Brief Original Article

Comparison of a DNA based PCR method with conventional methods for the detection of *M. tuberculosis* in Jos, Nigeria

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Abstract

Background: To achieve early diagnosis and effective treatment of pulmonary tuberculosis, simple and sensitive methods that enhance the detection of *Mycobacterium tuberculosis* (*M. tuberculosis*) from clinical specimens are needed. This study compared the effectiveness and suitability of an insertion sequence (IS 6110) based polymerase chain reaction (PCR) assay with conventional methods for the detection of *M. tuberculosis* from clinical specimens in a resource-limited setting.

Methods: Sputa from 101 HIV-positive patients and 40 clinical specimens (sputa, gastic wash out, ascitic fluid, pleural fluid and cerebrospinal fluid) collected from children (HIV status unknown), all suspected for pulmonary tuberculosis at the Jos University Teaching Hospital, Jos, (JUTH) Nigeria, were examined by Ziehl Neelsen (ZN) smear microscopy, Lowenstein Jensen's (LJ) egg-based culture, and PCR methods for the detection of *M. tuberculosis*

Results: Mycobacteria was detected in 45/101 (44.6%) of the specimens from the HIV-positive patients and comprised of 6% $ZN^+culture^+PCR^+$, 4% $ZN^-culture^+ PCR^-$, 16% $ZN^-culture^+ PCR^+$ and 19% $ZN^-culture^-PCR^+$. Twenty-two of forty (55%) children were positive with 0% smear microscopy; 4/40 (10%) $culture^+PCR^+$; and 18/40 (45%) $culture^- PCR^+$. The sensitivity and specificity of the PCR for the HIV-positive patients were 85% and 74% respectively against 23% and 100% for ZN smear microscopy.

Conclusion: The IS6110 PCR is a rapid and sensitive method that is specific for the *M. tuberculosis* complex group. It is simple in our experience and increased the detection of *M. tuberculosis* from the specimens examined. We suggest its use for the detection of *M. tuberculosis* in high TB and HIV burden areas.

Keywords: tuberculosis, PCR, HIV

Introduction

Tuberculosis continues to be a leading cause of death, especially in the developing countries where co-infection with HIV constitutes significant public health problems [1-2] In Nigeria, as in most resource-limited countries, the Ziehl Neelsen's (ZN) smear microscopy method is used in clinical laboratories for the confirmation of clinical TB. However, its limited sensitivity (detection limit: 1000 bacilli/ ml of sputum) and specificity (identifies only acid fast bacilli) [3] make it less dependable than other methods, especially in cases of poor sputum quality and low mycobacteria content [4-6]. The culture method is the gold standard because it is more sensitive than microscopy and highly specific [3] However, most routine laboratories do not culture for *Mycobacteria*

tuberculosis (*M. tuberculossis*) due to the slow turn-around time (three to eight weeks) of the Lowenstein Jensen (LJ) method [7] or the high cost and lack of advanced technology needed for the more sensitive automated methods [2].

Nigeria is one of Africa's largest countries with a population of about 140 million people, with a TB incidence rate of 57.5 per 100,000 population, case notification rates (new and relapse) of 59.2 per 100,000, and actual sputum smear positive detection rate of 31.7% [8]. The World Health Organization (WHO) ranked Nigeria as the fourth highest in global TB burden [6]. These findings underscore the need for improved methods for laboratory diagnosis of TB in Nigeria.

Although ZN microscopy is cheap, rapid, and easy to perform, its limited sensitivity appears to

compromise the program of National TB control, including directly observed treatment short course (DOTS) since only smear microscopy positive cases are eligible for DOTS enrolment [9-10]. Findings from a previous study in Jos, (North Central Region) had indicated that there is a need to review the procedures for TB diagnosis and treatment in Nigeria [11].

Reports on the use of PCR for the detection of *M. tuberculosis* in clinical specimens date over a decade [12-15]. Most of the studies, however, were performed in non-endemic western countries with only a few [16] from some endemic nations of sub-Saharan Africa [17]. The IS6110 sequence is specific for the *M. tuberculosis* complex (*M. tuberculosis, M. africanum, M. bovis, M. microti* and *M. canetti*) [18]. The sequence, which undergoes several inverted repeats in the bacterial chromosome, has been cloned and used for detection and for molecular epidemiological studies of the *M. tuberculosis* complex group [16].

The present study compared the IS6110 DNA based PCR assay with smear microscopy and LJ culture for the diagnosis of pulmonary tuberculosis (PTB) using clinical specimens from HIV-positive patients and children suspected for PTB at the Jos University Teaching Hospital, Jos, Nigeria. The aim was to determine the performance and the suitability of the IS6110 PCR assay in detecting *M. tuberculosis* in clinical samples in a resource-limited setting with high TB and HIV burden.

