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College of Graduate Studies

**Assessment of Sputum Preparation Techniques for
Diagnosis of Pulmonary Tuberculosis in Khartoum State**

تقييم طرق تحضير البلغم لتشخيص مرض الدرن في ولاية الخرطوم

**A Dissertation Submitted for the Partial Fulfillment for the Requirement of the Degree of
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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

QURAN

قال تعالى:

{ وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا }

سورة الإسراء الآية 85

DEDICATION

I would like to dedicate my thesis to the one who made my life colorful with

love and passion:

(My beloved husband)

And to whomever is encouraging me in every step in my life:

(My parents)

And to all the special people in my life

With love.

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ABSTRACT

This study was carried out in Khartoum State during the period between May 2013 to February 2014, aimed to evaluate two techniques smear preparation methods for Ziehl–Neelsen (ZN) staining for acid fast bacilli (AFB) in patients with signs and symptoms of tuberculosis and compared with PCR as reference method. Ninety sputum samples were collected from different TB centers in Khartoum State including Abu Anga Hospital, the National Reference Tuberculosis Laboratory, and Elsha'ab Teaching Hospital.

The results revealed that from the total of 33 positive samples by PCR, 8 specimens were found to be positive in direct, 24 positive with concentrated smear microscopy. The study concluded that use of the concentrated method for preparing smears for AFB microscopy increases sensitivity without a loss of specificity in identifying positive TB cases, which proved to be a better method for diagnosis of the disease.

الخلاصة

اجريت هذه الدراسة في ولاية الخرطوم في الفترة ما بين مايو 2013 وفبراير 2014 والتي هدفت لتقييم طريقتين لتحضير مسحة البلغم (البصقة) لتصبغ بصبغة زيل نيلسون للباكتريا الصائمة للحمض من مرضي باعراض وعلامات مرض الدرن وتمت مقارنتها بتقنية تفاعل البلمرة المتسلسل, حيث تم جمع 90 عينة بلغم من عدة مستشفيات في ولاية الخرطوم تتضمن مستشفى ابو عنجة, مستشفى الشعب والمعمل المرجعي للدرن.

اختبرت البكتريا العنقودية الصائمة للحمض تحت المجهر بعد التصبغ , مرة عن طريق المسح المباشر ومرة عن طريق التركيز بالطرد المركزي ومحلول هيبواكسيد الصوديوم والنتائج قرنتبتقنية تفاعل البلمرة المتسلسل . اظهرت الدراسة ان 33 عينة موجبه تفاعل البلمرة المتسلسل و8 عينات وجدت موجبه في طريقة المسحة المباشرة بينما 24 عينة موجبه بطريقة التركيز. واستخلصت الدراسة ان استخدام تقنية التركيز لعمل المسحة للفحص المجهرى زادت الحساسيه والتخصصيه في الكشف عن الحالات الموجبه لمرض الدرن, مقارنة بالطريقة المباشرة. مما يبرهن ان هذه التقنية اكثر جوده في تشخيص هذا المرض.

TABLE OF CONTENTS

DEDICATION.....	III
ACKNOWLEDGEMENT	IV
ABSTRACT.....	V
الخلاصة.....	VI
LIST OF FIGURES	IX
LIST OF TABLES	IX
ABBREVIATION	IX

CHAPTER ONE

1. INTRODUCTION

1.1 Background	1
1.2 Rationale	3
1.3 Objectives	3
1.3.1 General Objectives.....	3
1.3.2 Specific Objectives	4

CHAPTER TWO

2. LITERATURE REVIEW

2.1 History.....	4
2.2 The Genus <i>Mycobacterium</i>	5
2.2.1. Taxonomy	5
2.2.2. Classification.....	5
2.3 Biological Characteristics of the Genus <i>Mycobacterium</i>	7
2.3.1 Morphology	7
2.3.2 Clinical manifestation	8
2.4. Tuberculosis	11
2.4.1 Definition	11
2.4.2 Epidemiology of the Disease	11
2.4.3 Transmission.....	13
2.4.4 Risk Factors for Acquiring TB.....	14
2.4.5 Effective vaccines for protection against tuberculosis	15
2.5 Diagnosis	17
2.5.1 Clinical Signs of TB	17
2.5.2 Conventional Methods.....	17
2.5.3 Biochemical Tests	20

2.5.4 Molecular Technologies.....	20
2.5.5. Tuberculin Skin Testing (TST).....	22
2.5.6 QuantiFERON-TB Testing.....	22
2.6 Treatment of Tuberculosis.....	23
2.6.1 Tuberculosis Control Strategy	23
2.6.2 Drug Resistance.....	24

CHAPTER THREE

3. MATERIALS & METHODS

3.1 Study Design.....	25
3.1.1 Type of study	25
3.1.2 Study Area	25
3.1.3 Study Duration	26
3.1.4 Study population	26
3.1.5 Sample size.....	26
3.1.6 Ethical consideration	26
3.1.7 Inclusion and exclusion criteria	26
3.2 Collection of specimens	26
3.3 Methods of Identification of <i>Mycobacterium</i>	27
3.3.1. Direct smear preparation	27
3.3.2 Concentration Smear Preparation.....	27
3.3.3 Ziehl-Neelsen (ZN) Staining Method.....	28
3.3.4 Molecular identification using (PCR)	28
3.3.5 Calculation of Sensitivity and Specificity.....	30

CHAPTER FOUR

4. RESULTS

4.1 Epidemiological Findings.....	31
4.1.1. Gender	31
4.1.2 Frequency of TB among age groups.....	32
4.2.1. Sputum microscopy results	35
4.3 Molecular method (PCR) results	36
4.4 Statistical Analysis	38
4.4.1 Correlation of Direct smears and concentrated	38
4.4.2 Sensitivity and specificity of Direct smears and concentrated with PCR	40

CHAPTER FIVE

5. DISCUSSION

CONCLUSION & RECOMMENDATIONS.....	45
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REFERENCES	46
APPENDIX	56

LIST OF FIGURES

Fig. 1: Frequency of gender of patients with pulmonary TB infection	32
Fig. 2: Distribution of pulmonary TB infection among age groups.	33
Fig. 3: Acid-fast bacilli bacteria (red colour) with ZN stain using direct smear preparation.	34
Fig. 4: Acid-fast bacilli (red colour) with ZN Stain using concentrated smear preparation... ..	35
Fig. 5: Detection of M. tuberculosis band by (2%) agarose gel electrophoresis.....	38

LIST OF TABLES

Table 1: Zeil-Nelsen stain results of sputum with direct smear for TB patient.....	36
Table 2: Zeil-Nelsen stain results of sputum with concentrated smear for TB patient.....	36
Table 3: PCR results of sputum samples of TB patient	37
Table 4: Direct and Concentrated Method Cross Tabulation.....	39
Table 5: Chi-Square Tests.....	39
Table 6: correlation of direct and PCR results of TB patient	40
Table 7: correlation of concentrated and PCR results of TB patient	41

ABBREVIATION

AD Anno Domino

AFB Acid Fast Bacilli

AIDS Acquired Immunodeficiency Syndrome

BC Before Common Era

BCG Bacille Calmette-Guérin

CD Cluster of Differentiation

CDCs Communicable Disease Coordinators

DCs Dendritic Cells

DNA Deoxyribonucleic Acid

DOTS Directly Observed Treatment Strategy

ELISA Enzyme Link Immunosorbant Assay

FDA Food and Drug Administration

FMOH Federal Ministry of Health

HIV Human Immunodeficiency Virus

HPLC High Performance Liquid Chromatography

IFN Interferon

IL Interleukins

INH isoniazid

LAM Lipoarabinomannan

LPA Line Prop Assay

LTBI latent TB infection

MDR Multi Drug Resistance

MØs alveolar macrophages

MOTT mycobacteria other than *M. tuberculosis*

NRAMP Natural Resistance-associated Macrophage Protein

NTCP National Tuberculosis Control Programme

NTM Non-tuberculosis *Mycobacteria*

PBMCs peripheral blood mononuclear cells

PCR Polymerase Chain Reaction

RLP Restriction Length Polymorphism Analysis

PNB p-Nitrobenzoic acid

PPD Purified Protein Derivative

RMP rifampicin

RNA Ribonucleic Acid

Rpm Revolutions per minute

SCC Short- Course Chemotherapy

SSM Sputum Smear Microscopy

TB Tuberculosis

TBCP Tuberculosis Control Programme

TNF Tumor Necrotic Factor

TST Tuberculin skin testing

UNDP United Nations Development Programme

VDR vitamin D receptor

WHO World Health Organization

XDR Xtrem Drug Resistance

ZN Ziehl-Neelse

CHAPTER ONE

1. INTRODUCTION

1.1 Background

Tuberculosis (TB) is second to Human Immunodeficiency Virus (HIV)/Acquired Immune Deficiency Syndrome (AIDS) as the greatest killer worldwide due to a single infectious agent. In 2011, 8.7 million people fell ill with TB and 1.4 million died from the disease. Over 95% of TB deaths occur in low-and middle-income countries. Tuberculosis, or TB, is an infectious bacterial disease caused by *Mycobacterium tuberculosis*, which most commonly affects the lungs. It is transmitted from person to person via droplets from the throat and lungs of people with the active respiratory disease. (WHO, 2013)

The diagnosis of TB relies primarily on the identification of acid-fast bacilli (AFB) in sputum smears using a conventional light microscope. The sputum specimens are smeared directly onto the slides (direct smears) and subjected to Ziehl-Neelsen (ZN) staining. The requisites for the staining procedures are; basic fuchsin, phenol, absolute alcohol; sulphuric acid and methylene blue. Microscopic examination under oil immersion objective reveals *Mycobacterium* as red bacilli.

