1. INTRODUCTION AND OBJECTIVES

1.1. Introduction

Tuberculosis is a chronic granulomatous disease affecting man, many other mammals, bird, fish, amphibians, and reptiles. Mammalian TB is caused by five closely related species, collectively termed *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti*) (Grange and Yates, 1992).

Moreover, continues to be as it has been for centuries, one of the most prevalent infectious diseases of humans and is the leading cause of mortality from a single disease world wide.

One -third of the world's population is currently infected with the Tubercule bacilli, 5-10% of people who are infected with Tubercle bacilli but who are not infected with HIV, become sick or infectious at some time during their life. people with HIV and TB infections are much more likely to develop Tuberculosis (Bloom and Murray,1992).

Antituberculuos drugs can be divided into first -line drugs and second line drugs. First line drugs include isonizid, rifampin, pyrazinamide, ethambutol, and streptomycin. Second line-drugs include p-aminosalicyclic acid, ethionamide, cyclosyrine, capreomycin, and amikacin (Bloom and Murray, 1992).

Multi drugs therapy is used to prevent the emergence of drug resistant mutants during the long 6 to 9 month duration of treatment, organisms that become resistant to one drug will be inhibited by the other, isoniazid (INH) bactericidal drug is main stay of treatment.

Treatment for patients with pulmonary tuberculosis is with three drugs; INH, rifampin, and pyrazinamide. INH and rifampin are given for 6 months but, pyraziamide is stopped after 2 months in patients who are immune compromised (e.g., AIDS patient), who have disseminated disease, or who are likely to have INH- resistant organism and a fourth drug, ethambutol is added and all four drugs are given for months. Although therapy is usually given for months, the patient sputum becomes non infectious within 2-3 weeks after start of treatment (Levinson and Jawetz, 2002).

Drug resistance in *M. tuberculosis* is a worldwide problem. Mechanisms explaining the resistance phenomenon for many but not all of the resistant strains have been defined (Levinson and Jawetz, 2002).

Isoniazide resistance has been associated with deletion or mutation in catalase-peroxidase gene. These isolates become catalse negative or have a decrease catalase activity. It is also associated with alternation in the inh gene, which encodes an enzyme that function in mycolic acid synthesis (Levinson and Jawetz, 2002).

Streptomycin resistance has been associated with mutation in genes encoding the ribosomal S12 protein and 16S rRNA, and rifampin resistance has been associated with alteration in the b subunit of RNA polymerase (Brook, *et al.*, 2004).

Multi drug resistant *M. tuberculosis* MDR (resistance to both isoniazid and rifampin) is a major and increasing problem in tuberculosis treatment and control. There have been many outbreaks of tuberculosis with multi drug resistant strains. Multi-drug resistant tuberculosis becomes a major problem in Khartoum State where there is increasing evidence of MDR (WHO, 1998).

These are particularly important in persons with HIV infections. Persons infected with multi drug- resistant organisms or who are at high risk for such

infection, including exposure to another person with such an infection, should be treated according to susceptibility test result for the infecting strain. If susceptibility results are not available, the drugs should be selected according to known pattern of susceptibility in the community and modified when the susceptibility test results are available. Therapy should include a minimum of three and preferably more than three drugs to which the organisms have demonstrated susceptibility (Brook, *et al.*, 2004).

1.2. Rational

Multi-drug resistant tuberculosis is considered a challenge for clinicians and the frequency is not well determined in Sudan as well as in some other neighbouring countries.

1.3. Objectives

1.3.1. General objective

The present study aimed to determine the frequency of multi-drug resistant *M. tuberculosis* among patients with pulmonary tuberculosis in Khartoum State.

1.3.2. Specific objectives

- To isolate *Mycobacterium tuberculosis* from patients with pulmonary infection.
- To identify *Mycobacterium tuberculosis* by using conventional methods.
- To determine the frequency of multi-drug resistant strain among pulmonary tuberculosis patients.

2. LITERATURE REVIEW

2.1. Tuberculosis

2.1.1. Definition

Tuberculosis is a chronic granulomatous disease affecting man and many other mammals. It is an infection, which most commonly affects the lung where it is called pulmonary tuberculosis, but it can also affect the central nervous system (meningitis), circulating system and gastrointestinal tract (miliary tuberculosis), genitourinary system, bones and joint where it is called extra pulmonary tuberculosis (Grange, 2002).

2.1.2. Historical background

Tuberculosis is a disease of great antiquity, having been found in the mummaries of ancient Egypt

M. tuberculosis was probably first seen in tissues by Baumgarten and Koch in 1882. Koch cultivated *M. tuberculosis* and reproduced the disease in the period from 1882 to 1884 (Carter, *et al*., 1986).

The bacterium first infected animals possibly by inhalation or ingestion from the soil, and passed to humans through the animals flesh and milk. It affects many kinds of animals including cows, birds, fish and reptiles.

The earliest evidence of Tuberculosis in humans is from a Neolithic grave near Heidelberg, Germany, dating back to 5000 Bc,Examination of the spines of mummies and of tomb paintings from 4000 Bc confirms Tuberculosis was a common disease in ancient Egypt.and Skeletal remains in Italy from the same date, also show Tuberculosis in the spine by the mid 17th century, One in five death in London as recorded in the Bills of mortality was

due to Tuberculosis consumption. TB soon became and epidemic in Britain and major cities in the USA and Europe, commonly known as the white plaque (Betty, et al., 1998).

2.2. Classification of *Mycobacterium*

2.2.1. Mycobacterium tuberculosis complex

Members of MTC are the causative agents of tuberculosis in human and animals. Despite their genetic relationship, the species of the MTC show a large variability in their phenotypic properties, epidemiology and importance for human TB (Table 2-1 & 2-2) (Kubica, *et al.*, 2003).

Table 2-1. Differentiation of members of the *M. tuberculosis* complex from other cultivable mycobacteria (Brook, *et al.*, 2004)

Growth in PNB medium	Growth at 25°C	Growth within 3 days	Pigment production	Identification
-	-	-	None	*M.. tuberculosis* complex
+	+	-	Light only	Photochromogen
+	+	-	In dark and light	Scotochromogen
+	+ or -	-	None	Non chromogen
+	+	+	Variable	Rapid grower

2.2.1.1. *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is considered primarily a human pathogen, but it cause infections in a wide range of domestic and wildlife animal species, most frequently living in close contact with humans (Ocepek, *et al*., 2005).

2.2.1.2. *Mycobacterium bovis*

Mycobacterium bovis principally affects cattle, but it can cause disease in a range of wild and domestic animals and also humans (Smith, *et al.*, 2004). Cervical lymphadenopathy. intestinal lesions, chronic skin Tuberculosis (Lupus vulgaris) and other non pulmonary forms in human due to *M. bovis* Howeve , the clinical picture of pulmonary Tuberculosis caused by *M. bovis* is identical to Tuberculosis due to M. tuberculosis (Kubica, et al., 2003)both subspecies of *M. bovis*.

Caprae are reported to infect humans, while the vaccine strains *M. bovis* BCG resistance to pyrazinamid (PZA) is a major criterion for the differentiation of *M. bovis*, but some studies report susceptibility to PZA among *M. bovis* isolates (Niemann, *et al*., 2000).

2.2.1.3. *Mycobacterium africanum*

Since its first description in 1968, *M. africanum* has been found in several regions of Africa, where it represents up to 60% of clinical strains from patients with pulmonary tuberculosis. Two major M. africanum subgroups have been described, according to their biochemical characteristics. these subgroups correspond to their geographic origins in West Africa (subtype I) or East Africa (subtype II). Numerical analysis of biochemical characteristics revealed that M. africanum subtype I is more closely related to *M. bovis*, whereas subtype II more closely resembles M. tuberculosis (Niemann, et al., 2004).

