passing the slides through a flame 3 to 4 times with the smear uppermost. (Do not overheat).
ii. File smears not for immediate staining in slide storage boxes for future staining.

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iii. Place numbered smears on the staining rack in batches (maximum 12) ensuring that the slides do not touch each other.

iv. Include a known positive and a known negative smear slide in the first batch of the day or whenever a freshly prepared stain is to be used. This is done as a Quality Control measure to ensure the staining capability of the solutions and of the staining procedures and to confirm that acid fast contaminants are not present in the stains.

v. Flood entire slide with Auramine O and allow staining for 15 minutes, ensuring that staining solution remains on smears. (Do not heat or use filter paper strips)

vi. Rinse with distilled water (tap water contains chlorine which may interfere with fluorescence).

vii. Decolourise with 3% acid-alcohol for two minutes.

viii. Rinse with distilled water and drain.

ix. Flood with either Potassium permanganate or Acridine orange and allow counterstaining for 2 minutes. Time is critical here because counterstaining for longer may quench the fluorescence of acid- fast bacilli.

x. Rinse with distilled water and drain.

xi. Allow smears to air dry. Do not blot. Read as soon as possible after staining.

xii. Confirm all Auramine positive smears by ZN.

8.1.2.2 ZN Staining Procedure

a) Principle of test:

When stained with ZN, tubercle bacilli will take up the red colour of the stain. Upon decolourisation with acid alcohol, the bacilli will remain red whereas everything else that is not colourfast will lose the colour and take up the colour of the counter-stain.

On examination under x100 objective, the bacilli will appear like red rods, slightly curved, more or less granular, isolated, in pairs or in groups, standing out clearly against a blue or green background depending on which counter-stain has been used.

b) Reagents

i. Carbol fuchsin
ii. 3% Acid alcohol
iii. Methylene blue
iv. Tape water

c) Method

a. Proceed as in a) up to d) of 8.1.2.1 c) above.

b. Flood entire slide with ZN Carbol Fuchsin which has been filtered prior to use, or cover each slide with a piece of filter paper, and pour over, if unfiltered Carbol Fuchsin is used.

c. Heat the slides slowly until they are steaming. Do not boil. Maintain steaming for 3 to 5 minutes by using low or intermittent heat. (In no case must the stain boil dry).

d. Rinse each slide individually in a gentle stream of running water until all free stain is washed away.

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e. Flood the slides with the decolourising solution for a maximum of 3 minutes.
f. Rinse the slides thoroughly with water and drain excess water from the slides.
g. Flood the slides with counter-stain.
h. Allow the smear to counter-stain for 60 seconds.
i. Rinse the slides thoroughly with water. Drain excess water and allow smears to air dry. Do not blot dry.

8.2 Culture

8.2.1 Principle of test:

Mycobacterium tuberculosis, unlike other bacteria, proliferates extremely slowly and does not grow on simple chemically defined media. The only media allowing abundant growth of *M. tuberculosis* is egg-enriched media containing glycerol and asparagines and/or liquid medium supplemented with serum or albumin.

8.2.2 Materials

a) 4% Sodium hydroxide (NaOH)
b) Pre-Buffered Saline (PBS)
c) Centrifuge tubes
d) Lowenstein Jensen Slopes (LJ)
e) Kirchner's medium
f) Slides
g) Cryotubes
h) Cryotube boxes
i) Timer
j) Centrifuge

8.2.3 Procedure

8.2.3.1 Decontamination

a) To a volume of sputum in a 50ml centrifuge tube add an equal volume of 4% sodium hydroxide (e.g.: 2ml sputum and 2ml NAOH) and tightly close the tube.
b) Set timer to 15 minutes at this stage and allow the sputum to decontaminate, vortexing twice at intervals.
c) Dilute the contents with sterile phosphate buffer (Ph 6.8) up to the neck of the container. This dilution stops the action of sodium hydroxide and reduces the specific gravity for centrifugation.
d) Recap the container tightly and mix the contents by inversion several times.
e) Centrifuge at 3000xg for 20 minutes.
f) Pour off all the supernatant fluid into a suitable disinfectant and re-suspend the deposit in the residual fluid that runs back into the container.
g) Inoculate the culture medium.

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8.2.3.2 Decontamination for microscopy purposes only

a. Add X ml of 4% Sodium hydroxide (NaOH) to Xml of specimen in a 15ml centrifuge tube.
b. Set timer to 15 minutes at this stage.
c. Allow decontaminating while standing for 15 minutes, vortexing twice at intervals.
d. Centrifuge at 2800rpm for 15 minutes.
e. Pour off supernatant.
f. Vortex the sediment, add 15ml of sterile PBS and then mix the suspension.
g. Centrifuge at 3000xg (3700rpm) for 15 minutes.
h. Decant supernatant and re-suspend sediment in 0.75ml of PBS.

