

بسم الله الرحمن الرحيم
Sudan University of Science and Technology
College of Graduate Studies

**Comparison Between Conventional methods and Molecular
Techniques for Detection of Tuberculosis Microbe in
Sputum Specimens**

مقارنة بين التقنيات التقليدية والجزئية للكشف عن ميكروب الدرن الرئوي في
عينات القشع

A thesis submitted for partial fulfillment of the requirements of M.Sc. in Medical
Laboratory Science (Microbiology)

By:
Telal Refat NagimEldin Mohamed

Supervisor:
Prof.Humodi Ahmed Saeed

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الآية

بسم الله الرحمن الرحيم

قال تعالى:

وَكُلُوا فَضْلُ اللَّهِ عَلَيْكَ وَرَحْمَتُهُ لَهَمَّتْ طَائِفَةٌ مِنْهُمْ أَنْ يُضِلُّوكَ وَمَا يُضِلُّونَ إِلَّا أَنْفُسَهُمْ ۖ وَمَا يَضُرُّونَكَ مِنْ شَيْءٍ ۚ وَأَنْزَلَ اللَّهُ عَلَيْكَ الْكِتَابَ وَالْحِكْمَةَ وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ ۚ وَكَانَ فَضْلُ اللَّهِ عَلَيْكَ عَظِيمًا

صدق الله العظيم

سورة النساء الآية 113

DEDICATION

To my parents, family and friend

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Great thanks for **ALMIGHTY ALLAH** for his immortal grace and endless blessing.

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ABSTRACT

Tuberculosis remains a global epidemic, especially in developing countries. Rapid diagnosis of active *Mycobacterium tuberculosis* infection plays a critical role in controlling the spread of tuberculosis. Conventional methods may take up to several weeks to produce results.

The objective of the present study was to compare between conventional and molecular techniques in detection of tuberculosis microbe in sputum specimens.

This study was conducted on a total of 100 clinically suspects pulmonary tuberculosis patients. Out of 100 patients, 58 were males and 48 were females. Direct smear examination with Ziehl-Neelsen (ZN) and polymerase chain reaction (PCR) were applied for sputa. Culture on Löwenstein-Jensen medium was used as the “gold standard.”

The number of positive cases by three different diagnostic techniques was determined. Out of 100 specimen investigated, 16 was Z-N staining positive, 25 were standard culture positive and 24 were PCR positive. The sensitivity and specificity of PCR using culture method as gold standard were 96.1% and 100% respectively. The sensitivity and specificity of Z-N technique using culture method as gold standard were 64% and 100% respectively.

It is concluded that PCR is a rapid, sensitive and specific technique for detection of tuberculosis microbe in sputum. Further study with large number of specimens is required to validate the results of the present study.

المستخلص

لا يزال السل وباء عالمي، وخصوصا في البلدان النامية. التشخيص السريع للعدوى يلعب دورا حاسما في السيطرة على انتشار مرض السل. قد تستغرق الأساليب التقليدية فترة طويلة تصل إلى عدة أسابيع لتحقيق نتائج.

الهدف من هذه الدراسة كان للمقارنة بين الأساليب الجزيئية والتقليدية في تشخيص السل الرئوي.

أجريت هذه الدراسة على ما مجموعه تتألف من 100 مشتبه فيهم بالسل الرئوي. شمل الاستطلاع 58 من الذكور و 48 من الإناث. تم تطبيق فحص اللطاخة بشكل مباشر مع تسيل-نلسن وتفاعل البلمرة المتسلسل (PCR) للبلغم. كما تمت أيضا زراعة البلغم باعتبارها "المعيار الذهبي".

تم تحديد عدد من الحالات الإيجابية من قبل ثلاثة طرق التشخيص المختلفة. من أصل 100 شملهم الاستطلاع، 16 كان Z-N تلطيخ إيجابي، 25 منهم كانوا إيجابي بالمعيار الذهبي و 24 كانوا ايجابين بالبلمرة (PCR). وكانت حساسية وخصوصية وفقا للمعيار الذهبي (التزريع) 96.1% و 100% على التوالي. وكانت حساسية وخصوصية اللطاخة باستخدام وفقا للمعيار الذهبي (التزريع) 64% و 100% على التوالي.

خلصت الدراسة إلى أن تقنية البلمرة المتسلسلة سريعة وحساسة ومحددة للكشف عن ميكروب الدرن الرئوي، وأن مزيداً من الدراسات بعدد أكبر من العينة مطلوبة للتحقق من صدقية نتائج هذه الدراسة.

