Comparative Assessment of Five Laboratory Techniques in the Diagnosis of Pulmonary Tuberculosis in Abuja

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Abstract: *A total of 340 specimens from 192 (56.5%) male and 148 (43.5%) females attending tuberculosis clinics in Abuja metropolis were analysed by five different laboratory techniques (ZN Direct, ZN Bleach, LJ slants, BACTEC and Serology TB) for the diagnosis of Mycobacterium tuberculosis.*
Comparative analysis of results at P=0.05, revealed that there was a statistical significant (X^2=127.1, P<0.001) difference between the diagnosistic performance of the five laboratory techniques. A follow-up analysis based on the 95% confidence interval of pair differences in proportion between the five techniques indicated that the BACTEC assay was the major source of the difference(P<0.001) in pair methods. Comparism of the 95% confidence limit of pair differences in diagnostic specificity of Mycobacterium tuberculosis between ZN-BACTEC and other methods confirmed (P<0.001) the high detection rate of BACTEC. It was observed that BACTEC had the highest detection rate (61.2%), followed by LJ (31.2%), then ZN Bleach (30.3%) and ZN Direct (28.8%), while Serology had only 25.3% ZN BACTEC appeared to the most reliable, and time effective combination. ZN Bleach should be encouraged in poor resource settings in lieu of the conventional three standard smears for ZN Direct. The use of Serologic TB kit alone for the diagnosis of tuberculosis should be discouraged.
Keywords: Mycobacterium tuberculosis, BACTEC, Laboratory and Serology.

I. Introduction

Pulmonary tuberculosis is an infection caused by the human tubercle bacillus called Mycobacterium tuberculosis. It is a long, curved rod at least 1-4um long and arranged either singly or in small clumps.
[1]. They do not grow on ordinary media, preferring egg media such as Lowensterin-Jensen media. The most important source of Tuberculosis (TB) is an untreated Pulmonary Tuberculosis (PTB) patient. When such a person coughs, spites or sneezes, tiny droplet nuclei containing the tubercle germs are released. Transmission is through inhalation of these droplet nuclei. It is also transmitted by swallowing the bacteria from hands and infected utencils and materials. Mycobacterium tuberculosis is contagious but only 5% - 10% of infected normal individuals develop active disease [2]. The clinical signs and symptoms of Tuberculosis include: Chronic cough productive of mucoid or purulent sputum, pneumonia, fever worse at night, night sweats, chest wall pains, breathlessness, localized wheeze frequent cold, loss of weight etc.

Pulmonary tuberculosis remains an important socio-economical and medical problem throughout the world. According to the Centre for Disease Control and Prevention [3], the incidence of pulmonary tuberculosis is expected to increase from 7.5 million cases per year in 1995 to 11.9 million in 2005. The case fatality rate is estimated at 55% for untreated people and 15% for the treated patients.

Mycobacterium tuberculosis (MTB) infects about one-third of the world's population (i.e. 1.8 billion people) and causes more death worldwide than any single infectious diseases. It is the leading cause of infectious mortality worldwide accounting for three million (3million) deaths per year [4]. Majority of these individuals live in developing countries where the prevalence of Human Immunodeficiency Virus (HIV) infection is also high . However, eight million people are newly infected annually and each active TB patient is capable of infecting 10-15 persons yearly [5]. TB is associated with poverty, overcrowding, alcoholism, stress, drug addiction and malnutrition. The disease spreads easily in overcrowded, badly ventilated places among

people who are undernourished. The population at risk are the prisons, hospitals, overcrowded homes, homeless shelters, schools, medical clinics etc [6]. A diagnosis of active disease is based on clinical manifestations of an abnormal chest radiograph, Acid-fast Bacilli (AFA) in sputum or bronchioscopic specimens and recovery of the organism. Assays based upon amplification of mycobacterial gene in clinical specimens are also currently being tested. According to the prevalence of tuberculosis, there are four times more cases of sputum negative pulmonary TB than sputum positive. In such cases, usually a therapeutic trial of ant tubercular drugs is instituted by many physicians. It is unlikely that some patients with inactive disease or radiographic abnormalities entirely unrelated to tuberculosis have been treated unnecessarily. It is observed that about half of such patients actually have active TB and need treatment. Therefore the need for the comparative assessments of the diagnostic techniques.