8.2.3.3 Inoculation and Incubation

a. Label LJ slopes, (Kirchner's medium if in use), and slides appropriately to include client ID number, Laboratory number and date.
b. Discard condensed moisture at the bottom of the LJ medium.
c. Inoculate using sterile pipettes, disposable loops or nicrome wire loops, 0,2ml-0,4ml (2-4 drops or loopfuls) of resuspended sediment onto LJ slope and Kirchner's media.
d. Place a drop of sediment onto the labeled slide.
e. Incubate all cultures at 37⁰C, in a slanted position, with loosened caps (for LJ) for at least 24 hours to ensure even distribution of inoculum and ventilation.
f. Place bottles thereafter in an upright position to save incubation space and with caps tightened to avoid contamination.
g. Allow further incubation for 8 weeks, checking for growth every week.

9.0 Examination and validation

9.1 Auramine stained smears

9.1.1 Examination

Allow each slide to be examined twice by two different readers, independent of each other. Call a third reader (senior technologist or manager) to carry out a third examination where results are in discordance.
a. Examine Auramine stained smears with 40x objective.
b. Examine fluorochrome stained smears within 24 hours of staining as the fluorescence may fade with time. Smears that cannot be examined immediately after staining should be kept in the dark, preferably in a refrigerator, for a maximum of 24 hours.
c. Start by examining the known positive and negative smear slides as a Quality Control measure to check for false positives and negatives.
d. Make a series of systematic sweeps over the length of the smear. Move the slide longitudinally after examining a microscopic field so that the neighbouring field to the right can be examined and search each field thoroughly.
e. Examine a minimum of 70 fields before reporting as negative.
f. Take the slide from the microscope stage at the end of examination, check the identification number and note the result.
g. Store the slides in boxes for further references and for external QC.

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9.1.2 Interpretation of microscopy results

a. Look for rod shaped, bright yellow fluorescence emitting bacilli, against a dark (Potassium permanganate or Acridine orange) background.

b. Interpret the results as follows:

Number of acid fast bacilli (AAFB)	Fields	Report
0	70	Negative
1-2	70	Doubtful
2-18	50	1+
4-36	10	2+
10-90	1	3+

9.2 ZN stained smears

9.2.1 Examination

Examine as for Auramine stained smears with the following exceptions:

a. Examine Carbol fuchsin stained smears with 100x oil immersion objective.

b. Examine a minimum of 100 fields before reporting negative.

c. Wipe the immersion oil objective with a piece of lens tissue before examining the next slide.

9.2.2 Interpretation of Microscopy results

a) Look for fine red rods, slightly curved, approximately $1 - 10$ λm long, more or less granular, isolated, in pairs or in groups, standing out clearly against the blue background.

b) Interpret the results as follows:

No of Acid Fast Bacilli (AFB)	Fields	Report
No AFB	Per 100 immersion fields	No acid bacilli observed
1-9 AFB	Per 100 immersion fields	Record exact figure
10 – 99 AFB	Per 100 immersion fields	1+
1 – 10 AFB	Per field	2+
More than 10 AFB	Per field	3+

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9.3   Cultures

9.3.1   Examination

a. Examine cultures on LJ 24 hours after inoculation, to check that liquid has completely evaporated and to detect contaminants. Tighten caps in order to prevent drying out of media.

b. Examine the cultures thereafter weekly.

c. Look out for rough, crumby, waxy, non-pigmented, (cream-coloured) colonies appearing approximately 3 weeks after inoculation.

d. Allow a senior technologist or the lab manager to confirm growth) by examining the growth macroscopically using the naked eye.

e. Carry out a microscopic examination on the cultures using ZN staining to confirm that the growth is that of Acid and Alcohol fast bacilli (AAFB).

9.3.2   Interpretation of Results

Interpret results as follows:

Number of colonies	Report
No Growth	Negative
1 -19 colonies	Positive scanty
20 - 99	Positive 1+
100 - 199	Positive 2+
> 200 colonies	Positive 3 +
Contaminated	Contaminated

10.0   Species identification

There are 2 methods available for use

10.1   Method 1: Rapid testing using MTB antigen detecting kits

10.1.1 Principle of test

M. tuberculosis has been found to secrete 33 different proteins. In this rapid Immunochromatographic test, one of these predominant proteins, MPT64, is detected using mouse monoclonal antibodies to *M. tuberculosis* antigen and the assay is used to discriminate between the *M. tuberculosis* complex and MOTT bacilli. The test cassette consists of a sample pad, a gold conjugate pad, a nitrocellulose membrane and an absorbent pad. Mouse monoclonal ANTI-MPT64 is immobilised on the nitrocellulose membrane as the capture material (test line). Other antibodies, which recognise another epitope of MPT64, conjugated with colloidal gold particles were used for antigen capture and detection in a sandwich type assay. As the test sample applied in the sample well flows laterally through the membrane, the antibody-colloidal gold conjugate binds to the MPT64 antigen in the sample, liquid media.

