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DETECTION FAILURE OF SMEAR MICROSCOPY TO CONFIRM PULMONARY TUBERCULOSIS

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ABSTRACT

Background: The laboratories in poor resource settings commonly use smear microscopy for the diagnosis of pulmonary tuberculosis, because it is rapid, inexpensive and easy to perform. The commonly use Lowenstein Jensen culture method for the diagnosis of tuberculosis is slow and usually not very sensitive. Polymerase Chain Reaction (PCR) in the diagnosis of tuberculosis (TB) has come to stay as an effective diagnostic tool for effective pulmonary TB case detections.

Objectives: This is to promote and encourage utilization of molecular diagnostic method (PCR) for the diagnosis of tuberculosis especially in smear negative microscopy suspected cases to reduce the rate of missed diagnosis.

Material and Methods: This was an observational cross sectional prospective study among patients who presented at tuberculosis reference laboratory with request form for the diagnosis of pulmonary tuberculosis. Consent forms were administered and only consented clients were enrolled in the study. Standard sample collection criteria were used for all collected samples subjected to standard smear microscopy, Lowenstein Jensen culture and PCR studies.

Results: Direct smear microscopy (AFB) was positive in 13 (13.5%), 24 (23.3%) were detected by PCR and 20 (19.4%) were culture positive. All positive smears were found to be positive for both culture and PCR. All culture positive samples were positive by PCR, culture growth were also positive by PCR. Most of the PCR results were ready within the first day of the analysis with average of 1.2 days ($P = 0.014$) of the 103 samples processed. The result of smear microscopy is only ready on the second day after analysis of the second and third samples submitted with reported average of 1.7 days ($P = 0.035$). The fastest observable growth of positive culture was seen only within ten days and only three cases showed such. It took eight weeks for a negative growth to be regarded as negative. The culture has an average turn around time of 50.6 day ($P = 0.932$). The sensitivity, specificity, Positive Predictive value (PPV), Negative Predictive Value (NPV), and degree of accuracy for smear microscopy and PCR were 65.0%, 100% 100% 92.2% 93.2% and 100% 95.4%, 83.3%, 96.2% 96.3% respectively.

Conclusion: Employing PCR as the routine tool to confirm all suspected cases of smear negative microscopy improves case detection vis a vis an effective tuberculosis control measures.

Keywords: *Detection failure, Smear negative microscopy, Confirm, Pulmonary Tuberculosis*

Key words: awareness, use, stimulants, security personnel, Sokoto, Nigeria

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INTRODUCTION

Tuberculosis (TB) is one of most important public health problems worldwide and stands seventh in the ten leading causes of global disability adjusted life years (DALYS).¹ One-third of the world's populations are affected by tuberculous bacilli and every year eight million people become sick with TB of which 95% are in the developing countries.¹ Tuberculosis is the second leading cause of death from an infectious disease and the seventh-highest cause of mortality in poor countries.²³ Twenty-six percent of the avoidable adult deaths in the developing world are due to TB. More than 100,000 children die from TB every year and up to

50 million people are likely to be infected with drug-resistant TB. Thirteen of the fifteen countries with the highest TB incidence are in Africa. An average TB patient loses three to four months of work and up to 30% of yearly household earnings.⁴ It was estimated by the World Bank that the disease diminishes 4-7% of Gross Domestic Product (GDP) in some of the worst-affected countries.⁵ Africa has a complex problem especially in relation to accurate and rapid diagnosis of infectious diseases including TB.⁶ Provision of reliable confirmatory diagnostic testing for TB is severely limited in this region, and misdiagnosis commonly occurs.⁶ Inaccurate laboratory diagnostic testing may promotes the perception that laboratory testing

is unhelpful, and this compromises patient care. There is still need for more resources to assist in the diagnosis which is essential to the prevention and treatment of cases.

