Technical Article

Direct molecular detection of *Mycobacterium tuberculosis* complex from clinical samples – An adjunct to cultural method of laboratory diagnosis of tuberculosis

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Abstract

Background: Tuberculosis, a communicable disease with significant morbidity and mortality, is the leading cause of death in the world from bacterial infectious disease. Because of its public health importance, there is need for rapid and definitive method of detecting the causative organism. Several approaches have been attempted, but the molecular methods, especially Polymerase Chain Reaction assays are the most promising for rapid detection of *Mycobacterium tuberculosis* complex from clinical samples. **Aim:** This study was aimed at using Polymerase Chain Reaction for detection of *Mycobacterium tuberculosis* complex from clinical samples using universal sample processing methodology. **Subjects and Methods:** Two hundred clinical samples sent to Tuberculosis laboratories in Ibadan and Osogbo, Nigeria, were enrolled in this study. The samples were processed by universal sample processing methodology for PCR; smear microscopy was carried out on sputum samples by Ziehl Nelseen staining technique; and cultured on Middlebrook agar medium containing oleic acid albumin dextrose complex supplement after decontamination of samples. **Results:** Ninety six (48%) samples were detected positive for *M. tuberculosis* complex by polymerase chain reaction using the combination of boiling and vortexing and microscopy detected 72 (36%) samples positive for acid fast bacilli. Using culture method as gold standard, it was found that polymerase chain reaction assay was more sensitive (75.5%) and specific (94.8%) than microscopy (sensitivity of 48.5% and specificity of 85.7%) in detecting *M. tuberculosis* complex from clinical samples. There was significant difference in detecting *M. tuberculosis* from clinical samples when compared to microscopy ($p<0.05$).**Conclusion:** The study recommends that direct molecular detection of *M. tuberculosis* complex is sensitive and specific and polymerase chain reaction method should be used as an adjunct to other methods of laboratory diagnosis of tuberculosis.

Keywords: Molecular detection, Polymerase chain reaction, *Mycobacterium tuberculosis* complex; *Mycobacterium tuberculosis*, Nigeria.

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Introduction

Tuberculosis (TB) is the leading cause of mortality and morbidity due to bacterial infections in the world and ranks second of all infectious agents due to microorganisms with HIV taking the first spot. The aetiological agent of tuberculosis is the group of mycobacteria known as *Mycobacterium tuberculosis* complex. *Mycobacterium tuberculosis* complex comprises of *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. canetti*, and the vaccine strain of *M. bovis* otherwise known as Bacillus Calmette Guerin (BCG). Each member of the TB complex is pathogenic, *M. tuberculosis* is pathogenic for humans while *M. bovis* is usually pathogenic for animal kingdom long before invading humans. TB was declared a global health emergency by the World Health Organization (WHO) in 1993. Statistics has put TB to claim approximately 1.7 million lives per annum [1]. It is estimated that one-third

of the world's population is infected with *M. tuberculosis* complex [2], with around 9 to 10 million new cases reported annually [1]. The problem of TB has been compounded by the emergence of multi-drug resistance *M. tuberculosis* and human immunodeficiency virus (HIV).

Epidemiologically, African countries have not been faring well since late 1980s and this has coincided with the HIV pandemic. Co-infection of people living with HIV with *M. tuberculosis* has been shown to increase the mortality rate in sub-Saharan African countries like South Africa, Botswana, and Zambia [3]. Nigeria was ranked fifth as high burden country with tuberculosis according to WHO report of 2008 [1]. The frightening statistics calls for new strategies to stem the rate of infection in the community and one of such strategies is the rapid and accurate laboratory diagnosis of TB especially among the patients with pulmonary tuberculosis, which constitute the most infectious population that aid the spread of the disease in a community. The current method of laboratory diagnosis of TB in developing countries like Nigeria relies on microscopy i.e. the ability to demonstrate acid fast bacilli by Ziehl Nelseen staining technique. Culture that is known to be "gold standard" in laboratory diagnosis of TB usually takes 3 to 8 weeks. The identification of the isolates on the culture media and susceptibility testing to anti TB drugs add another 2 to 3 weeks to the time it takes to make a definitive laboratory diagnosis of TB. DNA amplification-based methods overcome delays caused by the need to culture sufficient biomass and are amenable to high-throughput analysis, thus improving detection. Polymerase chain reaction (PCR) has been shown to play important role as an alternative diagnostic tool in developed countries [4, 5] and has yielded variable results, with sensitivities ranging from 42% to 100% and specificities from 85% to 100% using various PCR targets such as IS6110, 65 kDa, TRC4, GCRS, 16S, to mention a few [4, 6, 7]. Immunochromatographic technique for identification of *M. tuberculosis* complex from broth culture of mycobacteria has also joined methods for rapid diagnosis of TB [8]. We have recently shown that the identification of *M. tuberculosis* complex from culture using PCR [9] can reduce the time it takes for identification in this environment but this has not reduced the time it takes for culturing the aetiological agent from clinical samples. It is on the premise that we evaluated the detection of *M. tuberculosis* complex from clinical samples by PCR in order to determine the suitability of the test in our environment. Therefore, this study was aimed at molecular detection of *M. tuberculosis* complex from clinical samples submitted at some of the tuberculosis laboratories in South Western of Nigeria using PCR technology.