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The complex then flows further and binds to the mouse monoclonal antibody on the solid phase in the test line, producing a red to purple colour band. In the absence of MPT64, there is no line in the test band region.

10.1.2 Materials
a) Test device individually foil pouched with a desiccant
b) Extraction buffer (for sample preparation from solid cultures)
c) Micropipette measuring 100-200µl
d) 1.5ml cryovials for sample preparation

10.1.3 Method
a) Remove the test device from the foil pouch and place it on a flat dry surface.
b) Add 100µl of liquid culture (or 100µl of suspended solid culture in buffer) into the sample well.
c) As the test begins to work, a purple colour moves across the result window in the centre of the test device.
d) Interpret the test results in 15 minutes after sample application.

10.1.4 Interpretation of results
a) A colour band will appear at the left section of the result window to show that the test is working properly. This band is the Control Band.
b) The right section of the window indicates the test results. If another colour band appears at the right section of the result window, this band is the Test Band.
c) **Negative Result**: The presence of only control band ("C" band) within the result window indicates a negative result.
d) **Positive Result**: The presence of two colour bands ("T" band and "C" band) within the result window, no matter which band appears first, indicates a positive result.

10.1.5 Validation of the method

a) **Purpose**: This procedure describes how the Tann Capilla kit was validated.

b) **Materials**:

    i.   Mycobacterium isolates from LJ slopes
    ii.   Plain LJ slopes
    iii. PNB
    iv. Tann Capilla Kit

c) **Method**

    i. Mycobacterium isolates grown from routine DetecTB project samples were speciated using the method described in the Tann Capilla kit.
    ii. The same samples were speciated using the method that was currently in use in the lab i.e. growth at different temperatures.

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d) Results:

	Routine method		Tann Capilla kit	
	MTB	46	MTB	46
	MOTT	7	MOTT	7
	MTB	2	MOTT	2
	MOTT	0	MTB	0
Total strains tested		55		55
Specificity	=	97%		

The kit was therefore adopted because of its high specificity and quick turn around time of results.

10.1.6 Method Source
Not applicable.

10.1.7 Variations from standard method
Not applicable.

10.2 Method 2: Growth at different temperatures

10.2.1 Setting up tests
a) Inoculate colonies onto 3 LJ media and one P-Nitro benzoic Acid LJ (PNB) media
b) Incubate the 3 LJs at 45^0C, 37^0C and 25^0C respectively and PNB at 37^0C.
c) Check for growth after every week for a maximum of 3 weeks.
d) Include positive and negative controls as a Quality Control measure.

10.2.2 Interpretation of Results

a) Classify no growth at 45^0C, 25^0C and PNB but growth at 37^0C as MTB.
b) Classify any growth at 45^0C, 25^0C or PNB and is ZN positive as Mycobacterium other than TB (MOTT)
c) Interpret results as follows:

	PNB	Growth at 45^0C	Growth at RT	Growth at 370C
Positive Control	NG	NG	NG	Growth
Negative Control	Growth	Growth	Growth	Growth
MTB	NG	NG	NG	Growth
MOTT	Variable	Variable	Variable	Growth

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11.0 Post examination procedures

11.1 Storage of Samples

11.1.1 Stained/unstained smears
a) Store these in slide storage boxes in numerical order starting from 1 up to the end of the study.
b) Note the Box number and position of the smear in the box on the Patient's request form, (see page 17 of 34, Appendix I).
c) Store boxes, in numerical order, in a room assigned for slide storage.

11.1.2 Decontaminated samples
a) Keep a minimum of 0.75mls of the remainder of the cultured sample in a cryotube, clearly labelled with the patient's ID Number and date processed.
b) Store these (in numerical order) in cryotube boxes labelled with the code corresponding to the type of specimen (see codes assigned to specimens in 4.2.2) Boxes should be numbered from 1 up to the end of the project.
c) Note the Box number and the position of the Cryotube under Sputum Location on the Patient request form, (see page 17 of 34, Appendix I).
d) Store boxes in a –20°C refrigerator.
e) Keep the centrifuge tubes containing the rest of the decontaminated sample in a plastic bag labelled with the day the samples were processed and store in –20°C for 2 weeks.

11.1.3 Positive TB cultures
a) Suspend as many colonies into 2 cryotubes, containing Tryptone Soya Broth (TSB) as the storage media, and clearly labelled with the Patient's ID, the Laboratory number and the date of storage.
b) Store these in cryotube boxes labelled with the Box number and the code corresponding to the type of specimen that yielded the culture.
c) Note the Box number and the position of the cryotube on the Patient's request form under Culture Location, (see page 17 of 34, Appendix I), when all positive cultures have been preserved.
d) Store boxes in a –70°C refrigerator
e) Keep all mother cultures/subcultures on LJ slopes in boxes labelled with the code corresponding to the type of specimen. Arrange these in numerical order for easy retrieval.