Polymerase chain reaction (PCR) has proved to be the best method in the laboratory diagnosis of pulmonary tuberculosis regardless of wide variation in the performance of the test and it give true picture of the burden of tuberculosis.⁷ This study was conducted to encourage clinicians of the continuous need to confirm all smear negative cases with strong clinical suspicious of pulmonary tuberculosis using molecular diagnostic methods.

MATERIAL AND METHOD

This was an observational cross sectional prospective study. The study population was drawn from patients who presented at Jos University Teaching Hospital (JUTH) TB reference laboratory with laboratory request form for the diagnosis of pulmonary tuberculosis. All subjects involved have consented for the study and appropriately filled and signed the consent form. One hundred and three (103) subjects consented and were enrolled for the study.

Specimen Collection

Three sputum specimens were collected from each patient. A spot-morning-spot-specimen (SMS) collection criterion was strictly adhered to. Patients were taught on how good quality sputum can be produced by rinsing his/her mouth with water, taking a deep breath and then coughing to produce the sputum. Spot specimens were collected on first visit in a clean, sterile, transparent, wide mouth, screw-capped universal container. Second sputum containers were given to the patient and were advised on how to collect early morning specimen on the next day to be immediately brought to the hospital. Third morning specimens spot were collected immediately upon receiving the second (morning) specimen.

Ziehl Neelsen Staining

A smear of the specimens were prepared on a clean grease free properly labeled glass slide and allowed

to air dried. The air dried smears were passed over Bunsen burner flame for at least three times to heat fixed it. The entire slides were flooded with Carbon Fuchsin. The flooded slides were heated slowly over Bunsen burner flame until steaming. Steaming was maintained for five minutes by occasionally passing the Bunsen burner flame under the slides. The slides were rinsed with water and then flooded with 3% acid-alcohol and allowed to decolourized for five minutes. Throughout the five minutes, the slides were continuously flooded with 3% acid alcohol until the slides were cleared of stain visible to the naked eyes. The slides were then thoroughly rinsed with water and any excess water from the slides was drained. The slides were then flooded with the counter stain (methylene blue) for one minute. The slides were finally rinsed thoroughly with water. The stained slides were allowed to air dry on a drying rack. The stained slides were then viewed under light microscope for acid fast bacilli at x10 x 40 and x 100 magnifications.

Quality Control Parameters

Positive and negative control slides smear were included with each run of stains. This verified the correct performance of the procedure as well as the staining intensity of the acid fast organisms. Control slides were reviewed before patient smears were examined to confirm that the mycobacteria stain acid-fast (Plate 1).

CULTURE

Modified Petroff's Procedure

This was carried out by dispensing a portion of sputum into centrifuge tube and a double volume of sterile 4% NaOH solution added. The caps of the

tubes were tightened, shaken well. The bottles were then vortexed until homogeneously mixed. The mixtures were kept in 37°C incubator for 15 minutes, the tubes were then removed from the incubator and 15 ml of sterile distilled water were added. It was then mixed well by shaking and centrifuged at 3000G for 15 minutes. The tubes were carefully removed from the centrifuge without shaking. The supernatant were discarded slowly into a container with 5% phenol solution. The pellets then washed with sterile distilled water by centrifugation at 3000G for 15 minutes, and the supernatants decanted. From the sediment, two slopes of LJ medium were inoculated aseptically. The inoculated media were labeled with patients' identification, study number and date of inoculation. Inoculated media were incubated at 37°C and examined after 48-72 hours to detect gross contaminations. The growths were checked weekly for eight weeks.

Culture Examination and Identification

Typical colonies of *M. tuberculosis* are rough, crumbly, waxy, non-pigmented (buff coloured; Plate 3) and slow-growers. The acid-fastness of the growth was confirmed by Ziehl-Neelsen (ZN) staining. Very small amount of growth removed from the cultures using a loop were gently rubbed into one drop of sterile saline on a glass slides. The ease with which the organisms emulsified in the liquid were noted, tubercle bacilli do not form smooth suspension, unlike some other mycobacteria. The smears were allowed to dry, fixed by heat and stained by the ZN method. Growth with no AFB on microscopy was reported as contamination.