Subjects and Methods

Clinical samples and clinical information

All clinical samples were drawn from those submitted to the TB laboratories of Department of Medical Microbiology of University College Hospital (UCH), Ibadan, and Osun State Hospital, Asubiaro, Osogbo, Nigeria for tuberculosis diagnosis. A detailed clinical history, sex, and age were collected from the requisition form that accompanied the samples. Two hundred (200) samples from two hundred patients were included in the study. Majorities of the samples were sputum samples. The results of the study did not have any bearing on the treatment schedule administered to the subjects at the respective centres.

Processing of samples

Four millilitres of the sputum sample was collected for processing. Half of the aliquot was analyzed by universal sample processing (USP) method for polymerase chain reaction as described previously [10, 11] and conventional methods for smear microscopy by Ziehl-Neelsen (ZN) stain and culture for mycobacteria. All clinical samples for PCR were rendered non-infectious by subjecting them to temperature of 80^{o}C for 40 minutes.

Ten microlitre of dithiothreitol (DTT) in 2 ml of distilled water was added to the viscous samples and incubated at 37^{o}C for 40 minutes. The resultant solution was ready for DNA extraction after washing with USP solution (4 M guanidinium hydrochloride, 50 mM Tris-Cl [pH 7.5], 25 mM EDTA, 0.05% Tween 20). Sputum samples were centrifuged at 2200 × g. for 2 minutes. The supernatants were discarded and 0.5 ml of USP solution was added to the sediments. The USP solution was used to wash the sample sediments. The resultant sediments after washing with the USP solution were suspended in USP solution and heated at 100^{o}C for a period of 10 minutes in a water bath.

PCR for Mycobacterium tuberculosis complex

PCR to detect the presence or absence of IS6110 of *M. tuberculosis* complex DNA was carried out as described previously [4, 9] was used for molecular detection of *M. tuberculosis* complex from clinical samples. After processing of sample using USP technology, 2 µl of the processed sample was added to the 18 µl PCR master mix containing *Taq* polymerase and IS6110F (cctgcgagcgtaggcgtcgg) and IS6110B (ctcgtccagcgccgcttcgg) primers. The reaction mixture was made up of 1.5 mM $MgCl_2$, 0.2 mM dNTP, 50 mM KCl, 5% glycerol, 1 µM of IS6110F, 1 µM of IS6110B and 25 units of *Taq* polymerase (New England Biolab, USA) in a 20 µl reaction volume. The reactions were subjected to 5 min. at 94^{o}C, followed by 30 cycles each of 30 sec. at 94^{o}C (denaturation), 1 min. at 68^{o}C (annealing), and 30 sec. at 72^{o}C (extension), with a final extension of 7 min. at 72^{o}C.

Reagent and sample preparation, PCR amplification and product detection were performed in separate areas of the laboratory using dedicated equipment, aerosol-resistant filter guard pipette tips, and a unidirectional work flow scheme to minimize the possibility of any false-positive result due to carryover of amplicon contamination. Positive (100 pg of *M. tuberculosis* H37Rv DNA) and negative controls for both sample preparation and PCR assays were utilized in every experiment. Samples and

control were loaded with loading buffer; DNA ladder (size marker) was also loaded along with each set of PCR assay. The amplified DNA products were visualized under UV light after agarose gel electrophoresis after staining with ethidium bromide.