2.2.1.4. *Mycobacterium microti*

The exact nature of *M. microti* is not currently known, but morphological and serological studies suggest that it belong to the tuberculosis complex. *M. microti* causes tuberculosis in small rodents, and although it has been reported to cause infections in cats and pigs, it is not considered to be an important human pathogen (Liebana, et al., 1996).

2.2.1.5. *Mycobacterium canetti*

All *Mycobacterium canetti* infection have been reported from Africa, it differs from all other members of MTB in having smooth ,round and glossy colonies.(Metchok,*et al* ., 2003**(.**

Table 2-2.Speciation within the *M. tuberculosis* complex (Brook *et al.* 2004)

Species and variant	**Nitratase activity**	**Oxygen preference**	**Pyrazinemide susceptibility**	**Pyrazina-midase**	**Thiophane carboxalic acid hydrazid**
M. tuberculosis					
- Classical	Positive	Aerobic	Sensitive	Positive	Resistant
- Asian	Positive	Aerobic	Sensitive	Positive	Sensitive
M. Africanium:					
- Type I	Negative	Microaerophilic	Sensitive	Positive	Sensitive
- Type II	Positive	Microaerophilic	Sensitive	Positive	Sensitive
M. bovis					
- Classical	Negative	Microaerophilic	Resistant	Negative	Sensitive
- BCG	Negative	Aerobic	Resistant	Negative	Sensitive

2.2.2 Mycobacteria other than tuberculosis bacilli (MOTTs)

This group has been given several collective names: atypical, anonymous, non-tuberculous, tuberculoid, opportunist and mycobacteria other than

tuberculosis bacilli. Many MOTTs are found in the environment, but they can also colonize man (e.g., in parts of a previously damaged respiratory tract) and cause clinical infections (Table 2-3) (Walt, *et al.* 1993)

Table 2-3. Principal types of disease caused by opportunist Mycobacteria in man (Ananthanaryanan and Piker, 1990)

Species	Natural habitat	Type of infection in man
M. asiaticum	Primates	Pulmonary.
M. avium SPP intracellular	Soil-sea water, animals	Pulmonary, systemic, gastrointestinal, lymphadenitis.
M. cheloneii Spp-Cheloneii	Soil-sea water, animals	Pulmonary, surgical wound.
M. fallax	Water, soil	Pulmonary, surgical wound.
M. fortuim	Water, soil	Pulmonary, surgical wound cutaneous systemic bone and joint.
M. haemophilium	Unknown	Cutaneous, subcutaneous.
M. malmoense	Unknown	Pulmonary.
M. marinum	Aquirium, water fish	Cutaneous (swimming pool granuloma), joint
M. scrofulacium	Soil, water, fomites	Lymphadenitis (usually cervical) pulmonary disseminated
M. schimoidei	Unknown	Pulmonary.
M. simiae	Primates, water	Pulmonary.
M. sulagi	Unknown	Pulmonary, lymphadenitis, cutaneous, subcutaneous, bunsitis
M. ulcerans	Unknown	Cutaneous.
M. xenopia	Soil, water	Pulmonary, epidymitis.
M. kansasii	Water, animals	Pulmonary, systemic, skin, joints

2.2.3. Environmental Mycobacteria

Atypical mycobacteria represent 80 species of *Mycobacteria* that normally exist as saprotypes in soil and water and are termed environmental or non tuberculous Mycobacteria (Greenwood, *et al.*, 2002). Some species occasionally cause opportunistic diseases in animals and man. The four groups of Mycobacteria associated with human disease are classified accordingly to their production of yellow or orange pigment And their rate of growth as follows :-

2.2.3.1. Photochromogens

These are colorless when incubated in the dark, but develop a bright yellow or orange coloration if young cultures are exposed to a light source for an hour or more and then re-incubated. The caps of the culture bottles must be loosened during exposure to light, as oxygen is essential for pigment production. they grow well at 37°C and are principally isolated from cases of pulmonary disease (e.g. *Mycobacterium kansaii, M. simiae, M. marinum*), (Greenwood, *et al.,* 2002).

2.2.3.2. Scotochromogens

produce pigmented colonies even when grow in the dark. Most slowly growing scotochromogens are isolated from sputum or urine and are of no clinical significance (e.g. *M. gordonae, M. scrofulaceum, M. szulgai*), (Greenwood, *et al.,* 2002).

2.2.3.3. Nonchromogens

These are unpigmented and the most prevalent and important opportunistic pathogens of man are *M. avium* and *M. intracellulare*. These two species are usually grouped together as the *M.avium* complex. Other non-chromogens includes *M. ulcerans, M. shinshensis, M. xenopi, M. malmoense, M. sylvoticum, M. lepraemurium, M. ternae, M. haemophilum* and *M. genevense* which is very slow growing organism occasionally isolated from AIDS patient's with disseminated mycbacterial disease and from birds (Greenwood, *et al.,* 2002).

2.2.3.4.Rapid growers

May be photochromogens, scotochromogens or non-chromogens. They produce visible growth on Lowenstein Jensen medium within one week on subculture Only two of the rapidly growing species are well recognized

human pathogens. *M. chelonae and M. fortuitum* .which are non-chromogenic. They occasionally cause pulmonary disseminated disease but are principally responsible for post infection abscesses and wound infections including corneal ulcers.

Most of the many other rapidly growing species are pigmented. Disease due to these are exceedingly rare, but they frequently contaminate clinical specimens. They are found normally on the genitalia, e.g. *M. smegmatis and M. flavescens* (Greenwood *et al*., 2002).

2.3. Antigenic Structure

Mycobacterium tuberculosis possesses, ahighly complex antigenic structure, and humoral immune responses are mounted to many of these antigens during the course of a tuberculosis infection. Antibody responses do not appear to be involved in immunity to infection, however, and no serological tests has so far proved sufficiently sensitive and specific to be useful as a diagnostic procedure. As there are no consistent antigenic differences between strains of *M. tuberculosis*, there is no practical serological typing procedure (Kenneth *et al.*, 1984).

2.4. Pthogenicity

2.4.1. Virulence Mechanisms

The organism owes its virulence to its ability to multiply within macrophages and under the physical and chemical conditions (low pH, high lactic acid, high CO_2) that are obtained in developing lesions. It is essentially affected by the humoral antibody response that it elicits, but it can survive for long periods in

macrophage that have been activated by the cellular immune response (Gibbons *et al.*, 1989).

Intracellular survival of *M. tuberculosis* appears attributable, at least in part, to inhibition of lysosomes effusion to the phagocytic vacuoles. This effect is caused by sulfatides in *Mycobacterium* cell wall. Disease manifestations result primarily from hypersensitivity to T.B. (Gibbons *et al.*, 1989).

2.4.2. Mode of Transmission

There are two sources of infection; the first is drinking infected milk and direct contact with infected cattle. Nowadays these sources are rare. The second one is human cases; this is common, usually the human type of *Mycobacterium* originates from an open cases. The spread of infection starts from the open human case when the patient disseminates a large numbers of bacilli in sputum and by coughing, speaking and grand sneezing. The bacilli may settle on the dust and infect people directly or indirectly via dried dust particulars. Sputum dried in hands, clothing, bed cloths, furniture or flowers may be broken, when dry, into fine dust and in the course of body movement and disturbance of these articles, it is inhaled by the victim (Gibbons *et al.*, 1989).