Currently, mycobacterial culture is the golden standard for detecting *M. tuberculosis*, but it is time-consuming and requires specialized safety procedures in laboratories. Serological methods are convenient but lack sensitivity and specificity. Although the polymerase chain reaction (PCR)

technique is rapid, it is costly for routine use in developing countries where most tuberculosis cases occur.

Conventional smear microscopy with the ZN stain is a rapid and practical method for detecting AFB, especially in low-income countries, due to its rapidity, low cost, and high positive predictive value for tuberculosis. However, the ZN method is severely handicapped by its low detection rate. One of the main reasons behind this is that *M. tuberculosis* can hardly be stained by acid-fast dyes once it enters the cells. Another important reason is that the ZN method requires a large volume of sputum, as it is incapable of detecting bacilli that are fewer than 10,000 in number per slide or per ml of specimen. Therefore, it is important to develop an alternative, cost-effective sputum pretreatment methods (Daley *et al.*, 2009).

Direct ZN sputum smear microscopy for the detection of AFB remains the most important diagnostic test for tuberculosis in high burden countries. Although rapid, specific, and appropriate for laboratories with minimal infrastructure, the analytical sensitivity of direct sputum smear microscopy is approximately 10,000 bacilli per milliliter of sputum, much less than culture methods (Dinneset *et al.*, 2007). The Global Plan to Stop TB recognizes the limitations of sputum smear microscopy and mandates further research into the optimization of this technique (WHO, 2006; Steingart *et al.*, 2007)

Although all mycobacterial species are acid fast, ZN assay is highly specific for *M. tuberculosis* in countries where TB is endemic because of the high burdens of this disease (Steingart *et al.*, 2007)

One approach to the improvement of sputum smear microscopy is the application of chemical or physical pretreatment (sputum processing)

procedures to disrupt sputum structure, separate clumps of mycobacteria, and concentrate bacilli, thereby increasing the probability of their detection. The sensitivity of microscopy is influenced by numerous factors, and this study was to assess the efficiency of two sputum preparation and pretreatment techniques for the detection of tuberculosis by comparing with PCR direct detection as standard method.

1.2 Rationale

Tuberculosis is a significant infectious disease having put 8.7 million patient worldwide under deleterious condition. TB is known to be endemic in Sudan. The present study was conducted to assess efficiency of the conventional ZN staining technique by direct and concentrated smear microscopy for detection of AFB from sputum sample in terms of correlation of positivity, sensitivity and specificity. We compared an improved method based on liquefaction of sputum with 4% NaOH followed by centrifugation with direct and no treatment smears and compared with PCR as reference method.

1.3 Objectives

1.3.1 General Objectives

The aim of the present study was to assess the effectiveness of direct smear preparation and concentrated smear preparation after Ziehl-Neelsen staining

and using PCR as standard and reference method in diagnosis of pulmonary TB.

1.3.2 Specific Objectives

- 1) To detect the present of *Mycobacterium tuberculosis* bacilli in sputum by microscope.
- 2) To compare the sensitivity and specificity of direct smear microscopy in TB patients with PCR result as the gold standard.
- 3) To compare the sensitivity and specificity of concentrated smear microscopy in TB patients with PCR result as the gold standard.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 History

The tubercle bacilli, the causative agent of tuberculosis (TB), were discovered by Robert Koch in 1882 disproving the notion that the disease

had a purely hereditary etiology, or was caused solely by the unhealthy living conditions of the lower classes of the society in the early industrial age. However, there is still too much to be learned about the nature of the organism, its virulence and the response of the host to the infection. TB is as old as humanity itself, clear evidence of TB was found in skeletal remains of the pre-historic humans dating back to 8000 BC in Germany. Egyptian skeletons (2500 to 100 BC) revealed evidence of Pott's disease of the spine. Best proof of TB, however, has been from an Inca mummy of 8- year-old boy at 700 AD (Stead and Dutt, 1994).

2.2The Genus Mycobacterium

2.2.1. Taxonomy

Prokaryotic

KINGDOM: Bacteria.

PHYLUM: Actinobacteria.

ORDER: Actinomycetales.

SUBORDER: Corynebacterineae.

FAMILY: Mycobacteriaceae.

GENUS: *MYCOBACTERIUM*.

2.2.2. Classification

Two major periods were distinguished in the prokaryotic taxonomy. The first period utilized the phenotypic studies, while the second one was focused on genotypic characteristics. The first period lasted from the dawn of mycobacterial studies (1880s) to the end of the 1980s, the second, started

late in the 20th century up to now. The rationale of genotypic taxonomy is linked to the presence within the genome of highly conserved regions harboring hypervariable sequences in which species-specific deletions; insertions or replacements of single nucleotides are present (Tortoli, 2003).

The genus *Mycobacterium* comprises a large number of organisms, including obligate parasites causing serious human and animal diseases, opportunistic pathogens and saprophytic species found in nature (Kim *et al*, 2003). Mycobacterial species, other than *M. tuberculosis* and *M. leprae*, are generally free-living organisms. They have been recovered from water, soil, domestic and wild animals, milk and food (Wolinsky and Rynearson, 1986). As the incidence of TB is declining in areas of the world where socio-economic conditions have rapidly improved, the frequency of isolating non-tuberculosis *mycobacteria* (NTM) was increased, and their relevance to human disease became apparent (Wolinsky, 1979). Currently, identification of annulated mycobacteria is achieved by standard culture and biochemical tests. Determination of phenotypic features is time-consuming, difficult to assimilate into a precise diagnosis concerning closely related taxa, not always highly reproducible and may result is in ambiguous or erroneous diagnosis.

With the availability of 16S ribosomal DNA sequencing, high-performance liquid chromatography (HPLC) and polymerase chain reaction-restriction length polymorphism analysis (PCR-RLPA), the number of new species of NTM has risen dramatically with the naming of species such as *M. genavense*, *M. interjectum*, *M. triplex*, *M. celatum* and *M. lentiflavum*. At present, over 100 species have been recognized (Brown *et al*, 2002). There are four species of tubercle bacillus (*M. tuberculosis*, *M. bovis*, *M.*

africanum and *M. microti*) referred to as *M. tuberculosis* complex. (Rogal et al, 1990). *Mycobacterium avium*, complex is a source of confusion as not only does it contain two closely related species *M. avium* and *M. intracellulare*, but also *M. paratuberculosis*, the causative agent of Johne's disease (Grange, 1996).

2.3 Biological Characteristics of the Genus *Mycobacterium*

Mycobacteria are aerobic, characteristically acid-alcohol fast (Ryan and Ray, 2004). All species share a characteristic cell wall, thicker than any other bacteria. It is hydrophobic, waxy, and rich in mycolic acids. Cell wall consists of a hydrophobic mycolate and a peptidoglycan layers held together by polysaccharide and arabinogalactan. The cell wall is responsible for the hardness of this genus. The bio-synthetic pathways of cell wall components are potential targets for new drugs. Many *Mycobacterium* species adapt to growth onto simple substrates, using ammonia or amino acids as nitrogen sources and glycerol as a carbon source in the presence of mineral salts (Bhamidi, 2009). Lipoarabinomannan (LAM) binds the cell wall together and anchors it to the cell membrane. LAM has two forms, one terminates in the arabinose and the other terminates in mannose. The former induces a number of immune responses, including the release of tumor necrosis factor (TNF), a key inducer of both protective and tissue destroying immune reactions. LAM plays for mycobacteria range between a role in pathogenicity (Besra and Chatterjee, 1994). Optimum growth temperatures (25°C -50°C) (Bhamidi, 2009).