Detection of PCR inhibition

Spiking of samples with 100 pg of *M. tuberculosis* DNA was used to determine the presence of PCR inhibitors. The inability to amplify the DNA after spiking of sample was considered to be the presence of PCR inhibitor in PCR negative samples from above. In order to reduce the inhibitor(s) in samples, re-amplification of all the PCR products were done with 1/10 of the PCR products. The PCR products re-amplification methodology and conditions were the same as that of the first amplification. PCR products and the reagent preparation were done in different areas to avoid cross-contamination.

Microscopy

A loopful of sputum was spread on a clean glass slide uniformly in oval shape by smearing repeatedly in coil-like patterns, approximately 2-3 cm in size. After smearing, the slide was air dried completely. Then, flame-fixed by passing through the flame carefully so that it would not washed off during staining. The smear was stained for presence of acid fast bacilli using Ziehl-Nelseen staining as described previously [12].

Culture

Culture was performed by a modified Petroff method [13]. Briefly, 10 ml of 4% NaOH was added to about 5 ml or equivalent proportion, the cap of the container was tightened and shook to digest. It was allowed to stand for 15 min at room temperature with occasional shaking and centrifuged at 2000 x g for 10 min, after which the supernatant was poured off into a stericol disinfectant jar. The deposit was re-suspended in sterile distilled water and centrifuged at 2000 x g for 15 min. The resultant deposit was inoculated onto Middlebrook agar containing oleic acid albumin dextrose complex (OADC) supplement. The culture was incubated at 35-37^{o}C until growth was observed for maximum period of 8 weeks. All cultures were compared with the control organism (H37Rv). Every week after the inoculation, Middlebrook agar slopes were removed from incubator and observed for 4 characteristics: aspect/consistency of the culture media; growth rate of the colonies; morphology of the colonies; and colour of the colonies (presence of pigmentation).

DNA Extraction

DNA was extracted from all the colonies suspected to grow organism irrespective of whether the culture resembled *M. tuberculosis* complex as described before [9, 14]. Briefly, the Middlebrook agar slope containing suspected colonies was heat sealed at 80^{o}C for 1 h to kill bacteria. Thereafter 3 ml of extraction buffer (50 mM Tris-HCl, 25 mM ethylene diamine tetra acetic acid (EDTA), 5% mono-sodium glutamate, pH 7.4) was added and the colonies were carefully scraped using a disposable loop and homogenised using a vortexer for 2-3 min to disrupt colonies. Four hundred microlitre of 50 mg/ml lysozyme stock and 10 µl of 10 mg/ml RNAase were added to the suspension and incubated at 37^{o}C for 2 h. Subsequently, 600 µl of 10 × proteinase K buffer (100mM Tris-HCl, 50 mM EDTA, 5% SDS, pH 7.8) was added with 150 µl of 10 mg/ml proteinase K and incubated at 45^{o}C for 16 h. The DNA was purified using phenol/chloroform/isoamyl alcohol and precipitated from upper aqeous phase using equal volume of isopropanol in the presence of 0.1 volume of 3 M sodium acetate, pH 5.5 at -20^{o}C for 30 min. The resulting DNA pellet after centrifugation at 3000×g for 30 min was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20^{o}C.

Polymerase Chain Reaction (PCR) for Identification of M. tuberculosis complex

After the DNA extraction, about 50 ng of the DNA sample was added to the PCR master mix prepared in the presence of *Taq* polymerase and IS6110F (cctgcgagcgtaggcgtcgg) and IS6110B (ctcgtccagcgccgcttcgg) primers to amplify 123 bp insertion sequence element present in all *M. tuberculosis* complex as described above for direct detection of *M. tuberculosis* complex from clinical samples.

Statistics Analysis

Data were analysed using statistical package within the Microsoft Excel and Epi-info software from Centre for Disease control and prevention, USA. Chi square was used to compare the differences in diagnostic yields. The p value less than 0.05 was considered to be significant.

Results

Clinical samples submitted between March, 2009 and May, 2009 were enrolled in this study, with total numbering two hundred (106 females and 94 males, cutting across several age groups). The major presenting clinical feature was cough (n=24), fever and weight loss (n=2), pulmonary tuberculosis (n=156), diabetes mellitus with pulmonary tuberculosis (n=8), pneumonia (n=4), and follow-up examinations (n=6) were included.

