East African Medical Journal Vol. 95 No. 4 April 2018
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EVALUATION OF PCR FOR DIRECT DETECTION OF MYCOBACTERIUM TUBERCULOSIS COMPLEX IN SPUTUM SAMPLES IN KAMPALA, UGANDA

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ABSTRACT

Objective: **The aim of this study was to evaluate an in-house PCR for direct detection of Mycobacterium tuberculosis complex (MTC) in sputum samples.**
Design: **Across sectional study was conducted on samples isolates from suspected TB patients.** ***Settings:*** **Patients presenting to Mulago hospital Ward 5 TB Clinics for management and care from June 2014 to April 2015.**
Subjects: **Three sputum samples per patient were obtained from 30 patients with negative (N= 90) and 30 patients with positive (N= 90) ZN smear results for a total of 180 sputum samples was studied. The samples were processed using N-acetyl-L-cycteine and 4% NaOH. Genomic DNA was extracted from the sputum samples and used as template for IS6110-PCR. The prevalence of MTC bacilli in the sputum samples was determined.**
Results: **IS6l110-PCR detected MTC bacilli in 81% (73/90) sputum samples from patients with ZN-positive smears while it detected 51% (46/ 90) sputum samples from patients with ZN-negative. There were statistically significant associations between frequency in performing PCR on three sputum samples per patient and increased proportion of samples positive for MTC, $p<0.05$). All the 30 patients with ZN positive smears were positive on IS6110-PCR, while 80% (24/30) patients with ZN-negative smears were positive on IS6110-PCR. Chi square test revealed a statistically significant positive association between ZN positive and ZN-negative smears $p<0.05$.**
Conclusion: **In low income setting burden with high TB incidence and mortality, in-house IS6110-PCR efficiently detects MTC bacilli in sputum samples and can be introduced for routine detection of these bacilli in isolates form pulmonary TB suspects.**

INTRODUCTION

Tuberculosis (TB) is a serious public health problem with high incidence, morbidity and mortality particularly in developing countries and affects up to 2 billion people worldwide [1-2]. The global increase in incidence rate is attributed to the gradual rise of human immunodeficiency syndrome virus (HIV) infections and other bacterial drug resistance strains in the population [2-3]. Approximately, 75% of people infected with both HIV and TB live in Sub- Saharan Africa [3]. Nearly 3 million cases and 750,000 TB deaths are estimated to occur in the region each year [3]. Out of the estimated 6 million adults living with HIV in the Sub Saharan Africa, about half are likely to be affected with tuberculosis.

Uganda ranks 22nd among countries with the highest TB burden in the world with TB accounting for thousands of deaths especially among HIV/AIDS patients [1]. Prevalence rate indicates 50% of TB cases usually HIV related with 25/100 000 population of the co-infected persons dying of TB/HIV [3]. With increasing incidences of HIV/TB co-infections, particularly in developing countries, there is need for rapid and sensitive diagnostics test for early detection of the tubercle bacillus [3-4].

Routine diagnosis of TB requires the widely used Ziehl-Neelsen (ZN) microscopy, which is cheap, quick and easy to conduct but has low sensitivity detecting only low number of the organisms (~10,000 bacilli/mL) [4]. With ZN microscopy approximately half of new TB cases tested are usually smear negative [5]. Therefore, this renders the method ineffective for diagnosis of TB among HIV/AIDS patients. In addition, ZN microscopy cannot discriminate between the Mycobacterium tuberculosis complex (MTC) (a group of genetically related organisms) that causes TB and the non-tuberculous mycobacteria (NTM) which can cause disease in immunocompromised individuals. While culture is still the gold standard for confirmation of pulmonary TB, it is time consuming and require up to eight weeks for identification of mycobacteria [6]. Bactec, which is widely considered as the most rapid culture technique, requires an average of 13 days for at least a positive result [7].

Polymerase Chain Reaction (PCR), a more rapid and specific detection method is available for diagnosis of MTC bacilli [8]. In addition, automated/commercial detection methods for mycobacteria now exist, but are expensive and often unaffordable in many developing countries. In poor resource settings PCR use may require evaluation since the efficiencies may vary due to differences in laboratory conditions. In-house PCRs are cheap and may provide alternatives to the already available expensive commercial kits. Major draw backs of PCR include inhibitors in sputum samples [9] coupled with complex and lengthy DNA extraction procedures (which usually precede amplification) [10]. These are additional challenges threatening the use of in-house PCRs in routine practice. While Uracil-N-glycosylase and dUTP are proposed for minimizing PCR-inhibitors in sputum samples [9], these are still additional costs incurred particularly in resource limited developing countries. Thus, premixed PCR reagents are still the highly favored choice among the researchers.