2.3.1 Morphology

Mycobacterial Cells are straight or slightly curved rods, 1.0-10 µm long and 0.2-0.6 µm wide, do not contain endospores or capsules and are

usually considered Gram-positive. *Mycobacteria* do not fit in the Gram-positive category from an empirical standpoint. Mycobacterial genome consists of a single circular chromosome and some strains contain one or more plasmids of little known functions. The chromosome contains insertion sequences or jumping genes. Some insertion sequences are unique to species and some strains. The most widely investigated insertion sequences are those available in the members of *M. tuberculosis* complex. Fingerprinting of insertion sequence of *M. tuberculosis* has been used in epidemiological studies.(Van Embden *et al.*, 1993)

2.3.2 Clinical manifestation

2.3.2.1 Primary Tuberculosis

Most *Mycobacterium tuberculosis* infections are caused by inhaling droplets or dust particles containing the bacilli. Tuberculosis usually attacks the lungs but can also affect other organs of the body (Kumar *et al.*, 2007). The bacilli are ingested by alveolar macrophages (MØs), where they replicate to form the initial lesion which is referred to as “primary tuberculosis” lesion (Ellner, 1997). The primary site of infection in the lungs is called the “Ghon focus”, and is located in either the upper part of the lower lobe, or the lower part of the upper lobe (Kumar *et al.*, 2007). Then in post-infection, the Bacteria are picked up by dendritic cells (DCs), where no replication occurs, but they transport the bacilli to local lymph nodes. Further spread is hematogenous to other tissues and organs where “secondary TB” lesions can develop in other parts of the lung, peripheral lymph nodes, kidneys,

brain, and bone (Herrmann and Lagrange, 2005). All tissues of the body can be affected by the disease, though it rarely affects the heart, skeletal muscles, pancreas and thyroid glands (Agarwalet *al.*, 2005).

Alveolar macrophage, T-cells, B-cells and fibroblasts are among the cells that aggregate to form a granuloma, being surrounded by lymphocytes. The granuloma functions not only to prevent dissemination of the *mycobacteria*, but also provides a local environment for communication of cells of the immune system. Within the granuloma, T- lymphocytes secrete cytokines, interferon gamma, that activates MØs to destroy the bacteria within them (Kaufmann, 2002). Cytotoxic T- cells also can directly kill infected cells, by secreting perforin and granulysin (Hutmacheret *al.*, 2002).

It is worthy that bacteria are not always eliminated within the granuloma, but can become dormant, resulting in a “latent infection” (Kumaret *al.*, 2007). Another feature of human tuberculosis granulomas is the development of abnormal cell death (necrosis) in the center of the tubercles (Grosset, 2003).

2.3.2.2 Post-primary Tuberculosis

Primary TB can be divided into progressive-primary and post-primary forms on the basis of the natural history of the disease. Post-primary TB results from either reactivated of a latent primary infection or, less commonly, from repeat infection of a previously sensitized host. The term "post-primary" is preferred to "re-activation" when referring to the clinical diagnosis, because firmly distinguishing recurrence from an antecedent infection is impossible in most cases. Approximately 10% of all infected patients are likely to develop reactivation, and the risk is highest within the first 2 years or during periods of immuno-suppression. *Mycobacteria* entre to the blood stream from

an area of damaged tissue and then they spread throughout the body and set up many infection foci, mostly tiny white tubercles in the tissues. Patients with disseminated TB have a fatality rate 100% if untreated. However, if treated early, the fatality rate is reduced to about 10% (Kimet *al.*, 2003). During active TB, some cavities are joined to the air passages bronchi and this material can be coughed up. It contains viable bacteria and can therefore pass on infection. Treatment with appropriate antibiotics kills bacteria and allows healing to take place. Upon cure, affected areas are eventually replaced by scar tissue (Grosset, 2003). Post-primary cutaneous tuberculosis (lupus vulgaris) mostly affects the face and neck. Some cases are secondary to sinus formation between lymph nodes and the skin (scrofuloderma) (Grange, 1992).

2.3.2.3 TB Association with Acquired Immune Deficiency Syndrome (AIDS)

Since 1981, an epidemic spread of infection with human immunodeficiency virus (HIV) has been in progress. Since this virus kills T-helper cells (CD4 lymphocytes), it renders its victim defenseless against mycobacterial infection. Because of the loss of CD4 T4 and macrophages, the only defense against *M. tuberculosis* is lost (Stead and Dutt, 1994). TB in HIV-positive patients may present atypically, both clinically and radiologically, with a lower probability of sputum positivity, greater difficulty in diagnosis, and a more rapid clinical deterioration than TB in HIV-seronegative patients (Drobniewskiet *al.*, 1995).

HIV infection increases the risk of reactivation of dormant tuberculosis infection (Selwyn *et al.*, 1989) and also the risk of progressive disease from new infection (Di Perriet *al.*, 1989). The incidence of TB is 500 times higher

in persons infected with HIV than in general population (Fertel and Pitchenik, 1989). TB usually occurs in HIV-positive persons at an early stage of immunodeficiency (Theueret *al.*, 1990). All HIV- infected persons should be screened for TB infection with TST (Pitchenik *et al.*, 1988). Likewise, it is important to perform serologic testing for HIV infection with or without any known risk factors. Although many believe that HIV-positive patients and TB rarely exhibit a positive tuberculin test, a positive tuberculin test increases the likelihood of TB, but a negative test does not exclude the possibility. In HIV- infected persons, indurations of ≥ 5 mm with PPD testing is indicative of a positive reaction (Stead and Dutt, 1994).

2.4. Tuberculosis

2.4.1 Definition

Tuberculosis is a chronic granulomatous disease affecting man, many mammals, birds, fishes, amphibians and reptiles. Mammalian tuberculosis is caused by *M. tuberculosis* and the regional variants or sub-types of *Mycobacterium africanum* and "*M. canettii*" which are primarily pathogenic for man. *M. bovis* and *M. microti* are the causative agents of TB in animals, and can be transmitted to humans (Lucía, 2007).

2.4.2 Epidemiology of the Disease

2.4.2.1 Global Magnitude of TB

In 2006, the World Health Organization (WHO) estimated that *Mycobacterium tuberculosis* infects a third of the world's human population and kills two million people each year: there is a new infection every second. The number of active infection was 14.4 million, corresponding to a prevalence rate of 219/100,000 persons. The incidence of new cases was

estimated to be 9.2 million, corresponding to a prevalence rate of 139/100,000 and 12 of the 15 countries with the highest estimated TB incidence were in Africa, where the TB incidence rate was 363/100,000 (Lonnroth and Raviglione, 2008).

The epidemiology varies substantially worldwide. The highest rates (100/100,000 or higher) are observed in sub-Saharan Africa, India, China, the islands of South East Asia and Micronesia. Most cases are currently encountered in South East Asia, with one-third of new cases in this region each year. Intermediate rates (26 to 100 cases/100,000) occur in Central and South America, Eastern Europe and Northern Africa. Low rates (< 25 cases per 100,000) occur in the USA, Western Europe, Canada, Japan and Australia (WHO, 2009).

The principal reasons for the high infection in the developing countries are the incomplete coverage by control programs and inadequate cure rates. In the early 1990s, the WHO introduced the directly observed treatment short course (DOTS) strategy as a cost-effective way to control TB and improve health. DOTS has five components: Sputum smear microscopy (SSM), directly observed treatment with standardized short-course chemotherapy (SCC), a delivery drugs-system without interruption and free, standardized recording and reporting of cases and national political commitment (Emmett *et al.*, 2006).

The introduction and broad adoption of the DOTS strategy in 183 countries has substantially improved TB control. The WHO set worldwide targets to diagnose 70% of new a sputum smear (ss+) that is positive for TB cases, to cure 85% of these cases by 2005 and to maintain or improve this

performance from 2006 onwards. It was projected that if these goals are met, TB incidence would decline by 5–10% annually in countries without endemic HIV infection. However, meeting these targets, especially for case detection, has proven difficult, with only 53% of ss+ cases being detected in 2004 (WHO, 2007)(a).

In 2007, Africa accounted for an estimated 78% of tuberculosis cases among HIV-positive persons worldwide. However, the estimated incidence in sub-Saharan Africa is twice that of South-East Asia. Eastern Europe countries also are facing a serious epidemic; there were over 150,000 new cases in Russia alone in 2008 (WHO, 2010).

2.4.3 Transmission

Tuberculosis is an air-borne disease due to droplet infection from those who suffer from active pulmonary TB through cough, sneeze, speak or spit. One sneeze can release about 40,000 droplets (Cole and Cook 1998). Each one of infectious droplets may induce the disease, since the infectious dose of tuberculosis is quite low (inhaling less than ten bacteria) may cause an infection (Nicas *et al.*, 2005). People with prolonged, frequent, or close contact with patients (co-patients) are at a particular high risk of being infected, with an estimated 22% infection rate. A person with active, but untreated tuberculosis can infect 10–15 people every year (WHO, 2007)(a).

Transmission can only occur from people with active, latent TB (Kumar *et al.*, 2007). It depends upon the number of infectious droplets expelled by a carrier, the effectiveness of ventilation, the duration of exposure and the virulence of the *M. tuberculosis* strain (CDC, 2003). Other methods of transmission are rare. In the past, milk-borne transmission of

Mycobacterium bovis through consumption of milk from infected cows was common, but this route has been brought under control in all developed countries by elimination of infected cattle and pasteurization of milk and milk products. This route of transmission is still prevalent in developing countries if cow's milk is a common item of food (Stead and Dutt, 1994). Infection, however, can also occur when bacilli are introduced into or through the skin. Infection by this route is occasionally seen among pathologists and laboratory workers who handle infected tissues and culture mycobacterial species. (Stead and Dutt, 1994).