11.2 Disposal of samples

11.2.1 Sputum
a) Dispose of used sputum containers in black plastic bags and incinerate.
b) Dispose of used centrifuge tubes by autoclaving first then incineration.

11.2.2 Cultures
a) Dispose of LJ slopes by autoclaving and incinerating the medium. Keep the bottles for recycling.

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12.0   Reporting, alteration and release of results

12.1   Reporting

12.1.1 Positive Results (DetecTB Project)

Notify the Project Team Manager / Nursing Sisters in Charge at the Caravan sites and at Beatrice Road Infectious Disease Hospital (B.R.I.D.H) immediately, of any positive TB smears and cultures as follows:

a) Fill in Laboratory Result Report form, (see page 22 of 34, Appendix IV).

Note   These forms are study specific

b) Send;
  i.   a copy of results to the Nursing Sister in charge of the Cluster where the sample was collected/submitted, immediately. (Nursing sister will then disclose of the results to the client.)
  ii.   a copy of the results to the clinic site which is located at Beatrice Road Infectious Disease Hospital (B.R.I.D.H)

c)   Record all positive smears and cultures in a Positive Smear / Culture Book, (see page 21 of 34, Appendix II) which is kept in the lab, for easy access to all Project Managers.

12.1.2 Microscopy Results

a) Record all smear results:
  i. In the Specimen Accession Book (see page 20 of 34, Appendix II)
  ii On the Patient's request form, (see page 18 of 34, Appendix I)
b) Authorize (senior technologist or manager) the entry and release of results.
c) Make copies of results from the Specimen Accession Book, to all sisters in charge at the caravan sites and at B.R.I.D.H
d) Send all results entered on Patient request forms to the Data Capture team for data capture and generation of typed reports, if required.

12.1.3 Culture results:

a. Record all culture results in the Specimen Accession Book (see page 20 of 34, Appendix II)
b. Record all positive culture results on the Patient's request form under Interim Culture Report section, (see page 19 of 34, Appendix I). Final Culture Report will follow later when species identification and sensitivity testing is done.
c. Record all negative cultures results under Final Culture Report section on the Patient's request form, (see page 19 of 34, Appendix I)
d. Authorise (senior technologist or manager) the entry and release of results.
e. Make copies of results from the Specimen Accession Book, to all sisters in charge at the caravan sites and at B.R.I.D.H
f. Send all results entered on Patient request forms to the Data Capture team for data capture and generation of typed reports, if required.

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12.1.4 Referral results

In case of positive results in step **12.1.1** needing reference:

a) Fill in the Internal Referral Lab Report, (see page 23 of 34, Appendix V), when typed reports are returned to the Lab by the Data Capture team.

b) Attach the Internal Referral Lab Report to the typed report.

c) Authorise (senior technologist or lab manager) release of results to the Project Team Manager.

12.2 Alteration of Reports

12.2.1 In case of reports needing alterations before dispatch:

a) Correct any mistakes noted and send report to the manager for approval.

b) Once approved by manager, send results to the appropriate Sisters in Charge at the Caravan sites and at B.R.I.D.H and to the Data team.

12.2.2 In case of a report needing alteration when a copy has already been dispatched:

a) Show the time and date record has been altered.

b) Show the name of person responsible for the change

c) Retain original electronic records and add alterations to the record through appropriate editing procedures.

d) Send out report as Amended to clearly indicate that results have been altered.

12.3 Release of examination results

Number	Stage of results	Person releasing results	To whom released
12.3.1	After examination and recording	Technical staff	Lab Manager/Senior Lab Technologist
12.3.2	After authorization by the Manager or Senior Lab Technologist	Manager/Senior Technologist	Data Capture Team and Drivers to take urgent results to the site nurse and B.R.I.D.H.
12.3.3	Typed reports	Data Capturing team	Lab Manager
12.3.4	Approved and signed	Lab Manager	Data Capture team
12.3.5	Approved and signed	Data Capture team	Drivers
12.3.6	Approved and signed	Drivers	Where typed reports are required.

13.0 Criteria for test acceptance and quality control:

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13.1 QUALITY CONTROL MEASURES

13.1.1 Internal Quality Control

13.1.1.1 Confidence in results
This has been provided by applying the following:
a) Examining by another procedure in parallel with the main procedure

    i.   Auramine in parallel with ZN stain
    ii.   Initial smear examined in parallel with the concentrated   smear
    iii   Two readers examining the same set of slides independently
    iv   LJ cultures in parallel with Kirshner's media

13.1.1.2 Proper Preparation of Reagents
a)   Clearly documented methods are in place for the preparation of reagents and their storage.

13.1.1.3 Equipment
a) A program has been implemented for maintenance and calibration of measuring systems and verification of trueness so as to ensure that results are traceable to SI units. (A contract has been signed with Isocal).

b) Procedures on First Line Service have been put in place for every piece of equipment being used.

