Establishment of the diagnostic system of tuberculosis feasible in developing countries

（開発途上国で実施可能な結核診断システムの確立）

Bhagwan Maharjan

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CHAPTER III

ABBREVIATIONS

WHO	World Health Organization
MDR	Multi drug-resistant
JMM	Joint monitoring mission
OMS	OMNIgene SPUTUM
SOC	Standard of care
NALC	Nacetyl L-cysteine
GENETUP	German Nepal TB Project
MTB	Mycobacterium tuberculosis
DS-TB	Drug Susceptible TB
DR-TB	Drug resistant TB
DST	Drug susceptibility testing
L-J	Lowenstein-Jensen
AFB	Acid fast bacilli
MGIT	Mycobaterium growth indicator tube
OM-S	OMNIgene SPUTUM

SOC	Standard-of-care
MTB	*Mycobacterium tuberculosis*
NALC	Nacetyl L-cysteine
RIF	Rifampicin
RR TB	Rifampicin resistant TB
PB	Phosphate buffer
Ct	Threshold cycle
MeG	Methyl green
LAMP	Loop-mediated isothermal amplification
MTB	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
NTM	Nontuberculosis mycobacteria
HNB	Hydroxylnaphthol blue
MaG	Malachite green
FD	Fluorescent detection reagent
DDW	Double-distilled water

BCG	Bacillus Calmette-Guerin
DNA	Deoxyribonucleic acid
NATA	Nepal Anti-TB Association
GENETUP	German Nepal TB Project
POC	Point of care
HNB	Hydroxyl naphthol blue
UV	Ultra violet

PREFACE

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* complex (MTBC) is a global public health problem and remains a leading cause of death worldwide. Despite all the global efforts to control TB, the World Health Organization (WHO) has estimated 10 million new TB cases and 1.5 million deaths globally in 2018 (45).Geographically, most of the TB cases were in the WHO regions of South-East Asia (44%) and one third of world's total TB cases were only in Nepal's neibouring countries Indian and China (45).

Nepal is known as one of the successful country in the world for nationwide effective TB control program with 90% treatment success. Howevernumber of incidence is not falling down as expected. WHO has estimated 45000 new, 1500 RR/MDR TB and 7000 cases death in 2018(45). The preliminary result of recently conducted prevalence survey, 2018 has suggested two thirds higher than the estimated (11). In 2018, only 32,474 DS-TB and about 400 RR/MDR cases were able to notify. (11) A huge number of DS-TB and RR/MDR TB cases are still missing from diagnosis.

The WHO ambitious goal of the End TB strategy which aims to achieve 90% reduction in incidence and 95% reduction in mortality by 2035 is not in track in most of the WHO regions including many high burden countries(45). Early diagnosis and rapid initiation of treatment is the key strategy to control tuberculosis. However, TB controls programs had relatively less success, especially with the development and use of new point of care (POC) diagnostic test that is suitable for developing countries. So, one of the reasons for limiting to achieve the WHO's ambitious goal is may be due to readily available simple and cheap rapid diagnostic methods.The current commonly used key diagnostic tool in many TB burden countries is sputum microscopy which has been used for more than 100 years. Although, sputum microscopy is a simple and convenient method, is an insensitive technique and misses nearly half of all TB cases. Hence a new POCdiagnostic method that is simple, cheap,

quick, sensitive, and feasible to implement a decentralized level with minimum training of health workers is urgently required to achieve end TB target (point of care diagnostics for tuberculosis).

The burden of drug-resistant TB is another worrying aspect at global, regional, and country levels. In 2018, there were approximately half a million new cases of rifampicin (RIF)-resistant TB (45). The WHO's End TB strategy calls for universal access to drug susceptibility, systematic screening of contacts and high-risk groups. However rapid molecular test (first endorsed by WHO in 2010) and culture-based method; takes up to 12 weeks to provide results. Many resource constraint countries have limited culture and DST lab facilities and face tremendous challenges transporting samples from peripheral health to centralized testing laboratory. Currently, long term transport and storage of sputum samples typically require reliable and continuous access to refrigeration to maintain sample integrity at the level required to culture/DST and molecular test for diagnosis of drug resistant TB. Constraints in sputum sample transportation increase the costs associated with each patient diagnosis and inadequate sample preservation during transit can result in multiple diagnostic and therapeutic issues: culture contamination; invalid test result, need for repeated sampling and consequently significant delays in initiating effective treatment. These constraints have led to a challenging transport situation for Nepal.

In my PhD study, I studied to develop a new strategy for TB diagnosis in Nepal by focusing on effective sample collection and transportation, and by use of a rapid diagnostic method. My thesis consists of three chapters wherein the first chapter, I studied the feasibility of the use of a novel sputum transport reagent [OMNIgene Sputum (OMS), DNA Genotek) for transporting TB sputum samples without maintaining the cold chain for routine TB testing in Nepal. The main objectives were to assess the performance of OMS for transporting sputum from peripheral sites without cold chain stabilization and also compare with Nepal's

standard of care (SOC) for MTB smear and culture diagnostics. Compared with the SOC method in Nepal, transporting samples in OMS reduced culture contamination rates from 12% to 2%, and improved detection of MTB-positive patients by 9%. The results suggest that OMS performs well at maintaining sample integrity for smear and solid culture, and has potential as an easy-to-implement solution that could reduce costs of testing (at the laboratory and national program levels) and improve patient access to timely results and clinical decision-making.The results suggested that OMS could be used for long term transport solution for smear and culture testing.

In the second chapter, I did an evaluation of the same OMS-stabilized sputum for long term transport and further feasibility in molecular diagnosis of TB by GeneXpert MTB/RIF testing in Nepal. The main purpose of study was to evaluate whether transporting samples in OMS reagents from a peripheral collection site to a central laboratory in Nepal can improve TB detection and also increase the sensitivity of Xpert MTB/RIF testing. The findings suggested that the rate of smear-positive, MTB-positive sample, detection was identical for both treatment groups, at 95%. In addition, more smear-negative MTB+ samples were detected in the OM-S group (17% vs. 13 %, P=0.00655).

In my third chapter, I developed novel method of direct detection of *Mycobacterium tuberculosis* in clinical samples by a dry methyl green loop-mediated isothermal amplification (LAMP) method. The main purpose of this study was to develop a simple visual methyl green (MeG) based on dry LAMP method for early detection of MTB from clinical samples. The research findings suggested that LAMP method showed high sensitivity and specificity.I evaluated the dry MeG MTB-LAMP with 69 new TB suspected samples from patients that did not have a confirmed history of TB treatment and found the sensitivity in culture-positive samples as 92.8% (13/14) and specificity in culture-negative samples as

96.3% (53/55). Therefore, LAMP system has the potential to be a point of care test for early diagnosis of active TB in developing countries like Nepal.

CHAPTER I

A novel sputum transport solution eliminates cold chain and supports routine tuberculosis testing in Nepal

INTRODUCTION

The World Health Organization's End TB Strategy(34) calls for universal access to drug-susceptibility testing and systematic screening of contacts and high-risk groups, and identifies these elements as essential to eliminating *Mycobacterium tuberculosis* (MTB) infections. To achieve these targets, it is necessary to consider how sample transportation affects patient access to drug-susceptibility tests and how sample quality affects test results. Increasing pressure is being placed on countries to test more samples using an expanding list of techniques; however, constraints differ by setting and minimal attention has been paid to practical solutions that *i*) improve sample quality and *ii*) provide a flexible approach that functions seamlessly with established and novel diagnostic tests. Such solutions are critical to enable high-priority, resource-constrained countries to scale their tuberculosis (TB) testing programs.

The National TB Program in Nepal has mounted one of the most successful TB campaigns in Asia, an effort that increased the rate of successful TB treatment outcomes from 45% in 1990 to 90% by 2010 (30). However, Nepal faces an increasing threat from multidrug-resistant TB (MDR-TB), and faces tremendous challenges transporting samples from peripheral hospitals to a centralized testing laboratory (30).Remote collection sites can be as far as 400 km away from the country's two TB reference laboratories in the capital city Kathmandu, and Nepal's mountainous geography can delay sample transport by up to 6 days. Currently, long-term transport and storage of

sputum samples typically requires reliable and continuous access to refrigeration to maintain sample integrity at the level required for smear, culture, and molecular TB diagnostics (24). Constraints on sputum sample transportation increase the costs associated with each patient diagnosis, and inadequate sample preservation during transit can result in multiple diagnostic and therapeutic issues: culture contamination; invalid test results; need for repeated patient sampling (with inherent delays to re-access patients/collection sites and transport each sample); and, consequently, significant delays in initiating effective treatment. These constraints have led to a challenging transport situation for Nepal. Whereas the country's standard operating guidelines state that samples should be transported within 3 days, the transport process routinely takes 4 or more days, and cold-chain stabilization is not feasible due to high courier costs and the requirement for reference laboratories to return cold boxes to peripheral labs. National TB control programs need products that can help effectively scale their testing networks while maintaining established diagnostic algorithms and workflows. OMNIgene®•SPUTUM (OMS; DNA Genotek, Ottawa, Canada) is a novel sample transport reagent that decontaminates and liquefies sputum, that is compatible with all gold standard TB tests (e.g., smear microscopy, solid and liquid culture, Cepheid® GeneXpert®, HainLifescience line probe assay) and other molecular assays (3), and that does not require cold chain. Versatile, reliable, diagnostically beneficial products that can be easily integrated into laboratory systems can offer a variety of solutions for TB control programs: cost reduction; increased patient access to reliable tests; improved sample quality for testing; and more rapid administration of appropriate therapy leading to better patient outcomes.

According to WHO guidelines, sputum samples must be refrigerated if they are stored or transported more than 24 hours prior to testing (35); however, it is widely

known that many resource-limited countries cannot finance or logistically provide reliable cold-chain transport. The aim of this preliminary study was to evaluate the effectiveness of OMS in a real-world setting and determine the feasibility of conducting additional larger studies. Performance of OMS was compared to that of Nepal's current standard sputum collection, shipping and processing protocol with respect to results for smear microscopy and solid MTB culture.

MATERIALS AND METHODS

Sample Collection and Transport Methods

The study was conducted at the GENETUP TB Reference Laboratory in Kathmandu, Nepal in February and March 2015. Sixty raw sputum samples were collected from suspected TB patients at peripheral hospitals. An individual sterile swab stick was used to manually split each sample into two equivalent portions as it was poured from one container to another. Portions were randomly assigned to one treatment method prior to being packaged for transport. As per the standard procedures for sputum collection, shipping and processing in Nepal, one sample portion (hereafter referred to as the "standard-of-care [SOC] sample") was left untreated. The second sample portion (the "OMS sample") had an equal volume of OMS reagent added to it at time of collection. All samples were shipped via airline courier and without refrigeration. (Note that cold-chain stabilization is not required for OMS samples. Although cold-chain transport is the recommended standard for sputum samples collected in Nepal, this was not feasible due to the high cost associated with this transport method.) Transport times varied from 0 to 8 days depending on the distance from the collection site to the GENETUP laboratory. Temperatures during transport ranged from 4°C to 24°C, as recorded in Kathmandu during the study period.

Sample Processing and Testing

Upon arrival at the laboratory, each SOC sample was processed using the Nepal standard NaOH/NALC method: fresh preparation of a 4% NaOH, 2.9% trisodium citrate, 0.5g NALC solution, addition of an equal volume of solution to the sample, and 15 minutes of incubation at room temperature, followed by neutralization using sterile phosphate buffer and centrifugation to produce a sediment. The OMS sample required

no further processing and was directly centrifuged to produce a sediment.

Sediments were re-suspended in sterile phosphate buffer and were assessed by smear microscopy and cultured on Lowenstein-Jensen (LJ) slants in duplicate. Cultures were incubated at 37°C for up to 56 days. Smears were categorized as negative or as one of four levels of acid-fast bacilli detection: scanty, 1+, 2+, or 3+.

Data Collected and Analysis

Transport times from collection site to laboratory were recorded. For each OMS sample and SOC sample, smear results were reported as negative or positive (defined as scanty, 1+, 2+ or 3+). Culture results were reported as negative, positive (i.e., growth), or contaminated. For positive cultures, the interval from date of inoculation to date of observable growth (i.e., time to culture-positive status) was recorded in days. When the duplicate culture slants from a sample yielded discrepant results, a single outcome was reported as follows: Samples that yielded one contaminated and one negative culture were counted as negative; samples that yielded one contaminated and one positive culture were counted as positive; samples that yielded two contaminated cultures were counted as contaminated.

The OMS and SOC methods were compared with respect to proportions of TB cases detected by smear and by culture, respectively, and with respect to proportions of contaminated cultures. As well, average time to culture-positive status (in days) was compared for the two methods. Findings were compared relative to transport time, as appropriate.

RESULTS

Table 1 summarizes transport times and key diagnostic information and results for each of the 60 respective pairs of OMS and SOC samples. Transport times ranged from 0 to 8 days.

Sputum volumes ranged from 0.5 mL (*n*=2) to 4.5 mL, and the majority (*n*=50) were ≥2 mL. Of the 60 sputum samples collected, 41 (68% of total) were positive by smear microscopy with OMS and SOC, respectively. There were two discrepancies between the methods: Sample 1945 for which only the OMS portion was positive, and Sample 2287 for which only the SOC portion was positive. Both samples were graded as scanty by smear microscopy.

The impact of OMS on smear microscopy was negligible, as smear categorization was similar for the two methods. Note that low-positive sputum samples (i.e., those categorized as scanty or 1+) were not negatively affected by transport in OMS, even after 7 days in transit (Table 1).

Regarding culture results, of the 60 OMS samples, 37 (62%) were culture-positive, 22 (36%) were culture-negative, and 1 culture (2% of total) was contaminated (Figure 1). In contrast, 32 (53%) of the 60 SOC samples were culture-positive, 21 (35%) were culture-negative, and 7 (12%) of the SOC cultures were contaminated (Figure 1).

Overall average time to culture-positive was not significantly affected by treatment method (23 days for both treatment methods) (Table 1, Figure 1). The largest variation in time to culture-positive was observed in samples that were 2 days in transport. In this group, the OMS-treated samples took an average of 4 days longer to become culture-positive (range, 16-23 days) as compared to the SOC-treated samples (range, 16-18 days) (Table 1). However, only five samples were transported for 2 days and each treatment method had 80% detection. When numbers of culture positives per group were

compared relative to transport time, the OMS group had two more positives at 3 and 4 days of transport, and one more positive at 5 days of transport; the other transport category comparisons were identical.

For analysis, "rescued" was used to indicate instances where one portion of a sample (i.e., OMS or SOC) was identified MTB-positive or MTB-negative by culture, whereas the corresponding portion yielded no usable diagnostic results (i.e., a contaminated culture, which provides neither a negative nor a positive result). Use of the OMS method resulted in seven samples being rescued (i.e., seven additional actionable results that would have been missed using SOC alone) and one sample being "missed" (i.e., for one of the 60 total samples, the culture for the SOC portion was positive whereas the culture for the OMS portion was contaminated). In contrast, the SOC method missed seven positives and rescued one sample (Table 1, Figure 1).

Of the smear-positive samples (i.e., 41 total for each method), the proportions identified as culture-positive for MTB were 90% (n=37) for the OMS method and 76% (n=31) for the SOC method. Within the smear-positive subgroup, the numbers of culture-negative results with the two methods were comparable; however, there were more contaminated cultures with the SOC method (i.e., six for SOC *vs*. one for OMS) (Figure 2).

Within the smear-negative subgroup, the OMS treatment method identified 19 samples as culture-negative, while the SOC treatment method identified 17 culture-negative, 1 culture-positive and 1 culture-contaminated. Two discrepant smear microscopy results were identified: Sample 1945 was smear-positive for OMS only and Sample 2287 was smear-positive for SOC only. Sample 1945 was culture-positive for both methods (average times to culture-positive: 33 and 42 days for SOC and OMS, respectively), while Sample 2287 was culture-negative for both methods. One smear-negative sample was rescued by OMS treatment. Sample 2355 was culture-negative (i.e., an actionable

diagnostic result) following OMS treatment, but was culture-contaminated (i.e., an unusable diagnostic result) after SOC treatment (Table 1 and Figure 3).

DISCUSSION

This evaluation showed that using the OMS reagent at point of collection might improve diagnostic results and reduce the complexity associated with standard-of-care methods. The study demonstrated positive impacts on several key endpoints: *i*) Costs: OMS decreased culture contamination rates, thus potentially reducing the expense of repeat testing; *ii*) Improved Workflow: OMS ensured the highest quality sputum samples even after 8 days of transport at ambient temperature, eliminated the need for daily preparation of NaOH/NALC, and simplified laboratory processing procedures; *iii)* OMS reduces courier cost compared to SOC ice box cost and the cost of OMS is only one dollar per test; and *iv*) Improved TB Case Detection: OMS yielded a greater proportion of MTB-positive test results. Most importantly, in seven cases, the samples treated with the OMS reagent yielded usable diagnostic results, whereas the corresponding samples treated with the SOC method resulted in the need for a second sputum collection to enable repeat testing by culture. This difference has significant implications for patient care, as patients may be lost during follow-up, and repeat collection and re-testing will delay initiation of appropriate antibiotic therapy (25).