2.4.3. Pathogenesis

virulence of tubercle bacilli is due to its ability to survive within the macrophage rather than to the production of a toxic substance (Grange *et al.*, 1992). The mechanism of virulence is poorly understood and is almost certainly multifactorial. The immune response to the bacillus is of the cell – mediated type, which, if mediated by T helper cells, leads to protective

immunity but the presence of the cells facilitates tissue destruction. Hypersensitivity reactions lead to the progression of the disease process and the nature of the immune responses following infection changes with time human tuberculosis disease is divisible into primary and post-primary forms with quite different pathological features (Grange *et al*., 1992).

2.4.3.1. Post-primary tuberculosis

Post primary pulmonary tuberculosis presents a variety of appearances. Probably the earliest lesion is an aggregation of proliferative follicles progressively causing collapse and consolidation of alveoli. The wall lesion tends to remain relatively localized for a considerable time, confined to the lung apex by reason of fibrosis that accompanies the tuberculosis process. Thus it represents a compromised destruction and repair, it is a chronic proliferative process that tends to heal by fibrosis (Montgomery, 1965).

2.4.3.2. Miliary Tuberculosis

Miliary tuberculosis is the term applied to the presence of tubercles like small grey seeds throughout the tissues and organs of the body. They are believed to be the result of a relatively massive infection of the blood stream from an active focus or a related lymph node. It has been suggested that the development of miliary tuberculosis is a critical combination of massive bacillaemia together with a particular degree of hypersensitivity (Montgomery, 1965). Infection may enter blood directly or the blood stream may be infected from the recurrence, miliary tuberculosis may occur in tuberculous infection and it is particularly liable to happen in the course of primary lesion (Montgomery, 1965). During life, miliary tubercles may be observed on the cornea of the eye. Microscopically miliary tubercles is found

un the supporting tissue of the older, in the lungs, for examples, the follicles occupy the reticular framework and not the alveoli of the lung providing a streaking contrast with other form of pulmonary tuberculosis (Montgomery, 1965).

Miliary tuberculosis is always serious and leads to fatal complications tuberculosis infection because of the involvement of the meninges in tuberculous meningitis, a condition which before the introduction of specific therapy was invariably fatal and still has a considerable morbidity and mortality (Montgomery, 1965).

2.4.4. Epidemiology

The most frequent source of infection in humans are patients who excretes, particularly from the respiratory tract, large number of tubercle bacilli. connect (e.g., in the family) and massive exposure (e.g. in medical personnel) make transmission by droplet nuclei most likely (Brooks *et al*., 2004). The susceptibility to tuberculosis is a function of the risk of acquiring the infection and the risk of clinical appearance after infection has occurred (Brooks, *et al*., 2004). For the tuberculin negative person, the risk of acquiring tubercle bacilli depends on exposure to a source of infectious bacilli, principally sputum-positive patients. This risk with a proportionate to the rate of active infection in the population, crowding, socioeconomic disadvantage, and inadequacy of medical care, the development of clinical disease after infection may have a genetic component. It is influenced by age (high risk in infancy and in the elderly), by under nutrition, and immunological status, co-existing disease (e.g., silicosis, diabetes), and other individual host resistance factors (Brooks, *et al*., 2004).

Infection occurs at an earlier age in urban than in rural populations. Disease occurs only in small proportions of infected individuals. The incidence of tuberculosis is especially high in minority persons with HIV infections. Primary infection can occur in any person exposed to an infectious source. Patients who have had tuberculosis can be infected exogenously a second time. Endogenous reactivation of tuberculosis occurs most commonly among persons with AIDS and elderly malnourished or alcohol consumption (Brooks *et al*., 2004).

2.5. Laboratory diagnosis

The definitive diagnosis of tuberculosis is based on the detection of acid fast bacilli in clinical specimen by microscopy, cultural techniques or by the polymerase chain reaction (PCR) and it is various derivatives. Numerous attempts have been made to develop serological tests for the disease with little success (Greenwood *et al*, 2002)

2.5.1. Tuberculin Test

Cellular immunity and cell-mediated hypersensitivity to tuberculo-proteins develop during the course of tuberculous infection and contribute to both the pathology and the immunity of the disease (Kenneth *et al.*, 1984). The most studied antigens are heat stable proteins that are liberated into liquid culture media. The original preparation was termed tuberculin. Now, a purified protein derivative (PPD) of tuberculin is available for skin testing for hypersensitivity and is standardized according to skin test activity as tuberculin units. Hypersensitivity does not result from repeated PPD injections in those who are skin test negative. It is now recognized, the degree of hypersensitivity may be some what increased for a few weeks in those already allergic (Kenneth *et al.*, 1984).

2.5.2. Specimens

The most usual specimen of diagnosis of pulmonary tuberculosis is sputum but, if non is produced, bronchial washings, brushings or biopsies and early morning gastric aspirate (to harvest any bacilli swallowed overnight) may be examined. Tissue biopsies are homogenized by grinding for microscopy and culture. Cerebrospinal fluid (CSF), pleural fluid, urine and other fluids are centrifuged and the deposit examined (Greenwood, *et al*, 2002).

2.5.3. Microscopy

Use is made of the acid fast property of Mycobacteria to detect them in sputum and other clinical material. In the Ziehl Neelsen (ZN) staining technique, heat fixed smears of the specimens are flooded with a solution strong carbol fuchsin (a mixture of basic fuchsin and phenol) and heated until steam rised. After washing with water, the slide is flooded with diluted mineral acid (e.g., 3% hydrochloric acid) and after further washing, green or blue counter stain is applied. Red bacilli are seen against a contrasting background color. Fluorescence microscopy based on the same principle of acid fastness, is increasingly used and is much less getting the microscopist. Modifications of the various staining techniques are used to examine tissue sections (Greenwood *et al.*, 2002).

2.5.4. Culture

As sputum and certain other specimens frequently contain many bacteria and fungi that would rapidly overgrow any mycobacterium in the culture media, these must be destroyed. Decontamination methods make use of the relatively high resistance of mycobacteria to acids, alkalis and certain disinfections. in

the widely modified Petroff methods, sputum is mixed well with 4% sodium hydroxide for 15 to 30 minutes, neutralized with1N hydrochloric acid with phenol red and inoculated into LJ or a similar media. Specimens such as CSF and tissue biopsies, which are unlikely to be contaminated, are inoculated directly onto culture media. As an alternative to chemical decontamination, mixtures of antimicrobial agent that kill fungi and all bacteria other than mycobacteria may be added to the culture media. Inoculated media are incubated at 35°C to 37°C and inspected weakly for at least 8 weeks. Cultures of material from skin lesions should also be incubated at 33°C, then bacterial growth is stained by the ZN method and bacilli are detected they are acid fast, it is subcultured for further identification. A more rapid bacteriological diagnosis is achievable by use of commercially available automated systems. Systems that detect color changes in dyes induced by the release of carbon dioxide, on the unquenching of fluorescent dyes on the consumption of oxygen by metabolizing bacilli, have replaced the earlier radiometric method. The first step in identification is to determine wether an isolate is a member of *M. tuberculosis* complex, these organisms grow slowly, don't produce yellow pigment, fail to grow at 25°C and 41°C and don't grow on egg media containing para nitrobenzoic acid (500 mg/L). Strains differing in any of these properties belong to other species.

2.5.5. Biochemical Tests and Morphological Features

There are various kinds of biochemical tests and morphological features for identification of mycobacteria. Based on pigment production, mycobacteria are classified into three groups: photochromogens, scotochromogens and nonchromogens. Photochromogens produce pigmented colonies in the light.