AIM OF THIS STUDY
To have a comparative assessment of five laboratory techniques used in the diagnosis of pulmonary tuberculosis using culture as gold standard.

OBJECTIVES
1. To obtain data for a comparative assessment of five laboratory techniques used for the diagnosis of pulmonary tuberculosis.
2. Establish the clinical significance of potential variations in the vive laboratory techniques.
3. Outline a test algorithm to minimize the laboratory variations in the diagnosis of pulmonary tuberculosis.

II.   Materials And Methods

STUDY SITE
This study covers seven major hospitals in Abuja, FCT. The hospitals include Maitama District Hospital, Asokoro District Hospitals, Wuse General Hospital, Gwagwalada Specialist Hospital, Kubwa General Hospital, Gwarinpa General Hospital and Zankli Medical Centre all in Federal Capital Territory Abuja.

The Hospitals were chosen based on patient attendance, geographical location within Federal Capital Territory and the Directly observed therapy (DOT) Programme being run by these hospital. A total of 340 volunteers were registered for this study from the patients attending the above –named hospitals.

CHOICE OF KITS
Before the serology kit was selected, questionnaires were drafted and sent to various health institutions where TB diagnosis is carried out. The results from the questionnaires were collated and analyzed from which Clinotech Diagnostic was chosen.

SAMPLES COLLECTION:
Both blood and sputum samples were collected from each patient. Three sputum samples (1st –on-Spot, overnight and 2nd –on-Spot) were collected from each patient in samples pots, and these were tested for mycobacterium tuberculosis by direct sputum examination (Ziehl-Neelsen), and culture. The blood samples were collected from each patient and centrifuged. The sera were separated and stored at -20°C in freezer, prior to analysis..

SPUTUM MICROSCOPY
The microscopy was carried out using ZN staining methods. Two different Ziehl Neelsen (ZN) staining methods were used on the sputum samples. The direct Ziehl Neelsen and the short-term bleach digestion methods.

DIRECT METHOD:
Three consective sputum samples from each patient were analyzed. The first "On Spot" sample was collected in a sterile container. A second specimen container was given to the patient to collect an early morning "Overnight "Sample. The third sample "2nd Spot" was collected when patient comes to clinic with the "overnight "sample. The direct ZN method of microscopy was done by making a smear of the sputum samples (1cm x 2cm) on new, grease – free glass slide. The slide was placed on a hot plate at 85c for 15mins to dry. The smear was then flooded with carbol fuchsin and allowed to stand at room temperature for 5mins. Gentle flame

was applied form underside of the slide was then washed with distilled water, tilted to drain, decolourised with 3% acid alcohol and then rinsed with distilled water again. Finally, after tilting to drain, the slide was flooded with Malachite Green for 1min. and then rinsed with deionised water. It was allowed to air dry and examined microscopically under oil immersion lens (x100).

SHORT –TERM BLEACH DIGESTION OF SPUTUM METHOD

Equal volume 95ml of domestic bleach (5% NaOCl) was added to the 1st on spot sputum sample in a sample pot. The sample pot cap was closed and contents shaken by hand for about 20 seconds. The container was then tilted at an angle of 450 for 30mins to allow for sedimentation. The sediment was withdrawn using a 2ml pipette and then a drop of the sediment was placed on a labelled slide to make a smear. The slide was air dried and stained according to the ZN's methods as in "direct" above.

SPUTUM CULTURE

Both liquid and solid media were used for the culture of the sputum samples. However before the culture, the sputum samples were treated using sodium hydroxide (Modified Petroff) method of sputum decontamination.

SPUTUM DECONTAMINATION (MODIFIED PETROFF METHOD)

The decontamination process is as follows:-

To a know volume (5ml) of sputum was added twice the volume (10ml) of 4% NaoH in a

container, capped tightly and shaken to digest the mixture was allowed to stand for

 15mins at room temperature with occasional shaking. The mixture was thereafter

Centrifuged at 3000g for 15mins. The supernatant was poured off and sediment resuspended in 15ml of sterile saline. It was centrifuged again at 3000g for 15mins, the supernatant was decanted and sediment was inoculated onto each culture medium immediately.