Previously, a PCR-based assay for the identification of MTC bacilli based on Bactec® 12B vials (at a growth index of ≥10) was introduced in routine practice at a cost of approximately $5 per sample making it relatively a viable option for confirmation of TB[11]. Therefore, this study evaluated in-

house PCR for direct detection of MTC bacilli in sputum samples, obviating multiplication of the bacilli in Bactec or MGIT machines. The prevalence of MTC bacilli in sputum samples from patients with ZN- positive and ZN-negative smears was determined. Timing for sample collection influenced the prevalence of MTC bacilli in sputum samples.

MATERIALS AND METHODS

Study design: A cross sectional study was performed on sputum samples from pulmonary TB suspects using ZN microscopy and PCR for detection of MTC bacilli and IS6110 gene.

Settings: Between April 2014 June to 2015 TB suspects presenting to TB Ward B at Old Mulago Hospital, Kampala for care and management were enrolled for the study. These patients were examined by clinicians and samples were obtained from those who reported coughing for more than 3 weeks and/or hemoptysis. Samples from 30 patients with ZN-negative smears and 30 patients with ZN-positive smears were collected.

Subjects: A total of 180 sputum samples were examined from TB suspects. Three sputum samples were collected from each patient; the first sample was collected on spot (when the patient attended the clinic for the first time), while the other two samples were collected during the patients' second visit (an early morning sample collected by patients while at home) and the third sample was collected on spot at on third visits). Patients with uniform ZN status (negative or positive) on all the three sputum samples were included in the study, while those with discordant smears were excluded. The ZN microscopy and PCR analyses were done at the Molecular Biology laboratory, Department of Medical Microbiology, Makerere University College of Health Sciences.

Processing of sputum specimens: The sputum samples were digested and decontaminated in a Bio-safety cabinet Class II as previously described [11], with modifications. Briefly, the samples were re-suspended and vortexed in 10ml buffer containing 0.5% N-acetylcysteine (NALC), 2.9 % sodium citrate and 4% NaOH for 15min. A Phosphate buffer (pH 6.8) was added to a final volume of 50 ml. The mixture was centrifuged at 3500g for 15min. The supernatant was discarded, and the pellet re-suspended in 2ml Elution buffer (Qiagen, Hilden, Germany), and immediately heat killed at 80°C in a ProBlot hybridization oven (Labnet, Oakham, UK) for 2 hours. The heated suspension was cooled to room temperature and centrifuged at 3500g for 15min to obtain pellets that were used in subsequent procedures directly as templates for PCR and/or processed using a Master core kit to obtain pure genomic DNA (see below).

Extraction of genomic DNA from sputum samples: Genomic DNA was extracted from the processed pellet using the Master core DNA purification kit (Epicenter, Madison, USA) following the manufacturer's guidelines. The pellet containing genomic DNA was re-suspended in 35µl of TE (Tris-EDTA) buffer, pH 7.5 and used as template for PCR.

Molecular assays: PCR detection of MTC bacilli in sputum samples was done using primers P43 (forward, 5'-TCAGCC GCGTCCACGCCGCCA-3') and P53 (Reverse, 5'-CCGACCGCTCCGACCGACGGT-3') [11] that amplify 521bp of the MTC specific IS6110 insertion sequence. Each reaction contained 2µL of template (the extracted DNA or processed sputum sediment), 1µl of the Custom PCR-Master mix (10 mM Tris-HC1, pH 9.0, 2 mM MgCl2, 50 mM KCl, 200 µM of

dNTPs and 5% DMSO -Thermofisher, Surry, UK), 20pmoles each of the forward and reverse primers, and 0.5U of Taq Polymerase (Thermofisher, Surry, UK), in a total volume of 10μL. Amplification was done in the PTC-200 Peltier thermocycler (MJ Research, Waltham, USA) under the following conditions; initial denaturation at 94°C for 5min, followed by 34 cycles each consisting of 94°C, 30 sec; 65°C, 30 sec; and 72°C, 45sec, with a final extension at 72°C, for 10 min. The amplicons were electrophoretically analyzed using a 1% agarose gel in TBE buffer (Tris-Borate EDTA).