Scotochromogens produce pigmented colonies when grown in the dark. Nonchromogens are nonpigmented in both light and dark, but only have light or buff-coloured colonies (Geo. F. Brooks, 2001; Ve'ronique VBincent, 2003). Pigmented mycobacteria are classified as nontuberculous mycobacteria (NTM), because M. tuberculosis does not produce pigments (Vincent *et al.*, 2003).

Growth rate, colony morphology, and biochemical tests are other important differentiating factors among mycobacteria. Mycobacteria that grow in less than 14 days are called rapid growers, and those that grow after 14 days are called slow growers. Examination of the morphology of colonies is important especially in mixed cultures. After 15 days growth, MTB produces thin, nonpigmented, rough colonies on 7H11 agar.

Biochemical tests also aid in the identification of MTB from NTM. TB has the ability to reduce nitrate to nitrite. Other tests such as production of catalase or urease, arylsulfatase test, iron uptake, tween hydrolysis, tellurite reduction, and positive niacin test, aid in the diagnosis of tuberculosis (table 2-4)
(Vincent *et al.*, 2003).

2.5.5.1. Niacin accumulation test

Nicotinic acid or niacin is produced by all mycobacteria, but some species, such as *M. tuberculosis*, *Mycobacterium simiae* and *M. bovis* BCG, excrete it due to a blockade in their scavenging pathway. The excreted niacin accumulates in the culture medium and is evidenced in the presence of cyanogen halide with a primary amine. Niacin-negative *M. tuberculosis* strains are extremely rare (Vincent *et al.*, 2003).

2.5.5.2. Nitrate reduction test

This test is particularly useful for differentiating *M. tuberculosis*, which gives a positive reaction, from *M. bovis*, which is negative (Vincent *et al.*, 2003).

2.5.5.3. Catalase test

Catalase is an intracellular enzyme that transforms hydrogen peroxide to oxygen and water. At 68°C catalase is a heat-tolerance test measuring catalase activity at high temperature. Characteristically, *M. tuberculosis* gives negative results, as do other species in the *M. tuberculosis* complex (Vincent,*et al,.2003)*

Table 2-4. Biochemicalcharacteristics for distingish species of the genus *Mycobacterium* (Sommer,1987).

Organism	Niacin test	Nitrate reduction	Catalase (68°C)	Tween hydrolysis	Arysulfatase	Urease
M. tuberculosis	+	3- 5 +	-	-/+	-	+
M. africanum	V	V	-	-	-	+
M. bois	V	-	-	-	-	+
	-	-	+	-	-	-
M. kansaii	-	1-5 +	+	+	-	+
M. marinum	V	-	-/+	+	-/+	+
M. simiae	+	-/+	+	-	-	-
M. gordonae	-	-	+	+	-	-
M. xenopia	-	-	+	-	+/-	-
M. intracellulare ovium complex	-	-	+	-	-	-
M. fortuitum	-	2-5+	+	+/-	+	+

2.5.6. Nucleic acid technology

Nucleic acid probes for the identification of *M. tuberculosis* complex and, specifically, *M. tuberculosis*, and also for certain other species are commercially available. They are not sensitive enough to detect mycobacteria in clinical specimens and are used to identify *mycobacteria* cultivated by conventional techniques. Amplification of specific nucleic acid sequence is achievable by PCR and related techniques.. Problems of low sensitivity, false positive reactions and cross contamination have largely been overcome by the

introduction of closed – system, isothermal technique for amplification of species. Specific 16s ribosomal RNA. Most members of *M. tuberculosis* complex contain 1– 20 species of the insertion sequence *IS 6110*, which has been used to develop DNA fingerprinting methods for epidemiological purposes. Alternatively for detection of spacer oligonucleotides, short DNA sequence found around the site of the insertion sequence, are useful for typing isolates (spligotyping) (Greenwood *et al* , 2002).

2.5.7. Drug Susceptibility Testing

Several methods have been described: the first is the resistance-ratio method in which test strains and susceptible controls are inoculated on sets of LJ medium containing double dilution of drugs. The results are expressed as the ratio of the drug. concentration inhibiting the test strain to that inhibiting the control strain. Susceptible strains have ratios of one or two while higher ratios indicate resistance (Greenwood *et al.*, 2002). The second are the radiometric or non-radiometric automated systems which provide results more quickly and finally nucleic acid technology, which is even more rapid in this method, commercial kits are able to detect about 95% of mutations to rifampicin resistant caused by mutations in the *rpoB* gene. (Greenwood *et al*, 2002).

2.6. Prevention and control

- Careful follow up of their contacts with tuberculin test, X-rays, and appropriate treatment and the main stay of public health tuberculosis control.
- Drug treatment of asymptomatic tuberculin positive persons in the age groups most grown to develop complications (e.g., children) and in tuberculin positive persons who must receive immunosuppressive drugs greatly reduces reactivation of infection.

- Individual host's resistance: non-specific factors may reduce host resistance, thus favoring the conversion of asymptomatic infection into disease. Such factors include starvation, gastrectomy, and suppression of cellular immunity by drugs (e.g.; corticosteroids) or infection. HIV infection is a major risk factor for tuberculosis.
- Immunization: various living avirulent tubercle bacilli, particularly BCG (Bacillus Calmette and Guerin, an attenuated bovine organism), have been used to induce a certain amount of resistance in those heavily exposed to infection. BCG is given to children in many countries. Statistical evidence indicates that an increased resistance for a limited period follows BCG vaccination.
- The eradication of tuberculosis in cattle and the pasteurization of milk have greatly reduced *M.bovis* infections (Brook *et al*., 2004).

2.7. Vaccination

Bacillus Calmette Guerin (BCG) is a live attenuated vaccine derived from strain of *M.bovis* by repeated subculture between the years 1908 and 1920. This species was selected rather than *M. tuberculosis* on the assumption that was of limited virulence in man. The vaccine was originally given orally to neonates but is now given by intracutaneous injection. The protective efficacy of BCG varies enormously from country to country.

Being a live vaccine, serious infections and even disseminated disease may occur in immunocompromised persons. BCG should never therefore be given to persons known to be HIV positive (Greenwood *et al*., 2002).

2.8. Prophylactic Chemotherapy

In true Prophylactic chemotherapy, drugs are administered to infected an individuals who are in unavoidable contact with a patient with open

tuberculosis. The main example is a baby born to a mother with the disease. More usually, it refers to therapy, principally with isoniazide alone, given to an individual, who have been infected but shows no signs of the active disease. short (2 to 4 months) regimens of rifampicin with isoniazide or pyrazinamide give short or medium term protection to tuberculin positive, HIV positive persons (Greenwood *et al*., 2002).

2.9. Treatment

The anti-tuberculosis drugs are divisible into 3 groups:

- Bacteriocidal drugs that effectively sterilize tuberculous lesion.
- Bacteriocidal drugs that only kill tubercle bacilli in certains situations. Streptomycin is ineffective against bacilli within macrophage and acidic inflammatory tissue, and isoniazide kills bacilli only if they are replicating.
- Bacteriostatic drugs, which are of limited usefulness and are not included in standard drug regimens.
- Mutation to drug resistance occurs at a rate of about one mutation every 10 cell divisions. Successful therapy requires the prevention of the emergence of drug. resistant strains by the simultaneous use of at least two drugs to which the organism is sensitive. The earlier two year regimen of streptomycin, isoniazide and P.aminosalicylic acid has been replaced by much more acceptable orally administrated regimens based on an initial intensive two months phase and a 4 to 6 months continuation phase depending on WHO treatment recommendations

- The response to therapy of drug-susceptible tuberculosis is divisible into 3 phases:
- During the first week or two, a large number of actively replicating bacilli in cavity walls are killed, principally by isoniazide, but also by rifampicin and ethambutol. The patient rapidly ceases to be infectious and hospitalization with barrier nursing is now rarely necessary.
- The following few weeks the disactive bacilli within macrophage, caseous material and dense acidic inflammatory lesions are killed by rifampicin and pyrazinamide.
- In the continuation phase any remaining dormant bacilli are killed by rifampicin – resistant mutant that starts to replicate is killed by isoniazide. The emergence of a drug-resistant strain (acquired resistance) during adequately supervised short course chemotherapy is uncommon. And most relapses are due to drug-sensitive bacilli (Greenwood et al., 2002).