2.4.4 Risk Factors for Acquiring TB

Many factors may influence the risk of developing TB in an individual or a population and depend upon the point of time in regard to the TB epidemic wave, anyone can get tuberculosis, but certain factors increase the risk of acquiring the disease. Having a disease that suppresses immunity, such as, AIDS, diabetes, end-stage kidney disease, pregnancy, certain cancers or silicosis, can reduce the body's ability to protect itself. The risk also is higher in people on corticosteroids, certain arthritis medications, chemotherapy, radiotherapy drugs or other drugs that suppress the immune system and close contact with someone with untreated TB. In general, the person must spend an extended period of time with someone with untreated, active TB to become latent infected and to contract the disease from usually a family member, friend or close co-worker (Bass, 2008).

Recent transmission of TB was reported to be associated with ethnic minority, being a native of the country, residing in an urban area, drug use, excessive alcohol consumption, homelessness, previous incarceration, HIV

infection/ AIDS, young people, sputum smear positivity and gender males (Nava-Aguilera *et al.*, 2009).

There is substantial evidence from studies on racial variation in susceptibility to tuberculosis (Stead and Dutt, 1994) and studies showed that host genetic factors are important in determining susceptibility to infection with *M. tuberculosis* and the subsequent development of TB (Bellamy *et al.*, 1998). In a large case-control study among 800 Gambian subjects, (Bellamy *et al.*, 1998) showed that genetic variants of the natural resistance-associated macrophage protein (NRAMP) and vitamin D receptor (VDR) were associated with smear-positive pulmonary TB.

2.4.5 Effective vaccines for protection against tuberculosis

Bacille Calmette-Guérin (BCG), derived from *M. bovis* in 1902, is the only currently approved anti-tuberculous vaccine and is globally used (Andersen and Doherty, 2005). It has a good safety profile in immunocompetent persons. BCG vaccine-induced protective efficacy appears to be influenced by multiple factors (Rieder, 2008) including; age of the vaccinated person, background infection rates with ubiquitous mycobacteria, mycobacteria other than *M. tuberculosis* (MOTT), virulence of the strains, co-infection with helminths (Elias *et al.*, 2008), acquisition of cell immunity against helminths in utero and other conditions that alter immune responses (e.g. malnutrition) (Malhotra *et al.*, 1999). Recent studies in household contacts support the notion that BCG may protect against *M. tuberculosis* infection (Soysa *et al.*, 2005). Concerns about potential adverse post vaccine reactions from the live BCG vaccine in HIV-1-infected persons have led to the development of new BCG vaccination guidelines by the WHO (WHO, 2007)(b).

The current planning for future prime boost vaccination strategies incorporates conventional BCG and/or new rBCG-derived vaccines as the priming backbone followed by booster vaccines that are not based on BCG. Some of the rBCG vaccines improve antimycobacterial T-cell responses through engagement of an alternative antigen presentation pathway (endosome escape).

Thus, current TB vaccine development efforts aiming at preventing primary *M. tuberculosis* infection through infection vaccination, or at interrupting the transition from latent TB infection (LTBI) to an active disease through post-BCG booster vaccination approaches (Barker *et al.*, 2009).

As high incidence areas for TB most often are also areas with high HIV-1 infection rates, HIV-1 infection in vaccine recipients must be considered in the choice of vaccines for future vaccination campaigns (Keertanet *al.*, 2010). Helminth co-infections that cause a cytokine bias must also be considered as they can alter and responses to TB vaccines (Elias *et al.*, 2008). The urgency to identify and use correlates of protective immunity is particularly obvious in the vaccine development field in which the study of immunological correlates is a crucial component of vaccine efficacy assessments, prior to embarking on large-scale efficacy field trials. It is clear that the induction of no single cytokine (IFN- γ , TNF, IL-2) or effector molecule (granzyme, nitric oxide, granulysin) can predict immunological protection. Poly functional immune responses have recently been considered useful correlates of protection (Soareset *al.*, 2008). Attempts to determine functional assays that would allow measurement of vaccine-induced immunological protection in in vitro growth inhibition assays are in progress. In such assays whole blood or peripheral blood mononuclear cells

(PBMCs) from vaccinated people (before and after vaccination) will be incubated with isolates of virulent *M. tuberculosis* and the vaccine induced inhibitory effect on *M. tuberculosis* growth assessed in vitro (Keertanet *al.*, 2010).

Novel tuberculosis vaccination approaches may also involve the lungs. As human infection with *M. tuberculosis* most often occurs via inhalation, and TB pathology most often affects the lungs, it is logical to induce pulmonary protective immunity. An aerosolized TB vaccine candidate is currently undergoing safety and immunogenicity studies in non-human Primates (Keertanet *al.*, 2010).

2.5 Diagnosis

2.5.1 Clinical Signs of TB

Active TB may be considered as a possible diagnosis when findings on a chest radiograph of a patient being evaluated for respiratory symptoms are abnormal, as occurs in most patients with pulmonary tuberculosis. The radiographs may show the characteristic findings of infiltrates with cavitations in the upper and middle lobes of the lungs (Thruppet *al.*, 2004). Both groups of patients, such as the elderly and patients with advanced HIV infection may not have these typical findings. Compared with other patients, both groups have the classic cavitation less often and may have lower-lobe infiltrates as a prominent finding (Thruppet *al.*, 2004).

2.5.2 Conventional Methods

2.5.2.1 Microscopy

Traditionally, the first laboratory test used to detect active tuberculosis in a patient with abnormal findings on chest radiographs is the examination of a

sputum smear for the presence of acid-fast bacilli. According to the CDC, two sputum specimens should be used for detection of pulmonary tuberculosis, with specimens collected in the morning on two consecutive days. The acid-fast staining reaction of mycobacteria with the Zeil-Nelsen staining technique, along with their beaded and slightly curved shape is valuable in the early detection of infection and monitoring of therapy (El-Sony *et al.*, 2000). Leonard *et al* (2005) concluded that examination of 2 specimens is just as sensitive. For the test, sputum is smeared on a slide, dried, stained and then treated with alcohol. Any bacilli that are present will remain red because they will not destain. The test is not specific for tuberculosis, because other mycobacteria give the same results, but it does provide a quick method to determine if respiratory precautions should be maintained while more confirmatory testing is performed. Results of sputum smears should be available within 24 hours of the specimen collection (CDC, 2007).

Principle of Zeil-Nelsen staining technique

The property of acid-fastness is based on the presence of mycolic acids in the cell wall of mycobacteria. Primary stain (fuchsin) binds to cell-wall mycolic acids. Intense decolourization (strong acid or acid/alcohol) does not release the primary stain from the cell wall and the mycobacteria retain the red colour of fuchsin – hence acid-fastness. Counterstaining (with methylene blue) provides a contrasting background.

While mycobacteria are AFB, very few other bacteria possess the property of acid-fastness, and then only weakly (e.g. *Nocardia*). AFB found in respiratory specimens of patients from countries with high TB prevalence are almost always TB bacilli. Non-TB mycobacteria are more commonly

found in countries where TB prevalence is low. In high-burden countries, however, some patients suspected of having MDR-TB may actually have disease caused by non-TB mycobacteria. AFB found in extrapulmonary specimens, particularly gastric washings, stool or urine, should never be automatically assumed to represent TB bacilli.

2.5.2.2 Isolation of Mycobacteria

The standard definitive diagnosis of TB requires the identification of *M. tuberculosis* in a culture of a diagnostic specimen. Sputum was the most frequent sample used from a patient with a persistent and productive cough. Because most mycobacteria grow slowly, 3 to 6 weeks may be required for detectable growth on solid media. Conventionally, three sputum specimens as mentioned earlier for smears, also applies for cultures (Leonard *et al.*, 2005). A patient is considered to have achieved culture conversion when a culture is negative for the mycobacteria after a succession of cultures have been positive; culture conversion is the most important objective evaluation of response to treatment (CDC, 2007).

Additionally, many critically ill patients have trouble in producing the necessary material from the lungs and instead produce saliva or nasopharyngeal discharge. For patients who have difficulty producing sputum, inhalation of an aerosol of normal saline can be used to induce sputum for collection (Porth, 2002; CDC, 2007). However, if sputum specimens are still inadequate, or the index of suspicion for tuberculosis is still high despite cultures negative for *M. tuberculosis*, alternative approaches are available.

Bronchoscopy with bronchial washings or bronchi-alveolar lavage can provide sputum for diagnosis (CDC, 2007).