Quality control: To avoid laboratory cross-contamination, sputum samples were processed in small batches ensuring one sample is opened at a time. The study used sputum samples from patients with only uniform ZN smears (negative or positive) for the three samples. For each sample processed, separate tubes containing the digestion mixture and phosphate buffer were used to avoid cross transfer of specimens. Sterile phosphate buffer was used in every batch processed. The molecular laboratory was designated into separate rooms housing facilities for sample preparation, amplification and analysis of amplicons. Positive control (Mycobacterium tuberculosis H37Rv genomic DNA) and negative controls (Mycobacterium smegmatis and Streptococus pneumoniae, which lack the IS6110 sequence, and water) were always included in the amplification reactions. To detect the presence of PCR-inhibitors (which may result in false-negatives), reactions with spiked genomic DNA (H37Rv) were included in the runs.

Data analysis: All data was entered in access database and analyzed using STATA 11.0. Pearson's Chi square test was used to determine levels of significance between means of proportions. The data are presented as mean and standard deviation and percent frequency, with $p < 0.05$ indicating statistical significance.

Ethical considerations: This study was reviewed and approved by the institutional review boards of Makerere University College of Health Sciences, Mulago National and Referral Hospital, and the Uganda National Council of Science and Technology. Sputum samples were from only consenting patients

RESULTS

This cross sectional included a total of 180 sputum samples from two groups of TB suspects screened between June 2014 to April 2015. Ninety sputum samples from ZN-negative and 90 from ZN-positive smear were evaluated for direct PCR detection of MTC bacilli. The PCR detected bacilli in all the samples from patients with ZN-positive smears, while only 51% (46/90) sputum samples from patients with ZN-negative smears were IS6110-positive. There was statistical significant difference between PCR positive (ZN positive smears) and the ZN negative smears ($P<0.05$). Out of the 30 ZN smear negative TB patients, PCR detected 16 (53.3%), 17 (56.7%), and 13 (43.3%) as positive for IS6110 sequence in the first spot, early morning and second spot sputum samples respectively (Table 1). Early morning sputum specimens had the highest detection by PCR (57%).

Table 1

PCR-detection of MTC bacilli in ZN-Positive and ZN-Negative sputum samples[1]

		(N=90) ZN[b]	(N=90) ZN[c]
	***IS6110*-PCR[a]**	**Positive**	**Negative**
First Spot samples	Positive (%)	25 (83)	16 (53)
	Negative (%)	5 (17)	14 (47)
	Sub-total	30	30
Early Morning samples	Positive (%)	23 (77)	17 (57)
	Negative (%)	7 (23)	13 (43)
	Sub-total	30	30
Second Spot samples	Positive (%)	25 (83)	13 (43)
	Negative (%)	5 (17)	17 (57)
	Sub-total	30	30
Total[d]		**90**	**90**

a: MTC bacilli detected by PCR IS610,

b: ZN positive sampled detected by PCR IS610

c: ZN negative sampled detected by PCR IS610

d: Total number of ZN positive sputum samples.

When we analyzed the role of multiple samples in detection of M.tb in ZN negative sputum samples, out of the 30 ZN smear negative patients, PCR was positive in 16 (53.3%) with the first samples. When the first two samples were analyzed, 20 (67%), out of 30 patients had at least one of their results positive by PCR. When all the three samples were analyzed, 24 (80%) patients had at least one of their samples positive by PCR (Table 2). Therefore, by including a second and third sample there was a statistical significant difference in the incremental yield of 14% and 13% respectively, ($p<0.05$). Our finding indicates that eighty percent (80%) of patients with ZN negative specimens were actually TB patients.

Table 2

Multiple samples in detection of M.tb in ZN negative sputum samples by PCR

Sample category	**PCR IS6110-Positive samples (N=30)**	
	Smear negate (n)	**Percentage (%)**
First sample	16	53
One of two samples	20	67
One of three samples	24	80

**ZN smears negative sputum samples tested positive in 24 out of 30 patients sampled.*

Additionally, we further examined the role of multiple samples in detection of M.tb in ZN positive sputum samples by PCR. Our findings indicate that, out of the 30 ZN smear positive patients, PCR was positive in 25 (83.3%) with the first samples. When the first two samples were analyzed, 28 (93.4%) out of 30 patients had at least one of their results positive by PCR. When all the three samples were analyzed, 30 (100%) patients had at least one of their samples positive by PCR. Therefore, by including a second and third sample, there was a statistical significant incremental yields of 10% and 7% respectively, $p<0.05$ (Table 3). PCR detected all ZN positive smears (100%) when 3 samples were analyzed.