Plate 2: Rough, crumbly, waxy, non-pigmented characteristic growth of *M. tuberculosis*

POLYMERASE CHAIN REACTION (PCR)

One hundred and three (103) sputum samples were subjected to PCR analysis based on the amplification of a 123bp fragment of the repeated insertion sequence IS6110 using the forward primer IS1 (5'-CCTGCGAGCGTAGGCGTCGG-3') and reverse primer IS2 (5'-CTCGTCCAGCGCCGCTTCGG-3').⁸ The reagents and primers were purchased from Inqaba Biotec, South Africa and the procedure were conducted at the molecular laboratory of National Veterinary Research Institute (NVRI) Vom, Plateau state, Nigeria. The sputa and cultures were lysed by boiling at the regional TB reference laboratory, Jos University Teaching Hospital (JUTH). Manufacturer's instructions were strictly followed in carrying out PCR procedure for the molecular diagnosis of PTB.

Sputum Preparation and Deactivation for Deoxyribonucleic Acid (DNA) Extraction

1 ml of each sputum sample were dispensed into Eppendorf tube and capped properly to avoid spillage. The Eppendorf tubes containing sputum samples were immersed using a floating perforated rack in a water bath at 80°C for one hour to lyse and deactivate the bacilli. The tubes were centrifuged at 14000 g for 10 minutes. The supernatant were carefully decanted. The pellets were used for DNA extraction.

DNA Extraction

1. 500 µl of lysis buffer were added to each sample in Eppendorf tube, mixed well by pipetting up and down until fully homogenized and then vortexed for 30 seconds. Samples were left to stand at room temperature for 10-15 minutes.
2. The mixtures were then centrifuged at 10,000 g for 1 minute.
3. The supernatants were transferred to a Zymo-spin™ column in a collection tube then centrifuged at 10,000 g for one minute. The collection tube containing the flow through were discarded
4. The Zymo-spin™ columns were transferred in

a new collection tube. 200 µl of DNA pre-Wash buffer were added to the spin column and centrifuged at 10,000 g for one minute.

5. 500 µl of g-DNA Wash Buffer were added to the spin column. Centrifuged at 10,000 g for one minute.
6. The spin columns were then transferred into micro-centrifuge tubes and 50 µl of DNA Elution Buffer were added to each column and allowed to stand at room temperature for 1 minute, then centrifuged at maximum speed (23,000 g) for 30 seconds to elute the DNA.
7. The eluted DNA was used for amplification of IS110 insertion sequence for PCR detection of *Mycobacterium tuberculosis* complex.

Preparation of Master - Mix

In preparation of master-mix the volume and units of reagents supplied was put into consideration to calculate the 50 µl reaction volume of master-mix. We used stocks of 10X buffer, 10 mM of each nucleotide, 20µM primers and Taq DNA polymerase at 5,000 Units/ml. All reagents and enzyme were purchased from Inqaba Biotec, South Africa. The final concentrations are highlighted below:

10 X buffer: 10X stock X (5 µl) = (1X) final concentration in a 50 µl reaction

Nucleotides: 10 mM stock of each nucleotide X (1 µl) = 200 µM final concentration in a 50 µl reaction (1mM=1,000µM)

Primers: 20µM stock X (0.5µl) = 0.2µM final concentration in 50 µl reaction mixture.

Taq DNA polymerase (5Units/µl) X (0.5 µl) = (2.5 Units) in (50 µl)

The primer sequences are as follows:

Is1 5'-CCTGCGAGCGTAGGCGTCGG-3' and S2 5'-CTCGTCCAGCGCCGCTTCGG-3' (Quick-gDNATM MiniPrep)

The amount of each reagent was calculated based on the numbers of reactions including the samples and controls.