OMNIgene®•SPUTUM offers several key advantages over the sputum collection and processing method currently used in Nepal. The OMS reagent is a highly stable product (1 year shelf life) that requires no additional preparation in the laboratory. This means that capturing efficiencies through task shifting of technician time would be easily achievable. Further, the ability to add the reagent at point of collection helps ensure that the highest-quality sample is received by the laboratory, since the product reduces putrefaction and downstream culture contamination rates. Maintaining sample integrity facilitates accurate and timely TB diagnosis, which is critical for countries that are implementing large-scale testing networks.

This study identified another advantage of the OMS reagent; it allows samples to be transported for extended periods of time without the need for cold chain. This could significantly reduce costs associated with sample transport (4) and could simplify TB testing algorithms for countries with remote populations that, prior to this advancement, have been difficult to access for testing due to sample transportation challenges (26). In addition to markedly facilitating transport and maintaining sample integrity, samples prepared using OMS are easily integrated into existing diagnostic workflows without the need for costly infrastructure investment or re-tooling of established laboratory methods. As the present study indicates, sediments from OMS-treated sputa are amenable to smear microscopy and solid culture methods. Other sputum decontamination solutions have been evaluated as either lab-added reagents (1) or transport alternatives (2), but these products have limitations related to shelf-life stability or compatibility with liquid culture systems. We have previously demonstrated that OMS is stable for 1 year prior to use and is compatible with abroad range of additional diagnostic methods, including liquid culture (Mycobacteria Growth Indicator Tube [MGIT]) (3), Cepheid® GeneXpert® MTB/RIF assay (12), HainLifescience line probe assays (3), and other molecular applications (12). Further, the versatility and fundamental sample-preservation features of the OMS method lend it not only to the latest current diagnostic advancements for TB, but also to test platforms that will be developed and used in the foreseeable future.

Minor differences were observed between the OMS and SOC methods with respect to smear categorization and time to culture-positive, and these likely reflect the imprecision of manually splitting a complex biological sample in half prior to liquefaction. The study had limitations: *i*) manually splitting sputum samples can lead to uneven distribution of bacilli, which is of particular importance with low-positive

samples; *ii*) a considerable number of samples that were identified as MTB-negative (by smear and culture) had required no transport prior to testing (i.e., 9 of 10 samples with 0 days in transport were negative on both diagnostic tests; Table 1); *iii*) most samples underwent 3 to 5 days of transport, whereas only a small number of samples underwent prolonged transit (7 or 8 days). The higher-than-expected proportion of culture-negative results for smear-negative samples was most likely an artifact of sample splitting, which would have greatest impact on samples with negative or scanty smear grades. Additional studies will be required to further evaluate this reagent in different regional settings; however, extended transport in OMS (i.e., longer than 4 days) demonstrated that this reagent performed better than Nepal's SOC method in ensuring the integrity of sputum samples for culture, as two of the six OMS-rescued samples were in this transport-time category.

Challenges with long-term transport of sputum samples from peripheral sites to a centralized laboratory include high cost and logistics of providing cold-chain stabilization, loss of samples through putrefaction, reduced case detection due to loss of viable MTB, and high rates of culture contamination. These issues exacerbate delays in reporting clinically relevant results to the clinician, and they can impact a patient's health when repeat testing is required prior to initiating antibiotic therapy.

Our preliminary findings from this in-country study suggest that OMS could negate or substantially mitigate key challenges associated with traditional sputum sample transport. Compared to the SOC method in Nepal, transporting samples in OMS reduced culture contamination rates from 12% to 2%, and improved detection of MTB-positive patients by 9%. The results suggest that OMNIgene®•SPUTUM performs well at maintaining sample integrity for smear and solid culture, and has potential as an easy-to-implement solution that could reduce costs of testing (at the laboratory and national program levels)

and improve patient access to timely results and clinical decision-making. Future investigations with larger sample sizes will be valuable, and will ideally include testing via liquid culture, testing smear-negative sputa with extended transport, and analysis of cost savings.

Table 1. Summary of transport time and diagnostic results for the OMNIgene®•SPUTUM and Standard-of-Care methods

Sample ID	**Days in Transport**	Smear		LJ Culture: Time to Positive (days)		OMS Impact on TTP (days)	Avg TTP SOC	Avg TTP OMS	Δ TTP for OMS
		SOC	OMS	SOC	OMS				
4600	**0**	1+	1+	21	24	3			
4796	**0**	neg	neg	NG	NG	NG			
4820	**0**	neg	neg	NG	NG	NG			
4547	**0**	neg	neg	NG	NG	NG			
4582	**0**	neg	neg	NG	NG	NG	21	24	3
4649	**0**	neg	neg	NG	NG	NG			
4671	**0**	neg	neg	NG	NG	NG			
4661	**0**	neg	neg	NG	NG	NG			
4817	**0**	neg	neg	NG	NG	NG			
2081	**2**	2+	1+	17	23	6			
2071	**2**	2+	1+	17	23	6			
2336	**2**	3+	3+	16	16	0	17	21	4
2366	**2**	2+	2+	18	23	5			
2374	**2**	1+	Scanty	NG	NG	NG			
1966	**3**	2+	1+	Contaminated	28	**Rescued**			
2064	**3**	1+	Scanty	25	25	0			
2067	**3**	1+	3+	36	29	-7			
2068	**3**	1+	1+	Contaminated/29	25	-4			
2093	**3**	1+	1+	6	19	13			
2099	**3**	3+	2+	25	19	-6			
2114*	**3**	2+	1+	Contaminated	23	**Rescued**			
2186	**3**	neg	neg	NG	NG	NG			
2200	**3**	3+	3+	12	18	6	22	23	1
2355	**3**	neg	neg	Contaminated	NG	**NG/Rescued**			
2356	**3**	neg	neg	NG	NG	NG			
2357	**3**	neg	neg	NG	NG	NG			
2360	**3**	neg	neg	NG	NG	NG			
2370	**3**	neg	neg	NG	NG	NG			
2371*	**3**	neg	neg	NG	NG	NG			
2372	**3**	3+	3+	18	18	0			
1872	**4**	2+	3+	16	23	7			
1879	**4**	1+	1+	Contaminated/NG	NG	NG			
1880	**4**	3+	3+	16	25	9			
2054	**4**	3+	2+	Contaminated	34	**Rescued**			
2060	**4**	3+	2+	18	18	0	22	22	0
2062	**4**	2+	Scanty	29	25	-4			
2075	**4**	2+	1+	29	18	-11			
2105	**4**	2+	2+	Contaminated/23	17	-6			
2117	**4**	3+	1+	Contaminated	14	**Rescued**			
2183	**4**	neg	neg	NG	NG	**NG**			
1911	**5**	1+	1+	25	25	0			
1929	**5**	Scanty	Scanty	NG	NG	NG			
1941	**5**	neg	neg	NG	NG	NG			
1945	**5**	neg	Scanty	33	42	9	28	28	0
1946	**5**	1+	2+	41	41	0			
2007	**5**	1+	Scanty	28	37	9			
2014	**5**	3+	3+	25	21	-4			

2027	**5**	1+	1+	37	28	-9			
2034	**5**	1+	Scanty	21	25	4			
2167	**5**	3+	3+	19	19	0			
2165	**5**	3+	3+	Contaminated	19	**Rescued**			
2326	**5**	1+	2+	25	20	-5			
1932	**6**	neg	neg	NG	NG	NG	19	18	-1
2038	**6**	3+	3+	18	22	4			
2287	**6**	Scanty	neg	NG	NG	NG			
2289	**6**	1+	2+	26	Contaminated	**Missed**			
2292	**6**	3+	3+	14	17	3			
2307	**6**	2+	1+	16	16	0			
2181	**7**	1+	1+	21	21	0	21	21	0
2244	**8**	3+	3+	Contaminated	21	**Rescued**	n/a	21	n/a

LJ: Lowenstein-Jensen; neg: negative; NG: no growth after 56 days; OMS: OMNIgene®•SPUTUM; SOC: standard-of-care; TTP: time to culture-positive; Δ TTP: difference in TTP between the methods.
**Collected sample volume was 0.5 mL.*

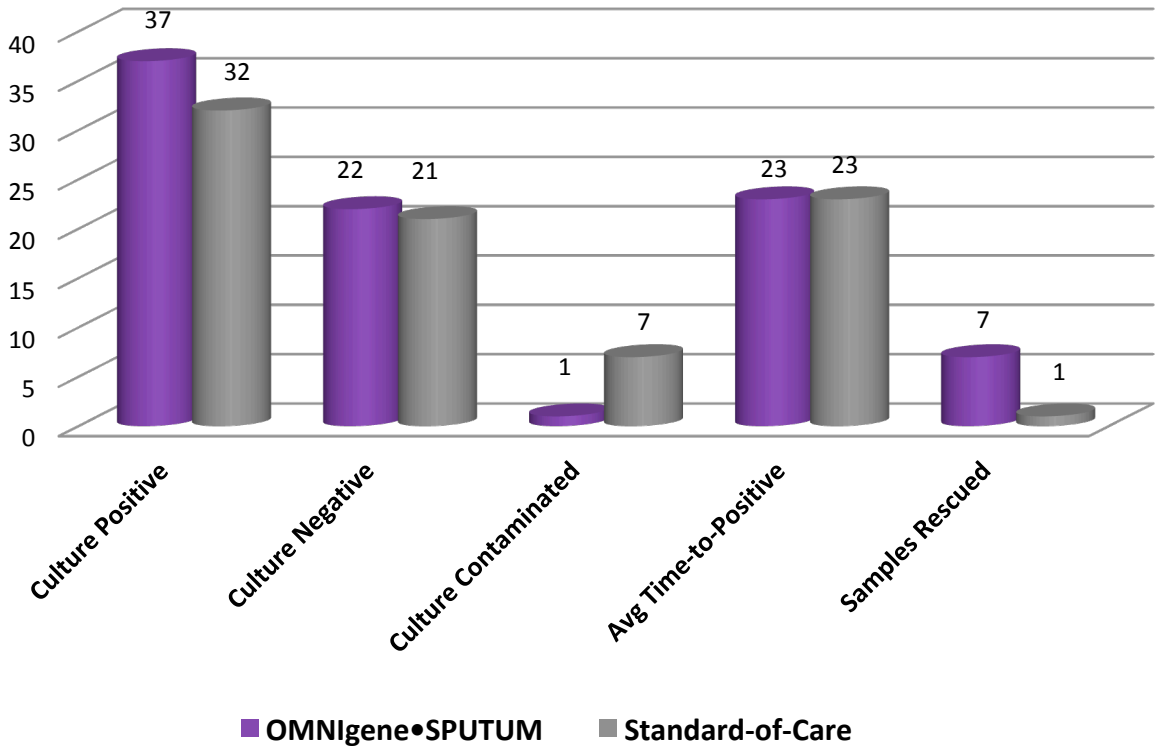

Figure 1. Comparison of OMNIgene SPUTUM and standard of care: solid culture results overall

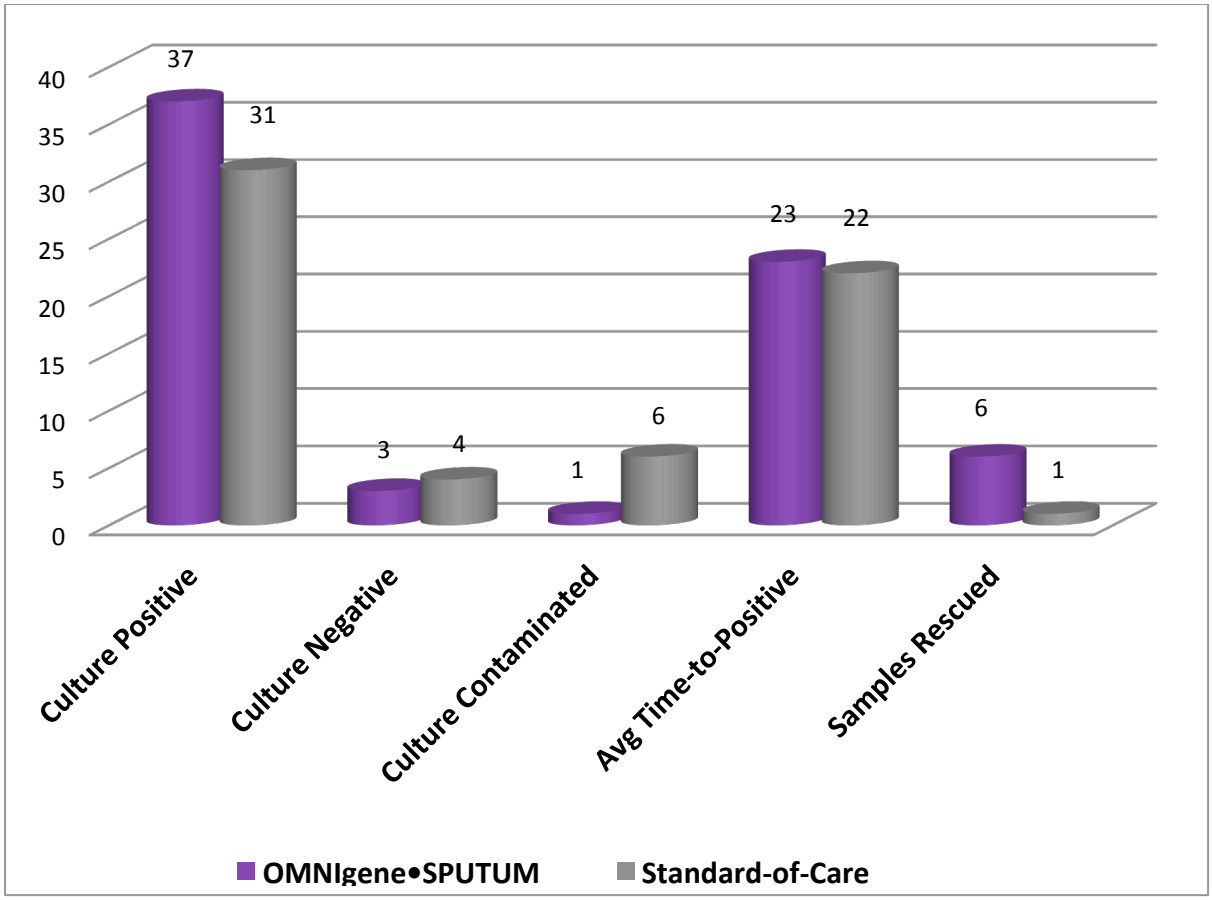

Figure 2. Comparison of OMNIgene SPUTUM and standard of care: solid culture results for smear-positive samples

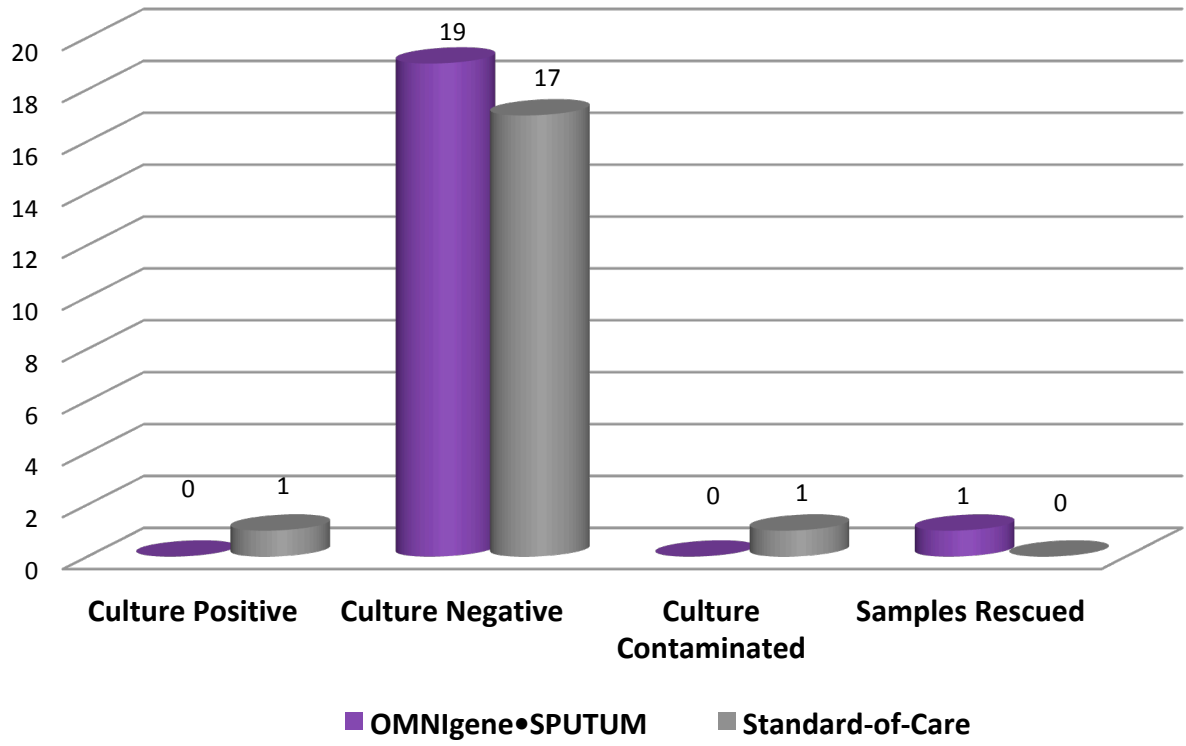

Figure 3. Comparison of OMNIgene SPUTUM and standard of care: solid culture results for smear-negative samples.