CHAPTER ONE

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Tuberculosis (TB) is an infectious disease which is caused by an acid-fast bacillus, which belongs to the *Mycobacterium tuberculosis* complex. TB transmission begins with a human source, most often a person with cavitary, pulmonary TB. When an infectious patient coughs, sneezes or talks, aerosols are formed in the lungs and expelled. These aerosols contain the micro-particles that carry the bacilli, and can be inhaled by others. The disease affects the lungs in approximately two thirds of cases, but almost all other organs can be the site of TB infection. It is estimated that about one third of the world's population is infected with TB. However, the infection is contained by the immune system in about 90 % of those infected. The TB bacilli can lie dormant for years, being protected by a thick waxy coat. If the immune system is weakened, for example by an HIV infection or treatment with immunosuppressive agents, the chances of developing active TB become much higher(Bernitz, 2008).

Tuberculosis (TB) is still a major health problem worldwide,with almost 9 million new cases and around 1.4 million deaths each year (WHO, 2011).

Although the conventional procedures are irreplaceable diagnostic tools, the poor sensitivity of acid-fast bacillus detection by microscopy and the slow growth of the tubercle bacillus in culture media limit the usefulness of these methods for rapid

diagnosis of TB. Two important concerns complicate the issue further: (i) infection with HIV has changed the clinical presentation of tuberculosis and reduced the sensitivity of classical microbiology methods and (ii) almost one-fifth of bacillus transmission is due to smear-negative pulmonary cases. Therefore, other accurate and rapid techniques are needed for early diagnosis of active TB (Moure, et al 2012).

Tuberculosis (TB) continues to be a global public health problem. In industrialized countries, four main conditions are currently referred to as promoting the spread of TB: human immunodeficiency virus pandemic, immigration from high-TB prevalence areas, the worsening of economic and social conditions (including an increase in homelessness), and the emergence of multidrug-resistant strains. Successful TB control depends on effective case finding and rapid detection of *Mycobacterium tuberculosis* complex (MTB). Conventional methods include staining smears for acid-fast bacilli (AFB) and culture by liquid and solid media. However, AFB staining lacks sensitivity and specificity, whereas culture results are usually not available earlier than 2 to 3 weeks.(Piersimoni *et al.*, 2002)

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-Neelsen 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 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. (Alli, *et al.*, 2011)

1.2. Justification

One of the principles of tuberculosis control is rapid and accurate diagnosis of the disease in order to allow prompt initiation of antimicrobial therapy and to prevent transmission. Amplification techniques have attracted considerable interest since they offer the opportunity to shorten the time required to detect and identify MTB organisms in both respiratory and extrapulmonary specimens. The purpose of the present study is to evaluate the molecular methods for diagnosis of tuberculosis in comparison with conventional methods. This may result in an introducing of rapid, specific and sensitive method for TB diagnosis.

1.3. Objectives

1.3.1. General objective

To compare between conventional and molecular techniques for Detection of tuberculosis microbe in sputum specimens in United Arab Emirates.

1.3.2. Specific objectives

- A) To perform Ziehl-Neelsen stain for the detection of tuberculosis microbe in sputum.
- B) To investigate similar specimens using PCR technique for detection of tuberculosis microbe.
- C) To cultivate sputum specimens on Lowenstein-Jensen medium.
- D) To determine sensitivity and specificity of the two techniques using culture as gold standard.

CHAPTER TWO

LITERATURE REVIEW

2.1. Tuberculosis microbe

2.1.1. General properties

Tuberculosis complex organisms are obligate aerobes growing most successfully in tissues with high oxygen content, such as the lungs. MTC is a facultative intracellular pathogens usually infecting mononuclear phagocytes (e.g. macrophages), slow-growing with a generation time of 12 to 18 hours. They are hydrophobic with high lipid content in the cell wall. Because the cells are hydrophobic and tend to clump together, they are impermeable to the usual stains, e.g. Gram's stain. They are known as "acid-fast bacilli" because of their lipid-rich cell walls, which are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, the cells resist decolorization with acidified organic solvents and are therefore called "acid-fast" (Joklik et al. 1992). The *M. tuberculosis* complex is the cause of TB and is comprised of *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canettii* and *M. microti*. The mycobacteria grouped in the complex are characterized by 99.9 % similarity at the nucleotide level and identical 16S rRNA sequences but differ widely in terms of their host tropisms, phenotypes and pathogenicity (Boddinghaus et al. 1990).