Table 3

Multiple samples in detection of M.tb in ZN positive sputum samples by PCR

Sample category	PCR IS6110-Positive samples (N=30)	
	Smear positive n (%)	Percentage (%)
First sample	25	83.3
One of two samples	28	93.3
One of three samples	30	100

**PCR IS610 detected MTC bacilli in all 30 patients with ZN positive samples*

We examined the PCR results using ZN as the gold standard for ZN positive sputum samples, the sensitivity and specificity was 83.3% and 46.7% with PPV and NPV of 60.9% and 73.7% respectively (Table 4). The McNemer x2 square test for small samples sizes revealed that the percentage of samples positive with PCR significantly differed from ZN, $\chi 2$ (1, N= 180) = 0.03, $P<0.05$. When analyzed for kappa values, its value at 95% CI was 0.668 (0.545-0.791) with SE of confidence was 0.063. Therefore, the strength of agreement between PCR and was good.

Table 4

Analysis of PCR results Vs ZN as gold standard for positive sputum samples

Sample category	ZN gold standard		
	Positive	Negative	Total
PCR positive	25	16	41
PCR Negative	5	14	19
Total	30	30	60

*Sensitivity = 25/30 * 100 = 83.3%, PPV = 25/41*100 = 60.9%*
*Specificity = 14/30 * 100 = 46.7%, NPV = 14/19*100 = 73.7%*

We further examined the PCR results using ZN as gold standard for sputum negative samples, the sensitivity and specificity was 76.6% and 43.3% with PPV and NPV of 57.5% and 65.0% respectively (Table 5). The McNemer x2 square test for small samples sizes revealed that the percentage of samples positive with PCR significantly differed from

ZN, $\chi 2$ (1, N= 180) = 0.012, P<0.05. When analyzed for kappa values, its value at 95% CI was 0.771 (0.66-0.882) with SE of confidence was 0.057. The strength of agreement was moderate.

Table 5:

Sample category	ZN gold standard		
	Positive	**Negative**	**Total**
PCR positive	23	17	40
PCR Negative	7	13	20
Total	30	30	60

*Sensitivity = 23/30 * 100 = 76.7%, PPV = 23/40*100 =57.5%*
*Specificity = 13/30 * 100 = 43.3%, NPV =13/20* 100 = 65.0%*

Purified Genomic DNA in detection of MTC bacilli

Our initially approach was to use, PCR to directly detect MTC bacilli in sputum sediments. While PCR directly detected MTC bacilli the sensitivity and specificity were low (Figure 1A). However, amplification on purified genomic DNA from sputum samples was more sensitive and gave better yields of amplicons. A large proportion of sputum samples that were negative on direct PCR yielded positive results upon purification with the Master core kit (Figure 1B).