SUMMARY

This preliminary study evaluated the transport reagent OMNIgene®•SPUTUM (OMS) in a real-world, resource-limited setting: a zonal hospital and national tuberculosis reference laboratory, Nepal. Objectives were *i*) assess the performance of OMS for transporting sputum from peripheral sites without cold-chain stabilization, and *ii*) compare to Nepal standard-of-care (SOC) for *Mycobacterium tuberculosis* smear and culture diagnostics. Sixty sputa were manually split into an SOC sample (airline-couriered to the laboratory, conventional processing) and an OMS sample (OMS added at collection, no cold-chain transport or processing). Smear microscopy and solid culture were performed. Transport was 0-8 days. Forty-one samples (68%) were smear-positive with both methods. Of the OMS cultures, 37 (62%) were positive, 22 (36%) were negative, and 1 (2%) was contaminated. Corresponding SOC results were 32 (53%), 21 (35%), 7 (12%). OMS "rescued" six (i.e., missed using SOC) compared to one rescue with SOC. Of smear-positives, six SOC samples produced contaminated cultures whereas only one OMS sample was contaminated. OMS reduced culture contamination from 12% to 2%, and improved tuberculosis detection by 9%. The results suggest that OMS could perform well as a no-cold-chain, long-term transport solution for smear and culture testing. The findings provide a basis for larger feasibility studies.

CHAPTER II

Evaluation of OMNIgene sputum-stabilised sputum for long term transport and Xpert MTB/RIF testing in Nepal

INTRODUCTION

Tuberculosis (TB) continues to affect human populations worldwide and was identified as the number one infectious disease killer in 2015 (36). The World Health Organization's End TB Strategy(37) sets specific targets for the year 2030, but recent assessments indicate that these goals will only be met if TB is addressed in radically more effective ways over the next 5 years(38). The Global Plan To End TB 2016-2020 (Global Plan) articulates a 5-year strategy to reach 90% of all people with TB, including 90% of those in the most at-risk populations, and to achieve 90% TB treatment success (38). The plan urges development of critical new tools to fight TB, and specifically to help access and treat the "missing 4 million" TB cases that fall through health system gaps each year.

The Global Plan names three main categories of tools for development: a TB vaccine, better drug regimens, and rapid diagnostic tests (38). One category overlooked is pre-analytic tools, which can have striking impacts on the accuracy of diagnostic tests and, subsequently, patient treatment. OMNIgene®•SPUTUM (OMS; DNA Genotek Inc., ON, Canada) is a sputum transport reagent that liquefies and decontaminates sputum while preserving *Mycobacterium tuberculosis* viability for at least 8 days at temperatures up to 40°C (3). When this reagent is added, cold chain is not required during shipment and samples are directly compatible with all molecular assays and with gold standard TB tests, including smear microscopy, liquid culture (BACTEC™ MGIT™ 960 System;

Becton Dickinson, NJ, USA) and the Cepheid® GeneXpert® System (Cepheid, CA, USA) (3, 5, 19).

Nepal's National TB Program raised the rate of successful TB treatment outcomes from 45% in 1990 to 90% by 2010; however, transporting samples from peripheral hospitals to a centralized testing laboratory is an ongoing challenge (37).Remote collection sites can be hundreds of kilometers away from the two TB reference laboratories in Kathmandu, and mountainous terrain can delay sample transport. Currently, transport and storage of sputum samples in Nepal typically requires reliable cold-chain support to enable smear, culture and molecular TB diagnostics (12); however, routine use of cold-chain during transport is not always feasible. A recent preliminary study in Nepal provided support for feasibility studies of OMS as a no-cold-chain, long-term transport solution for smear and culture testing (24).

The aim of this study was to further evaluate OMS regarding its ability to stabilize sputum long-term for use with the CepheidXpert® MTB/RIF (Xpert) assay. Currently, the Xpert protocol requires that samples be stored at 4°C and tested within 3 days of collection (15). Alleviating this constraint by using OMS could allow long-term transport of sputum for molecular analysis while still maintaining the integrity of the sample for routine testing by smear and culture methods. Any country that is attempting to scale national testing programs to meet the End TB Strategy goals would consider it highly desirable to be able to introduce a single reagent that can achieve the key benefits that OMS offers: *i*) no need for cold-chain during transport, *ii*) no additional laboratory processing/decontamination required, *iii*) extended time window for sputa to remain fit for testing, and *iv*) enables a single sample to be tested using all established reference methods.

MATERIALS AND METHODS

The investigation was conducted at the GENETUP TB Reference Laboratory in Kathmandu, Nepal from December 2015 to February 2016. Raw sputum samples (2 mL minimum) were collected from 100 patients with TB symptoms who presented to Seti Zonal Hospital, a peripheral center. A sterile swab stick was used to manually split each sample into two equivalent portions as it was poured from one 50 mL Falcon™ tube (Becton Dickinson, NJ, USA) to another. The aliquots were randomly assigned to one of two treatment groups: OMS or SOC.

Treatments

OMNIgene•SPUTUM group: Each sample had an equal volume of OMS reagent added and was mixed by inverting 10 to 20 times, placed in a plastic bag, and stored at room temperature. As soon as possible, the samples were airline-couriered to the GENETUP laboratory at ambient temperature (i.e., in an envelope without ice). At the laboratory, the OMS samples required no other processing; they were directly centrifuged at 3,000 x *g* for 20 minutes to produce sediment. The supernatant was discarded and the sediment was re-suspended in 1 mL sterile phosphate buffer (PB).

Standard of Care group: Samples were placed in individual plastic bags and stored at 2°C to 8°C until they were airline-couriered to the laboratory in a cooler with ice. At the laboratory, each sample was treated for 20 minutes with an equal volume of NaOH/NALC (i.e., a freshly prepared solution of 2% NaOH/NALC, 2.9% Trisodium citrate and 0.5 g NALC), neutralized with sterile PB, and centrifuged at 3,000 x *g* for 20 minutes. The supernatant was discarded and the sediment was re-suspended in 1 mL PB.

Transport, Testing, Data and Analysis

Temperatures during transport ranged from 0°C to 28°C, as recorded in Kathmandu during the study period. At the laboratory, a smear was prepared from each re-suspended sediment and microscopy results were recorded as negative, scanty, or 1+, 2+, or 3+ acid-fast bacilli (AFB). As well, 0.5 mL of each sample was prepared using the sputum sediment method (H.1 procedure) described in the Xpert package insert[9]. Sample preparations for both treatment groups were loaded into individual Xpert cartridges and tested in the same instrument. If an error occurred, the error code and sample identifier were recorded and the sample was retested with a new cartridge. The final test result for each sample was recorded and error codes were tabulated.

The OMS and SOC treatment groups were compared with respect to proportions of smear-positive and smear-negative cases detected, and proportions with discordant and concordant Xpert results. Concordance between the two treatment methods was also calculated relative to transport time for smear grade, Xpert results, and for categories of overall smear results (positive or negative) and Xpert diagnostic results. Xpert results and Ct values by probe were evaluated for sample pairs with matched and unmatched smear grades, respectively.

RESULTS

Transport times and all test data for the 100 pairs of OMS and SOC samples are summarized in Table 2. Transport time ranged from 2 to 13 days.

Of the 100 OMS samples, 58% were smear-negative and 42% were smear-positive. Forty-eight (83%) of the OMS smear-negatives were also Xpert MTB-negative, whereas 10 (17%; samples 9, 18, 23, 41, 48, 50, 53, 60, 68, 88) were MTB-positive with nine rifampicin (RIF)-sensitive and one RIF-resistant. For seven of the 10 smear-negative MTB-positives, smear grades were concordant with the corresponding SOC samples. In the remaining three pairs, the SOC smear grades were 1+ or scanty (two samples). Forty (95%) of the OMS smear-positives were also Xpert MTB-positive, whereas two (5%; samples 76 and 87) were MTB-negative and both these were concordant with SOC Xpert results.

Of the 100 SOC samples, 56% were smear-negative and 44% were smear-positive. Forty-nine (87.5%) of the SOC smear-negatives were also Xpert MTB-negative, whereas seven (12.5%; samples 25, 41, 43, 50, 53, 68, 88) were MTB-positive with six RIF-sensitive and one RIF-indeterminate. For six of the seven smear-negative MTB-positives, smear grades were concordant with the corresponding OMS samples. In the remaining pair, the OMS smear grade was scanty. Forty-two (95%) of the SOC smear-positives were also Xpert MTB-positive, whereas two (5%; samples 76, 87) were MTB-negative or diagnosed as non tuberculosis mycobacteria (NTM) and, as noted above, both concordant with the OMS Xpert results.

Across the 4-, 5-, 6- and 7-day transport time categories, smear grade concordance between the OMS and SOC groups ranged from 61% to 89% (Tables 3 and 4); small *n*

values in the other categories precluded analysis. The corresponding range for Xpert results was 89% to 100% (Table 4). Table 5 lists the proportions of the groups that were in each respective diagnostic category for smear result (positive or negative) and Xpert. Only two RIF-indeterminate results occurred and both were in the SOC group (Table 5).

Twenty-eight Xpert run errors occurred (15 SOC sample runs, 13 OMS sample runs), including two during repeat runs of SOC sample 19 and OMS sample 91 (Table 6; Table 7), but an actionable Xpert result was obtained for all 200 samples (Table 2). Only one sample generated an error with both treatment methods (sample 41; Table 7). The most frequent error code was 5006/5007 Post-Run Analysis Error (*n*=8 for both groups; 53% of SOC group errors and 62% of OMS group errors) (Table 6).

Table 8 summarizes the Xpert results and probe Ct values for the 74 sample pairs with matched smear grades. The Ct results for individual probes are shown in Figure 2. Only three of the 74 sample pairs (IDs 23, 25, 48) had discordant Xpert results (Table 9). For sample pair 23, the final SOC result was RIF-indeterminate, whereas the OMS result was RIF-sensitive. In the other two discordant pairs, one sample was RIF-sensitive (OMS sample) or RIF-indeterminate (SOC sample) while the other was MTB-negative.

Table 10 summarizes the Xpert results and probe Ct values for the 26 sample pairs with mismatched smear grades. The Ct results for individual probes are illustrated in Figure 3. Only one of the 26 sample pairs (ID 43) had discordant Xpert results, with the SOC sample RIF-indeterminate and the OMS sample RIF-sensitive (Table 11).

DISCUSSION

Overall, OMS-treated sputum samples shipped at ambient temperature performed comparably to refrigerated SOC-treated samples in both the diagnostic tests. The OMS group had a greater proportion of smear-negative samples that were MTB-positive but the difference was not significant (17% vs 13%, respectively; p=0.0655). Previous evaluations have shown that OMS is compatible with all gold standard TB tests (e.g., smear microscopy, solid and liquid culture, Xpert [protocols H.1 for sediment as well as H.2 for expectorated sputum], HainLifescienceGenoType MTBC line probe assay) and other molecular assays (3, 5, 15, 19, 31, 33). Culture remains the reference standard for diagnosis of TB (23), and the versatility of OMS confirms this reagent's utility for supporting long-term, ambient-temperature transport of sputum samples within the established testing frameworks of national reference laboratories.

Whereas other sample transport reagents inactivate *Mycobacterium tuberculosis* and, thus, render the sample biosafe, such pre-analytic tools are significantly limited in that they confine diagnostic algorithms to molecular assays only (23). A key advantage of OMS is its ability to be used not only with molecular assays, but also with smear and culture. OMNIgene®•SPUTUM acts as a bridge that allows a single sample to be tested by all assays, thus making certain that the patient receives the most accurate and comprehensive result possible, and providing the clinician with a breadth of information to guide treatment decisions.

For samples transported 4, 5, 6 or 7 days, concordance of smear grade for the two treatment methods ranged from 61% to 89%. For the categories with less than 4 or more than 7 days in transport, low sample numbers precluded robust interpretation of concordance. The lower smear result concordance for samples transported 6 days (62%)

or 7 days (61%) is likely attributed to variability caused by splitting low-positive (i.e., 1+ or scanty) sputum samples as well as the low sensitivity of the smear method. These two cohorts had relatively large numbers of low-positive samples; however, note that in every case of discordant smear grades, the Xpert results were 100% concordant.

Across all transport times, concordance of Xpert results for the two methods was 89% or greater. This indicates that OMS-treated samples (which are transported at ambient temperature) perform as well as Nepal SOC samples (which require cold-chain transport) in the Xpert assay. Only two samples had indeterminate RIF resistance results and both were in the SOC group.

The overall Xpert system error frequencies for the two treatment methods were comparable (15 for SOC and 13 for OMS). Only one sample (ID 41) generated errors with both treatment methods. The most frequent error code was 5006/5007 (53% of SOC group errors and 62% of OMS group errors), which indicates that recurrent post-run analysis failures contribute to re-testing of sputum samples by Xpert and that the treatment methods themselves did not directly cause the errors. All samples with errors were successfully re-tested and provided actionable Xpert results.

Three (4.1%) of the 74 samples with matched smear grades had discordant Xpert results. The SOC-treated portion of sample ID 23 was determined to be MTB-positive but RIF-indeterminate, whereas the result for the OMS-treated portion of this sample was MTB-positive and RIF-sensitive. Sample pairs 25 and 48 each had one portion MTB-positive and the other MTB-negative, and these discrepancies likely reflect biological variability due splitting of the specimens.

In 25 (96%) of the 26 sample pairs with mismatched smear grades, the OMS-treated

portion had a lower smear grade than the SOC-treated portion, yet the Xpert results were concordant for all 25 of these pairs. Only one pair (ID 43) with mismatched smear grades (negative for SOC, scanty for OMS) had discordant Xpert results (RIF-indeterminate and RIF-sensitive, respectively) (Table 10). This likely reflects the biological variability of sample portions due to specimen splitting.

The ranges of Ct values for both methods were comparable within and across all probes. This indicates that the amounts of target MTB DNA present in the OMS-treated samples were similar to those present in the SOC-treated samples. Of the above-noted 25 sample pairs in which the OMS portion had lower smear grade than the SOC portion, 9 (36%) of the OMS portions had Ct values that were higher than those of the SOC portions (as would be expected), 10 (40%) had Ct values that were equivalent, and 6 (24%) had Ct values that were slightly lower. These data indicate that OMS treatment does not negatively affect Ct values or the overall ability to call results, even when smear grades of split samples vary.

OMNIgene®•SPUTUM is a versatile, ambient-temperature transport reagent that enables all forms of diagnostic testing for TB and fits seamlessly with established national program algorithms. This study demonstrates that sputum can be transported in OMS for up to 13 days without refrigeration or ice and still yield smear and Xpert assay results that are concordant with results for samples transported or stored cold. The results provide further confirmation that OMS-treated sputa are compatible with the H.1 (sediment) protocol of the Xpert assay (39). This builds on previous demonstration of compatibility with the H.2 (expectorated sputum) protocol as well (39).

Treating sputum with OMS does not interfere with results for any of the probes involved in the Xpert assay. Further, OMS-treated sputa do not generate Xpert cartridge error

codes that suggest reagent incompatibility with the GeneXpert System. All error codes that occurred indicated post-run complications or equipment failures that are independent of the sample treatment method.

The finding that a slightly greater proportion of smear-negative but Xpert-positive samples were detected after OMS treatment warrants further investigation with larger sample sizes. This could help determine whether adding OMS to sputa can increase the sensitivity of the Xpert assay by allowing more smear-negative samples (i.e., low-positives that may be graded smear-negative) to be detected as MTB-positive in the Xpert assay.