2.10. Multi-drugs resistant (MDR)

Multi-drug resistant strains are defined as those resistant to isoniazide and rifampicin, They are sometimes also resistant to other drugs and constitute aserious problem. The regimens for multidrug-resistant tuberculosis are based on in-vitro drug susceptibility tests Useful agents include the newer

floroquinlones and macrolides and amoxycillin with a beta lactamase inhibitor such as sulbactam. Mortality rate in patients infected with MDR strains high, but with a high level of care can be reduced to around 15% concurrent infection of such patients with HIV provide a poor prognostic factor (Greenwood *et al.*, 2002).

3. MATERIALS AND METHODS

3.1. Study Design

3.1.1. Study Area

This Study was conducted during the period from November 2008 to May 2009, at the National Health Laboratory (Stack), Tuberculosis Reference Laboratory, Khartoum State.

3.1.2. Type of Study

Descriptive, cross-sectional laboratory based study.

3.2. Study population

Specimens were collected from 100 patients who were known to have TB infection,at Alshaab Teaching Hospital, Abu-Anga Hospital and the National Health Laboratory

3.3. Inclusion Criteria

Only patients who were positive with Ziehl-Neelsens were included in this study. Clinical data were collected by medical technicians using structured questionnaire after informed concent..

3.4. Ethical Consideration

All patients involved in this study participated after awritten agreement and All data were kept secret.

3.5. Collection of Specimens

One hundred (100) sputum specimens were collected randomly according to WHO criteria (1996) from TB patients at the tuberculosis reference laboratory (National Health Laboratory), ElShaab Teaching Hospital and Abu-Anga Hospital in Khartoum State. Sputum specimens were collected in wide-mouthed, screw-capped, plastic sputum containers and processed immediately in the Tuberculosis Reference Laboratory.

3.6. Equipment, plastic and glassware

Incubators (Fisher Scientific, USA), Olympus microscope (Olympus Co., Takio), safety cabinet class II for culture (Heraeus, Germany), glass

microslide with cut edges (Jisico, china), pH meter, plastic sputum containers (40 ml), vortex mixer, universal bottles, disposable sterile Appendorf tubes, plastic disposable pipettes, plastic disposable conical tubes (50 ml), (Cornindny Ecorparated, Canada) inspissator (Jisico China), ovens (Gallerhamh, England), refrigerators (Lec, Tokyo),and UV trnasilluminator (Biometry, Germany).

3.7. Disinfection and Sterilization

3.7.1 Flaming

Flaming was used to sterilize slides, cover slips and glass rods.

3.7.2. Red heat

Was used to sterilize wire loops by holding them over Bunsen burner flame until red.

3.7.3. Hot Air Oven

It was used to sterilize metals, glass ware such as test tubes, graduated pipettes and flasks. The holding period was one hours and the temperature was 160°C.

3.7.4. Moist heat (autoclaving)

Media, solutions and plastic ware were sterilized by autoclaving at 121°C (15 $Lb/inch^2$) for 15 minutes.

3.7.5. Irradiation

Ultraviolet irradiation for 20 minutes was used to sterilize the media pouring room.

3.7.6. Disinfectants

Phenol disinfectant and 70% alcohol were used for disinfecting the floor and working bench in the laboratories

3.8. Staining (Ziehl – Neelsen's stain)

Ziehl-Neelsen (ZN) stain was prepared and used to stain smears from clinical materials and from cultures (IUATLD, 1998) (Appendix I).

3.9. Medium for isolation: (Lowenstein Jensen medium)

Modified Lowenstein- Jensen (LJ) medium was used for the cultivation and differentiation of *Mycobacterium* species, specially *M. tuberculosis* and *M. bovis* (IUATLD, 1998), (AppedixII).

3.10. Decontamination of Sputum Specimens

Sputum was decontaminated by modified Petroff method without centrifugation (Mackie and McCartney, 1996) (AppendixIII).

3.11. Biochemical Testing

Selected biochemical tests were performed for the identification of the isolates. All the tests were done according to WHO criteria (1998) (Appendix IV).

3.12. Drug Susceptibility Testing

Susceptibility testing was performed by LJ proportion method which was described by (IUATLD, 1998) (Appendix V).

4. RESULTS

4.1. Epidemiological findings

4.1.1. Gender

Among the study population (100 patients), who showed AFB and had pulmonary infection 67% were found to be males, while 33% were females (Fig. 1).

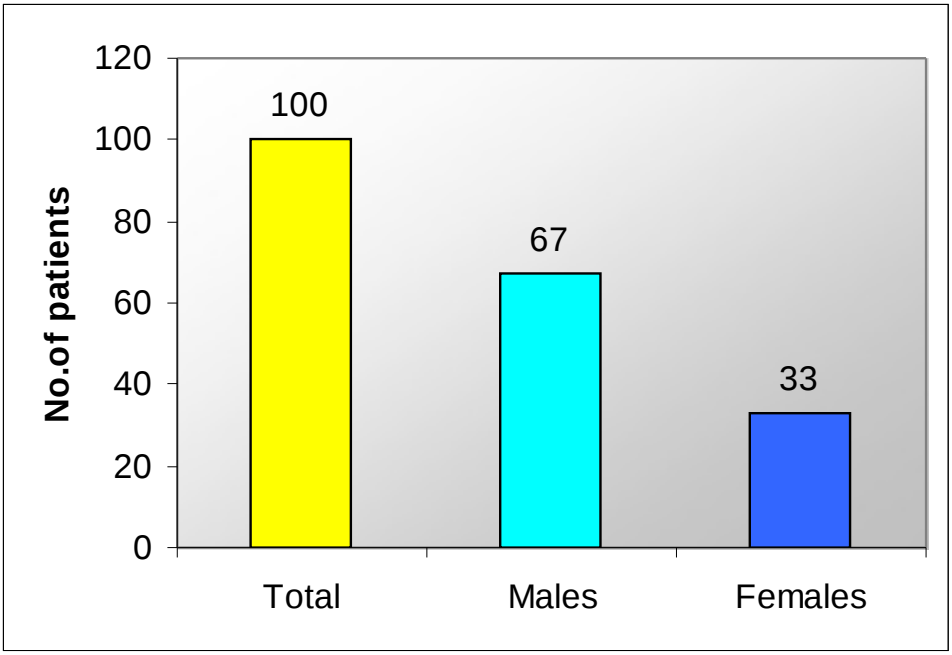

Fig. 1. Distribution of patients with suspected pulmonary TB according to gender

4.1.2. Age groups

All the ages were found to be affected with tuberculosis. highest frequency was among age group 21 – 50 years 72%, followed by age group 11

– 20 years 16% while The Lowest frequency was among age group 0 – 10 years 4% and among patients >5o years of age 8% (Fig. 2).