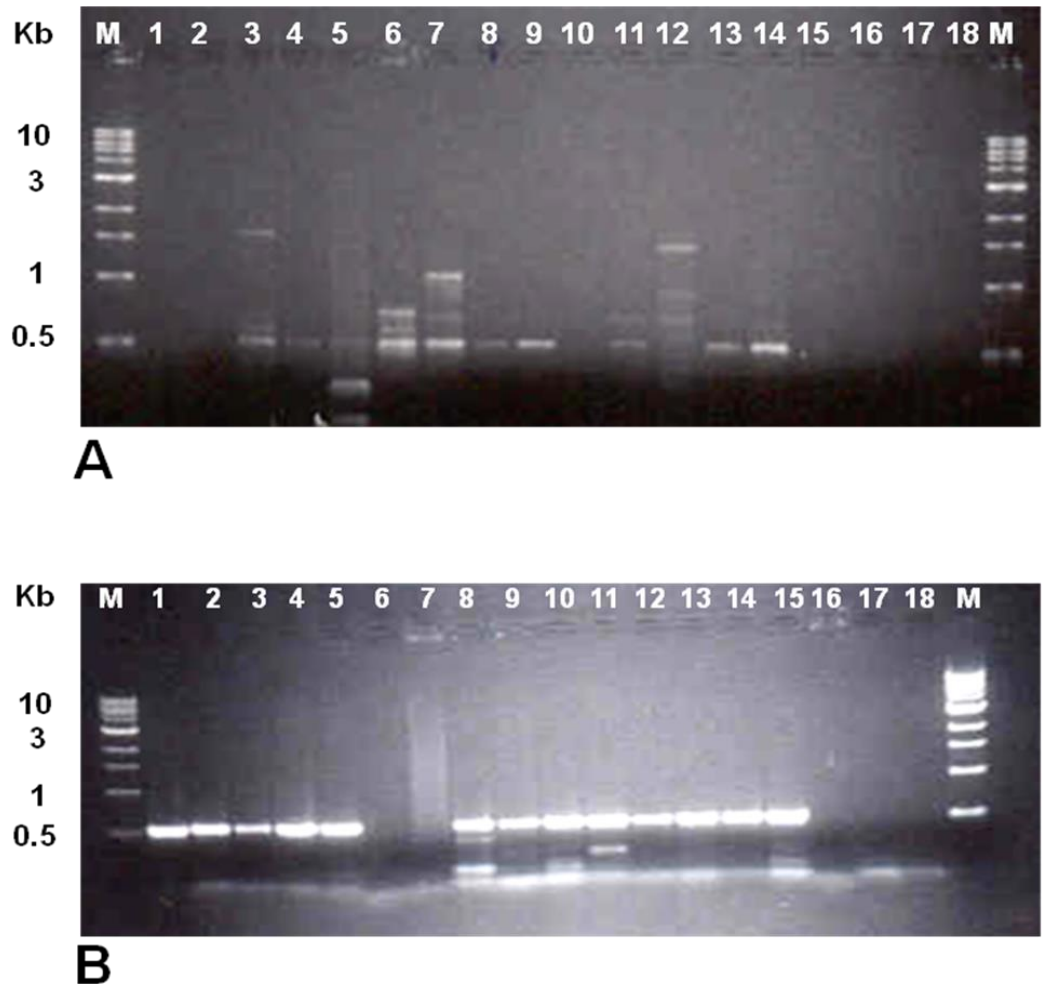

Figure 1: Agarose gel electrophoresis following *IS6110*-PCR on sputum samples

Panel A: Direct PCR-amplification on processed sputum samples (where DNA was not purified). Lanes: M, 1Kb DNA ladder; 1 to 15, amplification on sputum samples; 16 to 18, negative controls (*Mycobacterium smegmatis, Streptococcus pneumoniae* and water, respectively).

Panel B: *IS6110*-PCR-amplification using DNA purified from sputum samples as templates. Lanes: M, 1Kb DNA ladder; 1, Positive control (*Mycobacterium tuberculosis* H37Rv DNA); 2 to 15, PCR using DNA extracted from sputum samples; 16 to 18, negative controls (*Mycobacterium smegmatis, Streptococcus pnumoniae* and water).

DISCUSSIONS

A total of 180 sputum samples from two groups of TB suspects were evaluated for direct PCR detection of MTC bacilli in sputum samples. The PCR detected bacilli in all the samples from patients with ZN-positive smears, while only 51% (46/90) sputum samples from patients with ZN-negative smears were IS6110-positive. There was statistical significant difference between PCR positive (ZN positive smears) and the ZN negative smears ($P<0.05$). This is an indication of significant numbers of patients referred to the TB wards during the study period with TB although they were ZN negative. Additionally, significant number of PCR-positive sputum samples increased when all

the three samples for each patient were analyzed in both groups (ZN-positive and ZN-negative) P<0.05. This indicates that with increased number of ZN samples, PCR is more likely to detect bacilli in pulmonary sputum samples. Previous studies have reported similar findings where PCR results showed increased positivity with more sputum samples [12]. Additionally, Noordhoek et al [13] conducted a study which evaluated PCR for diagnosis of MTB and reported positivity in all the three samples. While the results in the current study is similar to those reported by Noordhoek et al [13], it is also at odds with those reported by Rodriguez et al [14] who reported increased positivity in only up to two sputum samples. Therefore, PCR incremental yields were attributed to positivity to at least all sputum samples. In the current study it can be concluded that all suspected patients with ZN smear negative have TB.