Table 2. Comparison of all data for the two treatment groups

				SOC							OMS						
Sample ID	Days in Transport	Smear		Xpert MTB/RIF Result	Ct Value by Probe						Xpert MTB/RIF Result	Ct Value by Probe					
		SOC	**OMS**		**A**	**B**	**C**	**D**	**E**	**SPC**		**A**	**B**	**C**	**D**	**E**	**SPC**
1	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.3	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.9
2	7	3+	1+	RIF-S	22.3	23.2	22.8	22.7	24.3	0.0	RIF-S	17.5	18.7	18.2	18.5	19.8	24.2
3	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.1	MTB Neg	0.0	0.0	0.0	0.0	0.0	29.9
4	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	28.8	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.7
5	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	28.9	MTB Neg	0.0	0.0	0.0	0.0	0.0	25
6	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.6	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.2
7	5	1+	1+	RIF-S	24.7	26.1	25.1	26.2	26.3	26.3	RIF-S	20.2	20.8	20.7	20.7	22.2	24.4
8	7	1+	1+	RIF-S	18.4	19.7	18.7	19.3	20.7	25.5	RIF-S	20.4	21.5	20.9	21.2	22.3	25.1
9	6	1+	Neg	RIF-S	20.9	22.7	21.3	22.6	22.5	23.8	RIF-S	22.2	23.2	22.7	23.7	23.7	25.1
10	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.2	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.1
11	3	1+	1+	RIF-S	17.9	20.2	18.4	19.8	19.6	22.6	RIF-S	20.1	20.4	20.4	21.6	21.6	26.4
12	4	2+	2+	RIF-R	17.4	19.3	18.2	19.4	0.0	24.5	RIF-R	15.1	16.5	15.5	16.9	0.0	24.8
13	3	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.2	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.5
14	3	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.0	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.8
15	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.2	MTB Neg	0.0	0.0	0.0	0.0	0.0	33.5
16	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.6	MTB Neg	0.0	0.0	0.0	0.0	0.0	28
17	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.5	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.2
18	6	5 AFB	Neg	RIF-S	19.1	20.6	19.5	20.8	20.7	27.0	RIF-S	21.3	22.9	21.8	23.3	22.6	23.9
19	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.9	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.1
20	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.9	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.2
21	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.9	MTB Neg	0.0	0.0	0.0	0.0	0.0	34.5
22	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	23.7	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.7
23	7	Neg	Neg	RIF-Ind	38.0	38.0	36.6	0.0	0.0	26.8	RIF-S	27.6	28.3	27.8	29.1	29.5	25.1
24	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	34.7	MTB Neg	0.0	0.0	0.0	0.0	0.0	30.3
25	5	Neg	Neg	RIF-S	29.8	30.2	29.4	31.3	33.0	28.1	MTB Neg	0.0	0.0	0.0	0.0	0.0	28.3
26	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.9
27	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	29.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.8
28	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.0	MTB Neg	0.0	0.0	0.0	0.0	0.0	31.5
29	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	30.1	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.4
30	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.9	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.1
31	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.6
32	5	3+	3+	RIF-S	10.9	13.4	11.9	12.4	13.5	0.0	RIF-S	13.3	15.0	13.7	14.2	14.9	25.6
33	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.7	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.2
34	6	3+	3+	RIF-S	16.5	18.2	17.3	17.6	18.6	28.0	RIF-S	12.7	14.3	12.9	14.2	14.7	22.6
35	4	2+	1+	RIF-S	13.6	15.4	13.9	14.4	15.4	26.0	RIF-S	12.3	13.4	12.5	13.2	13.8	24.9
36	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	28.1	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.3
37	5	3+	1+	RIF-S	11.6	13.3	12.4	11.9	13.8	29.7	RIF-S	14.2	15.7	14.3	15.3	16.3	25.4
38	5	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.3	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.1
39	6	1+	1 AFB	RIF-R	24.8	25.4	25.4	25.6	0.0	24.2	RIF-R	23.3	24.8	24.2	24.5	0.0	25.2

40	5	1+	1+	RIF-S	20.3	21.2	20.7	20.9	22.4	26.2	RIF-S	24.2	25.1	24.8	25.0	26.0	27.1
41	4	Neg	Neg	RIF-S	20.3	22.1	20.8	22.1	22.1	24.3	RIF-S	20.4	21.9	21.1	21.6	22.3	23.6
42	4	1+	1+	RIF-S	19.9	21.2	20.4	20.8	21.8	29.8	RIF-S	20.4	21.4	21.1	21.7	22.7	25.8
43	4	Neg	2 AFB	RIF-Ind	37.9	0.0	35.9	0.0	0.0	0.0	RIF-S	26.4	27.0	26.8	27.0	28.5	24.9
44	4	1+	1+	RIF-S	19.7	20.6	20.3	20.4	21.7	0.0	RIF-S	20.5	22.4	21.2	21.7	22.3	25.3
45	7	2+	1+	RIF-S	13.8	15.8	14.5	14.9	15.8	27.1	RIF-S	14.8	16.9	15.7	15.9	16.7	25.9
46	5	1+	1+	RIF-S	22.7	24.1	23.3	23.9	24.5	25.4	RIF-S	24.3	25.5	24.9	25.2	26.2	26.4
47	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.7	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.1
48	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.0	RIF-R	31.9	31.5	30.7	40.2	0.0	25.9
49	6	3+	1+	RIF-S	12.5	13.8	12.5	12.6	14.7	28.3	RIF-S	14.4	16.5	14.8	15.4	16.3	27.9
50	5	Neg	Neg	RIF-S	22.4	23.6	22.8	23.3	24.1	25.0	RIF-S	30.2	30.3	29.9	31.0	33.6	26.4
51	7	1+	1+	RIF-S	19.3	20.7	19.8	19.9	21.3	28.2	RIF-S	19.7	21.4	20.4	20.7	21.4	23.4
52	7	3+	1+	RIF-S	16.2	17.9	16.7	17.2	17.8	27.0	RIF-S	18.1	19.9	18.7	19.5	20.3	27.2
53	4	Neg	Neg	RIF-S	25.1	26.0	25.8	25.9	26.5	24.4	RIF-S	19.2	20.5	19.4	20.5	21.3	24.2
54	5	1+	1+	RIF-S	9.4	10.7	9.9	10.0	10.8	27.7	RIF-S	10.9	11.9	10.7	12.1	13.7	0.0
55	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.8	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.9
56	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.7	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.1
57	7	3+	2+	RIF-S	10.4	12.4	10.9	10.8	11.8	25.6	RIF-S	12.0	13.6	12.3	12.7	13.7	25.5
58	6	3+	2+	RIF-S	13.3	15.4	14.5	13.9	14.6	26.5	RIF-S	14.5	16.3	15.3	15.5	16.0	26.5
59	6	1+	9 AFB	RIF-S	21.4	22.8	22.3	22.4	22.7	25.1	RIF-S	20.9	22.1	21.6	21.8	22.6	23.4
60	6	3 AFB	Neg	RIF-S	19.7	21.7	20.7	20.7	20.9	26.0	RIF-S	25.0	25.6	25.6	25.7	26.5	24.9
61	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.0
62	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.6
63	6	3+	3+	RIF-S	11.6	14.4	12.5	13.5	13.5	26.5	RIF-S	13.3	15.6	14.4	14.9	14.9	28.0
64	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.6	MTB Neg	0.0	0.0	0.0	0.0	0.0	30.2
65	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.2
66	7	3+	1+	RIF-S	17.3	19.2	18.5	18.4	19.0	28.6	RIF-S	14.4	16.3	15.4	16.1	16.3	30.0
67	7	2+	1+	RIF-S	17.6	19.2	18.5	19.2	19.3	26.6	RIF-S	18.5	20.5	19.4	20.2	20.3	29.0
68	4	Neg	Neg	RIF-S	28.9	29.9	29.5	29.6	30.4	27.6	RIF-S	29.3	31.4	30.1	30.9	31.2	0.0
69	7	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.8	MTB Neg	0.0	0.0	0.0	0.0	0.0	29.8
70	6	3+	1+	RIF-S	9.4	11.9	10.5	11.4	11.4	27.3	RIF-S	11.9	13.9	12.9	12.8	13.5	27.5
71	5	3+	1+	RIF-S	15.0	17.8	16.0	16.9	16.9	27.5	RIF-S	13.8	16	14.7	14.6	15.3	25.0
72	7	2+	1+	RIF-S	18.5	20.5	19.5	19.9	19.8	26.3	RIF-S	18.1	19.1	18.6	19.1	19.5	23.7
73	5	2+	1+	RIF-S	17.4	19.4	18.3	19.3	18.9	25.4	RIF-S	17.2	18.4	17.5	18.2	18.5	22.6
74	6	2+	1+	RIF-S	14.8	16.8	15.6	16.4	16.7	26.8	RIF-S	12.2	14.7	13.4	13.9	13.9	26.4
75	5	3+	1+	RIF-S	15.0	17.3	15.7	16.9	16.5	26.1	RIF-S	16.2	17.7	16.7	17.4	18.1	27.2
76	9	1+	1+	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.4
77	9	3+	6 AFB	RIF-S	16.3	18.9	17.5	18.2	18.0	25.6	RIF-S	17.5	19.6	18.7	18.9	19.5	29.3
78	13	1+	1+	RIF-S	12.0	15.4	13.5	13.5	13.6	26.4	RIF-S	11.9	14.5	12.7	13.9	13.9	28.9
79	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.8	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.1
80	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.5	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.2
81	6	2+	1+	RIF-S	14.7	16.7	15.5	15.8	15.7	26.4	RIF-S	13.8	16.7	14.6	15.8	15.5	25.2
82	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	27.0	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.1
83	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.2	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.3
84	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.2	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.2

85	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.2	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.9
86	4	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.4	MTB Neg	0.0	0.0	0.0	0.0	0.0	23.5
87	2	1+	1+	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.0	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.0
88	1	Neg	Neg	RIF-S	32.2	32.2	32.3	32.4	33.8	26.6	RIF-S	28.7	29.5	29.5	30.0	30.5	27.5
89	2	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.0	MTB Neg	0.0	0	32.4	0.0	0.0	25.7
90	2	1+	3 AFB	RIF-S	20.6	22.9	21.5	21.5	21.5	23.7	RIF-S	12.9	15.6	13.6	14.8	14.8	26.0
91	2	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.2	MTB Neg	0.0	0.0	0.0	0.0	0.0	24.1
92	2	1+	1+	RIF-R	20.7	23.4	21.5	0.0	21.6	25.8	RIF-R	25.2	22.2	20.0	0.0	20.4	25.2
93	1	2+	1+	RIF-S	13.3	14.9	14.3	14.3	14.3	26.0	RIF-S	15.6	19.3	16.5	17.8	16.9	25.7
94	1	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.9	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.4
95	2	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.5	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.2
96	1	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	26.5	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.5
97	1	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.8	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.6
98	6	Neg	Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.6	MTB Neg	0.0	0.0	0.0	0.0	0.0	25.5
99	6	1+	1+	RIF-S	13.3	14.7	13.6	15.1	15.0	25.0	RIF-S	17.2	19.1	17.4	18.0	18.7	24.7
100	6	1+	1+	RIF-S	18.6	19.8	19.2	20.7	20.0	25.2	RIF-S	16.1	17.9	16.6	18.0	17.7	25.0

Abbreviations:AFB: acid-fast bacilli; Neg: Negative; OMS: OMNIgene•SPUTUM; RIF-Ind: MTB detected but rifampicin-indeterminate; RIF-R: rifampicin-resistant; RIF-S: rifampicin-sensitive; SOC: standard of care; SPC: control probe

Table 3. Smear and Xpert MTB/RIF assay results listed by number of days in transport

Sample ID	Days in Transport	Smear		Xpert MTB/RIF Result	
		SOC	OMS	SOC	OMS
88	1	Neg	Neg	RIF-S	RIF-S
93		2+	1+	RIF-S	RIF-S
94		Neg	Neg	MTB Neg	MTB Neg
96		Neg	Neg	MTB Neg	MTB Neg
97		Neg	Neg	MTB Neg	MTB Neg
87	2	1+	1+	MTB Neg	MTB Neg
89		Neg	Neg	MTB Neg	MTB Neg
90		1+	3 AFB	RIF-S	RIF-S
91		Neg	Neg	MTB Neg	MTB Neg
92		1+	1+	RIF-R	RIF-R
95		Neg	Neg	MTB Neg	MTB Neg
11	3	1+	1+	RIF-S	RIF-S
13		Neg	Neg	MTB Neg	MTB Neg
14		Neg	Neg	MTB Neg	MTB Neg
12	4	2+	2+	RIF-R	RIF-R
16		Neg	Neg	MTB Neg	MTB Neg
17		Neg	Neg	MTB Neg	MTB Neg
27		Neg	Neg	MTB Neg	MTB Neg
28		Neg	Neg	MTB Neg	MTB Neg
35		2+	1+	RIF-S	RIF-S
41		Neg	Neg	RIF-S	RIF-S
42		1+	1+	RIF-S	RIF-S
43		Neg	2 AFB	RIF-Ind	RIF-S
44		1+	1+	RIF-S	RIF-S
53		Neg	Neg	RIF-S	RIF-S
55		Neg	Neg	MTB Neg	MTB Neg
56		Neg	Neg	MTB Neg	MTB Neg
68		Neg	Neg	RIF-S	RIF-S
83		Neg	Neg	MTB Neg	MTB Neg
84		Neg	Neg	MTB Neg	MTB Neg
85		Neg	Neg	MTB Neg	MTB Neg
86		Neg	Neg	MTB Neg	MTB Neg
1	5	Neg	Neg	MTB Neg	MTB Neg
3		Neg	Neg	MTB Neg	MTB Neg
6		Neg	Neg	MTB Neg	MTB Neg
7		1+	1+	RIF-S	RIF-S
20		Neg	Neg	MTB Neg	MTB Neg
21		Neg	Neg	MTB Neg	MTB Neg
25		Neg	Neg	RIF-S	MTB Neg

29		Neg	Neg	MTB Neg	MTB Neg
30		Neg	Neg	MTB Neg	MTB Neg
31		Neg	Neg	MTB Neg	MTB Neg
32		3+	3+	RIF-S	RIF-S
33		Neg	Neg	MTB Neg	MTB Neg
37		3+	1+	RIF-S	RIF-S
38		Neg	Neg	MTB Neg	MTB Neg
40		1+	1+	RIF-S	RIF-S
46		1+	1+	RIF-S	RIF-S
50		Neg	Neg	RIF-S	RIF-S
54		1+	1+	RIF-S	RIF-S
71		3+	1+	RIF-S	RIF-S
73		2+	1+	RIF-S	RIF-S
75		3+	1+	RIF-S	RIF-S
4	6	Neg	Neg	MTB Neg	MTB Neg
5		Neg	Neg	MTB Neg	MTB Neg
9		1+	Neg	RIF-S	RIF-S
15		Neg	Neg	MTB Neg	MTB Neg
18		5 AFB	Neg	RIF-S	RIF-S
34		3+	3+	RIF-S	RIF-S
36		Neg	Neg	MTB Neg	MTB Neg
39		1+	1 AFB	RIF-R	RIF-R
49		3+	1+	RIF-S	RIF-S
58		3+	2+	RIF-S	RIF-S
59		1+	9 AFB	RIF-S	RIF-S
60		3 AFB	Neg	RIF-S	RIF-S
61		Neg	Neg	MTB Neg	MTB Neg
62		Neg	Neg	MTB Neg	MTB Neg
63		3+	3+	RIF-S	RIF-S
64		Neg	Neg	MTB Neg	MTB Neg
65		Neg	Neg	MTB Neg	MTB Neg
70		3+	1+	RIF-S	RIF-S
74		2+	1+	RIF-S	RIF-S
79		Neg	Neg	MTB Neg	MTB Neg
80		Neg	Neg	MTB Neg	MTB Neg
81		2+	1+	RIF-S	RIF-S
82		Neg	Neg	MTB Neg	MTB Neg
98		Neg	Neg	MTB Neg	MTB Neg
99		1+	1+	RIF-S	RIF-S
100		1+	1+	RIF-S	RIF-S
2	7	3+	1+	RIF-S	RIF-S
8		1+	1+	RIF-S	RIF-S
10		Neg	Neg	MTB Neg	MTB Neg
19		Neg	Neg	MTB Neg	MTB Neg

22		Neg	Neg	MTB Neg	MTB Neg
23		Neg	Neg	RIF-Ind	RIF-S
24		Neg	Neg	MTB Neg	MTB Neg
26		Neg	Neg	MTB Neg	MTB Neg
45		2+	1+	RIF-S	RIF-S
47		Neg	Neg	MTB Neg	MTB Neg
48		Neg	Neg	MTB Neg	RIF-R
51		1+	1+	RIF-S	RIF-S
52		3+	1+	RIF-S	RIF-S
57		3+	2+	RIF-S	RIF-S
66		3+	1+	RIF-S	RIF-S
67		2+	1+	RIF-S	RIF-S
69		Neg	Neg	MTB Neg	MTB Neg
72		2+	1+	RIF-S	RIF-S
76	9	1+	1+	MTB Neg	MTB Neg
77		3+	6 AFB	RIF-S	RIF-S
78	13	1+	1+	RIF-S	RIF-S

Abbreviations: AFB: acid-fast bacilli; MTB Neg: *M. tuberculosis* Negative; Neg: Negative; OMS: OMNIgene•SPUTUM; RIF-Ind: MTB detected but rifampicin-indeterminate; RIF-R: rifampicin-resistant; RIF-S: rifampicin-sensitive; SOC: standard of care

Table 4. Concordance between the treatment methods with results sorted by days in transport

Days in Transport	*n*	Concordance between SOC and OMS	
		Smear Grade	Xpert
1	5	80%	100%
2	6	80%	100%
3	3	100%	100%
4	18	89%	94%
5	21	81%	95%
6	26	62%	100%
7	18	61%	89%
9	2	50%	100%
13	1	100%	100%