In bronchial washing, a fiberoptic bronchoscope is inserted into the lungs, and fluid is squirted in and then collected, essentially washing out a sample of cells and secretions from the alveolar and bronchial airspaces. Aliquots obtained from subsequent lavages constitute bronchi-alveolar lavage specimens (Rutgers *et al.*, 2000). In patients with involvement of intrathoracic lymph nodes, as indicated by adenopathy suggestive of tuberculosis, who have sputum smears negative for *M. tuberculosis*, culture of specimens collected by transbronchial needle aspiration can be used to accurately and immediately diagnose the disease. With this technique, specimens are collected by inserting a 19-gauge flexible histology needle through a bronchoscopy tube; patients are sedated but conscious, and computed tomography scans are used for guidance (Bilaceroglu *et al.*, 2004).

2.5.3 Biochemical Tests

Unfortunately, there is no completely reliable single test that will differentiate *M. tuberculosis* from other mycobacteria. Nevertheless, the following tests e.g. Niacin test, Nitrate reduction test, catalase test and growth on medium containing p-Nitrobenzoic acid (PNB), will enable the identification of >95% of *M. tuberculosis* strains (WHO, 1998).

2.5.4 Molecular Technologies

Modern diagnostic techniques for faster detection of *M. tuberculosis* include nucleic acid amplification tests. In these tests, molecular biology technologies are used to amplify DNA and RNA, facilitating rapid detection of microorganisms; the tests have been approved by the Food and Drug

Administration (FDA) (Mazurek *et al.*, 2005). One method is the polymerase chain reaction (PCR) assay, which can be used to differentiate *M. tuberculosis* from other mycobacteria on the basis of genetic information and provides results within hours. Although the test can provide rapid confirmation of *M. tuberculosis* in sputum specimens positive for acid-fast bacilli.

PCR-band positive for *M. tuberculosis* in conjunction with a sputum smear positive for the organism indicates true tuberculosis, but in a patient with sputum smear negative for the organism, positive PCR should be considered carefully along with clinical manifestations. The results of these assays cannot be relied upon as the sole guide for isolation or therapy, once patients recover from a primary *M. tuberculosis* infection and the infection becomes latent, sputum specimens are negative for the organisms, and findings on chest radiographs are typically normal. These patients also do not have signs or symptoms of infection, and they are not infectious to others (Michos *et al.*, 2006).

Line probe assay reverse hybridization-based assays, referred to as line probe assays (LPAs), are available commercially and are being tested under field conditions in endemic areas for rapid detection of mutations resulting in resistance to INH and RMP. These assays are a useful tool for cost-effective detection of drug resistance in selected patients. In brief, LPAs are based on the hybridization of PCR products from patient specimens to specific probes for wild-type and mutant alleles of genes involved in drug resistance and have shown high specificity and sensitivity.

2.5.5. Tuberculin Skin Testing (TST)

Tuberculin skin testing (TST) is the most commonly screening test used for latent *M. tuberculosis* (Goldrick, 2004). The TST is performed by intradermal injection of 0.1 mL of intermediate-strength purified protein derivative (PPD) that contains 5 tuberculin units. 48 to 72 hours later, the injection site is examined for induration (5 mm or more is positive). Although the test is useful because the PPD elicits a skin reaction via cell-mediated immunity when injected in patients previously, it is limited because it is not *Mycobacterium* species specific. Many proteins in the PPD product are highly conserved in various species. Also, the test is of limited value in patients with active tuberculosis because of its low sensitivity and specificity. False-negatives can occur in patients who are immunocompromised. False-positives can occur in patients who have infections caused by mycobacteria other than *M. tuberculosis* or who have been vaccinated with BCG vaccine (Anderson *et al.*, 2006).

2.5.6 Quantiferon-TB Testing

Mantoux test was the only test available to detect latent tuberculosis until an interferon- release assay, called QuantiFERON-TB test, was approved by the FDA in 2001. Then, in 2005, a new interferon-assay, called QuantiFERON-TB Gold was approved and intended to replace the QuantiFERON-TB test, which is no longer commercially available. In both tests, the cell-mediated reaction to *M. tuberculosis* is determined by release of interferon- γ , by incubating whole blood with an antigen and then using an enzymelinked immune sorbent assay (ELISA) to measure the amount of interferon- γ released from the white blood cells. QuantiFERON-TB Gold provides results in less than 24 hours and can be used to detect both active and latent

tuberculosis. The results of the QuantiFERON-TB Gold test are similar to those of the tuberculin skin test, and the CDC has now recommend that the QuantiFERON-TB Gold test to be used in all instances in which the formerly tuberculin skin test would have been used (Mazurek *et al.*, 2005).

2.6 Treatment of Tuberculosis

Effective TB treatment is difficult, due to the unusual structure and chemical composition of the mycobacterial cell wall, which makes many drugs ineffective and hinders the entry of drugs (Brennan and Nikaido, 1995). The two antibiotics most commonly used are isoniazid and rifampicin. However, instead of the short course of antibiotics typically used to cure other bacterial infections, TB requires much longer periods of treatment (around 6 to 24 months) to entirely eliminate mycobacteria from the body (CDC, 2000). Latent TB treatment usually uses a single antibiotic, while active TB disease is best treated with combinations of several antibiotics, to reduce the risk of the bacteria developing resistance to antibiotic. People with latent infections are treated to prevent them from progressing to active TB disease later in life (O'Brien, 1994).

2.6.1 Tuberculosis Control Strategy

The internationally recommended TB control strategy is directly observed treatment short course (DOTS). DOTS are based on five elements: political will, case detection, observed therapy, effective drug supply and monitoring and evaluation. The intervention is based on achieving the maximum benefit of existing methods for diagnosis and treatment, and is a highly cost-effective intervention in developing countries (Murray *et al.*, 1991). An extended version of the strategy, DOTS-plus, is used to treat drug-resistant *M. tuberculosis* (Mukherjee *et al.*, 2004). Although the DOTS strategy has

contributed to the decline in incidence of TB in many parts of the world, as recognized in the recently published plan for global TB control, DOTS should be augmented by a combined approach, including new diagnostics, new drugs, new vaccines, reduction of incidence of HIV infection and advocacy (Raviglione and Uplekar, 2006).

DOTS completion rates of therapy are variable, partly because many patients discontinue treatment when their conditions improve. Failure to complete therapy is associated with long lasting infectious status, relapse and drug resistance (Zigno *et al.*, 2006). With every dose supervised by clinic or community health care workers, DOTS attempts to improve treatment completion. A recent meta-analysis has shown that DOTS did not improve the key outcomes of cure and treatment completion (Volmink and Garner, 2006). Provision of DOTS at home results in similar (Newell *et al.*, 2006), or slightly better outcomes than in the clinic or community (Volmink and Garner, 2006). Programmes that support home DOTS, either via family or community workers, should be encouraged, and new interventions to improve adherence should only be implemented with good evidence. High adherence rates have been achieved in antiretroviral roll-out programmes in the developing world with a patient-centered approach, which encourages the belief that empowerment of patients can promote adherence (Gary and Robert, 2007).

2.6.2 Drug Resistance

Drug-resistant *M. tuberculosis* is transmitted in the same way as regular TB. Primary resistance occurs in persons infected with a resistant strain of *M. tuberculosis*. A patient with fully susceptible TB develops secondary resistance (acquired resistance) during TB therapy because of inadequate

treatment, not taking the prescribed regimen appropriately or using low-quality medication (O'Brien, 1994). Drug resistant-TB is a public health issue in many developing countries, as treatment is longer and requires more expensive drugs. Multi-drug-resistant TB (MDR-TB) is defined as resistance to the two most effective first-line TB drugs: rifampicin and isoniazid. Extensively drug-resistant TB (XDR-TB) is also resistant to three or more of the six classes of second-line drugs (CDC, 2006).

CHAPTER THREE

3. MATERIALS & METHODS

3.1 Study Design

3.1.1 Type of study

This is a descriptive cross sectional laboratory based study.

3.1.2 Study Area

Different TB centers in Khartoum State including Abu Anga Hospital, the National Reference Tuberculosis Laboratory and Elsha'ab Teaching Hospital.

3.1.3 Study Duration

This study was done between May 2013 to February 2014.

3.1.4 Study population

A total of 90 patients were referred to different TB centers in Khartoum State for TB diagnosis and management.

3.1.5 Sample size

Ninety (90) sputum samples were collected from tuberculosis suspected untreated patients, treatment failure and relapse patients.

3.1.6 Ethical consideration

Approval of the National Ethics Committee, Ministry of Health (Sudan) was taken, verbal consent and informed about the importance of the study.

3.1.7 Inclusion and exclusion criteria

Patients with suspected pulmonary or extrapulmonary TB were prospectively recruited. Symptom criteria were outlined clearly. Pulmonary TB suspects had two or more of cough for two weeks, weight loss of 10% of healthy body weight, or symptoms of fever for two weeks, Patients with inability to submit and/ or report on subsequent visits were excluded.