DNA Amplification

The total reaction mixtures were subjected to 35 cycles at 94°C for 1 minute 30 seconds; 68°C for 1 minute 30 seconds; 72°C for 1 minute 30 seconds; after 94°C, 5 minutes initial denaturation; and final extension of 72°C for 10 minutes. All in thermocycler PCR machine

Preparation of Electrophoretic Gel

A 2% electrophoresis gel was prepared using dehydrated agarose gel mixed with agarose buffer according to manufacturer instructions. 2 g of agarose dehydrated powder was weighed, mixed well with 100 ml of buffer, and boiled in a microwave until fully dissolved. This was allowed to cool until about 60 degrees, and then two drops of ethidium bromide, a fluorescent compound was added. It was then poured on electrophoresis cast containing 2 x 20 comb which made loading well. It was then allowed to polymerize at room temperature for about 1 hour. The combs were gently removed. The gel was finally transferred to an electrophoretic tank.

Electrophoresis and Detection

The products of amplification were mixed with a loading dye, which is a dense viscous, blue liquid. Two µl of the loading dye were mixed with 10µl of amplicons. First wells were loaded ladder, 100bp DNA. Subsequent wells were loaded with amplified samples mixed with loading dye. The last two wells were used for positive and negative control respectively. After loading an electrical current was passed through the content of the gel box and the negatively charged DNA migrated towards the anode.^{9, 10} The larger pieces of DNA were retarded by the gel matrix, compared with smaller pieces of DNA. It was allowed to run for 40 minutes. The presence of a single band of the expected size (123bp) for *Mycobacterium tuberculosis* complex is good preliminary evidence of the specificity of amplification reaction.

After gel electrophoresis, the electrophoretic products were subjected to ultra violet (UV) light scanning procedure. The gels were transferred to the ultra violet light scanner (BIO-RAD) and the

images were captured and bands were viewed on the computer. The ladder which is 100bp was used to analyze the amplified DNA, the exact correspondent match were diagnostic for *Mycobacterium tuberculosis* complex .

Ethical Consideration

The ethical approval for this study was obtained from the Ethical Committee of Jos University Teaching Hospital, Jos.

Data Analysis

The data was analyzed using Epi Info™ 7.1.5.0, 2013 and IOS Wizard statistical package. In all, critical p value of < 0.05 was regarded as significant. Data was presented in tables and figures

RESULTS

A total 103 sputum samples were subjected to microscopy, PCR and culture in the study. Direct smear microscopy (AFB) were positive in 13 (13.5%), while 24 (23.3%) were detected by PCR and 20 (19.4%) were culture positive (Table 4.1). All positive smears were found to be positive for both culture and PCR. All culture positive samples were positive by PCR.

Table 4.1: Frequency of *Mycobacterium tuberculosis* detection by microscopy, culture and PCR of patients with pulmonary tuberculosis in Jos

(N = 103)

Result	Microscopy	PCR	Culture
Positive	13	24	20
Negative	90	79	83
P- value	0.293	0.012	0.030

Most of the PCR results were ready within the first day of the analysis (Table 4.2) with average of 1.2 days ($P=0.014$) of the 103 samples processed. The results of smear microscopy were ready on the second day after analysis of the second and third samples submitted with reported average of 1.7 days ($P=0.035$). The fastest observable growth of positive culture was seen only within ten days and

only three cases showed such. It took eight weeks for a negative growth to be regarded as negative. The cultures had an average turnaround time of 50.6 days ($P=0.932$)

Table 4.2: Turnaround time for the final results of microscopy, culture and PCR of sputum of patients with pulmonary tuberculosis in Jos

Procedure	Average duration (days)	P-value
Microscopy	1.7	0.035
Culture	50.6	0.932
PCR	1.2	0.014

The sensitivity, specificity, Positive Predictive value (PPV), Negative Predictive Value (NPV), and degree of accuracy for smear microscopy and culture (Table 4.3) were 54.2%, 100%, 87.8% 92.2%, 93.2% and 83.3%, 100%, 100%, 95.2% and 96.1% respectively. PCR had 100% performance test status. The disease prevalence for smear microscopy, culture and PCR were 13.5%, 19.4% and 23.3% respectively with average of 18.4%.