2.1.2. pathophysiology

Practically, the only reservoir of *M. tuberculosis* that contributes to spreading TB-infection is the patient with pulmonary TB, with their chronic respiratory symptoms such as cough and sputum production who produces aerosol droplets from the

bronchi. When they come in contact with air they dry rapidly and become very light particles that still contain live bacilli, and remain suspended for a long time. When the bacteria penetrate the pulmonary alveoli of a healthy person, they are phagocytized by macrophages, in which they multiply. This creates a response where other macrophages and monocytes are attracted and participate in defense against the infection. This creates a primary focus; an infectious focus made up of inflammatory cells. This is also called the Ghon focus.

The bacteria and its antigens are drained by the macrophages to the nearest lymph node through the lymphatic system. Inside the lymph node, the antigens are recognized by T-lymphocytes, leading to transformation into specific CD4 and CD8 lymphocytes and liberation of lymphokines such as interferon- γ . This activates macrophages to kill intracellular mycobacteria and/or inhibit the growth of the phagocytized bacilli. The TB bacteria usually attack the lungs. But, TB bacteria can attack any part of the body such as the kidney, spine, and brain. If not treated properly, bacteria are picked up by dendritic cells, which do not allow replication, although these cells can transport the bacilli to local (mediastinal) lymph nodes. Further spread is through the bloodstream to other tissues and organs where secondary TB lesions can develop in other parts of the lung (particularly the apex of the upper lobes), peripheral lymph nodes, kidneys, brain, and bone (Tang et al., 2006).

2.2 Laboratory diagnosis by Conventional methods

2.2.1 Microscopic examination

Mycobacteria are distinguished from other micro-organisms by thick lipid-containing cell-walls that retain biochemical stains despite.

Decolourisation by acid-containing reagents (so-called 'acid-fastness').

Microscopic examination of sputum is simple, inexpensive and quickly in detecting infectious cases of pulmonary TB but the disadvantages of direct smears microscopy is relatively insensitive and at least 5,000 bacilli per millilitre of sputum are required.

Microscopy for acid-fast bacilli (AFB) cannot distinguish *Mycobacterium tuberculosis* from NTM, nor viable from non-viable organisms, or drug-susceptible from drug-resistant strains.

2.2.2 Culture and species identification

Mycobacteria cultured in Lowenstein-Jensen (L-J) medium and incubated at 37°C and examined for growth twice weekly for the first 2 weeks and once weekly up to 8 weeks.

After which a definitive result obtained to provide diagnosis of *M.tuberculosis*.

Culture also provides the necessary isolates for conventional DST.

Specimens have to be decontaminated prior to being cultured to prevent overgrowth by other micro-organisms.

Decontamination methods are to some extent harmful to *mycobacteria*, and culture, therefore not 100% sensitive

Positive culture is identified by rapid immunochromatographic assays to differentiate *M.tuberculosis* from NTM. (WHO, 2011).

2.3 Laboratory diagnosis by molecular methods

2.3.1. Description of Polymerase chain reaction (PCR)

PCR is used to amplify specific regions of a DNA strand (the DNA target). This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although some techniques allow for amplification of fragments up to 40 kb in size. A basic PCR set up requires several components and reagents. These components include:

- a) DNA template that contains the DNA region (target) to be amplified.
- b) Two primers, which are complementary to the DNA regions at the 5' (five prime) or 3' (three prime) ends of the DNA region.
- c) Taq polymerase or another DNA polymerase with a temperature optimum at around 70°C.
- d) Deoxynucleoside triphosphates (dNTPs; also very commonly and erroneously called deoxynucleotide triphosphates), the building blocks from which the DNA polymerases synthesize a new DNA strand.
- e) Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- f) Divalent cations, magnesium or manganese ions; generally Mg^{2+} is used, but Mn^{2+} can be utilized for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.
- g) Monovalent cation potassium ions.

The PCR is commonly carried out in a reaction volume of 10-200 μ l in small reaction tubes (0.2-0.5 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. Many modern thermal cyclers make use of the Peltier effect which permits both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit favorable thermal conductivity to allow for rapid thermal equilibration. Most thermal cyclers have heated lids to prevent condensation at the top of the reaction tube. Older thermocyclers lacking a heated lid require a layer of oil on top of the reaction mixture or a ball of wax inside the tube. The PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps. The cycling is often preceded by a single temperature step (called *hold*) at a high temperature ($>90^{\circ}\text{C}$), and followed by one hold at the end for final product extension or brief storage. The temperatures used and the length of time they are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (T_m) of the primers (Lorenz 2012).