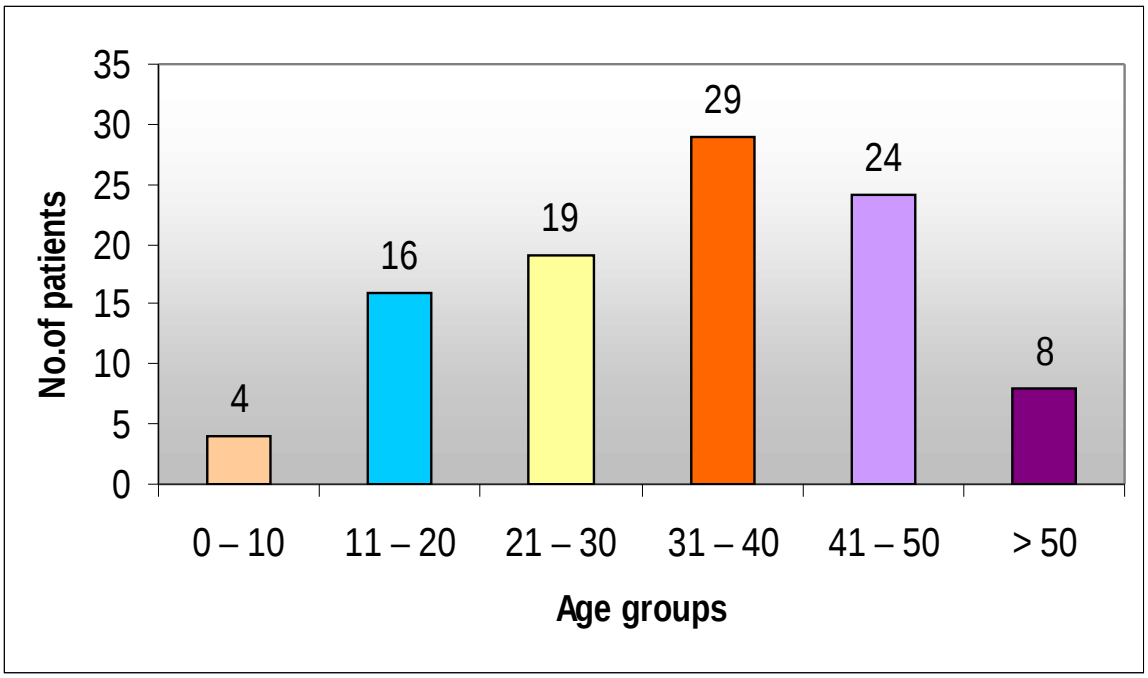

Fig. 2. Correlation between number of patients and age groups

4.1.3. Occupation

Among the enrolled patients 29% were found to be labourers, 18%were People with marginal jobs, 12% were housewifes, 11% were

students, 10% were officers, 9% were farmers, 4% were teachers and 7% were unemployed (Fig. 3).

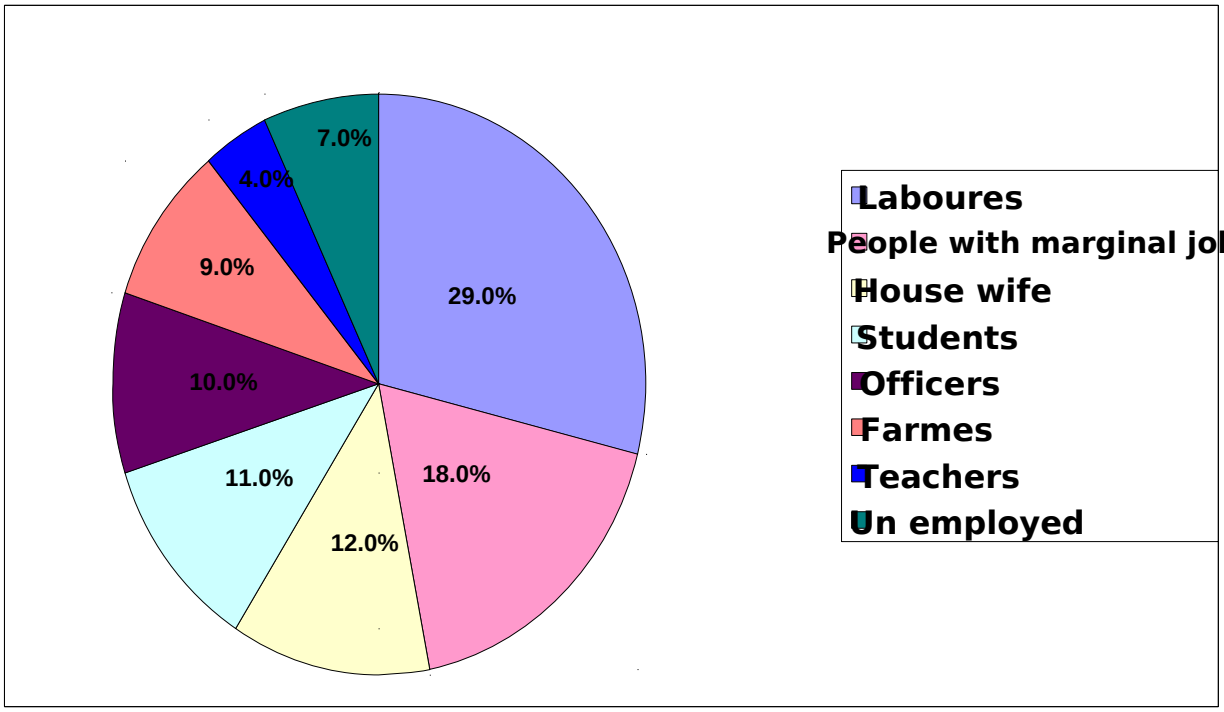

Fig. 3. Occupation of study population

4.1.4. Tribes

Fig 4.shows prevalence of tuberculosis according to tribes. Members from Deinka tribe were found to have highest prevalence rate 27%, followed

by Nuba 20%, Foar 15%, Gaalieen 12%, Gawamma 10% and fallata 9%, while rate other tribes had a low prevalence 7%.

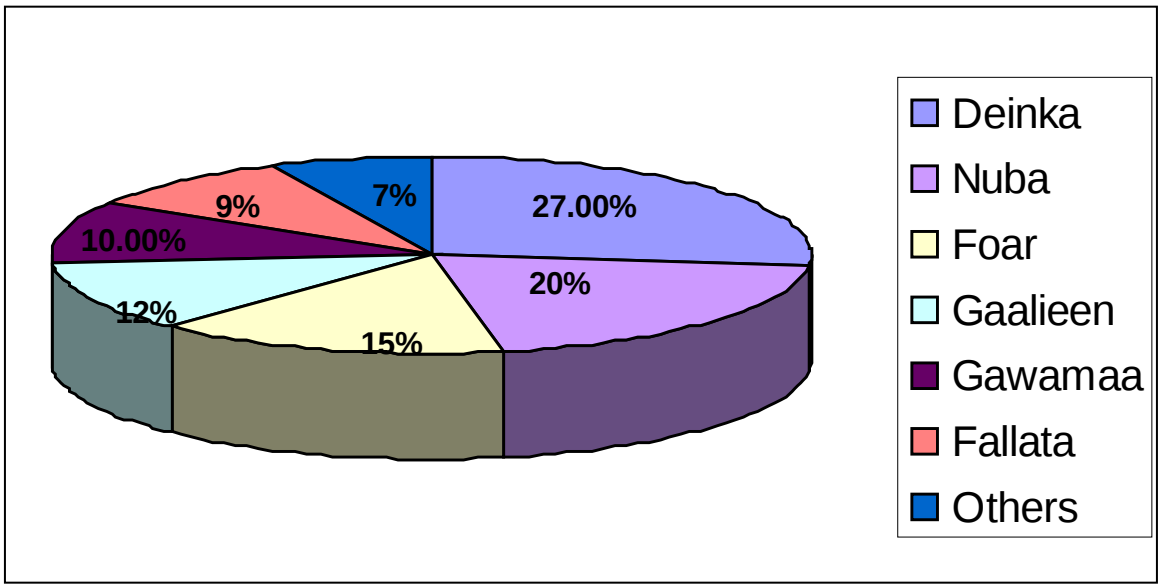

Fig. 4. Percentages of patients according to their tribes

4.1.5. Treatment status

The majority of the patients 30% were new cases, followed by previously treated cases 27%, relapses 22% and treatment failure 21% as shown in Fig. 5.