Materials and Methods
Specimens
Sputa from 101 HIV patients comprised of 70 new, 14 follow up, 9 fail, 2 re-treatment, and 6 'relapse' (Table 1) cases, and body fluids (11 gastric washout, 10 sputa, 9 pleural fluid, 5 ascitic taps and 5 cerebro spinal fluid [CSF]) from 40 children (HIV status not known), with clinical diagnosis of PTB at the Jos University Teaching Hospital Jos, Nigeria, were examined for detection of *M. tuberculosis* by smear microscopy, LJ culture, and the IS6110 DNA based PCR. All the specimens were collected between January and December 2008.

Specimen preparation
Sputum specimens were decontaminated with equal volume cetyl pyridinum chloride (CPC) and centrifuged for sedimentation of mycobacteria. Deposits obtained after centrifugation were used to prepare smears for microscopy and inoculated onto duplicate LJ slopes. All preparations were processed according to standard methods [7,19]. Centrifuged deposits of other specimens from the children excluding sputa were processed directly without CPC treatment.

DNA extraction
Two loops of *M. tuberculosis* cultures or 500 μl of deposits from decontaminated clinical specimens were suspended in 400 μl TE buffer in 1.5 ml microcentrifuge tubes. These were frozen at -20ºC for 15 minutes, heated up to 100ºC in a water bath for five minutes, frozen again at -20ºC for 15 minutes, and processed for DNA extraction.

Briefly, 100 μl of STEP (1ug/μl proteinase K in STE buffer pH 7.5) was added to frozen samples, mixed well, and heated at 50ºC in a water bath for 60 minutes with occasional gentle mixing. Next, 600 μl phenol chloroform/isoamyl alcohol (25:24:1) (SIGMA®) was added and mixed gently for five minutes to emulsify without vortexing. The mixture was centrifuged at 12,000 rpm for 10 minutes. The top aqueous DNA layer was transferred to a clean tube while avoiding the interface, and 0.1 volume of absolute ethanol was added, mixed by inversion to precipitate DNA, centrifuged at 1,200 rpm for 10 minutes, washed with 70% alcohol, and centrifuged again at 1,200 rpm for 10 minutes. The supernatant was discarded and extracts were dried in a heat block at 50ºC.

Table 1. Definition of TB cases for registration on diagnosis (WHO/CDC/2003/TB.313)

TB cases	Definition of cases
New	A patient who has never had treatment for TB or who has taken anti-tuberculosis drugs for less than 1 month
Follow-up	A patient who has been taking anti- tuberculosis drugs for two months or more
Fail	Patient who is sputum smear positive at 5 months or later during treatment
Re-treatment	A patient who returns with positive bacteriology following interruption of treatment for two months or more
Relapse	A patient previously treated for TB, who has been declared cured or treatment completed and is diagnosed with positive bacteriology (smear or culture) tuberculosis

Table2. Prevalence of Mycobacteria detected by ZN smear microscopy, LJ culture and IS 6110 PCR in HIV-positive patients.

TB Cases	Total No examined	ZN$^-$Cult$^-$ PCR$^-$	ZN$^+$Cult$^+$PCR$^+$	ZN$^-$Cult$^+$ PCR$^+$	ZN$^-$Cult$^+$ PCR$^-$	ZN$^-$ Cult$^-$ PCR$^+$
New	70	47(67)	3(4.3)	9(13)	4 (6)	7(10)
Follow-up	14	5(35)	2(14)	3(21)	0	4 (29)
Fail	9	4(44)	1(11)	2(22)	0	2(22)
Re-treatment	2	0		0	0	2(100)
Relapse	6	0		2(33)	0	4 (67)
Total	**101**	**56(55)**	**6(6)**	**16 (16)**	**4(4)**	**19(19)**

ZN = Ziehl Neelsen, Cult = Culture, PCR = Polymerase chain reaction

DNA amplification

PCR was performed in a 50µl reaction volume comprised of 5µl PCR 10X buffer (Fermentas®); 1µl, 10mM dNTPs (Fermentas®); 0.5µl each of 10µM oligonucleotide primers IS$_1$ 5'-CCTGCGAGCGTAGGCGTCGG-3' and IS$_2$ 5'-CTCGTCCAGCGCCGCTTCGG-3' (Whitefield Scientific® PTY LTD South Africa), 0.5µl Taq DNA polymerase (Fermentas®), 37µl Nuclease free water and 5µl extracted DNA. The total reaction mixture was submitted to 35 cycles of 94ºC for 1.30 minutes; 68ºC for 1.30 minutes; 72ºC for 1.30 minutes; after 94ºC, 5 minutes initial denaturation; and final extension of 72ºC for 10 minutes. Amplicons and 100 base pair (Fermentas®) molecular markers were loaded in 2% agarose gel stained in ethidium bromide 10 mg/ml (Promega®) with 6X loading dye (Fermentas®) and electrophoresed at 80V for one hour.