Table 4.3: Performance of smear microscopy, culture and PCR of patients with pulmonary tuberculosis in Jos

Test performance	Smear microscopy	PCR	Culture
Sensitivity	54.2%	100%	83.3%
Specificity	100%	100%	100%
PPV	100%	100%	100%
NPV	87.8%	100%	95.2%
Disease prevalence	13.5%	23.3%	19.4%
Accuracy	89.3%	100%	96.1%

Key

PPV = Positive Predictive Value

NPV = Negative Predictive Value

DISCUSSION

The laboratories in our environment commonly use smear microscopy for the diagnosis of pulmonary tuberculosis. It is rapid, inexpensive and easy to perform however, its associated low sensitivity limits its diagnostic accuracy. Sample should

contain at least 10,000 bacilli/ml for microscopy to be positive and doesn't differentiate between mycobacterium species.¹¹ This contributes to poor reflection of true burden of the disease when smear microscopy is being employed as the only readily available diagnostic tool. Cultural methods are slow and may give false negative in multibacillary pulmonary tuberculosis, paucibacillary pulmonary tuberculosis or non-pulmonary disease.¹² Polymerase chain reactions has an excellent sensitivity and has been found to be useful in diagnosing paucibacillary smear negative samples.¹² It can also detect samples with *M. tuberculosis* strains lacking IS6110¹³. PCR was able to detect missed cases of TB by subjecting the negative smear microscopic slides for *Mycobacterium tuberculosis* DNA amplification and detection and can also detect the DNA from processed sputum sediments and supernatant.¹⁴

Rapid and correct diagnosis warrant early initiation of appropriate treatment which are important for effective TB control. The use of molecular techniques such as the Polymerase Chain Reaction (PCR) in diagnosis of tuberculosis has been considered to be of overwhelming advantages for effective pulmonary TB case detections.^{15, 16} PCR method was reported to be a valuable, cost-effective and alternative tool for rapid diagnosis of active tuberculosis in different clinical specimens.¹⁷

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This study was performed among routine patients with suspected pulmonary TB on clinical evaluation who presented with laboratory request form at tuberculosis reference laboratory of Jos University Teaching Hospital (JUTH). This was a cross-sectional study conducted to promote utilization of PCR to improve case detection of pulmonary tuberculosis. We use PCR targeting IS6110 which was found to have better positivity than other molecular targets and it was simple and reproducible.

In this study the rate of *Mycobacterium tuberculosis* detection was found to be higher by PCR targeting IS6110, PCR detected 24 cases

(n=103) whereas only 13 cases (n=103) were smear positive. Culture detected 20 cases (n=103). PCR was able to detect 11 of the smear negative cases and this was in agreement with similar studies conducted in Bangladesh and Kenya.^{21, 22} In another study smear microscopy missed 51% of PTB cases that was detected by PCR in Ashanti and western region of Ghana.²³ This is almost similar to our finding which detected 45.8% of cases missed by microscopy.

The high sensitivity, specificity, positive predictive value, negative predictive value and excellent degree of accuracy are additional qualities of PCR over routine conventional methods that highly influence TB detection, which was also reported in similar studies.^{24, 25} Based on the aforementioned qualities of PCR, the prevalence of pulmonary tuberculosis was found out to be higher than other study that uses conventional methods in their assessment. This is enough justification for the use of PCR as the best tool to confirm all smear negative suspected tuberculosis. Turnaround time affect how early antiTB drugs can be initiated, regardless of its technicality we found that PCR has the best turnaround time which directly affect commencement of treatment.

CONCLUSION

This serve as a reminder to encourage utilization of PCR to confirm all cases of smear negative tuberculosis which improve case detections as well as a good tool for effective TB control programme.

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