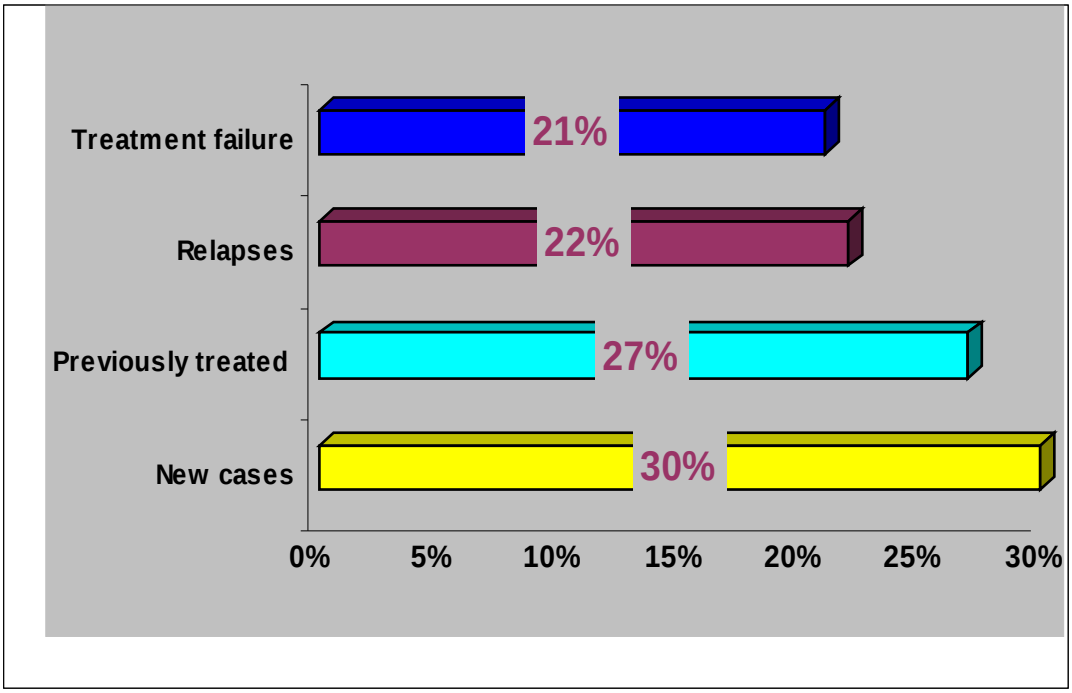

Fig.5. Different treatment statuses among enrolled patients

4.2. Isolation and identification

4.2.1.Isolation

From collected 100 sputum samples 83 % showed *Mycobacterium tuberculosius* complex like colonies in lowenstein Jensen medium, 10% were considered as rapidly growing, and 7% samples showed no growth. All isolates that showed growth were confirmed as acid fast bacilli by Zn staining (indirect smear). *Mycobacterium tuberculosis* complex like colonies were further confirmed by biochemical test (Fig. 6).

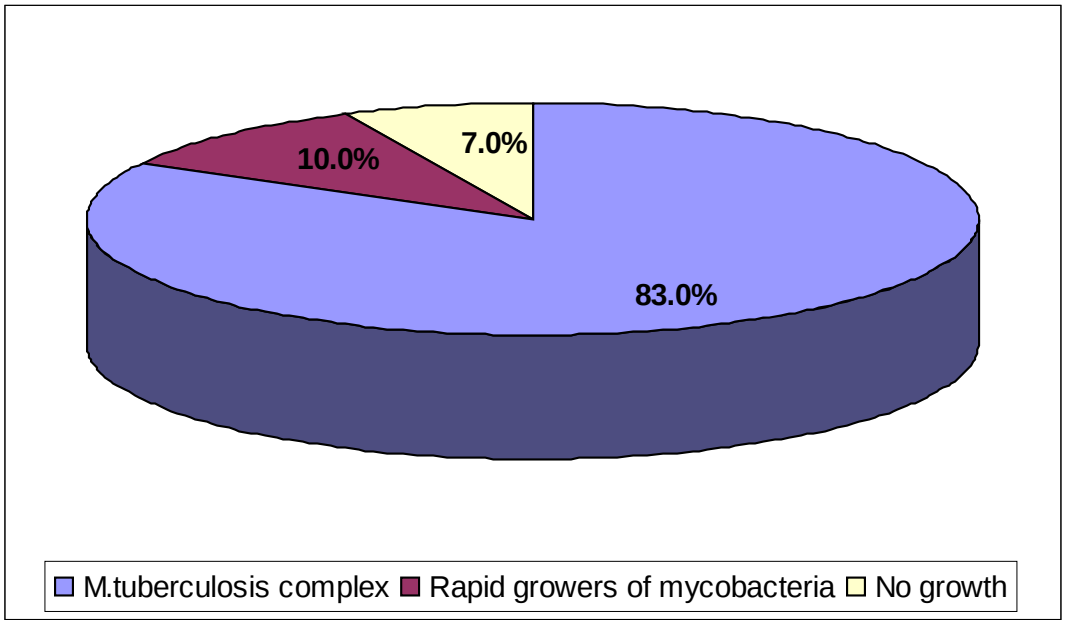

Fig. 6. Percentage of MTC and other isolates

4.2.2. Microscopic examination

Indirect Ziehl-Neelsen stain was done for all suspected growth. The results confirmed the appearance of acid fast bacilli (Fig. 7).

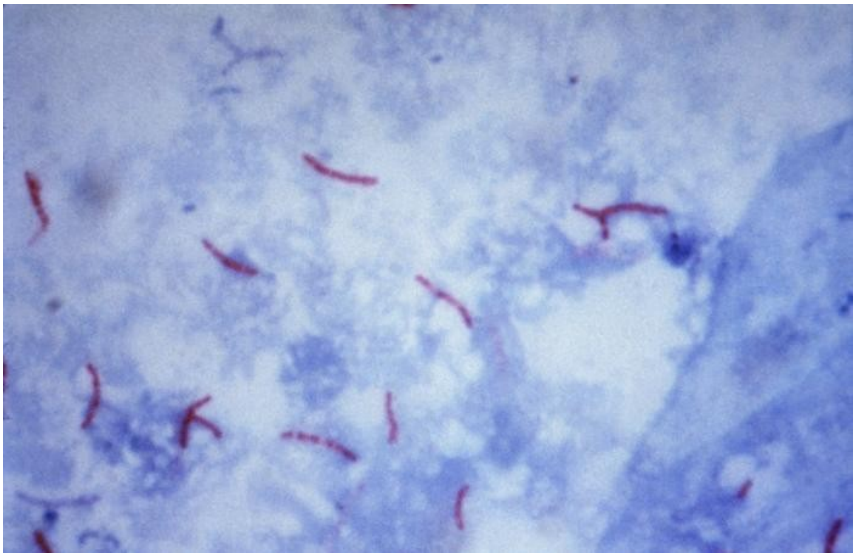

Fig. 7. Mycobacterium tuberculosis bacilli stained by Ziehl Neelsen stain (x100)

4.2.3. Culture and morphological characteristics

The growth rate of the isolates ranged between 3 days up to 8 weeks, most of the isolates showed visible growth after 2 weeks, 10 out of 100 isolates showed visible colonies within 3 days after inoculation, hence they were identified as rapid growers, while the growth rate of 83 isolates ranged between 2 to 5 weeks and they were identified as slow growers belonging to *Mycobacterium tuberculosis* complex species. Culture properties of the isolates on Lowenstein Jensen medium showed typical appearance of *Mycobacterium tuberculosis* complex as dry rough, raised tough and cream buff coloured (Fig. 8).