One hundred twenty-three base pair (bp) target DNA fragments specific for *M. tuberculosis* complex were viewed under ultraviolet illumination (Fig 1). An in-house *M. tuberculosis* strain was used as positive control.

Results

Mycobacterium species was detected in 45/101 (44.5%) of the specimens from HIV positive patients with; 6% ZN$^+$culture$^+$PCR$^+$, 4% ZN$^-$culture$^+$ PCR$^-$, 16% ZN$^-$culture$^+$ PCR$^+$, 19% ZN$^-$ culture$^-$ PCR and 39/45 (86%) ZN undetected cases that were culture$^+$PCR$^+$ or culture$^-$PCR$^+$ (Table 2).

The detection rate according to patients' TB cases showed the following results: new: 23/70 (33%); follow-up: 9/14 (64.3%); relapse: 6/6 (100%); fail: 5/9 (55.5%); and re-treatment: 2/2 (100%) (Table 2.)

PCR method detected 19% extra cases that were not detected by LJ culture or smear microscopy while 4/100 (4%) culture-positive cases were not detected by PCR (Table 2).

ZN smear microscopy had a low sensitivity of 23% against 84% for PCR and detected 6/101 (6%) cases (Tables 2, 3).

In the children, Mycobacteria was detected in a total of 22/40 (55%) clinical specimens with 0% smear microscopy; 4/40 (10%) culture$^+$PCR$^{+;}$ and 18/40 (45%) culture$^-$ PCR$^+$. Ten out of 10 (100%) sputum specimens examined were positive with 4/10 (40%) ZN$^-$culture$^+$PCR$^+$ and 10/10 ZN$^-$culture$^-$ PCR$^+$(100%) (Table 4). Other clinical specimens [Pleural effusion: 7/9 (78%); Gastric juice 3/10 (27%); and ascitic fluid: 2/5 (40%)] were positive by PCR only. Five of the CSF specimens were negative by all three methods (Table 4).

Discussion

Mycobacteria were detected in 44.55% of the HIV-positive patients in this study. The result is within 35-40% estimated range for TB in HIV patients in Nigeria [20-21]. The prevalence 22/31 (71%) [10/31 (32%) culture$^+$, 12/31 (39%) PCR$^+$] from the follow up, failed, re-treatment and relapse cases in this study is relatively high and requires further examination for drug susceptibility to rule out the presence of drug resistant strains. Acquired drug resistance in *M. tuberculosis* may occur in HIV-positive patients receiving concomitant treatment with anti-tuberculosis and anti-retroviral drugs due to intestinal mal-absorption or drug interactions [22-24].

Four of 101 (4%) LJ isolates were not detected by PCR (Table2). The isolates may be other mycobacteria species requiring the use of species specific primers for confirmation since the IS6110 sequence specifically targets the *M. tuberculosis* complex group [18,15]. Culture by LJ detected 16% more cases than the ZN smear microscopy. Although the procedure is cumbersome and slow, it is cheap and affordable for use in laboratories with the biosafety containments required for mycobacteria culture.

Table 3. Sensitivity and specificity of PCR and ZN smear microscopy in HIV-positive patients

		Culture			
		Positive	**Negative**	**Sensitivity**	**Specificity**
PCR	Positive	22	19	84%	74%
	Negative	4	55		
ZN	Positive	6	0	23%	100%
	Negative	20	74		