Abbreviations: OMS: OMNIgene•SPUTUM; SOC: standard of care

Table 5. Comparison of diagnostic results for the two treatment methods

	Proportion of SOC (*n*=100)	Proportion of OMS (*n*=100)
Smear (+)	44%	42%
Smear (-)	56%	58%
RIF-S	45%	46%
RIF-R	3%	4%
RIF-Ind	2%	0%
MTB Neg	50%	50%

Abbreviations: MTB Neg: *M. tuberculosis* Negative; OMS: OMNIgene•SPUTUM; RIF-Ind: MTB detected but rifampicin-indeterminate; RIF-R: rifampicin-resistant; RIF-S: rifampicin-sensitive; SOC: standard of care

Table 6. Summary of Xpert MTB/RIF run errors for the two groups

	# of Errors per Treatment Method	
Error Code	SOC (*n*=15)	OMS (*n*=13)
5006/5007 Post-Run Analysis Error	8	8
2127 Operation terminated	3	4
Invalid	4	1

Abbreviations: OMS: OMNIgene•SPUTUM; SOC: standard of care

Table 7. Xpert MTB/RIF run errors by sample

Sample ID	Treatment Method	Error Code	Error Notes
1	SOC	5006	Post-Run Analysis Error
3	SOC	5007	Post-Run Analysis Error
4	SOC	Invalid	SPC Failed, all probes invalid
5	OMS	5007	Post-Run Analysis Error
6	OMS	5007	Post-Run Analysis Error
7	SOC	5007	Post-Run Analysis Error
19	SOC	Invalid	SPC Failed, all probes invalid
19 - repeat	SOC	2127	Operation Terminated. Module communication loss detected
20	SOC	2127	Operation Terminated. Module communication loss detected
21	SOC	Invalid	SPC Failed, all probes invalid
22	SOC	2127	Operation Terminated. Module communication loss detected
23	*OMS	5007	Post-Run Analysis Error
24	SOC	Invalid	SPC Failed, all probes invalid
27	SOC	5007	Post-Run Analysis Error
28	SOC	5007	Post-Run Analysis Error
29	OMS	5007	Post-Run Analysis Error
35	OMS	5007	Post-Run Analysis Error
41	SOC and OMS	5007	Post-Run Analysis Error
52	SOC	5007	Post-Run Analysis Error
53	SOC	5007	Post-Run Analysis Error
91	OMS	2127	Operation Terminated. Module communication loss detected
91 - repeat	OMS	5007	Post-Run Analysis Error
92	OMS	2127	Operation Terminated. Module communication loss detected
93	OMS	2127	Operation Terminated. Module communication loss detected
94	OMS	2127	Operation Terminated. Module communication loss detected
96	OMS	5007	Post-Run Analysis Error
97	OMS	Invalid	SPC Failed, all probes invalid

Abbreviations:OMS: OMNIgene•SPUTUM; SOC: standard of care
*The SOC sample was also run twice due to a "MTB Very Low" result on first run (not an error code), and this result was considered final after the second run indicated MTB Neg.

Table 8. Xpert MTB/RIF results and probe Ct values for samples with matched smear grades (*n*=74).

					Ct Value by Probe											
Sample ID	Smear		Xpert MTB/RIF Result		A		B		C		D		E		SPC	
	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS
1	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.3	27.9
3	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.1	29.9
4	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	28.8	24.7
5	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	28.9	25
6	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.6	24.2
7	1+	1+	RIF-S	RIF-S	24.7	20.2	26.1	20.8	25.1	20.7	26.2	20.7	26.3	22.2	26.3	24.4
8	1+	1+	RIF-S	RIF-S	18.4	20.4	19.7	21.5	18.7	20.9	19.3	21.2	20.7	22.3	25.5	25.1
10	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.2	25.1
11	1+	1+	RIF-S	RIF-S	17.9	20.1	20.2	20.4	18.4	20.4	19.8	21.6	19.6	21.6	22.6	26.4
12	2+	2+	RIF-R	RIF-R	17.4	15.1	19.3	16.5	18.2	15.5	19.4	16.9	0.0	0.0	24.5	24.8
13	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.2	24.5
14	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.0	24.8
15	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.2	33.5
16	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.6	28
17	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.5	26.2
19	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.9	25.1
20	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.9	25.2
21	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.9	34.5
22	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	23.7	24.7
23	Neg	Neg	RIF-Ind	RIF-S	38.0	27.6	38.0	28.3	36.6	27.8	0.0	29.1	0.0	29.5	26.8	25.1
24	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	34.7	30.3
25	Neg	Neg	RIF-S	MTB Neg	29.8	0.0	30.2	0.0	29.4	0.0	31.3	0.0	33.0	0.0	28.1	28.3
26	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.4	25.9
27	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	29.4	25.8
28	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.0	31.5
29	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	30.1	26.4
30	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.9	26.1
31	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.4	26.6
32	3+	3+	RIF-S	RIF-S	10.9	13.3	13.4	15.0	11.9	13.7	12.4	14.2	13.5	14.9	0.0	25.6
33	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.7	25.2
34	3+	3+	RIF-S	RIF-S	16.5	12.7	18.2	14.3	17.3	12.9	17.6	14.2	18.6	14.7	28.0	22.6
36	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	28.1	24.3
38	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.3	27.1
40	1+	1+	RIF-S	RIF-S	20.3	24.2	21.2	25.1	20.7	24.8	20.9	25.0	22.4	26.0	26.2	27.1
41	Neg	Neg	RIF-S	RIF-S	20.3	20.4	22.1	21.9	20.8	21.1	22.1	21.6	22.1	22.3	24.3	23.6
42	1+	1+	RIF-S	RIF-S	19.9	20.4	21.2	21.4	20.4	21.1	20.8	21.7	21.8	22.7	29.8	25.8
44	1+	1+	RIF-S	RIF-S	19.7	20.5	20.6	22.4	20.3	21.2	20.4	21.7	21.7	22.3	0.0	25.3
46	1+	1+	RIF-S	RIF-S	22.7	24.3	24.1	25.5	23.3	24.9	23.9	25.2	24.5	26.2	25.4	26.4
47	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.7	27.1
48	Neg	Neg	MTB Neg	RIF-R	0.0	31.9	0.0	31.5	0.0	30.7	0.0	40.2	0.0	0.0	26.0	25.9
50	Neg	Neg	RIF-S	RIF-S	22.4	30.2	23.6	30.3	22.8	29.9	23.3	31.0	24.1	33.6	25.0	26.4

51	1+	1+	RIF-S	RIF-S	19.3	19.7	20.7	21.4	19.8	20.4	19.9	20.7	21.3	21.4	28.2	23.4
53	Neg	Neg	RIF-S	RIF-S	25.1	19.2	26.0	20.5	25.8	19.4	25.9	20.5	26.5	21.3	24.4	24.2
54	1+	1+	RIF-S	RIF-S	9.4	10.9	10.7	11.9	9.9	10.7	10.0	12.1	10.8	13.7	27.7	0.0
55	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.8	24.9
56	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.7	26.1
61	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.4	26.0
62	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.4	25.6
63	3+	3+	RIF-S	RIF-S	11.6	13.3	14.4	15.6	12.5	14.4	13.5	14.9	13.5	14.9	26.5	28.0
64	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.6	30.2
65	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.4	26.2
68	Neg	Neg	RIF-S	RIF-S	28.9	29.3	29.9	31.4	29.5	30.1	29.6	30.9	30.4	31.2	27.6	0.0
69	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.8	29.8
76	1+	1+	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.4	25.4
78	1+	1+	RIF-S	RIF-S	12.0	11.9	15.4	14.5	13.5	12.7	13.5	13.9	13.6	13.9	26.4	28.9
79	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.8	27.1
80	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.5	26.2
82	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	27.0	25.1
83	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.2	26.3
84	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.2	26.2
85	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.2	26.9
86	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.4	23.5
87	1+	1+	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.0	25.0
88	Neg	Neg	RIF-S	RIF-S	32.2	28.7	32.2	29.5	32.3	29.5	32.4	30.0	33.8	30.5	26.6	27.5
89	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0	0.0	32.4	0.0	0.0	0.0	0.0	25.0	25.7
91	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	24.2	24.1
92	1+	1+	RIF-R	RIF-R	20.7	25.2	23.4	22.2	21.5	20.0	0.0	0.0	21.6	20.4	25.8	25.2
94	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.9	25.4
95	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.5	25.2
96	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	26.5	25.5
97	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.8	25.6
98	Neg	Neg	MTB Neg	MTB Neg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	25.6	25.5
99	1+	1+	RIF-S	RIF-S	13.3	17.2	14.7	19.1	13.6	17.4	15.1	18.0	15.0	18.7	25.0	24.7
100	1+	1+	RIF-S	RIF-S	18.6	16.1	19.8	17.9	19.2	16.6	20.7	18.0	20.0	17.7	25.2	25.0

Abbreviations: MTB Neg: *M. tuberculosis* Negative; Neg: Negative; OMS: OMNIgene•SPUTUM; RIF-Ind: MTB detected but rifampicin-indeterminate; RIF-R: rifampicin-resistant; RIF-S: rifampicin-sensitive; SOC: standard of care; SPC: control probe

Table 9. Summary of samples with matched smear grades but discordant Xpert MTB/RIF results (*n*=3).

					Ct Value by Probe											
Sample ID	Smear		Xpert MTB/RIF Result		A		B		C		D		E		SPC	
	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS
23	Neg	Neg	RIF-Ind	RIF-S	38.0	27.6	38.0	28.3	36.6	27.8	0.0	29.1	0.0	29.5	26.8	25.1
25	Neg	Neg	RIF-S	MTB Neg	29.8	0.0	30.2	0.0	29.4	0.0	31.3	0.0	33.0	0.0	28.1	28.3
48	Neg	Neg	MTB Neg	RIF-R	0.0	31.9	0.0	31.5	0.0	30.7	0.0	40.2	0.0	0.0	26.0	25.9

Abbreviations: MTB Neg: *M. tuberculosis* Negative; Neg: Negative; OMS: OMNIgene•SPUTUM; RIF-Ind: MTB detected but rifampicin-indeterminate; RIF-R: rifampicin-resistant; RIF-S: rifampicin-sensitive; SOC: standard of care; SPC: control probe

Table 10. Xpert MTB/RIF and probe Ct values for samples with mismatched smear grades (*n*=26).

le ID	Smear		Xpert MTB/RIF Result		Ct Value by Probe											
					A		B		C		D		E		SPC	
	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS
2	3+	1+	RIF-S	RIF-S	22.3	17.5	23.2	18.7	22.8	18.2	22.7	18.5	24.3	19.8	0.0	24.2
9	1+	Neg	RIF-S	RIF-S	20.9	22.2	22.7	23.2	21.3	22.7	22.6	23.7	22.5	23.7	23.8	25.1
18	5 AFB	Neg	RIF-S	RIF-S	19.1	21.3	20.6	22.9	19.5	21.8	20.8	23.3	20.7	22.6	27.0	23.9
35	2+	1+	RIF-S	RIF-S	13.6	12.3	15.4	13.4	13.9	12.5	14.4	13.2	15.4	13.8	26.0	24.9
37	3+	1+	RIF-S	RIF-S	11.6	14.2	13.3	15.7	12.4	14.3	11.9	15.3	13.8	16.3	29.7	25.4
39	1+	1 AFB	RIF-R	RIF-R	24.8	23.3	25.4	24.8	25.4	24.2	25.6	24.5	0.0	0.0	24.2	25.2
43	Neg	2 AFB	RIF-Ind	RIF-S	37.9	26.4	0.0	27.0	35.9	26.8	0.0	27.0	0.0	28.5	0.0	24.9
45	2+	1+	RIF-S	RIF-S	13.8	14.8	15.8	16.9	14.5	15.7	14.9	15.9	15.8	16.7	27.1	25.9
49	3+	1+	RIF-S	RIF-S	12.5	14.4	13.8	16.5	12.5	14.8	12.6	15.4	14.7	16.3	28.3	27.9
52	3+	1+	RIF-S	RIF-S	16.2	18.1	17.9	19.9	16.7	18.7	17.2	19.5	17.8	20.3	27.0	27.2
57	3+	2+	RIF-S	RIF-S	10.4	12.0	12.4	13.6	10.9	12.3	10.8	12.7	11.8	13.7	25.6	25.5
58	3+	2+	RIF-S	RIF-S	13.3	14.5	15.4	16.3	14.5	15.3	13.9	15.5	14.6	16.0	26.5	26.5
59	1+	9 AFB	RIF-S	RIF-S	21.4	20.9	22.8	22.1	22.3	21.6	22.4	21.8	22.7	22.6	25.1	23.4
60	3 AFB	Neg	RIF-S	RIF-S	19.7	25.0	21.7	25.6	20.7	25.6	20.7	25.7	20.9	26.5	26.0	24.9
66	3+	1+	RIF-S	RIF-S	17.3	14.4	19.2	16.3	18.5	15.4	18.4	16.1	19.0	16.3	28.6	30.0
67	2+	1+	RIF-S	RIF-S	17.6	18.5	19.2	20.5	18.5	19.4	19.2	20.2	19.3	20.3	26.6	29.0
70	3+	1+	RIF-S	RIF-S	9.4	11.9	11.9	13.9	10.5	12.9	11.4	12.8	11.4	13.5	27.3	27.5
71	3+	1+	RIF-S	RIF-S	15.0	13.8	17.8	16	16.0	14.7	16.9	14.6	16.9	15.3	27.5	25.0
72	2+	1+	RIF-S	RIF-S	18.5	18.1	20.5	19.1	19.5	18.6	19.9	19.1	19.8	19.5	26.3	23.7
73	2+	1+	RIF-S	RIF-S	17.4	17.2	19.4	18.4	18.3	17.5	19.3	18.2	18.9	18.5	25.4	22.6
74	2+	1+	RIF-S	RIF-S	14.8	12.2	16.8	14.7	15.6	13.4	16.4	13.9	16.7	13.9	26.8	26.4
75	3+	1+	RIF-S	RIF-S	15.0	16.2	17.3	17.7	15.7	16.7	16.9	17.4	16.5	18.1	26.1	27.2
77	3+	6 AFB	RIF-S	RIF-S	16.3	17.5	18.9	19.6	17.5	18.7	18.2	18.9	18.0	19.5	25.6	29.3
81	2+	1+	RIF-S	RIF-S	14.7	13.8	16.7	16.7	15.5	14.6	15.8	15.8	15.7	15.5	26.4	25.2
90	1+	3 AFB	RIF-S	RIF-S	20.6	12.9	22.9	15.6	21.5	13.6	21.5	14.8	21.5	14.8	23.7	26.0
93	2+	1+	RIF-S	RIF-S	13.3	15.6	14.9	19.3	14.3	16.5	14.3	17.8	14.3	16.9	26.0	25.7

Abbreviations: AFB: acid-fast bacilli; Neg: Negative; OMS: OMNIgene•SPUTUM; RIF-Ind: MTB detected but rifampicin-indeterminate; RIF-R: rifampicin-resistant; RIF-S: rifampicin-sensitive; SOC: standard of care; SPC: control probe

Table 11. Summary of samples with mismatched smear grades but discordant Xpert MTB/RIF results (*n*=1).