13.1.2 External Quality Control

13.1.2.1 Participating in a suitable program of inter-lab comparisons

a) National Health Lab Services in South Africa (International)
    for comparison of TB Microscopy, Culture and species identification)
b) Mpilo (National) Reference Laboratory in Bulawayo for comparison of Species Identification.
c) Zimbabwe National Quality Assurance Programme (ZINQUAP) in Harare for comparison of TB Microscopy

14 Test validation (Procedure, records and acquisition validation):
14.1 Microscopy and culture

Adopted method

15.0 Method source:

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WHO/TB/98.258

16.0 Variations from standard method: N/A

17.0 Amendment history:

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Appendix I

Patient request form

Place Client bar code stick here

EARLY MORNING SPUTUM SPECIMEN

DL01. Cluster Code & Name: _____

DL02. Client ID:

DL03. Process as: _____

DL04. Client Name:

    1 = for results immediately ➜ <u>symptomatic patient</u>
    2 = for results immediately ➜ follow-up specimen from
       TB suspect

DL05 Collected by (Nurse ID) :

DL06 Date: /   /
            d   d   m   m   y
       y   y   y

(FOR LAB USE ONLY below this line)

DL07. Specimen Quality:
  1 = Salivary

  2 = Mucoid

  3 = Mucopurulent
  4 = Blood stained

DL08. Specimen processing
  1 = Processed and stored

  2 = Stored and not processed

  3 = Discarded

DL09. Smear 1 location: Box   Position

DL10. Smear 2 location: Box   Position

DL11. Sputum location: Box   Position (-20^0C)

DL12. Culture location: Box   Position (-70^0C)

MICROSCOPY

Initial Exam by: Checked by:

DL14 Date examined   /   /
   d   d   m   m   y   y   y   y

DL13. Auramine 1   **DL14.** Auramine 2

DL15. ZN1 result   **DL16.** ZN2 result

DL17. Check Auramine1

DL18. Check Auramine 2

DL19. Check ZN1   **DL20.** Check ZN2

DL21. Final Auramine Result (highest of DL13➜14)

  DL22. Number of +ve auramine smears

DL23. Final ZN Result (give highest of DL15➜ 16)

DL25 .Date reported (dd/mm/yyyy)   /   /

1 = smear negative
2 = scanty positive
3 = 1+
4 = 2+
5 = 3+
9 = Not done

DL24. Number of +ve ZN smears

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WK	Date	LJG	LJP	Kirchner's	Colonial Morphology ?	AFB+ve	Comments
1							
2							
3							
4							
5							
6							
7							
8							

DL26 3 WK CULTURE
DL27 INTERIM CULTURE

1 = contaminated
2 = compatible with *M.tb*
3 = suggestive of MOTT
4 = No growth

IDENTIFICATION

DL28 Growth at Room temp.

DL29 Growth at 45^{0}C

DL30 PNB

1 = no growth
2 = growth
7 = contaminated
9 = not done

FINAL CULTURE REPORT

1 = negative
2 = scanty positive
3 = positive
7 = contaminated *(culture only)*
9 = not done

Smear: -ve / scanty +ve / +ve / not done **DL31**

Culture:-ve / scanty +ve / +ve / contaminated / not done **DL32**

Colony count (1 week after first growth seen: leave blank if no growth) **DL33**

1 to 20: give exact n
21 to 99:   = 021
100 to 199 = 100
200 to 500 = 200
500 or more = 500

CULTURE ID

1 = *M. tuberculosis*
2 = MOTT
3 = Contaminant
4 = Mixed MTB/MOTT
9 = Not done (negative culture)

DL34. DETECTB Culture ID...

DL35. BULAWAYO Culture ID.......................................

DL36. NHLS Culture ID...

DL37. FINAL Culture ID - Colonies of *Mycobacterium tuberculosis* / MOTT / Contaminant/ Mixed grown ....

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APPENDIX II

Specimen Accession Book

Patient Study Name ID	Lab number	Date Received	Received by	Smear result	Preliminary Culture result	Growth	Final Culture result

APPENDIX III

Positive Smear /Culture Book

Date	Lab ID	Patient's Name	Smear Result	Culture result

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APPENDIX IV

To be inserted on the result section of the **Laboratory Report Form (FM/QMS/84)**

**Cluster
Code**

Cluster Location ....
....

D / /

Laboratory No.

Client Id Number ┌─────────────────────────┐
                     └─────────────────────────┘

Client Name ....
....

Client Address ....
....
/ /

Smear Result **POSITIVE**   **Date**

Authorised by ....
....