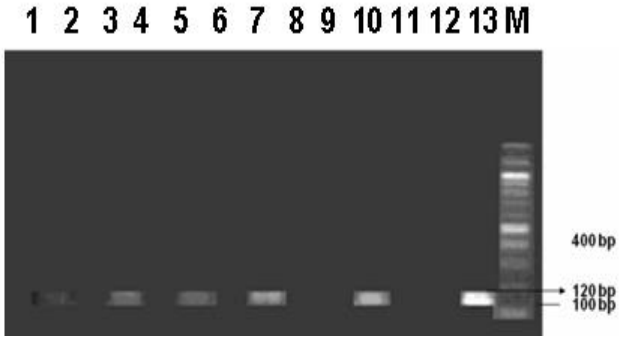

Fig. 1 PCR for detection of *M. tuberculosis* complex from clinical samples. Representative photograph of agarose gel electrophoresis following PCR. Lanes 1, 3, 5, 7 and 10 are positive samples, lanes 4, 6, 8, 9, and 11 are negative samples for *M. tuberculosis* complex. Lanes 12 and 13 are negative and positive controls respectively while lane 10 is 100 bp DNA

ladder (New England Biolab, USA).

Table 1 PCR, Microscopy, and Culture in diagnosis of TB

Clinical Diagnosis	Positive PCR	Positive Culture	Positive Microscopy
Productive and Chronic cough	7/15	9/15	0/15
Pulmonary tuberculosis	81/166	75/166	65/166
Pnumonia	2/3	2/3	0/3
Broncho-pneumonia	0/2	0/2	0/2
Fever and weight loss	0/3	0/3	0/3
Diabetes mellitus and PT	2/6	2/6	5/6
Follow up examination	4/5	2/5	2/5
	96/200	90/200	72/200

Table 2 Diagnostic yields for different methods of detecting *M. tuberculosis* complex from clinical samples

Diagnostic Parameter	PCR (boiling)	PCR (Boiling/vortexing)	Microscopy (ZN)
Sensitivity	14.0%	75.50%	49.10%
Specificity	74.8%	94.8%	85.70%
Positive predictive value	17.90%	100%	55.10%
Negative predictive value	70.80%	94.80%	82.10%

All 200 clinical samples were subjected to smear microscopy, culture on Middlebrook 7H9 agar medium, and PCR using USP methodology of processing samples. Details of the diagnostic categories of patients and samples are provided in Table 1. Table 1 also shows how the different methods of laboratory diagnosis of tuberculosis performed with respect to patients' diagnosis. Table 1 shows that PCR had the highest presumptive diagnosis of tuberculosis (48%); followed closely by cultural method (45%). Diagnosis of tuberculosis in this study was made when culture was positive and PCR was used to confirm the identity of the organism on agar slope.

Conventional microscopy by ZN detected 72 (36%) out of 200 samples as positive. Using boiling method as method of lysing mycobacteria in sputum samples after treatment with USP solution, PCR detected 52 (26%) as *M. tuberculosis* complex using the IS6110 sequence primers as shown by the amplification of 123 bp product (Fig. 1) in 200 samples; out of which initial PCR detection of *M. tuberculosis* complex DNA was 24 (12%) out of 200 clinical samples. Further twenty eight (28) (14%) samples were detected to be positive for *M. tuberculosis* complex DNA as indicated by the amplification of 123 bp product as a result of further dilution of the clinical samples to reduce the PCR inhibitor(s). Combining the boiling method of lysing mycobacterial DNA with 15 minutes of mixing the samples with a vortexer prior to amplification showed that 84 (42%) samples out of 200 samples tested were positive for *M. tuberculosis* complex DNA while additional 12 samples were detected after reducing the PCR inhibitor bringing the total to 96 (48%) positive samples for *M. tuberculosis* complex. The detection limit of the PCR for detection of IS6110 of *M. tuberculosis* complex DNA was found to be 10 fg. A total of 90 (45%) samples out of the 200 samples tested were positive by culture method for *M. tuberculosis* complex as indicated by the characteristic growth on Middlebrook 7H9 agar, rate of growth (average growth of about 4 weeks), and confirmation of colonies on Middlebrook 7H9 agar medium by PCR for *M. tuberculosis* complex. Comparison of culture with microscopy showed that 32 (35%) of 90 were detected positive by microscopy while PCR using combination of boiling and vortexing detected 70 (78%) out of 90 culture positives. The diagnostic yield of the different laboratory methods for diagnosis of TB using culture as gold standard is shown in Table 2.