Sample ID	Smear		Xpert MTB/RIF Result		Ct Value by Probe											
					A		B		C		D		E		SPC	
	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS	SOC	OMS
43	Neg	2 AFB	RIF-Ind	RIF-S	37.9	26.4	0.0	27.0	35.9	26.8	0.0	27.0	0.0	28.5	0.0	24.9

Abbreviations: AFB: acid-fast bacilli; Neg: Negative; OMS: OMNIgene•SPUTUM; RIF-Ind: MTB detected but rifampicin-indeterminate; RIF-S: rifampicin-sensitive; SOC: standard of care; SPC: control probe

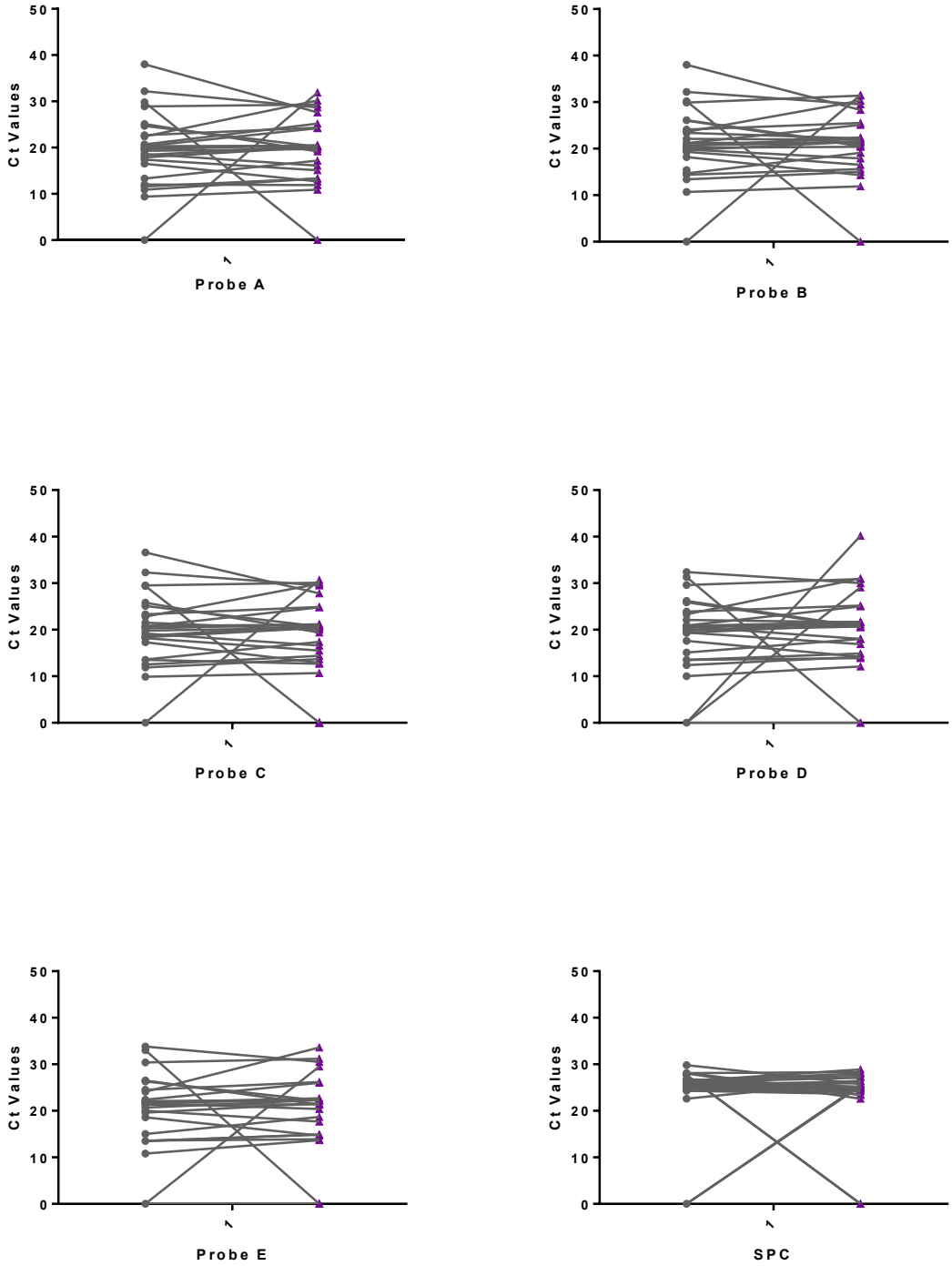

Figure 4. Ct values by probe for the two methods for sampleswith matching smear grades (*n* ¼ 74). Graphs exclude sampleswith MTB; grey circles ¼ SOC; black triangles ¼ OMNIgene**W**-SPUTUM. Ct ¼ threshold cycle; MTB ¼*M.tuberculosis*negative; SOC ¼ standard of care.

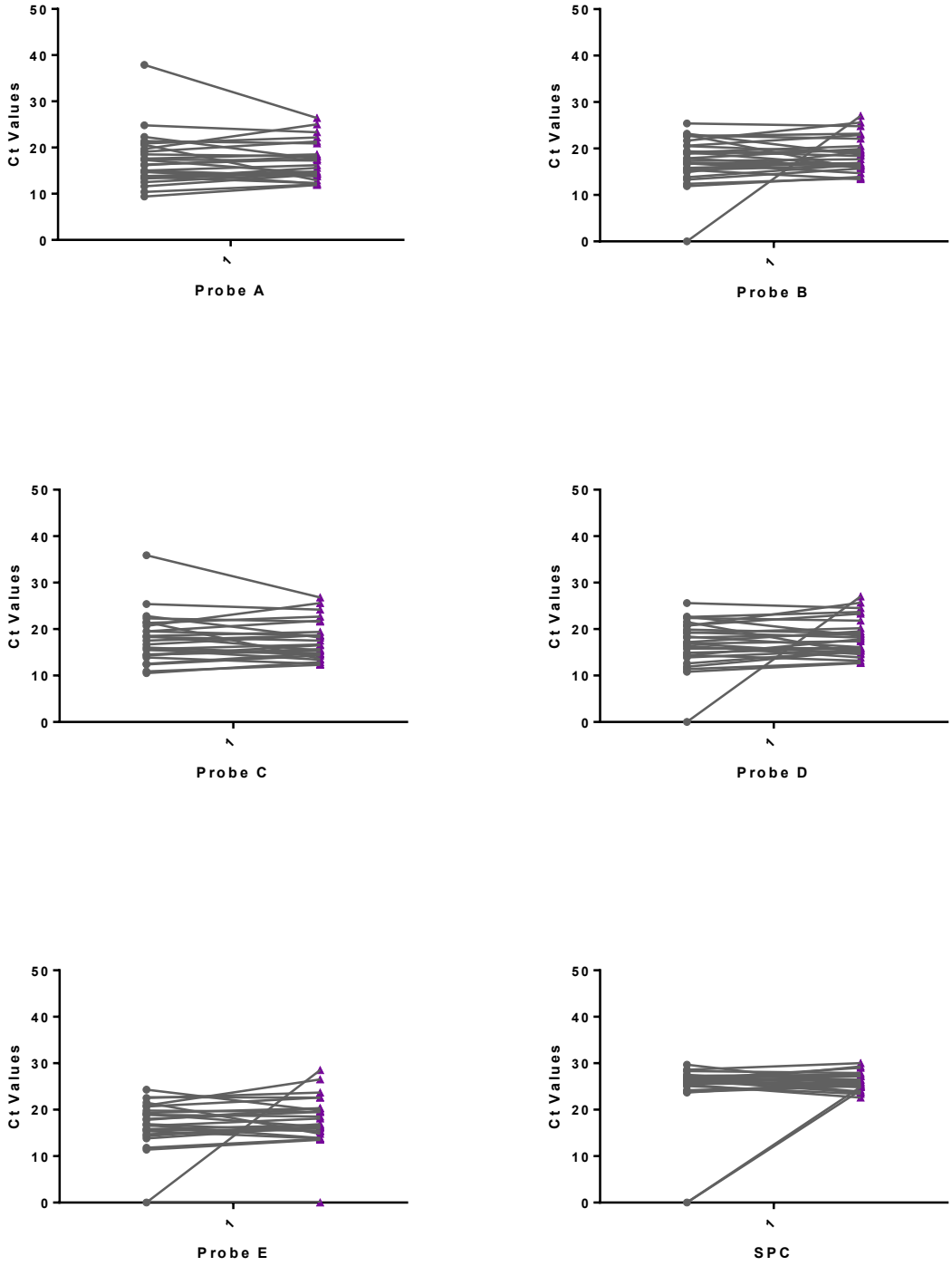

Figure 5. Ct values by probe for the two methods for sampleswith mismatched smear grades (*n* ¼ 26). Graphs excludesamples with MTB; grey circles ¼ SOC; black triangles ¼OMNIgene**W**SPUTUM. Ct ¼ threshold cycle; MTB¼ *M.tuberculosis*-negative; SOC ¼ standard of care**.**

SUMMARY

This study was conducted atGENETUP national tuberculosis reference laboratory in Nepal to evaluate whether transporting samples in OMNIgene®•SPUTUM (OMS) from a peripheral collection site to a central laboratory in Nepal can improve tuberculosis detection and increase the sensitivity of Cepheid® Xpert® MTB/RIF testing. One hundred sputa were manually split. Each portion was assigned to the OMS group (OMS added at collection, airline-couriered without cold chain, no other processing required) or the standard-of-care (SOC) group (samples airline-couriered on ice, NaOH+NALC processing required at the laboratory). Smear microscopy and Xpert® MTB/RIF testing were performed.Transport time was 2-13 days. Overall smear results were comparable (58% and 56% smear-negative in the OMS and SOC groups, respectively). The rate of smear-positive MTB-positive sample detection was identical for both treatment groups at 95%. More smear-negative MTB-positive samples were detected in the OMS group (17% versus 13%; p=0.0655). The study suggests thatsputa treated with OMS can undergo multi-day ambient-temperature transport and yield comparable smear and Xpert® MTB/RIF results to those of SOC samples.

Chapter III

Direct detection of *Mycobacterium tuberculosis* in clinical samples by a dry methyl green loop-mediated isothermal amplification (LAMP) method

INTRODUCTION

With an estimated 10 million new cases and 1.6 million deaths from tuberculosis (TB) in 2017, TB is the leading cause of death from an infectious disease globally, particularly in developing countries (40). The WHO has proposed an 'End TB' strategy that seeks to end the global TB epidemic by reducing new cases by 90% and by decreasing TB deaths by 95% between 2015 and 2035; WHO has strongly emphasized the need for development of accurate and rapid point of care (POC) diagnostic methods as a part of this strategy (41).

The conventional diagnosis of TB comprises clinical examination, chest x-ray and bacteriological examination. Bacteriological examination involves direct observation of acid-fast bacilli in sputum samples and further processing of sputum samples for culture (13). In spite of the low sensitivity and specificity of sputum microscopy, and the limitation in its ability to differentiate *Mycobacterium tuberculosis* complex (MTBC) from other nontuberculosis mycobacteria (NTM), sputum microscopy is still widely used in developing countries. Bacteriological culture, while considered a gold standard test for TB diagnosis, requires skilled manpower, infrastructure and a 6-8 weeks to get results (42). In a similar vein, molecular-based rapid diagnostic tools, such as the Genexpert (Cepheid, CA) and Hain line probe assay (Hain life sciences GmbH), might not be convenient for routine diagnosis of TB in developing countries due to their high cost and insufficient laboratory infrastructure.

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification assay that can detect DNA or RNA with high specificity, efficiency and rapidity under isothermal

amplification conditions (20). In 2012, WHO recognized the feasibility of molecular detection of TB using LAMP, citing its advantages of being simple and having the potential as a POC diagnostic method (43). In a policy document released on 2016, WHO advised that a commercial MTB-LAMP (such as Loopamp MTBC detection kit, Eiken chemical company, Japan) may be used as a replacement test for sputum microscopy for the diagnosis of pulmonary TB in adults with clinical signs and symptoms consistent with TB (44). Thus, the development a LAMP-based diagnostic test for TB that combines the simplicity and rapidity of sputum microscopy with the reliability of bacterial culture is a clear priority.

Efforts around the research and development of LAMP technology have focused mainly on two areas. First is with the practical application of LAMP to various pathogens in clinical settings, and second is the development of simple and rapid methods for detection of positive LAMP reactions (16). Several methods have been used to detect positive LAMP reactions: i) by using a real-time turbidimeter that detects turbidity arising from magnesium pyrophosphate formation (17, 27); ii) by using a real-time PCR machine which detects fluorescence (6); iii) by visual detection by agarose gel electrophoresis (6, 20); iv) by visual detection using the commercial Eiken fluorescent detection reagent (FD) which requires a UV illuminator to detect positive LAMP reactions (32); v) by visual detection using other nucleic acid binding fluorescent dyes such as pico green (32), propidium iodide (10), SYBR green (8, 10); vi) by non-fluorescent dyes that can visually detect positive LAMP reactions such as hydroxylnaphthol blue (HNB) (7), malachite green (MaG) (21) and leuco crystal violet (18).

In this study, we have identified methyl green (MeG) as a novel dye that can be used to visually detect positive LAMP reactions without the aid of a UV illuminator. We have employed a dry form of MeG based MTB-LAMP to overcome some of the more usual issues with the LAMP reaction such as contamination due to frequent opening of reaction tubes and

the need for maintenance of a cold chain for the reagents. Our simple colorimetric, dry-MeG based MTB-LAMP reaction mixture was validated to detect DNA of TB bacteria using clinical samples submitted for TB testing in Nepal.

METHODS

Preliminary testing of dyes

Thirteen leuco dyes were selected to assess their potential as dyes for the detection of a positive LAMP reaction. They were: crystal violet; fuchsin basic; bromophenol blue (Wako, Japan); phenol red sodium salt; basic violet; pararosaniline hydrochloric acid; bromo-cresol green; bromocresol purple sodium salt; comassie brilliant blue; thymol blue; bromothymol blue (all from Tokyo Chemical Industries, Japan); and methyl blue and MeG (both from Sigma-Aldrich, USA). The parameters that were used to assess suitability of the 13 dyes were that tested dyes should not inhibit the LAMP reaction and should be able to visually differentiate between positive and negative LAMP reaction by a change in color.Dyes with proven utility in a LAMP reaction, namely MaG, HNB, SYBR green and Eiken FD (from Eiken Chemical Co. Ltd.) were used as comparators in this study.

Dilution of dyes

Dyes were dissolved in double-distilled water (DDW) to prepare a stock solution of 1%. The stock solution was further diluted in DDW to prepare different concentrations (0.05%, 0.1%, 0.2% & 0.4%) of working solution to be used for LAMP reactions.

Screening of dyes for MTB-LAMP reaction

A previously developed and validated MTB-LAMP system that targets the MTB16S rRNA gene (27) was used for the LAMP reaction. The MTB-LAMP reaction was performed in 25 µl of reaction mixture consisting of: 0.2µM of each outer primer (F3 & B3); 1.6 µM each inner primer (FIP & BIP) and 2.2µM of loop primer (FLP & BLP); 1 mMdNTPs; 0.8 M betaine; 20 mMTris-HCl (pH 8.8); 8 mMKCl; 8mM $(NH_4)_2SO_4$; 0.08% Tween-20; 3 mM $MgSO_4$; 8 units of *Bst* DNA polymerase (New England Biolabs), and 1 µl of dye working

concentrations. *M. bovis* Bacillus Calmette-Guerin (BCG) Tokyo 172 DNA at a concentration of 5 picogram (pg), 500 femtogram (fg) or 50 fg per tube was used for the LAMP reactions. The mixture was incubated at 64°C for 60 minutes in a Loopamp real time turbidimeter (LA-200, Teramecs). The reaction was deemed positive when the turbidity was greater than 0.1 unit within 60 minutes. Subsequently, the change in color and time required for the turbidity to be greater than 0.1 was recorded. The reaction mixture was then heated at 95°C for 5 minutes to terminate the reaction. Each reaction was performed with technical duplicates and repeated in three independent experiments.

Development of the dry MTB-LAMP method

For the preparation of dry LAMP reagents, 10.7 µl of reaction mixture consisting of: 2 µl of primer mix [consisting of 100 µM of outer primers (F3 & B3), inner primers (FIP & BIP) and loop primers (FLP & BLP) mixed at 1:8:11 ratio]; 1.4 µl of dNTPs (25mM each); 2.5 µl of 2M trehalose; 1 µl of 25× LAMP buffer (500 mMTris-HCl [pH 8.8], 250 mMKCl); 1.8µl of 100 mM $MgSO_4$; 8 units of *Bst*2.0 warm start DNA polymerase (New England Biolabs) and 1 µl of 0.1% of MeG was used. To dry the mixture, the prepared reaction mixture was placed on the periphery of the inner side of a 0.2 ml tube lid and kept under a flow of clean air on a clean bench for 3 hours (Fig. 6). Then, the tubes were stored in boxes with zeolite molecular sieves. Boxes were wrapped in an opaque plastic bag and stored at room temperature.

For the MTB-LAMP reaction with dried reagents, 23 µl DDW and 2 µl DNA containing *M. bovis*BCG Tokyo 172 DNA at a concentration of 5 pg, 500 fg or 50 fg per tube was added into the bottom of tubes. In negative control tubes, 25 µl DDW was added into the bottom of tubes. The tubes were turned upside down and placed for 3 minutes in the inverted

position to allow for the reconstitution of reagents. Then the reaction mixture was mixed by inverting five times (Fig. 6). The reaction was performed and recorded as described above.

Validation of dry MTB-LAMP system using clinical TB samples in Nepal

After development of MeG MTB-LAMP method and further simplification of the system by preparing the dry MeG MTB-LAMP method, we validated our dry MTB-LAMP method in Nepal from June to December 2016. Validation of the dry MeG MTB-LAMP method was performed using clinical samples and only simple visual inspection of color development was used to detect positive or negative LAMP reaction.

A total of 69 clinical samples from new TB suspected Nepalese patients who had been referred for TB testing to the National Anti-Tuberculosis Association – German Nepal TB Project (NATA-GENETUP) reference laboratory were evaluated to determine sensitivity and specificity of our developed method. Smear microscopy, decontamination and concentration of the samples were performed as previously described (27, 28). The processed samples were used for inoculation in culture media as described, and 500 µl of the remaining sample was used for DNA extraction. The DNA was extracted by alternate cycles of boiling (10 minutes at 95°C) and freezing (30 minutes at -20°C) for three times (33). The extracted DNA was stored at -20°C until analysis.