Initialization step: This step consists of heating the reaction to a temperature of 94-96 $^{\circ}\text{C}$ (or 98 $^{\circ}\text{C}$ if extremely thermostable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Denaturation step: This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

Annealing step: The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand.

Final elongation: This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended (Hoseini and Saueret ., 2015).

2.3.2 The PCR process can be divided into three stages

Exponential amplification: At every cycle, the amount of product is doubled (assuming 100% reaction efficiency). The reaction is very specific and precise.

Leveling off stage: The reaction slows as the DNA polymerase loses activity and as consumption of reagents such as dNTPs and primers cause them to become limiting.

Plateau: No more products accumulate due to exhaustion of reagents and enzyme (Garibyan and Avashia., 2013).

Ichiyama and his colleagues evaluated the clinical utility of an rRNA amplification-based Gen-Probe Amplified *M.tuberculosis* Direct Test (AMTD) system and a PCR-based Roche AMPLICOR MYCOBACTERIUM system for direct detection of *M. tuberculosis*, *M. avium*, and *M. intracellulare*. Of the 422 sputum samples from 170 patients, 137 (121 of *M. tuberculosis*, 14 of *M. avium*-*M. intracellulare* complex (MAC), 2 of mycobacteria other than *M. tuberculosis* or MAC) were culture positive with the Septi-Chek AFB system. One sample of a contaminated culture results was excluded for further analyses. The AMTD system detected all of the 121 samples which grew *M. tuberculosis* (sensitivity, 100%). Of the 284 culture negative samples, 28 were positive by this system (specificity, 90.1%). After resolution of the discrepant samples, based on a positive history for culture of the patient, the specificity of this system increased to 99.3%. On the other hand, the AMPLICOR system gave a positive result for 132 out of the 135 culture positive samples for *M. tuberculosis* or MAC (sensitivity, 97.8%). Of the 284 culture-negative samples, 37 were positive by this system (specificity, 87.0%). The specificity for this system after resolution of the discrepant samples increased to 98.9%. The agreement between the results from the AMTD system and the AMPLICOR system was 98.7%. Both of the systems are highly sensitive and specific for detecting *M. tuberculosis* and/or MAC directly from sputum samples within hours, and they should be recommended for routine use in the clinical microbiology laboratory (Ichiyama *et al.*, 1996).

The polymerase chain reaction (PCR) using oligonucleotides based on the repetitive sequence (IS986) of *M. tuberculosis* as a primer and the Gen-Probe Amplified *M.tuberculosis* Direct Test (MTD), which combines an *M. tuberculosis* rRNA amplification method with the hybridization protection assay format, were evaluated for detection of *M. tuberculosis* in clinical samples. The detection limits of these two assay systems based on nucleic acid amplification for cultured *M. tuberculosis* were less than 10 cells per reaction. A total of 135 sputum specimens were examined by the two assay systems. The PCR and the MTD systems for detection of *M. tuberculosis* gave overall positivity rates of 84.2% (32 of 38) and 91.9% (34 of 37), respectively, as compared with 71.9% (23 of 32) by smear and 96.9% (31 of 32) by culture in the liquid medium MB-Check. Procedures for sample preparation used in the two methods were different. Although the sensitivities of the PCR and MTD appeared to be similar to that of culture with the MB-Check system, the two methods based on nucleic acid amplification should be very useful for rapid detection of *M. tuberculosis* infections without the long time required for culture of *M. tuberculosis*.

Pounder and his coworkers assessed the performance of a real-time polymerase chain reaction (PCR) assay using the Smart Cycler instrument and a minor groove binding MGB Eclipse probe (Epoch Biosciences, Bothell, WA) for identification of *Mycobacterium tuberculosis* complex in acid-fast bacillus smear-positive and smear-negative clinical specimens by comparing results to the Amplified *M. tuberculosis* Direct Test (MTD) and mycobacterial culture plus clinical diagnosis. After initial testing, the overall sensitivity, specificity, and positive and negative predictive values of PCR for the 172 specimens submitted for mycobacterial culture were 86.3%, 100%,

100%, and 94.5%, respectively. These same values for MTD were 98.0%, 99.2%, 98.0%, and 99.2%. For 83 additional specimens, only MTD and PCR were performed; 5 specimens were positive and 78 were negative by both tests. The sensitivity of the PCR assay was improved by using different primers and probes. The time to a result for real-time PCR, starting with a decontaminated sample, was less than 3 h compared with 5-6 h for the MTD(Pounder, *et al.*, 2006)

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-Nelsen staining technique; and cultured on Middlebrook agar medium containing oleic acid albumin dextrose complex supplement after decontamination of samples. 96 (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 ($p < 0.05$). 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(Alli *et al.*, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1.Study design

This was a cross-sectional study.