Using culture as the gold standard, the sensitivity and specificity of microscopy was 48.5% and 85.7%, respectively while that of PCR using boiling method was 14% and 69%, respectively. There were significant differences in sensitivity (75.5%) ($\chi 2 = 36.59$; $P < 0.05$) and specificity (94.8%) ($\chi 2 = 27.85$; $P < 0.05$) when PCR was carried out using boiling method compared to microscopy (ZN). Furthermore, when PCR was carried out using boiling / vortexing method of preparing crude DNA for PCR compared to boiling method, there were significant differences in sensitivity (75.5%) ($\chi 2 = 6.79$; $P < 0.05$) and) specificity (94.8%) ($\chi 2 = 9.49$; $P < 0.05$).

Discussion

The conventional methods for laboratory diagnosis of *M. tuberculosis* include microscopy (acid fast staining) and culture. For enhancing treatment strategies and reducing the potential of spreading TB in a community by pulmonary TB patients, new diagnostic methods for *M. tuberculosis* complex are needed to help combat this deadly disease, in which the use of nucleic acid amplification and detection in sputum, blood and body fluids may provide quick and specific results for identifying the *M. tuberculosis* complex [15].

Using culture as the gold standard, 53 samples out of 200 were confirmed to be positive for *M. tuberculosis* complex. Out of these, 26 samples were positive by microscopy and

40 samples were positive by PCR assay after boiling/vortexing of samples – the full optimised method of preparing clinical samples prior to PCR. The sensitivity of PCR assay using boiling method of extracting DNA in this study was 14% while that of microscopy was 36%. While marked improvement was seen when the boiling method was combined with vortexing of the samples for PCR as indicated by 84.8% sensitivity using culture as gold standard, suggesting that the yield of DNA is dependent on the physical disruption of the cell cum proper lysing of samples following boiling of the acid fast bacilli in the sample. This is in agreement with previous studies where physical method of disruption of mycobacteria has been shown to increase the RNA yield when sonicator or beadbeater or FastPrep machine was used [16, 17]. Various researchers have reported variable sensitivities (53.6 to 96.0%) and specificities (38.7 to 99.0%) using PCR for detection of *M. tuberculosis* complex from clinical samples [18-20]. Using the USP technology of processing, fully optimised DNA extraction method, and taken into consideration the PCR inhibition, we found that the sensitivity and specificity of PCR were 75.5% and 94.8%, respectively, which is in agreement with other studies [10, 15].

PCR technology is prone to contamination and PCR inhibition. The way round the PCR contamination in our study was the use of barrier filtered tips and separation of sample preparation bench from where the PCR products were analysed. Strict adherence to workflow in the laboratory also helped to combat the problem of PCR contamination. We also observed high degree of PCR inhibition despite the fact we used USP technology that has been shown to reduce PCR inhibition [7, 10] in processing the samples. Dilution of the samples and spiking of the processed samples with DNA helped in identifying this phenomenon. Failure to identify PCR inhibition could lead to false negative result. However, there are numerous examples in the literature of amplification-based test performances being marred by inhibitory substances present in clinical samples, which could include blood, host proteins and even eukaryotic DNA that can inhibit amplification when present in a high concentration [15, 21, 22].

Clinical samples contain inhibiting substances that interfere with the performance of the PCR [23, 24]. Therefore, a routine procedure suitable for removal of all inhibitors simultaneously is highly desirable [15, 22]. Owing to the extremely variable nature of inhibitors, no single ideal procedure exists yet [25, 26].

In a study carried out by Bennedsen *et al*., [27], it was found that 51% of the samples (22 of 43 samples) were inhibited, half of PCR diagnoses would have been missed without the silica membrane protocol of removing inhibitors used in that study, thereby increasing the sensitivity from 66% to 87% in sample processed without silica membrane protocol and those processed with silica membrane protocol of removing inhibitors respectively.