Freshly dried MeG MTB-LAMP tubes were transported from Japan to Nepal and were stored at room temperature prior to use. For the MTB-LAMP reaction, 20 µl of DDW and 5 µl of extracted DNA were added to the bottom of tubes, whereas for the negative controls only 25µl of DDW was added. The presence or absence of a blue-green color was used to determine positive or negative MTB-LAMP reactions (Fig. 6). The LAMP reaction was always performed by using positive and negative controls to facilitate interpretation of the test results.

In this study, MeG MTB-LAMP was not used for clinical diagnosis of TB. Our study was retrospectively performed after collecting all the samples so as to evaluate the efficacy of our developed method.

RESULTS

Preliminary screening of dyes for MTB-LAMP detection

Our preliminary experiment to assess the suitability of dyes to detect positive MTB-LAMP reactions identified only MeG as being suitable. Using MeG, a positive LAMP reaction developed a blue-green color with an increment of turbidity greater than 0.1 in a Loopamp real time turbidimeter (LA-200, Teramecs) indicating amplification of DNA; meanwhile, the negative reaction mixture was colorless and did not show turbidity, indicating the absence of DNA amplification.

Comparison of dyes used for detection of MTB-LAMP reaction

We assessed the suitability of MeG in comparison with other proven dyes used for visual detection of LAMP reactions (Table 12). The standard dyes showed the expected utility in the LAMP reaction. We also wanted to compare the relative speed of detecting positive LAMP reactions when these dyes were used. Both MeG and malachite green (MaG) (Nzelu et al, 2014) showed a similar speed in developing a positive LAMP reaction, but the reaction mixture with MeG had a more intense color (Table 13, Fig. 7). For the comparative study, we used the lower concentration [(0.05% stock, final concentration 0.002%) and (0.1% stock, final concentration 0.004%)] of dyes to reduce the likelihood of any inhibition of the reaction and to ensure a minimal effect on turbidity (17).

Development of dry methyl green MTB-LAMP

We identified a concentration of 0.1% MeG (final concentration of 0.004% in a 25µl reaction volume) to be optimal for the design of dried reagents for LAMP reactions, based on its speed of color development and superior color intensity. (Table 13, Fig.7). Using this

concentration of MeG, the dry MTB-LAMP system successfully detected LAMP reaction using our developed conditions with BCG DNA (Table 14, Fig.6).

Validation of dry MeG MTB-LAMP system using clinical samples of TB in Nepal

We compared the performance of our dry MeG MTB-LAMP system with microscopy and culture. The sensitivity and specificity were evaluated using 69 new TB suspected clinical samples that were submitted for TB testing at GENETUP Nepal. Although we tested 142 clinical samples, we excluded 67 samples from patients that were under treatment, or re-treatment or treatment failure status and also excluded 6 samples with unknown treatment history (Table 15).The sensitivity of our MeG MTB-LAMP system in culture positive samples was 92.8% (13/14) and specificity in culture negative samples was 96.3% (53/55); and the sensitivity and specificity when compared with sputum microscopy was 92.8% (12/13) and 94.6% (53/56) respectively (Table 16) indicating its potential for POC MTB detection method.

DISCUSSION

The simplest method to detect a LAMP reaction is by observing the turbidity using the naked eye; however, it is often difficult to distinguish turbidity and it would be unreliable as the basis for a POC test. Although a Loopampturbidimeter can be used to detect turbidity in real time, using a Loopampturbidimeter for a POC test is an inconvenient option in developing countries. Thus, developing a simple visual detection system for LAMP is an attractive option. Some of the reported visual detection systems, such as Eikenfluorescent dye and HNB (Table 12), only show a small color difference between positive and negative reactions (Fig. 7), proving difficult to distinguish with the naked eye.

We identified MeG as a suitable dye to detect MTB-LAMP positive reactions. As with the previously reported MaG(21), MeG could be used for visual detection of LAMP reactions, where the positive reaction was a blue-green color and the negative reaction was colorless. While both MeG and MaG had similar visual detection properties, the color intensity of MeG in positive LAMP reaction was superior to that of MaG (Table 13, Fig. 7), thus making it easier to differentiate between positive and negative reactions. Using the lowest concentration (50fg) of DNA, the MeG-based MTB-LAMP proved more sensitive (detected 4 of 6 reactions, both with 0.05% and 0.1% dye) than the MaG-based MTB-LAMP (1 of 6 with 0.05% dye and 2 of 6 with 0.1% dye) (Table 13). The visual detection of the LAMP reaction using MeG was both reproducible and consistent. Thus, MeG was selected as a novel candidate for developing a simple POC using an MTB-LAMP detection system. We hypothesize that MeG detects changes in DNA concentration, whereby color development is through binding of MeG to the major groove of DNA as previously suggested (14, 29) and in a similar way as previously reported with crystal violet (18).

To simplify the LAMP system as a POC test, we dried the reaction mixture using a vitrification technique as previously described (9). The preparation of dried reagents was

simple and could be easily implemented in developing countries (Fig. 6). Like wet LAMP reagents, dried LAMP reagents successfully yielded LAMP positive reactions (Table 14). These dried LAMP reagents could be stored at room temperature and were stable for up to 4 months (data not shown). Both the wet and dry MTB-LAMP system could detect up to 50fg of TB DNA (Table 13 &14, Fig. 6 & 7). However, the time detection speed for dry reagents was lower than the wet LAMP system. When we compared the real time detection speed of both our wet and dry MeG MTB-LAMP (Table 13 &14) with that of real time turbidity result of the previously reported MTB-LAMP (27), the time detection speed was equivalent suggesting that 0.004% MeG does not interfere with the LAMP reaction. It is estimated that the weight of 4.4 mega base pairs of one MTB bacterial genome is 5 fg. As our LAMP method detected to 50 fg of DNA, this suggest that as few as 10 MTB bacilli could be detected using our method.

Initially, we validated our in-house developed dry MeG MTB-LAMP system by using 142 clinical samples [105 sputum and 37 extra-pulmonary (pus, urine, pleural fluid, biopsy tissue, broncho-alveolar lavage, fine needle aspiration sample, bone marrow)] that were submitted for TB testing at GENETUP Nepal (Table 15). However, to calculate sensitivity and specificity in comparison with the culture results, we excluded 73 samples from patients who were under-treatment, re-treatment or treatment failure, or those samples from patients with unknown treatment history; these samples can provide discrepant results when compared with culture results because of the possibility of the presence of dead MTB bacteria that can be detected by MTB-LAMP but not by culture (Table 15). Thus, for a targeted evaluation of our method, we included only 69 samples from new TB suspected cases and found the sensitivity and specificity when compared with culture results to be 92.8% and 96.3% respectively (Table 16). Our result is superior than the results of a summary of 26 different MTB-LAMP studies that were conducted globally where overall

sensitivity and specificity was 89.6% and 94.0% respectively (22). When we compared the result of MTB-LAMP and smear microscopy of those 69 new suspect TB samples, sensitivity and specificity was 92.3% and 94.6% respectively (Table 16). The advantage of our MTB-LAMP system over smear microscopy is its ability to detect only MTB complex bacteria and to distinguish MTB complex from nontuberculous mycobacteria (NTM). Two retreatment samples that were smear positive/negative, both culture positive and later identified as NTM by a line probe assay (HainLifesciences GmbH) were negative by our MTB-LAMP system (Table 15). From these results, we suggest that our dry MeG MTB-LAMP method could be performed together with smear microscopy as a POC TB diagnosis method.

In this study, we have identified MeG, methyl green, as a novel candidate for simple and visual detection of LAMP reactions. We have simplified and validated our MeG-based MTB-LAMP visual detection system by preparing dried reagents with a low cost (less than one dollar per test) and demonstrated its potential for the diagnosis of TB in developing countries. Further improvement of our dry MeG-based MTB-LAMP system will help to develop it as an effective POC test. Furthermore, MeG is a suitable candidate for simple visual detection of LAMP reactions and can be applied to wide range of other infectious diseases or other diagnostic areas that employ LAMP reactions.

Table 12. Comparison of dyes used for visual detection of LAMP reaction

	Dyes	Detection methods			
		Naked eye	Ultraviolet fluorescence	Inhibit LAMP if added before reaction	Reference
	Propidium Iodide	Yes	Yes (enhanced)	Yes	[13]
	Pico green	Yes	NA	Yes	[11]
*	Hydroxynaphthol blue	Yes	Not required	No	[15]
*	Malachite green (MaG)	Yes	Not required	No	[16]
*	Eiken fluorescent dye	Yes	Yes (enhanced)	No	[12]
*	Methyl green (MeG)	Yes	Not required	No	This study

*: Dyes selected for comparison in this study
NA: Not available

Table 13. Time in minutes required for the LAMP solution to exceed the turbidity of 0.1 in real time turbidimeter(LA-200, Teramecs) with different concentrations of dyes and DNA

DNA concentration	0.05%			Eiken fluorescent dye
	Methyl green	Malachite green	Hydroxynaphthol blue	
5 pg	14.4±2.4	14.3±1.4	14.5±1.4	15.4±0.3
500 fg	18.6±2.1	21.9±7.4	18.8±3.7	21.1±4.4
50 fg	37.6±16.5[§](4 of 6)	21.6[*] (1 of 6)	31.6[*] (1 of 6)	23.7±2.4[#] (5 of 6)
NC	NA	NA	NA	NA
	0.1%			Eiken fluorescent dye
	Methyl green	Malachite green	Hydroxynaphthol blue	
5 pg	16.5±1.4	15±1.9	17.6±4.1	15.4±0.3
500 fg	20.4±1.3	20.2±1.8	25.3±7.3[#] (4 of 6)	21.1±4.4
50 fg	32.6±13[§] (4 of 6)	25±4.5[†] (2 of 6)	54.6±3.3[†] (2 of 6)	23.7±2.4[#] (5 of 6)
NC	NA	NA	NA	NA

Data is presented as mean ± standard deviation
LAMP reaction was performed in duplicate in three independent experiments. Sample size is 6, unless it is indicated. Information in parenthesis indicate number of times results of LAMP reaction were obtained out of total 6 reactions.
*sample size = 1, † sample size = 2, §sample size = 4, # sample size = 5, these sample size indicate result of positive LAMP reaction.
NC= Negative control, DDW
NA= not available, inability of turbidity to exceed 0.1 within 60 minutes of reaction.

Table 14. Time in minutes required for the dried LAMP system to exceed the turbidity of 0.1 in real time turbidimeter (LA-200, Teramecs) with different concentration of DNA

DNA concentration	Time
5 pg	20.7±1.7
500 fg	27.5±5.6
50 fg	31±4.2[§](4 of 6)
NC	NA

Data is presented as mean ± standard deviation
Sample size is 6, unless otherwise indicated. Information in parenthesis indicates the number of times results of the LAMP reaction were obtained out of a total 6 reactions.
§sample size = 4 as 4 reactions were positive.
NC= Negative control, DDW
NA= not available, inability of turbidity to exceed 0.1 within 60 minutes of reaction

Table 15. All clinical specimens examined in Chapter III

S. No	Lab ID	Patient treatment category	Smear	Culture	Dry MTB-LAMP	Sample
1	1260A	New	Negative	Negative	Negative	sputum
2	1382A	New	Negative	Negative	Negative	sputum
3	1448A	New	1+	1+	Positive	sputum
4	1451A	New	1+	3+	Positive	sputum
5	1512A	New	1+	1+	Positive	sputum
6	1549A	New	1+	1+	Positive	sputum
7	1867B	New	Negative	Negative	Negative	sputum
8	1871A	New	Negative	Negative	Negative	sputum
9	1880A	New	Negative	Negative	Negative	sputum
10	1900A	New	Negative	Negative	Negative	sputum
11	1901A	New	Negative	Negative	Negative	sputum
12	1841A	New	Negative	Negative	Negative	sputum
13	1941A	New	1+	2+	Positive	sputum
14	1966A	New	Negative	Negative	Negative	sputum
15	1978A	New	Negative	Negative	Negative	sputum
16	2053A	New	Negative	Negative	Positive	sputum
17	2108A	New	1+	1+	Positive	sputum
18	2175B	New	6AFB	1+	Positive	sputum
19	2233A	New	Negative	Negative	Negative	sputum
20	2192A	New	Negative	Negative	Negative	sputum
21	2404A	New	Negative	Negative	Negative	sputum
22	2402A	New	Negative	Negative	Negative	sputum
23	2279A	New	Negative	Negative	Negative	sputum
24	2285	New	Negative	Negative	Negative	sputum
25	2568A	New	Negative	Negative	Negative	pus
26	2534A	New	Negative	Negative	Negative	urine
27	2499A	New	Negative	Negative	Negative	pleural fluid
28	2380A	New	Negative	Negative	Negative	pleural fluid
29	2216A	New	Negative	Negative	Negative	urine
30	2086A	New	Negative	Negative	Negative	pleural fluid
31	2074A	New	Negative	Negative	Negative	pleural fluid
32	1446A	New	Negative	Negative	Negative	pus
33	1868A	New	Negative	Negative	Negative	pleural fluid
34	1884A	New	Negative	Negative	Negative	urine
35	2000A	New	Negative	Negative	Negative	pleural fluid
36	2001A	New	Negative	Negative	Negative	pleural fluid
37	2061A	New	Negative	Negative	Negative	pleural fluid
38	1856A	New	Negative	Negative	Negative	bonemarrow
39	1827A	New	Negative	Negative	Negative	Biopsy tissue

40	1800A	New	Negative	Negative	Negative	BAL
41	1341A	New	2+	1+	Positive	sputum
42	1341B	New	5 AFB	2+	Positive	sputum
43	1312B	New	Negative	Negative	Negative	sputum
44	1512B	New	1 AFB	1+	Positive	sputum
45	2053B	New	Negative	Negative	Positive	sputum
46	1866A/B	New	Negative	Negative	Negative	sputum
47	1833A	New	Negative	Negative	Negative	sputum
48	1992F	New	Negative	1+	Positive	sputum
49	2183A	New	Negative	Negative	Negative	sputum
50	1251A	New	Negative	Negative	Negative	sputum
51	1964A	New	Negative	Negative	Negative	sputum
52	1880B	New	Negative	Negative	Negative	sputum
53	1435	New	Negative	Negative	Negative	pleural fluid
54	1387A	New	Negative	Negative	Negative	endometrial tissue
55	1939	New	Negative	Negative	Negative	urine
56	2141A	New	Negative	Negative	Negative	pleural fluid
57	1985A	New	Negative	Negative	Negative	pus
58	2200A	New	Negative	Negative	Negative	bone marrow
59	1193A	New	Negative	Negative	Negative	sputum
60	1260	New	Negative	Negative	Negative	sputum
61	1867A	New	Negative	Negative	Negative	sputum
62	2510A	New	Negative	Negative	Negative	sputum
63	2412B	New	4 AFB	1+	Negative	sputum
64	H41	New	Negative	Negative	Negative	urine
65	H42	New	Negative	Negative	Negative	urine
66	3401A	New	Negative	Negative	Negative	sputum
67	3395A	New	1+	3+	Positive	sputum
68	2299C	New	Negative	Negative	Negative	sputum
69	3270C	New	3+	3+	Positive	sputum

Table 16. Comparison of sensitivity and specificity of dry MTB-LAMP, microscopy and culture for diagnosis of TB

		Culture			Sensitivity %	Specificity %
		Positive	Negative	Total		
MTB-LAMP	Positive	13	2	15	92.8	96.3
	Negative	1	53	54		
	Total	14	55	69		
		Microscopy				
		Positive	Negative	Total		
MTB-LAMP	Positive	12	3	15	92.3	94.6
	Negative	1	53	54		
	Total	13	56	69		

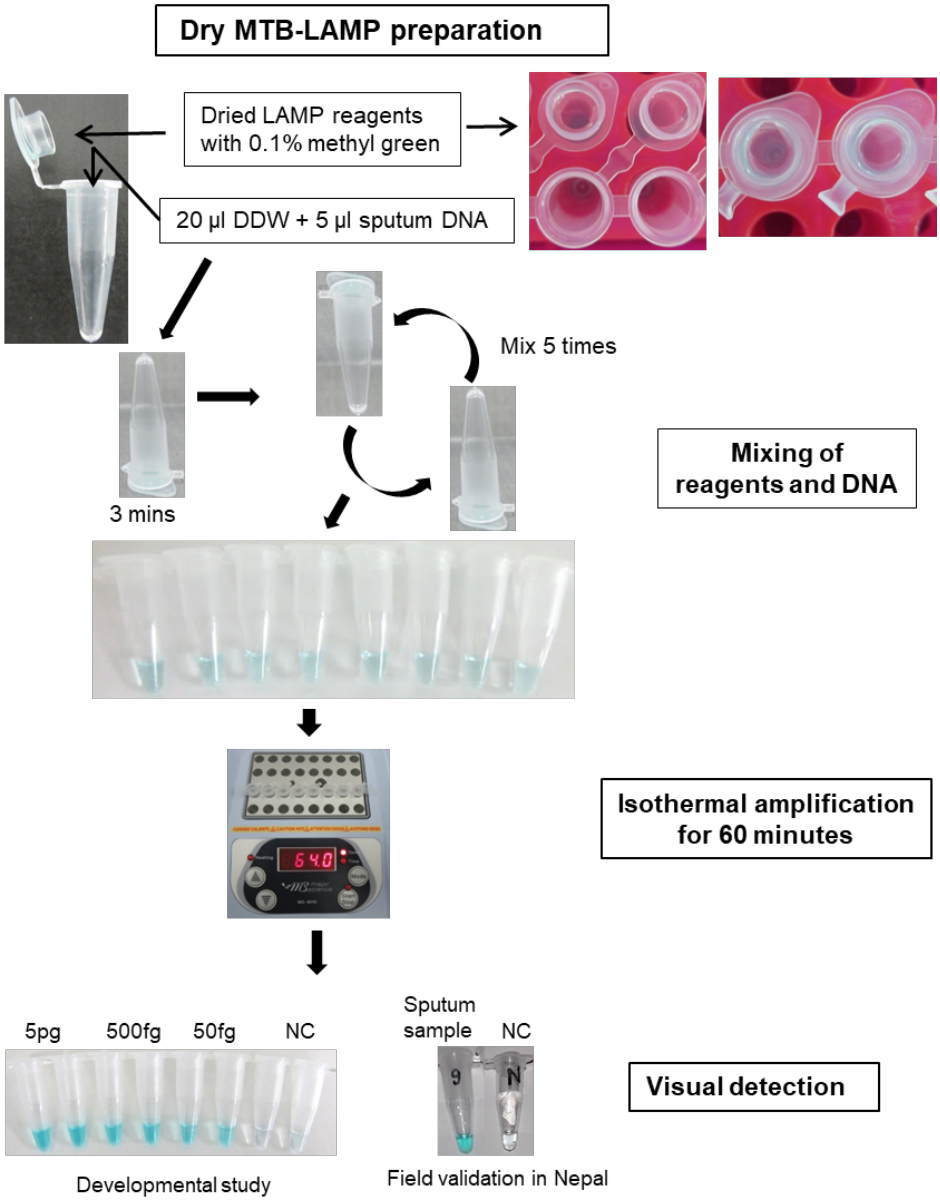

Figure 6.Flow chart of procedure of methyl green based dry *Mycobacterium tuberculosis*-loop-mediated isothermal amplification (MTB-LAMP). The LAMP reagents are dried on the of inner side of a micro tube lid. The dried reagents and DNA are reconstituted, mixed and the reaction mixture incubated at 64°C for 60 minutes to perform the LAMP reactions.