Figure 1. Agarose gel electrophoresis of IS6110 based Polymerase chain reaction for detection of *M. tuberculosis* from clinical specimens
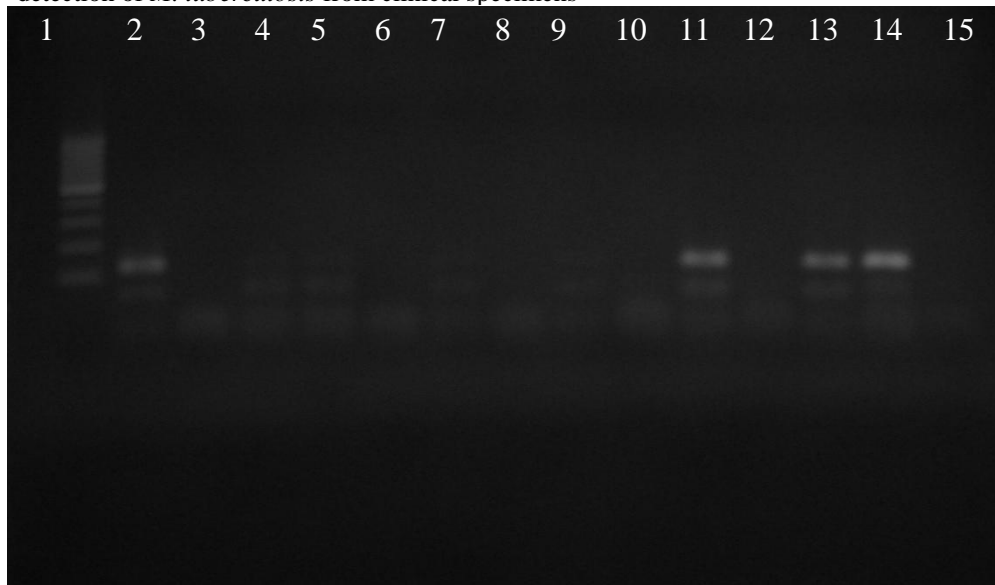
Lanes: 1 = 50 bp molecular marker; 2 = positive control (123 bp); 4, 5, 7, & 9 = positive from direct PCR using clinical specimens; 11,13,14 = Positive from culture isolates of *M. Tuberculosis;* 3, 6, & 10 = negative from clinical specimens; 15 = negative control.

Table 4. The prevalence of Mycobacteria detected by ZN smear microscopy, LJ culture and IS 6110 PCR in clinical specimens of children N (%)

Specimens	Total No Examined	Cult⁺ PCR⁺	Cult⁻ PCR⁺
Sputum	10	4 (40)	6 (60)
Gastric wash	11	0	3 (27)
CSF	5	0	0
Ascitic fluid	5	0	2 (40)
Pleural effusion	9	0	7 (78)
Total	**40**	**4 (10)**	**18 (45)**

ZN = Ziehl Neelsen, Cult = Culture, PCR = Polymerase chain reaction

The detection of 6/101 (6%) acid fast bacilli (AFB) with 39/101 (39%) undetected cases which were otherwise culture/PCR positive (Table 2) calls for the improvement of TB diagnostic methods in Nigeria, particularly in HIV patients, because they are more likely to produce poor sputum quality. False negative TB cases constitute a risk to public health as long as they remain undetected and untreated.

In the children, mycobacteria was detected in a total of 22/40 (55%) clinical specimens Four out of

10 (40%) and 10/10 (100%) sputum specimens were culture[+] PCR[+] and culture[-]PCR[+] respectively (Table 4). Although sputum is considered most appropriate for laboratory diagnosis of PTB, children are often unable to expectorate sputum [25]. The possibilities of false negative results by conventional methods in this regard are high because other body fluids (gastric washout, pleural effusion and ascitic taps) used as alternatives [26] usually do not contain sufficient quantities of mycobacteria that could be detected by routine methods. Positive results 12/40 (75%) were obtained for the clinical specimens other than sputum by PCR only (Table 4). The use of DNA PCR has been reported to improve the detection of Mycobacteria species in clinical specimens from children and adults [27-29].

In our experience, PCR increased the detection rate of *M. tuberculosis* but was cost intensive. It was estimated to cost about $15 per test against $4 for LJ culture and $2 for smear microscopy at 110 Nigerian Naira to US $1 exchange rate. In spite of the price differences, the sensitivity and short turn-around time of PCR outweighs its costs. While cost effectiveness and affordability are significant in the choice of diagnostic methods, especially in resource-limited settings, the cost of losses in terms of life is much more expensive.

A common problem with PCR assays is the high risk of false positive results due to laboratory contamination or the presence of killed or dormant bacilli in the patient's specimen [30-31]. Proper control checks, good laboratory practice, and conformity of laboratory results with clinical findings would optimally minimise the chances of errors.

The overall low prevalence of ZN positive smear microscopy (4.2%) in this study reflected the 31% actual sputum smear positive detection rate reported in Nigeria [9], which is less than the 70% global target recommended by the World Health Organisation [32].

We suggest that TB reference centres in countries such as Nigeria with high TB and HIV burdens should consider the use of PCR in combination with smear microscopy to enhance prompt detection of *M. tuberculosis* in HIV-positive patients, in children, and in other cases with low mycobacteria sputum content.

Acknowledgements

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