SPUTUM CULTURE ON LOWENSTEIN JENSEN (LJ) MEDIUM

The decontaminated sputum sample from each patient was cultured using a sterilized wire loop onto the surface of a protein-enriched Lowenstein-Jensen medium. The cultured LJ medium was then incubated at 35-37°C using the mermmet incubator for a period of six weeks (42days) before being discarded. Within this period, a raised, dry cream (buff) coloured colonies characteristic of mycobacterium tuberculosis was observed for the isolates were identified by ZN staining method as in "Direct" above.

CULTURE ON BACTEC MGIT 960 SYSTEMS

The mycobacterium growth indicator tube (MGIT) contains 7ml of modified middlebrook 7H9 Broth base. It is a liquid medium for the cultivation of mycobacterium. The decontaminated/processed specimen (sputum) was inoculated into the MGIT tube and placed into the BACTEC MGIT 960 system for continuous monitoring and incubation at 37°C as recommended by the manufacturer and tested until positive samples were detected within the recommended 42 day testing protocol. Positive tubes identified by the BACTEC MGIT 960 instrument was stained with the direct ZN method as earlier enumerated to obtain the ZN-BACTEC results.

PRINCIPLES OF THE PROCEDURE (BACTEC)

A fluorescent compound is embedded in silicone on the bottom of 16 x 1000mm round bottom tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected.

Tubes entered into the BACTEC MGIT 960 System are continuously incubated at 37°C and monitored every 60 min for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive; i.e., the test sample contains viable organism. An instrument positive tube contains approximately 10^5 to 10^6 colony forming units per millilitre (CFU/ml). Culture vials which remain negative for a minimum of 42 days (up to 56 days) and which show no visible signs of positivity are removed are removed from the instrument as negatives and sterilized prior to discarding.

The BACTEC MGIT Growth Supplement is added to each MGIT tube to provide substances essential for the rapid growth of mycobacteria. Oleic acid is utilized by tubercle bacteria and plays an important role in the metabolism of mycobacteria. Albumin acts as a protective agent by binding free fatty acids which may be toxic to Mycobacterium species, thereby enhancing their recovery. Dextrose is an energy source. Catalase destroys toxic peroxides that may be present in the medium. Contamination is reduced when supplementing the

BBL MGIT broth base with BACTEC MGIT Growth Supplement/BBL MGIT PANT antibiotic mixture prior to inoculation with a clinical specimen.

TB SEROLOGY (CLINOTECH DIAGNOSTICS)

About three millilitres (3ml) of whole venous blood was drawn from the autecubital fossa of all patients whose sputum has been collected they were centrifuged using DIAN YUAN Bucket bench centrifuge. The sera were them separated and stored at $20^{o}C$ in the GHT freezer model MXM 2706.

PROCEDURE

The test device and specimen was brought to room temperature prior to testing. Then the test device was removed from the pouch and placed on a clean, dry level surface. 100ul of specimen was pipetted into the sample application well. The results were read at 15mins after sample application.

PRINCIPLE OF CLINOTECH DIAGNOSTICS

Clinotech TB diagnostics is a direct binding, double sandwich antigen immunochromatographic test for the detection of TB antigens conjugated to a colloidal gold particle and recombinant TB antigens immobilized on the membrane. Once the specimen is applied to the sample well, the colloidal gold particle conjugated antigens forms sandwich complexes with TB antibodies present in the specimen. The complexes pass through the membrane, which is pre-coated with recombinant TB antigens on the test line (T) and anti-TB antibodies on the control line ©. As the complexes move along the membrane, the immobilized TB antigens on the membrane, if any antibodies to tuberculosis are present the TB antigens capture them in turn. The produces a pink/purple band in the test line (T), indicating a positive result. The remaining complex that did not bind anti-TB anti bodies in specimen continues to migrate and capture anti-TB antibodies in specimen continues to migrate and capture anti-TB antibodies immobilized on the control line ©. This control band indicates that the test has been performed properly.