3.2 Collection of specimens

Sputum specimens from the first productive deep cough of the morning were obtained without being contaminated by saliva or nasal secretions. A total of ninety samples were collected in clean one-use, disposable screw capped plastic sputum container and instructed to produce the specimen by deep coughing in a well-ventilated area. The specimen was transferred to National Reference Laboratory immediately and subjected to microscopic examination and extraction.

3.3 Methods of Identification of *Mycobacterium*

3.3.1. Direct smear preparation

After disinfecting the working area, the slides were labeled properly using laboratory register serial number marked on the sputum container. Then Proceeding to smearing; taking the labeled slides and opening containers one by one. Smearing was done behind the flame of a Bunsen burner or spirit lamp. Then for a direct sputum smear, selection of small portion of purulent or mucopurulent material with the stick and transferred to the slide, The material was spread carefully over an area equal to about 2–3 cm x 1–2 cm using repeated circular movements, without touching the edge of the slide. Making the smear as even as possible by continuing this process until no thick parts remained.

3.3.2 Concentration Smear Preparation

Sodium hydroxide (NaOH), a decontaminating agent, also acts as an emulsifier was added. Because of its potential toxicity, NaOH was used at the lowest concentration that effectively digests and decontaminates the specimen.

Method

Work was done under a biological safety cabinet and protective clothing, gloves, and masks were worn. A maximum of 10 ml of sputum was transferred to a sterile, disposable, plastic 50 ml conical centrifuge tube with a leak-proof and aerosol-free plastic screw-cap. An equal volume of freshly prepared digestant (4% NaOH) was added to the tube, and tightened the cap completely. The specimens were vortexed for approximately 15 seconds or for a maximum of 30 seconds. After 15 minutes of digestion, enough

phosphate buffer was added to reach within 1 cm of the top, the tube was inverted to mix the solutions and stop the digestion process. Centrifugation was made to all tubes at 36,000 revolutions per minute (rpm) for 15 minutes, using aerosol-free sealed centrifuge cups. Then carefully the supernatant was poured off into a splash-proof container. Then resuspended the buffer, (PH 6.8) and then smears were prepared.

3.3.3 Ziehl-Neelsen (ZN) Staining Method

Staining procedure

Slides were placed; smeared upwards, on the staining rack over a sink, about 1 cm apart. A new filter paper was placed in a small funnel. It was kept over the first slide and filled up with carbol-fuchsin staining solution. All slides were heated by torch, keeping the torch a little below them and moving it continuously back and forth along the line until steam rises. This was repeated twice at intervals of 3–5 minutes. Contact time of the staining solution with the smear was 10 minutes. The slides were rinsed well with distilled water or clean tap water from a beaker (not directly from the tap). The acid solution was poured over the smears, and acting for 3 minutes. After wash, smears flooded with methylene blue solution for 1 minute (60 seconds). Stain washed, using forceps, each slide was taken from the rack and the water drained off by air.

Reagents and Solutions (see Appendix III)

3.3.4 Molecular identification using (PCR)

Polymerase chain reaction was used as a rapid tool to diagnose pulmonary tuberculosis from sputum samples.

3.3.4.1 DNA extraction from sputum samples

DNA was extracted using CinnaPure DNA kit (CinnaGenInc, Cat. No.PR881613 form Iran) which was designed to isolate double stranded DNA form cell culture, tissues, Gram-negative bacteria.

100µl of sputum were added to 400 µl of lysis buffer and vortexed for 20 seconds, and then transferred to spin columns with collection tube. Then centrifuged at 13.000 rpm for 1 min. Collection tubes were discarded, and replaced with new one and step repeated. Then spin columns were washed with 400 µl of wash buffer II and centrifuged at 13.000 rpm two times and collection tubes were discarded, Then columns were transferred to new 1.5ml tubes and 50 µl of 65 °C pre-heated elution buffer were added to the columns and incubated for 3-5 min at 65 °C. Then centrifuged at 13.000 rpm for 1 min. The elution contained purified DNA.

3.3.4.2 Polymerase Chain Reaction (PCR) Protocol

Mycobacterium tuberculosis PCR detection kit (CinnaGenInc Iran (cat.No. PR7935C)) was used for diagnosis in addition to negative and positive controls were provided. The reaction mix with a total volume of 25 µl included: 20 µl 1x PCR MIX, 0.4 µl Taq DNA polymerase and one drop mineral oil, followed by the addition of 5 µl of the template DNA. The mixture was gently mixed, then the tubes were transferred to preheated thermocycler machine, the program started as followed, the following temperature profile: 93 °C for 1 min for denaturation, 72 °C for 50 sec for annealing, 72 °C for 50sec for extension followed by 37 cycles, then 93 °C for 20 sec and ended with 72 °C for 120sec.

3.3.4.3 Visualization of PCR product

The PCR product (10 µl) was loaded into the gel wells (2% agarose gel) and was visualized by electrophoresis at 80V for 30 min. With 5 µl of ethidium bromide and photographed.

The presence of 163bp fragments comparing with 5 µl DNA size marker (ladder) indicated positive samples.

3.3.5 Calculation of Sensitivity and Specificity

When evaluating a clinical test, the terms sensitivity and specificity are used. They are independent of the population of interest subjected to the test.

Sensitivity: The sensitivity of a clinical test refers to the ability of the test to correctly identify those patients with the disease. And calculated to the following equation

$$\begin{aligned}\text{sensitivity} &= \frac{\text{number of true positives}}{\text{number of true positives} - \text{number of false negatives}} \\ &= \frac{\text{number of true positives}}{\text{total number of sick individuals in population}} \\ &= \text{probability of a positive test, given that the patient is ill}\end{aligned}$$

Specificity: The specificity of a clinical test refers to the ability of the test to correctly identify those patients without the disease. and was calculated according to the following equation

$$\begin{aligned}
\text{specificity} &= \frac{\text{number of true negatives}}{\text{number of true negatives} - \text{number of false positives}} \\
&= \frac{\text{number of true negatives}}{\text{total number of well individuals in population}} \\
&= \text{probability of a negative test given that the patient is well}
\end{aligned}$$

CHAPTER FOUR

4. RESULTS

4.1 Epidemiological Findings

4.1.1. Gender

Out of the 90 patients, suspected to have pulmonary tuberculosis, 61 were found to be males (67.8%) and 29 were females (32.2%) (Fig1).

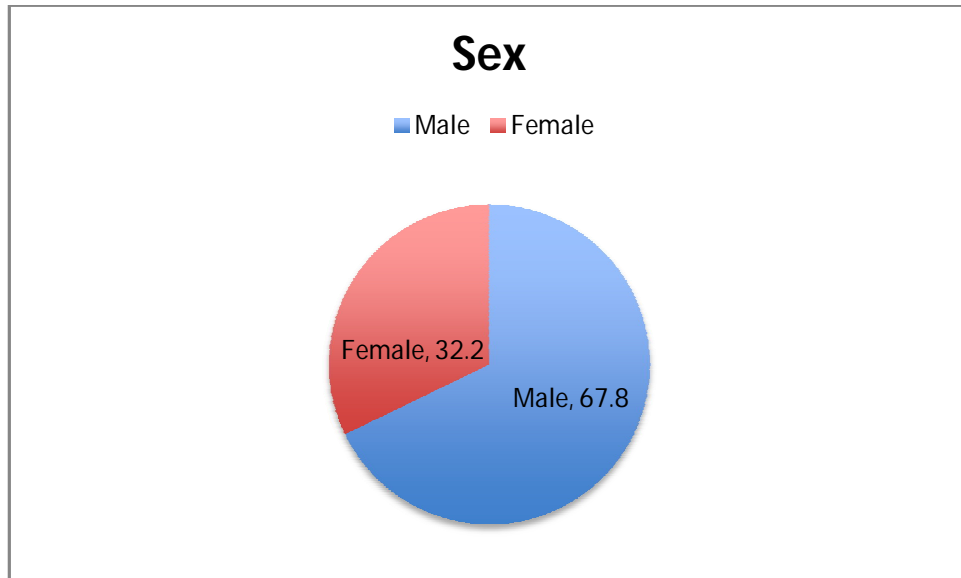


Fig.1: Frequency of gender of patients with pulmonary TB infection

4.1.2 Frequency of TB among age groups

All the ages were found to be infected with tuberculosis. Highest frequency was among age group 31 -49 years 45 patients (50%), followed by age less than 30 was 31 patients (34.4%) while the lowest frequency was age group more than 50 was 14 patients (15.6%) (Figure 2).