3.1.Study area

The study was conducted in the Disease Prevention and Screening Centre (DPSC), Abu Dhabi, United Arab Emirates.

3.3. Study population

Individuals in United Arab Emirates suspected of having tuberculosis (TB).

3.4.Study duration

This study was conducted during the period from June 2015 to December, 2015.

3.5.Sample size

One hundred patients were enrolled during this study.

3.6. Experimental work

3.6.1. Collection of specimens

Patients were counseled to deliver morning sputum specimens for three successive days. The specimens were collected in robust, leak-proof and clean containers. A volume of 3-4 ml was collected from each patient.

3.6.2. Digestion and decontamination of the specimens

3.6.2.1. Reagents

Digestants: NaOH solution (2 to 4%), sterilized by autoclaving.

2N HCl: Diluted 33 ml of concentrated HCl to 200 ml with water. Sterilized by autoclaving.

Phenol red indicator: Combined 20 ml of phenol red solution (0.4% in 4% NaOH) and 85 ml of concentrated HCl with distilled water to make 1,000 ml.

Phosphate buffer: The buffer was 0.067 M and pH 6.8. It composed from two solutions A and B. Mix 50 ml of solution A (0.067 M Na₂HPO₄; 9.47 g of anhydrous Na₂HPO₄ in 1 liter of distilled water) and 50 ml of solution B (0.067 M KH₂PO₄; 9.07 g of KH₂PO₄ in 1 liter of distilled water). When the final buffer requires adjustment, solution was added to raise pH or solution B to lower it.

3.6.2.2. Steps

The digestion and decontamination were carried out according to (Zignol, *et al.*, 2006):

1. A volume of 3 to 4 ml of specimen was transferred to a sterile 50ml screw-cap plastic centrifuge tube, and then an equal volume of NaOH was added.

2. The contents were agitating vigorously for 15 min on a vortex mixture.
3. Phosphate buffer (pH 6.8) was added up to the 50 ml.
4. The mixture was centrifuged at $\geq 3,000 \times g$ for 15 min, the supernatant was decanted, and few drops of phenol red indicator were added to the sediment, then Neutralized with HCl.
5. The contents of the tube were thoroughly mixed; the acid addition was stopped when the solution is persistently yellow.
6. The sediments were re-suspended on 1 to 2 ml of phosphate buffer.
7. The suspension was stored at -20°C until used.

3.6.3. Acid fast/Z-N staining method

Reagents

- a) Carbon fuchsin stain.
- b) De-colorizing agent 25% sulphuric acid
- c) Counter stain: Methylene blue

Smear preparation

The smear was prepared on clean and oil free slide. It is then air dried and fixed by heating. The entire slides were flooded with carbon fuchsin and heated slowly by a Bunsen burner for 5 minutes after steaming. Then the slide was washed by clean water and then decolorized by 25% H_2SO_4 and allowed for 10 minutes. Then it was rinsed thoroughly with clean water and counter stained with Methylene Blue for 1 minute.

Slide was washed thoroughly with clean water, dried in air and examined under microscope by oil immersion lense.

Interpretation

Microscopic examinations were interpreted and recorded according to WHO as follows;

0 bacilli per 100 field reported as negative

1-9 bacilli per 100 field reported as scanty (in this case 200 field examined)

10 -99 bacilli per 100 field reported as 1+ positive

1-10 bacilli per field reported as 2+ positive

3.6.4. Culture and identification

A volume of 0.25 ml was inoculated on Lowenstein-Jensen (L-J) medium and incubated at 37°C and examined for growth twice weekly for the first 2 weeks and once weekly thereafter upto 8 weeks; after which a definitive result was obtained. Cultures that showed no growth after 8 weeks was scored as no growth. A patient was defined as a TB-positive case, if the sputum specimens will have a positive culture and as a non-TB case, if the sputum specimens showed no growth on the culture slant (Homorodean, 2005).