The effect of PCR inhibitors was demonstrated in this study, first amplification detected 60 as positive out of 200 samples whereas re-amplification after diluting out the inhibitor detected 36 more samples as positive out of the samples tested, making the total number of positive samples to be 96 out of 200 samples tested, this result is in concordance with other studies [27-31] where 51-65% of the positive samples were inhibited by the inhibitory substances in the clinical samples. Presence of inhibitors contributed to the lower sensitivity of PCR assay as compared with microscopy unless procedure that helps in identifying this phenomenon is incorporated into diagnostic methods of TB by PCR. The sensitivity of re-amplification was far superior to that of first amplification due to the dilution of the possible inhibitors. USP methodology and re-amplification of the amplicon increase the sensitivity and reliability of PCR assay by reducing the prospective inhibitors, and increasing the DNA copy in the PCR products. The DNA purification method using guanidinium thiocyanate and diatoms effectively removed most or all inhibitors of the PCR thereby increasing the sensitivity from 32% to 91% and specificity from 61% to 87% [32].

Various attempts have been made to reduce PCR inhibition in diagnostic tests, mostly with regard to a specific material [33, 34]. Inexpensive methods, such as boiling, have been effective with urine samples [35] and partially effective with cerebrospinal fluid, depending on the protein level [36]. Notably, boiling can also cause inhibition [37]. Boiling was found to be as effective as sample dilution with cervical samples [38]. Sample dilution worked particularly well with urine samples but was inadequate for respiratory tract samples [39]. Instead, in the case of the latter, the addition of bovine serum albumin neutralized inhibitors in 21 of 22 samples [40]. The addition of bovine serum albumin protects PCR from the effects of blood, but this procedure has been analyzed with only a few clinical samples.

Phenol-chloroform extraction has been shown to be highly effective [41] but uses toxic substances and is particularly laborious. Boiling method alone used in this study was not effective but we found it to be effective if combined with physical disruption of bacteria using vortexer to mix the sample for 15 mins without additional cost. The problem we came across was the cumbersome nature of the technique especially if many samples are needed to be processed at the same time. In this case, FastPrep machine or Ribolyzer can be substituted for vortexer in physical lysing of mycobacteria present in clinical samples so as to reduce the inconvenience in processing of samples and increase detection of mycobacterial DNA by PCR as a result of the increase in the release of DNA from mycobacteria. Alternatively, the use of silica membrane in processing samples can help in removing the PCR inhibitor. The silica membrane protocol adds approximately $1 per sample to costs but has been found to be effective in a variety of materials [27]. Therefore, it remains to be seen whether a given method or some

combination of methods will be best suited to the task of overcoming PCR inhibition [27-31].

Culture of samples on Middlebrook 7H9 agar would detect viable mycobacteria while PCR for *M. tuberculosis* complex would detect both viable and dead mycobacteria. More samples were detected to be positive for *M. tuberculosis* complex (96 for PCR; 90 for cultures) suggesting the possibility of PCR in detecting both viable and non viable mycobacteria after patients must have started TB treatment while culture indicates true reflection of patients' condition with respect to the presence of viable *M. tuberculosis* complex. The importance of this discourse is the need to combine PCR, culture, and clinical diagnosis in monitoring TB treatment. PCR has been shown from other studies to detect *M. tuberculosis* complex after 2 to 3 weeks of active TB treatment [42]. In order for PCR to be highly specific for patient's clinical condition, there is need for reverse transcription PCR (RT-PCR) which can differentiate between live and dead mycobacteria [5], hence, giving good indication of how the patient is responding to TB treatment with TB drugs. PCR in its present form as used in this study can be used as an adjunct to microscopy to diagnose TB in patients on their first visit to hospital. The inability of microscopy in differentiating *M. tuberculosis* complex from other acid fast bacilli necessitates this. CDC, USA has come up with guidelines for the use of PCR along with existing laboratory methods of diagnosing tuberculosis [43]. In the algorithm, multiple samples are advocated for in patient suspected of being infected with *M. tuberculosis* complex; with the interpretation that a patient can be assumed non-infectious if all smear and PCR tests results are negative. The ability of PCR in discriminating *M. tuberculosis* complex from other mycobacteria also helps in the choice of chemotherapy for TB treatment which will go a long way in improving patient's prognosis instead of waiting for culture that will take between 3 and 8 weeks.

Conclusion

We have demonstrated the use of PCR in detection of *M. tuberculosis* complex in clinical samples in this environment and on the basis of this study we advocated for the integration of molecular diagnosis of *M. tuberculosis* complex in diagnosis of tuberculosis especially the first visit of patients to TB/chest clinic as this will offer timely intervention in the treatment of TB and curbing the spread of this dangerous organism.

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