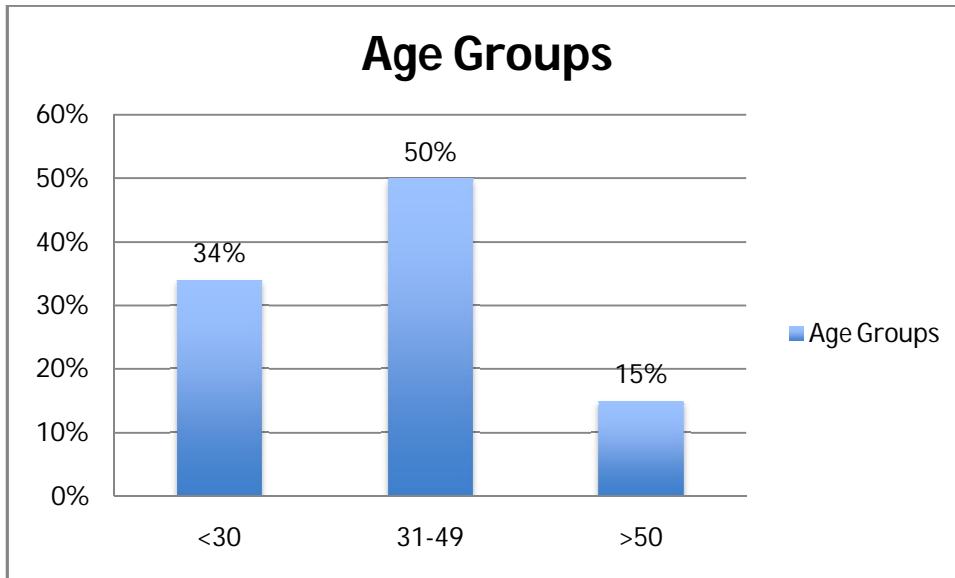


Fig.2: Distribution of pulmonary TB infection among age groups.

4.2. Morphological Findings

Zeil-Nelseenstain was applied and Acid-fast bacilli appeared bright red against the background material counterstained in blue. (Figure 3) show direct smear preparation with few numbers of bacilli and concentrated smear preparation with more and concentrated number of bacilli.

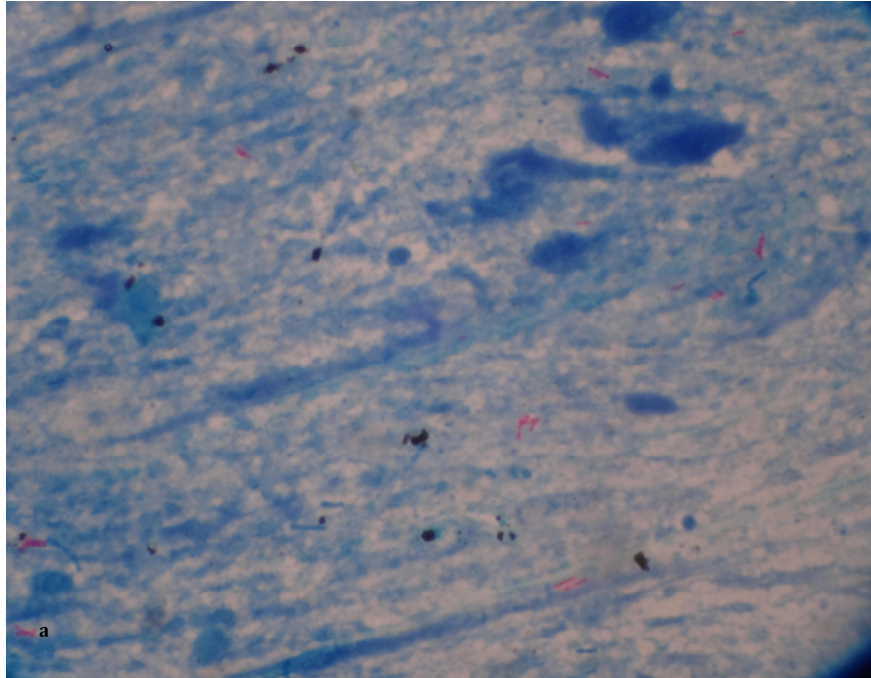


Fig.3:Acid-fast bacilli bacteria (red colour) with ZN stain using direct smear preparation.

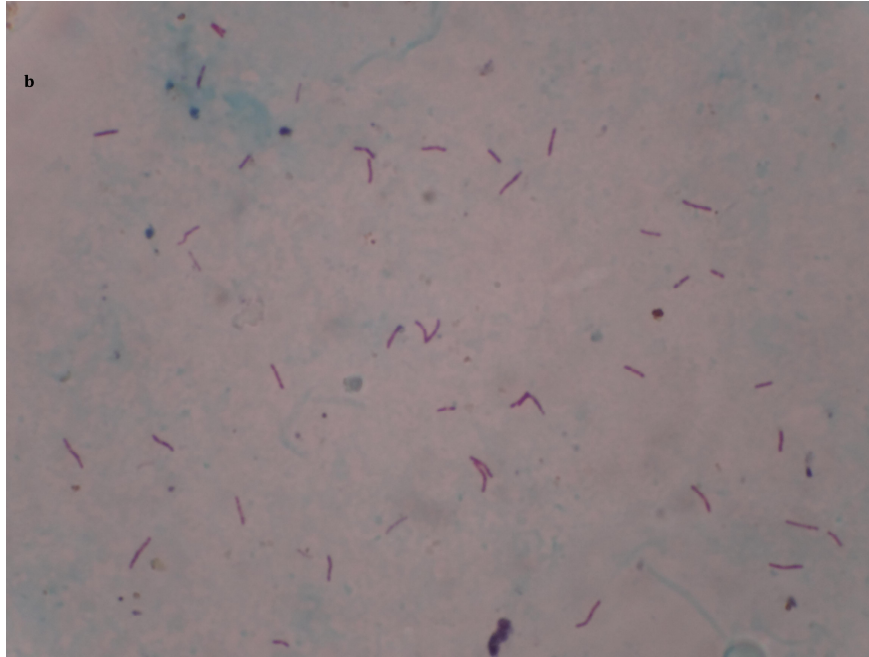


Fig.4: Acid-fast bacilli (red colour) with ZN Stain using concentrated smear preparation

4.2.1. Sputum microscopy results

When using direct smear preparation method, 8(8.9%) were positive while 82 (91.0%) were negative in Table1, in concentrated smear preparation method 24(26.7%) were positive while 66 (73.3%) were negativeas shown in Table 2

Table1:Zeil-Nelseen stain results of sputum with direct smear for TB patient

DIRECT

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid positive	8	8.9	8.9	8.9
negative	82	91.1	91.1	100.0
Total	90	100.0	100.0	

Table 2:Zeil-Nelseen stain results of sputum with concentrated smear for TB patient

CONCEN

	Frequency	Percent	Valid Percent	Cumulative Percent
Valid positive	24	26.7	26.7	26.7
negative	66	73.3	73.3	100.0
Total	90	100.0	100.0	

4.3 Molecular method (PCR) results

By PCR 33(36.7%) were positive, while 57 (63.3%) were negative results.as shown in Table 3 and figure 5.

Table 3: PCR results of sputum samples of TB patient

PCR				
	Frequency	Percent	Valid Percent	Cumulative Percent
Valid positive	33	36.7	36.7	36.7
negative	57	63.3	63.3	100.0
Total	90	100.0	100.0	

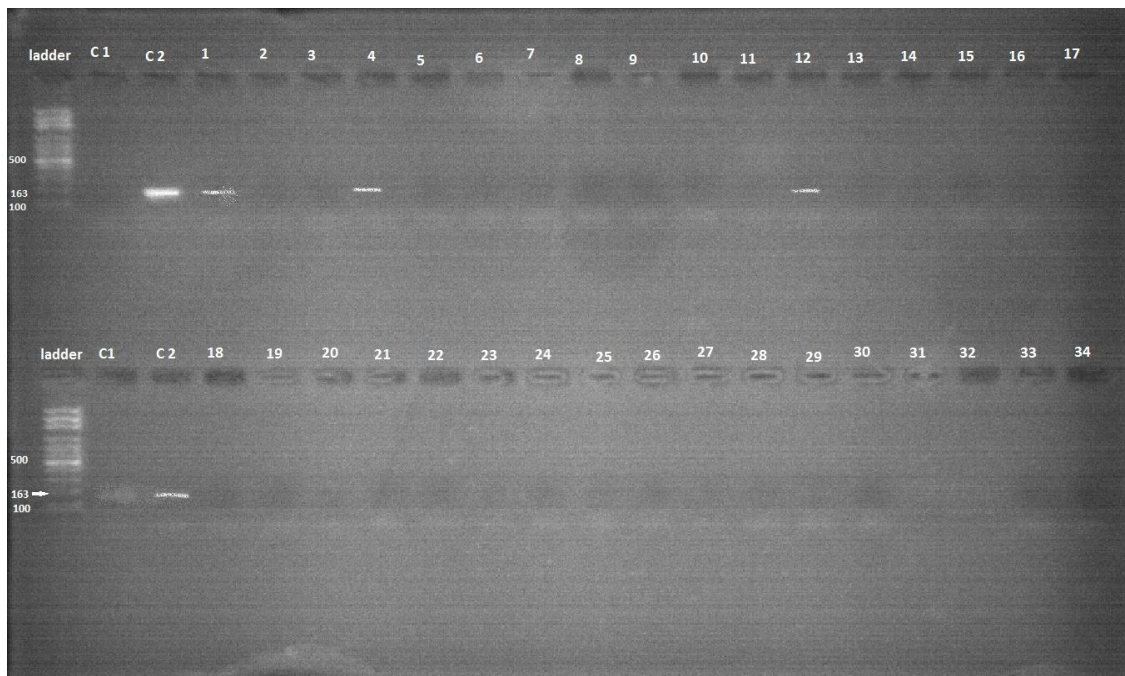


Fig.5: Detection of *M. tuberculosis* band by (2%) agarose gel electrophoresis.