3.6.5 Molecular study

a) DNA Extraction

DNA extraction was performed from sputum specimen. The sputum specimen was firstly concentrated. NaOH was used for decontamination. After a final centrifugation, the bacterial pellet used for the following DNA isolation.

b) PCR for detection *M. tuberculosis*

Detection of *M. tuberculosis* (MTB) was performed using PCR. The COBAS® TaqMan® MTB Test was an *in vitro* nucleic acid amplification test for the qualitative detection of MTB complex. DNA extracted from liquefied, decontaminated and concentrated human respiratory specimens, including sputum and bronchial alveolar lavages (BAL).

c) Principles of the Procedure

The COBAS® TaqMan® MTB Test was based on two major processes: (1) simultaneous PCR amplification of target DNA using complementary primers, and detection of target DNA through cleavage of dual fluorescent dye-labeled oligonucleotide probes^{15,16}. Together, these processes permit the detection of MTB target amplified product (amplicon) and *Mycobacterium* Internal Control DNA, which was amplified and detected simultaneously with the specimen. The Master Mix reagent contains a primer pair that was used to amplify a genus-specific region of the chromosome and specificity for the *M. tuberculosis* complex (*M. tuberculosis* *bovis*,

M. africanum and *M. microti*,) is provided by a dual labeled target detection probe.

The same primer pair is used to amplify the Internal Control DNA. The detection of amplified DNA is performed using target-specific and Internal Controlspecificdual labeled oligonucleotide probes that permit independent identification of MTB amplicon and Mycobacterium Internal Control amplicon.

d)Target Selection

The *Mycobacterium* genome contains a highly conserved region of approximately 1500 nucleotides encoding the gene for 16S rRNA. The COBAS®TaqMan® MTB Test uses *Mycobacterium*genus specific primers to define a sequence within this region.(Boddinghaus, *et al.*, 1990)

e) Target Amplification

PCR amplification occurs in K-tubes or K-trays in which prepared specimens are added to the amplification mixture. The reaction mixture is heated to denature the double stranded DNA and expose the primer target sequences. As the mixture cools, the primers anneal to their target sequences. Z05 DNA polymerase, in the presence of Mg²⁺ and excess deoxynucleotide triphosphates (dNTPs), extends the annealed primers along the target templates to produce a double stranded DNA molecule termed an amplicon .Real time PCR The Analyzer automatically repeats this process for a designated number of cycles, with each cycle ended to double the amount of amplicon DNA. The required number of cycles is preprogrammed into the COBAS®TaqMan® 48 Analyzer. Amplification occurs only in the region of the MTB genome between the primers; the entire MTB genome is not amplified.

f) Internal Control Amplification

In enzyme-based amplification processes such as PCR, efficiency can be reduced by inhibitors that may be present in the clinical specimen. The Internal Control permits the identification of prepared specimens containing substances that may interfere with PCR amplification. The *Mycobacterium* Internal Control was a noninfectious, recombinant linearized plasmid DNA with primer binding regions identical to those of the *M. tuberculosis* target sequence, a randomized internal sequence of similar length and base composition as the *M. tuberculosis* target sequence, and a unique probe binding region that differentiates the *Mycobacterium* Internal Control amplicon from target amplicon. These features are selected to ensure equivalent amplification of both *Mycobacterium* Internal Control and the *M. tuberculosis* target DNA. The *Mycobacterium* Internal Control Reagent is included in the COBAS® TaqMan® MTB Test and is introduced into each amplification reaction to be co-amplified with MTB DNA from the clinical specimen. The *Mycobacterium* Internal Control is designed to ensure identification of specimens that contain inhibitors that would interfere with the amplification and detection of the MTB target sequence.

g) Selective Amplification

Selective amplification of target nucleic acid from the specimen was achieved in the COBAS® TaqMan® MTB Test by use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine, but not DNA containing

thymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Any nonspecific product formed after initial activation of the Master Mix by the magnesium is destroyed by the AmpErase enzyme.

The AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at deoxyuridine residues by opening the deoxyribose chain at the C1- position. When heated in the first thermal cycling step at the alkaline pH of Master Mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme is inactive at temperatures above 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon formed during amplification.

h) Detection of PCR Products in a COBAS®TaqMan® Test

The COBAS®TaqMan® MTB Test utilizes real-time PCR technology. The use of dual-labeled fluorescent probes provides for real-time detection of PCR product accumulation by monitoring the emission intensity of fluorescent reporter dyes released during the amplification process. The probes consist of MTB and Mycobacterium Internal Control-specific oligonucleotides labeled with a reporter dye and a quencher dye. In the COBAS®

TaqMan® MTB Test, the MTB and Mycobacterium Internal Control probes are labeled with different fluorescent reporter dyes. When the dual fluorescent dye-labeled probes are intact, the reporter fluorescence was suppressed by the proximity of the quencher dye due to Förster-type energy transfer effects. During PCR, the probe hybridizes to a target sequence and was cleaved by the 5' to 3' exonuclease activity of the thermostable Z05 DNA polymerase. Once the reporter and quencher dyes are released and separated, quenching no longer occurs, and the fluorescent activity of the reporter dye was increased. The amplification of MTB DNA and Mycobacterium Internal Control DNA are measured independently at different wavelengths. This process is repeated for a designated number of cycles, each cycle effectively increasing the emission intensity of the individual reporter dyes, permitting independent identification of MTB DNA and Internal Control DNA.

3.6.6. Statistical analysis

A simple statistical analysis was done

Calculation of sensitivity = $\text{true positive} / (\text{true positive} + \text{false negative})$

Calculation of specificity = $\text{true negative} / (\text{true negative} + \text{false positive})$

CHAPTER FOUR

RESULTS

The present study was conducted on a total of 100 patients clinically suspected of having pulmonary tuberculosis. The patients were categorized into four age groups (Table 1). The majority of the patients (40%) belong to the age group of 36-50 years. Most of infected patients were aged between 36-50 years. Out of the 100 patients enrolled 58 (58%) were males and 48 (48%) were females. Age and gender distributions of the study population were shown in Table (2).

The number of positive cases by the three different diagnostic techniques was determined. These were 16 (16%) Z-N staining positive, 25(25%) culture positive and 24 (24%) PCRpositive (Table 3). Out of 25 positive cases, 16 cases were found within the age group 36-50 (Table 4). Males were found more infected compare to females(Table 5)

The sensitivity and specificity of PCR using culture method as gold standard were 96.1% and 100% respectively. The sensitivity and specificity of Z- N stain using culture method as gold standard were 64% and 100% respectively (Table 6).

Table 1. Distribution of patients according to age group

Age group (Year)	Number of Patients (%)
5-20	23 (23%)
21-35	23 (23%)
36-50	40(40%)
51-65	14 (14%)
Total	100 (100%)

The patients were divided into age groups; 0-20, 21-35, 36-50 and 51-70. The age group 36-50 constitutes the highest per cent of patients.

Table 2. Distribution of patients according to age and gender

Age group	Males	Females	Total
0-20	14 (14%)	9 (9 %)	23 (23%)
21-35	13(13 %)	10(10 %)	23 (23%)
36-50	21 (21 %)	19(19 %)	40(40%)
51-70	10 (10 %)	4(4%)	14 (14%)
Total	58 (58%)	42 (42%)	100 (100%)

This table describes the separate distribution of male and female according to the age group. The age group 36-50 years contains the highest per cent of male and female while the lowest per cent of both male and female were included in the age group 51-70 years.

Table 3. Number and percentage of positive cases according to diagnostic techniques

Technique	Positive cases	
	No.	%
Z-N stain	16	16
PCR	24	24
Culture	25	25
Total	100	100

Table 4. Distribution of infected TB patients according to age group

Age group	Infected Patients	
	No.	Percentage
0-20	2	8%
21-35	3	12%
36-50	16	64%
51-70	4	16%
Total	25	100%

Table 5. Distribution of infected TB patients according to gender

Gender	Infected Patients	
	No.	Percentage
Males	15	60%
Females	10	40%
Total	25	100%

Sixty per cent of infected subjects were males while 40% were female.

Table 6. Sensitivity and specificity of Z-N and PCR techniques

Result	Technique	
	Z-N stain	PCR
Sensitivity	64%	96.1%
Specificity	100%	100%

The specificity and sensitivity of ZN when compared with culture method as the gold standard method were 100% and 64% respectively.

The specificity and sensitivity of PCR when compared with culture method as the gold standard method were 100% and 96% respectively.