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APPENDIX V

Internal Referral Lab Report

To be inserted on the result section of the **Laboratory Report Form (FM/QMS/84)**

<u>**RESULTS**</u>

	RESULTS	DATE REPORTED
Specimen 1 (Lab No).......... Smear final report Culture Preliminary: Culture Final:	Pos/Scanty Pos/ Neg/ Awaiting/Not done	.../.../... .../.../... .../.../...
Specimen 2 (Lab No).......... Smear final report Culture Preliminary: Culture Final:	Pos/Scanty Pos/ Neg/ Awaiting/Not done	.../.../... .../.../... .../.../...
Specimen 3 (Lab No).......... Smear final report Culture Preliminary: Culture Final:	Pos/Scanty Pos/ Nag/ Awaiting/Not done	......./......./....... ..../......./....... ..../......./.......
Specimen 4 (Lab No).......... Smear final report Culture Preliminary: Culture Final:	Pos/Scanty Pos/ Neg/ Awaiting/Not done	.../.../... .../.../... .../.../...
Anonymous HIV specimen received	Yes/No	**Date received** ....

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Attachment 1

1.0   Preparation of Staining Reagents

1.1   Auramine O Staining reagent

1.1.1   Auramine

(a)   Solution 1 (Auramine)
Auramine   0.1g
95 %ethanol (technical grade)   10ml

i.   Dissolve auramine in ethanol...................................Solution █

Auramine is carcinogenic and direct contact with the powder or the solution should be avoided.

(b)   Solution 2 (Phenol)
                                                                     3.0g
Phenol crystals
Distilled water   87ml

i.   Dissolved phenol crystals in water ....Solution █

ii.   Filter solution █ into **2** and store in tightly stopped amber bottle away from heat and light.
iii.   Label bottle with the name of the reagent and dates of preparation and expiry.
iv.   Store at room temperature for three months. Turbidity may develop on standing but this does not affect the staining reaction.

1.1.2   Decolourising solution

a) Materials
                                                                     0.5
Concentrated hydrochloric acid
70% ethanol (technical grade)   100mls

b) Method
i.   Carefully add concentrated hydrochloric acid to the ethanol. Always add acid slowly to alcohol, not vice versa.
ii.   Store in amber bottle. Label bottle with name of reagent and dates of preparation and expiry.
iii.   Store at room temperature for three months.

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1.1.3 Counter-stains

Either potassium permanganate or acridine orange may be used as counter-stains

1.1.3.1 Potassium permanganate

a) Materials

Potassium permanganate (KmnO4)   0.5g
Distilled water   100ml

b) Method

i. Dissolve potassium permanganate in distilled water in a tightly stoppered amber bottle.
ii. Label with name of reagent and dates of preparation and expiry.
iii. Store at room temperature for three months.

1.1.3.2 Acridine orange

a) Materials

Anhydrous dibasic sodium phosphate (Na_2HPO_4)   0.01g
Distilled water   100ml
Acridine orange   0.01g

b) Method

i. Dissolve sodium phosphate in distilled water.
ii. Add acridine orange and label bottle with name of reagent and dates of preparation and expiry.
iii. Store at room temperature for three months.

1.2 Ziehl-Neelsen (ZN) stain

1.2.1 Carbol Fuchsin

a) Fuchsin
Basic fuchsin   1.5g
95% ethanol (technical grade)   100ml

i. Dissolve basic fuchsin in ethanol   **Solution** ▌

b) Phenol
Phenol crystals   22.5 g
Distilled water   450ml

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i. Dissolve phenol crystals in distilled water (gentle heat may be required)..**Solution** ▮

c) **Working Solution**

   i.   Combine 10ml of **Solution 1** with 90ml of **Solution 2.**
   ii.   Filter into an amber bottle.
   iii.   Label bottle with the name of reagent as well as preparation and expiry dates. Can be stored at room temperature for six to twelve months.

1.2.2 Decolourising agents

1.2.2.1 3% acid-alcohol

a) **Materials**

Concentrated hydrochloric acid (technical grade)	3mls
95% ethanol (technical grade)	97mls

b) **Method**

   i.   *Carefully* add concentrated hydrochloric acid to 95 % ethanol. *Always add acid slowly to alcohol, not vice versa.* The mixture will heat up.
   ii.   Store in an amber bottle.
   iii.   Label with name of reagent and dates of preparation and expiry. Can be stored at room temperature for six months.

In countries where the acquisition of alcohol may be problematic, a solution of 25% sulphuric acid may be used as decolourising agent. This is prepared as follows:

1.2.2.2 25% sulphuric acid

a) **Materials**

Concentrated sulphuric acid (technical grade)	25ml
Sterile distilled water	100ml

b) **Method**

   i.   *Carefully* add concentrated sulphuric acid to water.
   ii.   **Always add acid slowly to water, not vice versa.**
   iii.   The mixture will heat up.
   iv.   Store in amber bottle.

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v. Label bottle with name of reagent as well as preparation and expiry dates. It can be stored at room temperature for six to twelve months.

1.2.3 Counterstain:
1.2.3.1 Methylene blue

a) **Materials**

Methylene blue powder 3g
Distilled water 100mls

b) **Method**

i. Dissolve Methylene blue chloride in distilled water and store in amber bottle.
ii. Label bottle with name of reagent and dates of preparation and expiry. The reagent can be stored at room temperature for six to twelve months.