DATA ANALYSIS: Data were analyzed using chi square, at 95% confidence limit (Brown and Swanson, 1994)

III. Results

Table 1 show the age and sex distribution of volunteers who participated in this study. From the result, we observed that of the 340 individuals 192 (56.5%) were males while 148 (43.5%) were females. The age bracket of 21-30 years was the most represented with 144 (42.4%) followed by 31-41 years (19.5%), then 10-20 years (14.9%).

In table 2, the diagnostic performance assessment of the five laboratory techniques (ZN Direct, ZN Bleach, LJ medium, BACTEC MGIT 960 and Serology) reveals that there was a statistical significant difference ($X^2=127.1$, P <0.001) between the various methods.

The 95% confidence interval of pair differences in proportion between the five laboratory techniques in table 3 indicated that only pair methods involving BACTEC MGIT 960 system were statistically different (P<0.001) in their performance. This pointed to the fact that the BACTEC method was responsible for the difference observed in table 2.

Table 4 presents the 95% confidence interval of pair difference in diagnostic specificity to Mycobacterium tuberculosis between ZN-BACTEC and other methods. A statistically significant difference (PV<0.001) between pair methods was observed. This infers that ZN – BACTEC specificity to Mycobacterium tuberculosis was higher (P<0.001) than that of other methods.

Table 1:Age And Sex Distribution Of Patients

AGE	SEX	
	MALE	**FEMALE**
10 – 20 years	27 (7.4%)	25 (7.5%)
21 – 30 years	66 (19.4%)	78 (23%)
31 - 40 years	47 (13.8%)	22 (6.5%)
41 - 50 years	30 (8.8%)	14 (4.1%)
51 – 60 Years	154 (4.9%)	6 (1.8%)
61 and above	9 (2.7%)	3 (0.8%)
TOTAL	**192 (56.5%)**	**148 (48.5%)**

Table 2: Diagnostic Performance Of The Five Laboratory Techniques And Chi-Square (X^2) Of Significance In Performance

	ZnDirect	ZnBleach	LJ	Bactec	Serology	Total	X^2	Pv
Positive	98 (28.8%)	103 (30.3%)	106 (31.2%)	208 (61.2%)	86 (25.3%)	601 (176.8%)		
Negative	242 (71.2%)	237 (69.7%)	234 (68.8%)	132 (38.8%)	254 (74.7%)	1099 (323.%)		
Total	340 (100%)	340 (100%)	340 (100%)	340 (100%)	340 (100%)	1700 (500%)	127.1	<0.001

X^2 tabulated   =   18.5

X^2 calculated   =   127.1

P_V <0.001

X^2 calculated > X^2 tabulated. Therefore there is a statistical significant

(P_V < 0.001) difference between the five methods

Table 3:95% Confidence Interval Of Pair Differences In Proportion Between The Five Laboratory Methods And Their Chi-Square (X^2) And P Values]

PAIR METHODS	95% CIP	X^2	P_V
½	1.4 – 1.6	0.2	0.625
1/3	2.3 – 2.5	0.4	0.5
¼	32.3 – 32.5	71.9	<0.001
1/5	3.4 – 3.6	1.1	0.25
2/3	0.9 – 1.1	0.1	0.75
2/4	30.8 – 31.0	79.8	<0.001
2/5	4.9 – 5.1	2.1	0.175
¾	29.9 – 30.1	61.5	<0.001
3/5	5.8 – 6.0	2.9	0.075
4/5	35.8 – 36.0	88.5	<0.001

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KEY
1 = ZN DIRECT
2 = ZN BLEACH
3 = LJ MEDIUM
4 = BACTEC
5 = SEROLOGY
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Table 4:95% Confidence Interval, Chi-Square (X^-) And P Values Of Pair Differences In Diagnostic Specificity To Mycobacterium Tuberculosis Between ZN-BACTEC And Other Methods