CHAPTER FIVE

DISCUSSION

Despite long-standing intense efforts to detect and defeat the scourge of tuberculosis, drug-resistant MTB strains and non-tuberculous *Mycobacterium* (NTM) have emerged recently; tuberculosis incidence in HIV-infected patients and children has increased (Bodmer, *et al.*, 1997). The development of tests for rapid, sensitive, and specific identification of the causative agent of tuberculosis is crucial for successful control of this disease.

Regarding the distribution of the tuberculous patients among the age groups, this study revealed that the highest rate of infection was within the age group 36 to 50 (40%). This is in agreement with the results of Assad (1999) who reported that tuberculosis in the community is mainly a disease of young adults (Assad, 1999).

For sex distribution, results showed an infection ratio of 1.5:1 as males to females. This is comparable to the previous results (Verhagen *et al.*, 2011) who found a ratio of 2:1. However, Taskapan and his colleagues reported a ratio of 1:1 (Taskapan, 2000). This difference might be due to the group of patients selected to run the study on them.

The gold standard technique of identifying tuberculosis microbe is culture. However, this technique requires long time (El-Zammar and Katzenstein, 2007). The simplest rapid diagnostic technique for detection of tuberculosis microbe is Ziehl-Neelsen (Z-N) technique. However, microscopic examination with either Z-N stained smears is insensitive; detecting acid-fast bacilli only when there are $\geq 10^4$ mycobacteria per

ml(Bennedsen *et al.*, 1996). There is an urgent need for a reliable diagnostic test that meets all the requirements of rapidity, sensitivity and specificity. This was the motivation for the development of the PCR as a diagnostic tool for tuberculosis(Kehinde *et al.*, 2005).

The role of amplification assays as an adjunct to smear and culture in the diagnosis of tuberculosis has been questioned because of the low sensitivity (43 to 74%) for smear-negative specimens(Bergmann and Woods, 1996).

Acid-fast smear also has a relatively good positive predictive value, usually over 90% with culture-positive specimens. Thus, in clinical practice, amplification assays have been considered most beneficial in the rapid differential diagnosis between non-tuberculous mycobacteria and *M. tuberculosis* (Bodmer *et al.*, 1997).

Our results found that the PCR has a sensitivity and specificity of 96.1% and 100%, respectively as compared to the culture technique, the gold standard method for detection tuberculosis microbe in sputum specimens by using primers *mtb1* and *mtb2*, the primers commonly target IS 6110, an insertion sequence and the size of the amplified product 123bp(Hemal *et al.*, 2000).

Aslanjadeha *et al.* (2008) from USA showed in their study the sensitivity and specificity of PCR for the diagnosis of pulmonary tuberculosis from sputum specimens was 100% and 100% (Aslanzadeh *et al.*, 1998). Another study by Kocagoz *et al.* (1993), found the sensitivity and specificity of PCR for the diagnosis of pulmonary tuberculosis from sputum sample 87% and 96% which almost similar to our study(Kocagöz *et al.*, 1993).

In present study we found, all smear positive cases were culture and PCR positive and the sensitivity and specificity of Ziehl-Neelsen (Z-N) stain were 64% and 100%. No false positive result found when compared with gold standard culture technique.

A study done by El-Dawi (2004) at Sudan showed that Ziehl-Neelsen (Z-N) sensitivity was 65.4% and the specificity was 90.5% which is similar to findings of the present study (El-Dawi *et al.*, 2004). Another study by Abu-mostafa *et al.*, (2009) from Libya showed that the sensitivity and specificity for Z-N microscopy were 86.2% and 100% which is almost similar to our study (Abu-mostafa *et al.*, 2004). A study done by Chakravorty *et al.*, (2005) showed in their study the sensitivity and specificity of Ziehl-Neelsen (Z-N) staining 68% and 80% which is consistent to our study (Chakravorty *et al.*, 2005).

Analyzing the findings of the present study it can be easily understood that the PCR technique is a rapid, simple and alternative method of Ziehl-Neelsen (Z-N) staining and culture on egg based Lowenstein-Jensen medium for the diagnosis of pulmonary tuberculosis.

Conclusion

It is concluded that the PCR techniques is more sensitive than Z-Nstain with 100% specificity and it has advantages over culture by being very fast.

Recommendations

1. The molecular diagnosis of pulmonary TB is very reliable, rapid, specific and high sensitive and is recommended to be a principle tool for the diagnosis of pulmonary TB.
2. Cost-effectiveness studies and operational studies are required to support an evidence-based decision to introduce PCR for TB diagnosis.
3. Further study with large number of specimens is required to validate the results of the present study.

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