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Attachment 2

2.0 Culture media preparation methods

2.1 Lowenstein Jensen (LJ) Media

2.1.1 Mineral salt solution
a) **Materials**

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	1.2g
Magnesium sulphate ($MgSO_4 . 7h_2O$)	0.12g
Magnesium citrate	0.3g
Asparagine	1.8g
Glycerol	6ml
Distilled water	300ml

b) **Method**

i. Dissolve the ingredients *in order* in the distilled water.
ii. Autoclave at 121°C for 30 minutes to sterilise.
iii. Cool to room temperature. This solution keeps indefinitely and may be amounts in the refrigerator.

2.1.2 Malachite green solution, 2%
a) **Materials**

Malachite Green dye	0.3g
Sterile water distilled water	10ml

b) **Method**

i. Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will not store indefinitely and may precipitate or change to a less-deeply-coloured solution. In either case discard and prepare a fresh solution.
ii. Clean fresh hens' eggs not more than seven days old, by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap.
iii. Let the eggs soak for 30 minutes in the soap solution.
iv. Rinse eggs thoroughly in running water and soak them in 70% ethanol or methylated spirit for 15 minutes.
v. Before handling the clean dry eggs scrub the hands and wash them.
vi. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in sterile blender.

2.1.3 Preparation of complete medium

a) Pool the following ingredients aseptically in a large, sterile flask and mix well:

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ii.	Mineral salt solution	300mls
iii.	Malachite green solution	10mls
iv.	Homogenised eggs (10-12 eggs, depending on size)	500mls

b) Check pH (6.8 – 7.2). If too acid add alkaline.
  i. If too alkaline add acid.
c) Distribute the complete egg medium in 6-8mls in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20 x 150mm screw-capped test tubes, securely fasten the tops.
d) Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.
e) Place the bottles in a slanted position in the inspissator and coagulate the medium for 60 minutes at 85°C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilise it). Heating for a second or third time has a detrimental effect on the quality of the medium.

2.1.4 Quality Check

a) Allow media to cool to room temperature.
b) Check on the quality of the media:

  I   Quality of the egg media deteriorates when coagulation is done at   too high a temperature or for too long.
  Ii   Discolouration of the coagulated medium may be due to excessive temperature.
  Iii   The appearance of little holes or bubbles on the surface of medium also indicates faulty coagulation procedure.

c) Discard poor quality media.
d) Perform ten fold dilutions from a neat suspension of H 37 Strain of Mtb up to 1:1000.
e) Inoculate on a few slopes from each batch of freshly prepared LJ media and then incubate for 2-3weeks.
f) Check for growth every week.

2.1.5 Sterility Check

a) After inspissation, incubate the whole media batch or a representative sample of the culture media at 35°C – 37°C for 24 hours to check sterility.
b) Discard any media bottles with bacterial colonies growing on them.

2.1.6 Storage

a) Label the LJ medium with the date of preparation.

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b) Store in the refrigerator. These can be kept for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ medium should not be older than four weeks.

For cultivation of M. bovis, enrich LJ medium with 0.5% sodium pyruvate. Omit Glycerol and add 8.0g of sodium pyruvate to the mineral solution.

2.1.7 Precaution during media preparation

For media of the best quality, chemicals of certified purity, clean glassware and freshly distilled water and sterilised water should be used. Direction for preparing media must be followed precisely and without modification. A few general points to obtain good quality media and to avoid contamination of reagents and media are as follows:

a) Keep the environment as clean as possible. Swab the work surface with a suitable disinfectant (e.g. 5% methylated spirits) before dispensing sterile reagents and media.

b) Clean the floor with a wet mop to limit dust

c) Use sterile glassware and equipment

d) Use reagent grade chemicals and reagents unless otherwise specified

e) Check the temperature of the inspissators and hot air ovens

f) Follow strict aseptic techniques when preparing media, e.g. flaming flasks and tubes

g) When preparing egg based media, carefully clean egg shells before breaking

h) Do not overheat media during inspissation. Do not leave media exposed to light (including ultra-violet light), but store in the refrigerator in the dark when not in use.

i) Do not skimp on the volume of medium. Place 6-8mls of egg medium in each 20ml universal bottle

2.2 Kirchner's Media

a) Stock solution

i. Weigh 7.4g of powder
ii. Disperse in 1 litre of de-ionised water and 20mls of glycerol
iii. Mix well and heat gently to dissolve
iv. Autoclave at 121°C for 10 minutes
v. Store at 2°C to 8°C

b) Working solution

i. To 100mls of Kirchner's solution, add 11mls of heat inactivated horse serum by filter sterilisation and the following antibiotics/ selective agents.