PAIR METHODS	95% CIP	X^2	P_V
1/6	13.5 – 13.7	13.75	<0.001
2/6	12.0 – 12.2	10.67	0.001
3/6	11.1 – 11.3	9.14	0.006
4/6	18.7 – 18.9	24.1	<0.001
5/6	17.0 – 17.2	22.1	<0.001

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KEY
1 = ZN DIRECT
2 = ZN BLEACH
3 = LJ MEDIUM
4 = BACTEC
5 = SEROLOGY
6 = ZN-BACTEC
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IV.   Discussion

The rapid diagnosis of tuberculosis is important if necessary control and prevention steps are to be taken in due time, the spread of the disease limited, and the administration of inadequate therapy avoided and

the cost of hospitalization reduced. Clinical and radiological findings permit only a presumptive diagnosis of tuberculosis. For mycobacterial culture, use of both a liquid and a solid medium is recommended. The combination of both media should allow detection of growth within 14 days of receipt of the specimen in the laboratory.

The recently introduced fully automated BACTEC MGIT 960 has been shown to be a viable alternative to Lowenstein-Jensen slant for the rapid and reliable laboratory diagnosis of tuberculosis from this study. In contrast to BACTEC 460 system, BACTEC MGIT 960 is a non-radiometric assay and there is no need for each test and establishment of reading schedule. Therefore it is less labour-intensive and hence may free laboratory staff for other duties. In addition, the capacity of BACTEC MGIT 960 is much higher and therefore its application will be more useful for laboratory dealing with large numbers of specimen daily.

In this study five different diagnostic techniques for Mycobacterium tuberculosis were assessed. These include, ZN Direct, ZN Bleach, Lowenstein-Jensen slants, BACTEC MGIT 960 system and Serology using Clinotech diagnostic test kits. The study indicated that BACTEC MGIT 960 system displayed the highest detection rate (61.2%) to mycobacteria. This was follow by Lowenstein-Jensen slant (31.2%), ZN Bleach (30.3%), ZN Direct (28.8%) and Serology (25.3%). This clearly shows that serology has the least detection rate. A statistical significant difference (P<0.001) was found between BACTEC MGIT 960 and others.

In the course of this study, a total of 100 randomly selected BACTEC MGIT 960 instrument-negative vials (at the end of the 42 days protocol) were examined by AFB Smear. N false – Negative samples from this random terminal AFB Smear. No false-Negative samples from this random terminal AFB Smear of the instrument-negative tubes were detected. However, in this study, of a total of 92 samples which were positive for ZN Direct, ZN Bleach, LJ and BACTEC MGIT 960, only 34 showed positive for Serology. The remaining 52 samples out of the 86 samples that indicated positive for Serology may be false-positive. This study also suggests a higher sensitivity of ZN Bleach to ZN Direct.

The mean time to detection (TTD) of Mycobacterium tuberculosis isolates from this study were 14.1(6-26) days for BACTEC MGIT 960 and 20.2 (12-40) days for Lowenstein-Jensen slant.

V. Conclusion

The newly introduced BACTEC MGIT 960 system is a dependable, high-capacity, compact, fully automated continuous monitoring instrument for the recovery of mycobacterium from human clinical samples. When used in combination with a solid medium, the BACTEC MGIT 960 system shows high performance for the detection of mycobacterium complex while providing greater recovery of mycobacterium other than tuberculosis (MOTT). Despite the advantages of the broth-based cultivation system, traditional solid media still plan a role in the recovery of mycobacterium from clinical samples. The combination of a solid and liquid-based culture system increase the sensitivity of cultivation for mycobacterium. This combination should therefore be considered to be the "gold standard" rather than the conventional use of LJ alone. This finding agrees with that of [3], [4] and [5]. The use of serology test kits alone for the diagnosis of tuberculosis should be discouraged as the result cannot be reliable. However in poor resource settings, the use of ZN bleach should be encouraged rather than the conventional three standard smears for ZN Direct because of its higher performance. Finally, although AFB smears should be done on all BACTEC MGIT 960 instrument-positive samples to eliminate false instrument – positive cases, the BACTEC MGIT 960 system appears to be the most reliable diagnostic technique of the five being assessed in this study.

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