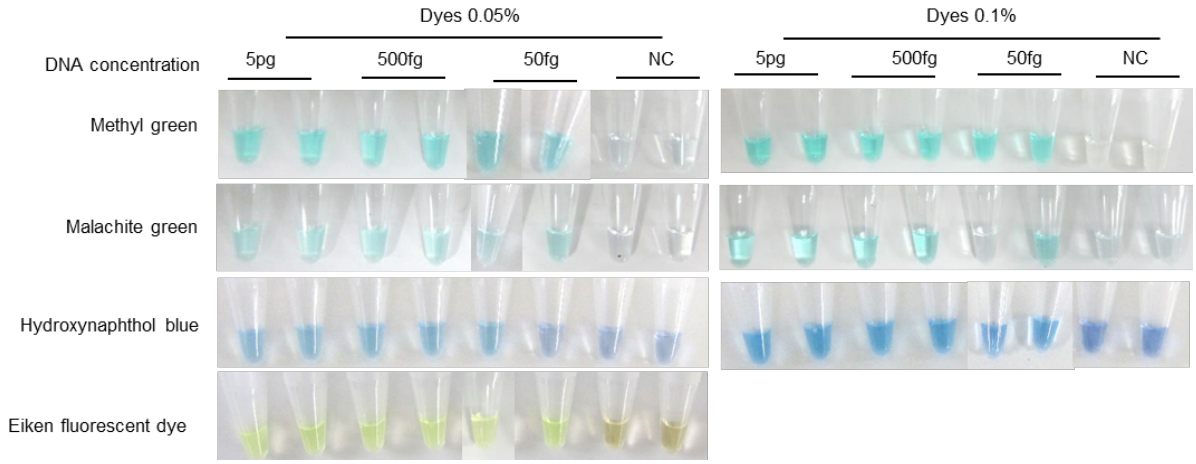

Figure 7. Comparison of colorimetric detection of *Mycobacterium tuberculosis*-loop-mediated isothermal amplification (MTB-LAMP) reaction by methyl green (MeG), malachite green (MaG), hydroxynaphthol (HNB) and Eiken fluorescent dye. Both MeG and MaG showed blue-green color with positive LAMP reaction and colorless with negative LAMP reaction, thus offering easy visual detection of LAMP positive reactions with the naked eye. However, HNB and Eiken dye generated smaller color differences between positive and negative LAMP reactions.

SUMMARY

The purpose of this study was to develop a simple visual methyl green (MeG) based dry loop-mediated isothermal amplification (LAMP) method for early detection of *Mycobacterium tuberculosis* (MTB) from clinical samples. We identified MeG as an indicator of a positive LAMP reaction, where a positive reaction gave a blue-green color while a negative reaction was colorless. The MeGMTB-LAMP system was further simplified by drying all reagents for ease of use, and was then validated for its ability to diagnose TB directly using Nepalese clinical samples. We evaluated the dry MeG MTB-LAMP with 69 new TB suspected samples from patients that did not have a confirmed history of TB treatment and found the sensitivity in culture positive samples as 92.8% (13/14) and specificity in culture negative samples as 96.3% (53/55). Our LAMP system has the potential to be a point of care test for early diagnosis of active TB in developing countries.

CONCLUSION

OMNIgene SPUTUM (OMS) is suitable solution for transporting samples from peripheral sites to central laboratory without cold chain and OMS treated smear and culture results were better than standard of care method and Methyl Green (MeG) dry lamp result were comparable to gold standard culture test.

In Chapter I, the novel sputum transport solution; OMS was examined to show higher case detection, minimized laboratory procedures, eliminated cold chain and can undergo multiday ambient-temperature for transporting TB suspected samples from periphery to central laboratory for smear and culture testing. It substantially helps to mitigate key challenges associated with traditional sputum transport of national tuberculosis control program of Nepal. However, future investigations with larger sample sizes seemed to be valuable. In addition, testing via liquid culture, testing smear-negative sputa with extended transport, and analysis of cost savings also seemed to be necessary.

In Chapter II, OMS-stabilized sputum for long term transport and GeneXpert MTB/RIF testing were evaluated in Nepal. The research findings suggested that sputum sample can be transported in OMS for at least 7 days without cold chain and still yield smear and GeneXpert MTB/RIF result that are concordant with sample transported with cold chain. A slightly greater proportion of smear-negative but GeneXpert MTB/RIF -positive samples were detected after OMS treatment requires further investigation with larger sample sizes. This could help determine whether adding OMS to sputum samples can increase the sensitivity of the GeneXpert MTB/RIF assay by allowing more smear-negative samples (i.e., low-positives that may be graded smear-negative) to be detected as MTB in the GeneXpert MTB/RIF assay.

In Chapter III, a simple visual methyl green (MeG) based dry loop-mediated isothermal amplification (LAMP) method for early detection of MTB from clinical samples was developed. MeG was identified as a novel dye for simple and visual detection of LAMP reactions. Additionally, MeG-based MTB-LAMP visual detection system was simplified by preparing dried reagents and validated to demonstrate it's potential for the diagnosis of TB in Nepal and potentially other developing countries. Further improvement of the dry MeG-based MTB-LAMP system will help to develop it as an effective POC test.

In summary, this study showed that the combination of novel sputum transport solution for increasing detection rate, OMS-stabilized sputum for long term transport and Xpert MTB/RIF testing, and the direct detection of MTB in clinical samples by dry MeG-based MTB-LAMP method can make diagnosis of TB in developing country feasible.

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REFERENCES

1. Asmar S, Drancourt M. Chlorhexidine decontamination ofsputum for culturing Mycobacterium tuberculosis. BMCMicrobiol 15, 6,2015.
2. Bobadilla-del-Valle M, Ponce-de-Leon A, Kato-Maeda M,Hernandez-Cruz A, Calva-Mercado JJ, Chavez-Mazari BA,et al. Comparison of sodium carbonate, cetyl-pyridiniumchloride and sodium borate for preservation of sputa forculture of Mycobacterium tuberculosis. J ClinMicrobiol41,4487–8, 2003.
3. DNA Genotek. Product Data Sheet PD-BR-00195. www. dnagenotek.com Accessed July 2016.
4. Dowdy DW, Lourenco MC, Cavalcante SC, Saraceni V, KingB, Golub JE, et al. Impact and cost-effectiveness of culturefor diagnosis of tuberculosis in HIV-infected Brazilianadults. PLoS ONE3, e4057, 2008
5. DNA Genotek. Product Data Sheet PD-BR-00196. www. dnagenotek.com Accessed July 2016.
6. Dukes JP, King DP, Alexandersen S. Novel reverse transcription loop-mediated isothermal amplification for rapid detection of foot-and-mouth disease virus. Arch Virol151,1093-106, 2006.
7. Goto M, Honda E, Ogura A, Nomoto A, Hanaki K. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxyl naphthol blue. BioTechniques 46, 167-72, 2009
8. Hill J, Beriwal S, Chandra I, Paul VK, Kapil A, Singh T et al. Loop-mediated isothermal amplification assay for rapid detection of common strains of *Escherichia coli*. J ClinMicrobiol46(8), 2800-04, 2008.

9. Hayashida K, Kajino K, Hachaambwa L, Namangala B, Sugimoto C. Direct blood dry LAMP: A rapid, stable and easy diagnostic tool for human African Trypanosomiasis. PloSNegl Trop Dis9(3), e0003578, 2015.
10. Iwamoto T, Sonobe T, Hyashi K. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J ClinMicrobiol41(6), 2616-22, 2003
11. Joint Monitoring Mission Report for Tuberculosis, Government of Nepal, 2019.
12. .Kelly-Cirino C, Niles J, Ray B, Stewart A. Maximizing centralized testing models and GeneXpert hubs with OMNIgene SPUTUM. DNA Genotek Whitepaper PD-WP-00044. Available at: www.dnagenotek.com. [Accessed 22 Mar 2016].
13. Kent PT, Kubica GP. Public health mycobacteriology: a guide for the level III laboratory. US department of health and human services, center for disease control; 1985.
14. Kim SK, Norden B. Methyl green: A DNA major-groove binding drug. FEEBS J 315(1),61-64, 1993.
15. Maharjan B, Shrestha B, Weirich A, Stewart A, Kelly-Cirino CD.A novel sputum transport solution eliminates cold chain and supports routine tuberculosis testing in Nepal. J Epidemiol Glob Health Apr 27, 2016 .
16. Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP): recent progress in research and development. J Infect Chemothe 19, 404-11, 2013.
17. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loopmediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. BiochemBiophys Res Commun 289, 150-4, 2001.
18. Miyamoto S, Sano S, Takahashi K, Jikihara T. Method for colorimetric detection of double-stranded nucleic acid using leucotriphenylmethane dyes. Anal Bioch 475, 28-33, 2015.

19. Niles J, Ray B, Curry P, Kelly-Cirino C. Compatibility of OMNIgene[w] SPUTUM and prepIT[w] MAX with molecular assays for tuberculosis: real-time PCR and HainLifescienceGenoType MTBC line-probe assay. DNA Genotek Whitepaper PD-WP-00051. www.dnagenotek.com Accessed July 2016.
20. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28(12), e63, 2000.
21. Nzelu CO, Gomez EA, Caceres AG, Sakurai T, Martini-Robles L, Uezato H et al. Development of a loop-mediated isothermal amplification method for rapid mass-screening of sand flies for *Leishmania* infection. Acta Trop 132, 1-6, 2014.
22. Nagai K, Horita N, Yamamoto M, Tsukahara T, Nagakura H, Tashiro K et al. Diagnostic test accuracy of loop-mediated isothermal amplification assay for Mycobacterium tuberculosis: systematic review and meta-analysis. Sci Rep 6, 39090, 2016.
23. Omar S V, Peters R P H, Ismail N A, et al. Field evaluation of a novel preservation medium to transport sputum specimens for molecular detection of Mycobacterium tuberculosis in a rural African setting. Trop Med IntHealth 21,776–782, 2016.
24. Pang Y, Du J, Zhang Z Y, et al. The feasibility of sputum transportation system in China: effect of sputum storage on the mycobacterial detection. Biomed Environ Sci 27, 982– 986, 2014.
25. Parsons LM, Somoskovi A, Gutierrez C, Lee E, ParmasivanCN, Abimiku A, et al. Laboratory diagnosis of tuberculosis inresource-poor countries: challenges and opportunities. ClinMicrobiol Rev 24, 314–50, 2011.
26. Paramasivan CN, Narayana A, Prabhakar R, Rajagopal MS,Somasundaram PR, Tripathy SP. Effect of storage of sputumspecimens at room temperature on smear and cultureresults. Tuber 64,119–24, 1983.

27. Pandey BD, Poudel A, Yoda T, Tamaru A, Oda N, Fukushima Y et al. Development of an in-house loop-mediated isothermal amplification (LAMP) assay for detection of *Mycobacerium tuberculosis* and evaluation in sputum samples of Nepalese patients. J Med Microbiol 57, 439-43, 2008.
28. Poudel A, Pandey BD, Lekhak B, Rijal B, Sapkota BR, Suzuki Y et al. Clinical profiling and use of loop-mediated isothermal amplification assay for rapid detection of *Mycobacterium tuberculosis* from sputum. Kathmandu Univ Med J 7(2), 109-14, 2009.
29. Prieto D, Aparicio G, Morande PE, Zolessi FR. A fast, low cost, and highly efficient fluorescent DNA labeling method using methyl green. Histochem Cell Biol 142(3), 335-45, 2014.
30. The Guardian. Nepal has lessons to teach on TB. Available at: <http://www.theguardian.com/global-development/2010/sep/27/who-fighting-tuberculosis-medical-nepal-health>. [Accessed 22 Mar 2016]; 2010
31. Training Package on Xpert[w] MTB/RIF. StopTB Partnership. Geneva, Switzerland: World Health Organization, 2016. http:// www.stoptb.org/wg/gli/TrainingPackage_XPERT_MTB_RIF. asp Accessed August 2016
32. Tomita N, Mori Y, Kanda H, Notomi T. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat Protoc 3(5), 877-82, 2008.
33. Thapa J, Nakajima C, Maharjan B, Poudel A, Suzuki Y. Molecular characterization of *Mycobacterium orygis* isolates from wild animals of Nepal. Jpn J Vet Res 63(3), 151-8, 2015.
34. World Health Organization Post—2015 Global TB Strategy. Global strategy and targets for tuberculosis prevention, care and control after 2015. Available at: http://www.who.int/tb/post 2015_strategy/en/. [Accessed 22 Mar 2016].

35. World Health Organization. Mycobacteriology Laboratory Manual. 1st ed. Global Laboratory Initiative; 2014. Avail-able at: www.who.int/tb/laboratory/mycobacteriology-laboratory-manual.pdf. [Accessed 22 Mar 2016].
36. World Health Organization. Global tuberculosis report, 2015. WHO/HTM/TB/2015.22. Geneva, Switzerland: WHO, 2015.
37. World Health Organization. Post-2015 Global TB Strategy. Geneva, Switzerland: WHO, 2015. http://www.who.int/tb/ post2015_strategy/en/. Accessed August 2016.
38. World Health Organization; Stop TB Partnership. Global Plan to End TB 2016–2020. Geneva, Switzerland: WHO, 2016. http://www.stoptb.org/global/plan/plan2/. Accessed August 2016.
39. World Health Organization. TB diagnostics fact sheet. Geneva, Switzerland: WHO, 2014. http://www.who.int/tb/publications/ tbDiagnostics_factsheet.pdf?ua¼1. Accessed August 2016.
40. WHO. Global tuberculosis report 2018, https://www.who.int/tb/publications/global_report/tb18_ExecSum_web_4Oct18.pdf?ua=1; 2018 [accessed 9 March 2019].
41. WHO. The end TB strategy, http://www.who.int/tb/post2015_TBstrategy.pdf?ua=12015[accessed 3 February 2019].
42. WHO. Xpert MTB/RIF implementation manual: technical and operational 'how-to': practical considerations, http://apps.who.int/iris/bitstream/10665/112469/1/9789241506700_eng.pdf; 2014 [accessed 3 February 2019].
43. WHO. The use of a commercial loop-mediated isothermal amplification assay (TB-LAMP) for the detection of tuberculosis, http://apps.who.int/iris/bitstream/10665/83142/1/WHO_HTM_TB_2013.05_eng.pdf?ua=1; 2013 [accessed 3 February 2019].

44. WHO. The use of loop-mediated isothermal amplification (TB-LAMP) for the diagnosis of pulmonary tuberculosis. http://apps.who.int/iris/bitstream/10665/249154/1/9789241511186-eng.pdf; 2016 [accessed 3 February 2019].
45. World Health Organization. Global tuberculosis report 2019.