Selective agents
Polymixin B   40µl
Carbenicillin   10µl

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Trimethoprim   2µl
Amphotericin B   20µl

c) Reconstituting antibiotics for Kirchner's

I. Reconstitute to 10mls for all antibiotics (Mitchson's antibiotic mixture).
V_1 = volume of reconstituted antibiotic to be added to media, which is unknown.
C_1 = concentration of antibiotic reconstituted in 10ml distilled water.
V_2 = volume of media to be prepared.
C_2 = final concentration of antibiotic in media.

ii. Prepare 100mls of media using the following:

Formulae $V_1 C_1$ = $V_2 C_2$
1mg =   1000µg
1g   =   1000000 µg
1ml =   1000µl

Amphotericin B V_1 =   ?
C_1 =   50000µg / ml
V_2 =   100ml
C_2 =   10µg / ml

Therefore V_1 x   50000µg / ml =   100ml x 10µg/ml
V_1 =   (100ml x 10µg/ml) / 50000µg/ml
=   1000ml /50000
=   0.02ml
=   20µl

Trimethoprim V_1 =   ?
C_1 =   500000µg/ml
V_2 =   100ml
C_2 =   10µg/ml

Therefore V_1 x   500000µg/ml =   100ml x 10µg/ml
V_1 =   (100ml x 10µg/ml) / 500000µg/ml
=   1000ml / 500000
=   0.002ml
=   2µl

Polymixin B V_1 =   ?
C_1 =   500000units/ml
V_2 =   100ml
C_2 =   200units/ml

Therefore V_1 x   500000units/ml =   100ml x 200units/ml

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$$V_1 = (100ml \times 200units/ml) / 5000000units/ml$$

$$= 20000ml / 500000$$

$$= 0.04ml$$

$$= \underline{\mathbf{40\mu l}}$$

Carbenicillin

$$V_1 = ?$$
$$C_1 = 500000\mu g/ml \text{ or } 5g/10mls$$
$$V_2 = 100ml$$
$$C_2 = 50\mu g/ml$$

Therefore

$$V_1 \times 500000\mu g/ml = 100 \times 50\mu g/ml$$
$$= (100ml \times 50\mu g/ml) / 500000\mu g$$
$$= 5000ml / 500000$$
$$= 0.01ml$$
$$= \underline{\mathbf{10\mu l}}$$

2.3 Culture Identification media

2.3.1 Paranitrophenol Benzoic Acid (PNB)

a) Materials

Same as for LJ but add 500 mg PNB dissolved in Dimethylsulphoxide (DMSO) to the salt solution.

2.4 Storage medium preparation

2.4.1 Tryptone Soya Broth

a) Mix 3g Tryptone Soya Broth (Oxoid, Basington, UK), 20 ml of Glycerol and 80 ml of distilled water.

b) Autoclave at 121°C for 15 minutes.

c) Store at 4°C for up to 6 months.

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Attachment 3

3.0 Preparation of decontamination and disinfectant reagents

3.1 Decontamination Reagents

3.1.1 Sodium Hydroxide (NaOH)

a) **Materials**

NaOH	4g
Distilled water	100mls

b) **Method**

i. Dissolve 4g NaOH in 100mls of distilled water.
ii. Autoclave at 121°C for 15 minutes.
iii. Allow to cool before use.

3.1.2 Phosphate buffered saline (PBS)

3.1.2.1 Using Sigma Tablets:

If using commercially prepared PBS such as SIGMA phosphate buffered saline tablets prepare according to manufacturers instructions i.e.

a) Dissolve 1 tablet in 200ml distilled water to obtain 0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride solution pH 7.4 at 25_OC.
b) Store solution at $0-5_OC$.
c) Discard if turbidity develops.

3.1.2.2 Using Salts:

These are best prepared from stock phosphate solutions A and B.

a) **Stock phosphate solution A**

Materials

Sodium dihydrogen phosphate, 1-hydrate ($NAH_2PO_4.H_2O$)	27.6g
Distilled water	to 1 litre

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Method

i. Weigh accurately the chemical, and transfer to a litre volumetric flask.
ii. Half fill the flask with water, and mix to dissolve the chemical. Make up to the 1 litre mark with distilled water, and mix well.
iii. Transfer to a clean bottle.
iv. Label the bottle 'Stock phosphate solution A'. Store in a cool place or
v. Preferably at $2-8_0$C. The solution is stable for several months.

b) Stock phosphate solution B

Materials

Di-Sodium hydroxide phosphate, Anhydrous (Na_2HPO_4)	28.38g
Distilled water	to 1 litre

Method

i. Prepare as described above for solution A.
ii. Label the bottle 'Stock phosphate solution B'. Store in a cool place or preferably at $2-8_0$C. The solution is stable for several months.

c) Phosphate buffered saline, ph 7.2

To make about 200ml:

Stock phosphate solution A	28ml
Stock phosphate solution B	72ml
Distilled water	100ml
Sodium chloride	1.7g

3.2 Disinfectant Reagents

3.2.1 5% Phenol

a) Materials

Phenol crystals	25g
Tap water	500mls

b) Method

i. Dissolve 25g of phenol crystals in 500mls tap water, in discard jar.
ii. Use within 24 hours.