Detection of MTC bacilli in sputum samples by IS6110-PCR

Among patients with ZN-Positive smears, IS6110-PCR detected 83% (25/30) first sputum samples, 77% (23/25) early morning and 83% (25/25) second spot samples (Table 1). The sensitivity and specificity for IS6110-PCR on 1st spot samples were 83% and 47%, respectively. For early morning samples, the sensitivity and specificity were 77% and 43%, respectively, while it was 83% and 57%, respectively, on 2nd spot samples. Thus, in patients with ZN-Positive sputum smears, the first and second spot samples were the most sensitive upon PCR. Conversely, among patients with ZN-negative smears, IS6110-PCR detected 53.3% (16/ 30 first spot samples, 57% (17/30) early morning and 43.3%, (13/30) second spot sputum samples (Table 5). Among these patients, the sensitivity and specificity for IS6110-PCR on 1st spot samples were 94% and 47%, respectively. For early morning samples and 2nd spot samples, the sensitivity and specificity were 100% and 43%, respectively. In this category of patients, early morning and second spot sputum samples were more sensitive but less specific than first spot samples. Therefore, PCR significantly detected more bacilli in ZN positive samples than the ZN positive smears (P< 0.05). Whereas the sensitivity was high and comparable between on spots samples (83%), early morning samples significantly differed in specificity (47%), p>0.05. These may be due to poor samples handling by patients. In contrast there was high sensitivity of on spots samples in ZN smear negative smears with corresponding low specificity. The sensitivity and specificity differed significantly between the two approaches (P<0.05).

PCR IS610 detection of MTC bacilli in sputum samples

Previous studies on PCR assays indicate sensitivities of 100% and 73.1% in smear-positive and smear-negative respiratory specimens, respectively with corresponding specificities of 100% and 99.8% [15, 16]. The high sensitivity and specificity, coupled with the potential for detecting a wide range of mycobacteria, make PCR a useful tool in the clinical management of mycobacterial infections [15]. Using culture as the gold standard, Van et al (2005) [16] obtained sensitivity of 77-95% and specificity of more than 95% in smear positive specimens, while the specificity in the smear negative TB patients was 51-71%. In our study, whereas the sensitivity agreed with that of Van et al (2005) [16, 17 and 18], the specificity was lower, probably due to the differences in the gold standards used (we used ZN as the gold standard).

Screening three sputum samples improves PCR-detection of MTC bacilli

PCR was positive in 83% (25/30) ZN-Positive sputum samples obtained at the first visit (Table 2). Analyzing results from the first two samples reveals that 93% (28/30) patients at least had one sample positive by IS6110-PCR. Overall, the 30 patients had at least one of the three samples positive by IS6110-PCR, implying that MTC bacilli were detected in all (100%). Therefore, screening three sputum samples for PCR increases the odds of detecting MTC bacilli. Similarly, PCR detected MTC bacilli in sputum from 53% (16/30) patients with ZN-Negative smears at the first visit (Table 2). Analyzing results from the first two samples reveals that 67% (20/30) patients had at least one of their sputum samples positive upon IS6110-PCR. Overall, 80% (24/30) patients had at least one of the three samples positive by IS6110-PCR. Likewise, screening three sputum samples for IS6110-PCR increased the odds of detecting MTC bacilli in sputum from patients with ZN-Negative smear results. Kivihya et al (2003) [3] reported maximum detection for PCR in the first sputum samples in comparison to subsequent samples from the same TB suspect. Similarly, in this study PCR was more sensitive in the early morning samples. Similarly, it was more sensitive in the first spot and second spot samples for samples from patients with ZN-positive smears (83.3%). Therefore, PCR significantly detected more bacilli in ZN positive samples than the ZN positive smears ($P< 0.05$). Whereas the sensitivity was high and comparable between on spots samples (83%), early morning samples had low specificity (47%). These may be due to poor samples handling by patients. In contrast, there was high sensitivity of on spots samples in ZN smear negative smears with corresponding low specificity. The sensitivity and specificity differed significantly between the two approaches ($P<0.05$).

CONCLUSIONS AND RECOMMENDATIONS

In a high disease-burden, low income setting, in-house PCR is useful for rapid and direct detection of MTC bacilli in sputum samples. Purification of DNA from sputum samples greatly improves the efficiency of IS6110-PCR. In addition, the chances of detecting MTC by IS6110-PCR are high when three sputum samples are tested. Further study should include cultures and large samples.

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