A 34 well gel electrophoresis result of sputum samples. C1; negative control, C2; positive control. *M. tuberculosis* DNA fragment is 163bp. Sample 1,4,12 is positive for *M. tuberculosis*. Compared to the DNA marker (ladder).

4.4 Statistical Analysis

4.4.1 Correlation of Direct smears and concentrated

Collected data were analyzed using the Statistical Package for Social Science (SPSS). Chi-square test was used to determine the relation between direct smear preparation and concentrated preparation. All 90 direct smears were compared with the concentrated ones after staining with ZN stain. Result showed 8 (8.9%) positive samples in direct method, while 24 (26.7%) showed positive results in concentrated method. This difference was

found to be statistically significant (less than 0.05) between direct & concentrated methods. (Table 4, 5)

Table 4: Direct and Concentrated Method Cross Tabulation

DIRECT * CONCEN Crosstabulation

		CONCEN		Total
		postive	negative	
DIRECT positive	Count	8	0	8
	% of Total	8.9%	.0%	8.9%
negative	Count	16	66	82
	% of Total	17.8%	73.3%	91.1%
Total	Count	24	66	90
	% of Total	26.7%	73.3%	100.0%

Table 5: Chi-Square Tests

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	24.146 ^b	1	.000		
Continuity Correction ^a	20.206	1	.000		
Likelihood Ratio	23.440	1	.000		
Fisher's Exact Test				.000	.000
Linear-by-Linear Association	23.878	1	.000		
N of Valid Cases	90				

a. Computed only for a 2x2 table

b. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 2.13.

Because the significant value is (.000) (less than (0.05)) so there is relation between the two method.

4.4.2 Sensitivity and specificity of Direct smears and concentrated with PCR

A 2 X 2 table was used to calculate for sensitivity and specificity of ZN negative and ZN positive smears for both preparation methods on PCR result. Positivity by ZN method was 8.9% for direct preparation and 26.7% for the concentrated preparation while PCR give 36.7% positivity. The sensitivity of ZN microscopy was 24%, 72% for direct and concentrated respectively and specificity was 100% for both. (Tab 6,7)

Table 6: correlation of direct and PCR results of TB patient

DIRECT * PCR Crosstabulation

Count

	PCR		Total
	postive	negative	
DIRECT positive	8	0	8
negative	25	57	82
Total	33	57	90

Table 7: correlation of concentrated and PCR results of TB patient

CONCEN * PCR Crosstabulation

Count

	PCR		Total
	postive	negative	
CONCEN positive	24	0	24
negative	9	57	66
Total	33	57	90

Sensitivity =

True positives / (True positives + False negatives)

Specificity=

True negative / (False positives + True negative)

Sensitivity of direct = $8/(8+25) * 100 = 24\%$

Specificity of direct = $57/(0+57)*100 = 100\%$

Sensitivity of conc. = $24/(24+9)*100 = 72\%$

Specificity of conc. = $57/(0+57)*100 = 100\%$

CHAPTER FIVE

5. DISCUSSION

Tuberculosis (TB) has been recognized as a major public health problem since the 1950s in Sudan, and still remains a health priority, with about 18,055 new cases of TB reported during 2004 (UNDP, World Development Report, 2004).

The low national income and the meager resources resulted in poor health services to the needy communities. The civil war and the displacement of population have complicated the health problems and have facilitated the spread of infectious diseases in different parts of the country. Although TB is known to be endemic in Sudan, meager information is available about the magnitude of the disease, its epidemiology, or the extent of drug resistance problem (El-Sonyet *al.*, 2000), TB is considered as a leading cause of health service utilization in the ambulatory services, ranking fourth (11.6%) of the most frequent diseases for hospital admission and the most frequent (16%) cause of hospital deaths.

Smear microscopy plays an important role in early diagnosis of mycobacterial infections as the method is highly specific, rapid and cheapest method used for detection of AFB in sputum. The only disadvantage of this method is low sensitivity (varying from 50%- 80%) relative to culture (Bruchfeldet *al.*, 2000); (Masoodet *al.*, 2008). The sensitivity of microscopy is influenced by the quality of specimen collection, the number of *Mycobacterium* present in the specimen, the method of processing, the

staining technique, and the quality of the examination (Somoskoviet *al.*, 2001). Hence the aim of this study was to evaluate two different smear preparation methods for sputum after stained with ZN stain and examined for presence of red bacilli under light microscope for diagnosis of tuberculosis.

Among the total of 90 specimens collected, males were more susceptible than females (68%) to (32%) respectively with high frequency among age group 31-49 years (50%),

Tuberculosis incidence and prevalence is higher in adult males than in adult females (WHO, 2001) and the reasons for the higher male prevalence and incidence are poorly understood, and need further research to identify associated risk factors, this may be due to the fact that men are out doors most of the time during working.

This study showed that the use of the concentrated method for preparing smears for AFB microscopy increases sensitivity (72%) without a loss of specificity (100%) in identifying positive TB cases, compared to the direct method with (24%) sensitivity and (100%) specificity according to PCR result as reference method.

From the 90 used samples, 8 were found positive when tested by direct method and 24 were positive when concentrated method was used by NaOH digestion and centrifugation which led to concentration of the bacilli in the tested samples, this result is in agreement with Farniaet *al.*,(2002) and Gebre-Selassie (2003) where as it was not in agreement with Cattamanchiet *al* (2009).

CONCLUSION & RECOMMENDATIONS

Conclusions:

We concluded from this study that the concentrated method yield more bacilli in the tested samples hence easy detection by microscopy and significantly more sensitive and obtaining the results in much less time than the direct method without losing specificity.

Recommendations:

- 1) All diagnosis of TB should pass through conventional methods including ZN stain.
- 2) Sputum preparation technique by 4% NaOH liquefaction methods of concentration is safe, relatively inexpensive and easy to perform.
- 3) We recommend implementation of concentrated preparation to enable rapid, sensitive laboratory diagnosis of pulmonary tuberculosis, especially in resource-poor settings where culture is not possible.
- 4) The impact of these findings should be substantiated in other populations and with a larger number of subjects at the country level.

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APPENDIX

إقرار المشاركة في البحث: Appendix I

أقر أنا
برغبتي في المشاركة في البحث والذي تم شرحه لي (أعلاه) والذي سيقوم به:

.....
في منطقة..... بولاية.....

توقيع المشارك.....

إسم المشارك:

.....
.....

أسم الشاهد عن المشارك أو من ينوب عنه

.....

.....

..... توقيع الشاهد:

عنوان الشاهد أو من ينوب عنه:

.....

.....

.....

في حال عدم قدرة المشارك على قراءة الإقرار ويحتاج إلى من يشرح أو يترجم له:

..... إسم الشارح.

..... توقيع الشارح أو (المترجم):

..... عنوان الشارح أو (المترجم):

AppendixII: Questionnaire

Name:

Serial Number:

Sex: Male Female:

Age:

Resident:

Requesting test for:

Diagnosis:

Follow up:

Appendix III: Reagents

1. Sputum Smear Reagents

1) 4% NaOH:

Sodium Hydroxide (NaOH)
..... 4 g

Distilled water
..... 100 ml

2) Distilled Water:

Distilled water autoclaved at 121C for 15 min.

2. Ziehl-Neelsen Staining:

1) Reagents:

Basic
Fuchsin.....10.0g

95%
Ethanol.....100ml

Phenol
crystals.....50.0g

Distilled
water.....850 ml

Dissolve the phenol in the alcohol; now dissolve the fuchsin powder in this mixture by swirling. If dissolving is difficult, add 50 to 100 ml of the water.

After complete dissolution, add the remaining water and mix again. Store in an amber bottle, it can be stored at R.T. for 6-12 months and filter before use.

2) Decolorizing Agent:

25% Sulphuric Acid

Conc.	Sulphuric acid
.....	250ml

Distilled water.....	750ml
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Carefully add conc. Sulphuric acid to water, store at R.T. for 6-12 months.

3) Counter stain:

Methylene Blue

Methylene blue.....	1.0g
---------------------	------

Distilled water.....	1000ml
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Store in an amber bottle at R.T. for 6-12 months.