Fig.8 Four Weeks Growth of *Mycobacterium tuberculosis* on L.J medium

4.2.4. Biochemical tests

Selected biochemical tests were performed; these tests included nitrate reduction, catalase production and growth in thiophane carboxylic acid hydrazid and para-nitrobenzoic acid (Fig. 9 and 10). The results of these tests were listed in table 4-1.(Appendix VI).

Fig.9. Nitrate reduction test for *Mycobacterium tuberculosis* complex organism from left to right. Purplish red = 5+, deep red = 4+, red = 3+, deep pink = 2+, clear pink = 1+, no color = negative.

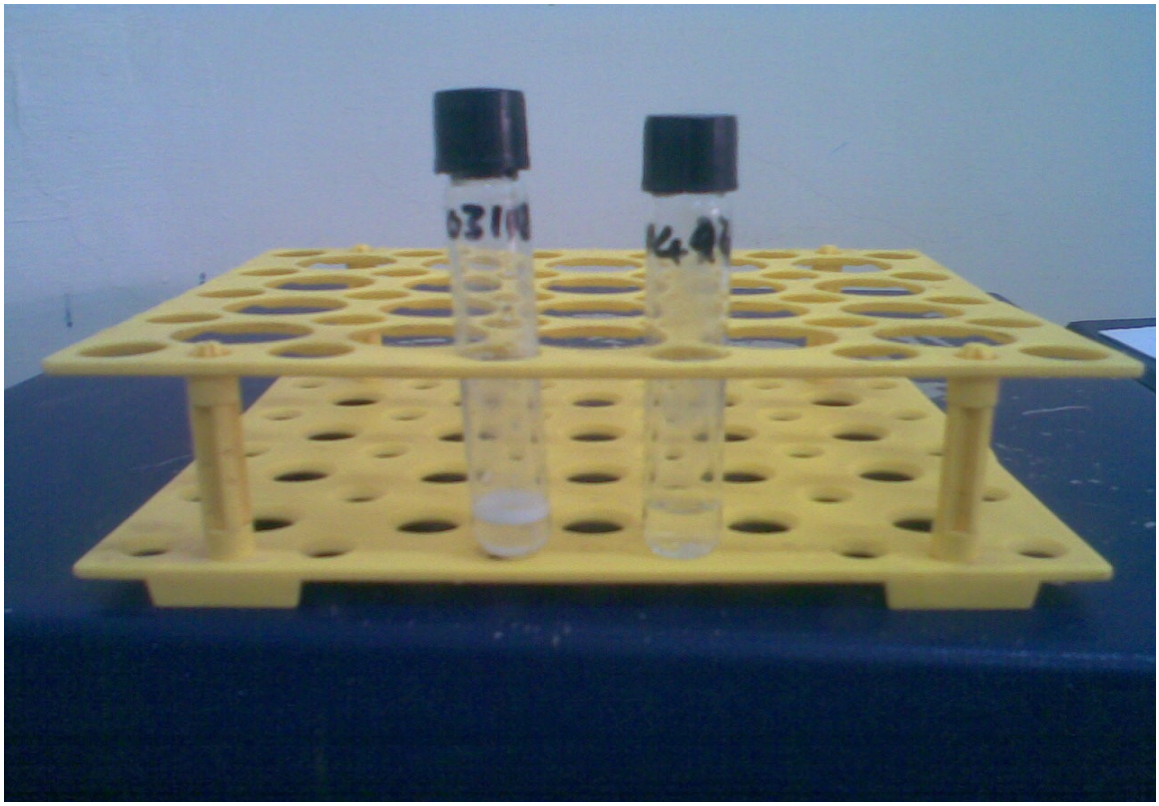

Fig. 10 Catalase test for MTC from left to right positive at room temperature and negative at 68°C

4.3. Drug susceptibility test

Eighty three isolates were subjected to drug susceptibility test using the proportion method, 38% of the isolates showed growth in INH, 24% of the isolates showed growth in RIF, 37% isolates showed growth in STM and 29% isolates showed growth in ETH, Three percent of the isolate showed growth in INH + RIF were resistant to different combination of three drugs, INH + RIF + STM 4%, INH + RIF + ETH 1%, INH + STM + ETH 2%, RIF + STM + ETH 1%, 22% isolates were resistant to four drugs and 36% were sensitive to the four drugs (Fig. 11).

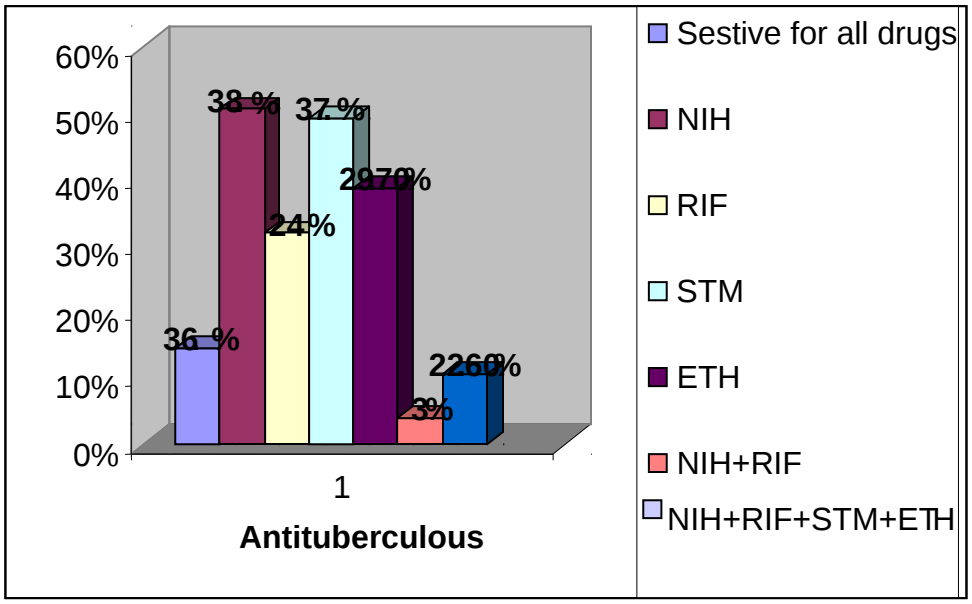

Fig.11. Senstive and Resistance of MTBC isolates to TB empirical drugs

5. DISCUSSION

Tuberculosis is an important public health problem, and the frequency of drug resistance is increasing, a major reason for the development of resistant infections and relapses is poor compliance with medical regimens (Stephen, *et al*., 1992). The increasing global burden of tuberculosis (TB) is linked to human immunodeficiency virus (HIV) infection (Elizabeth, *et al*., 2003).

Multi-drug resistant tuberculosis becomes a major threat that increase day by day and it's prevalence is still unknown in Sudan and Some other countries, so new drugs are required for treating multi-drug resistance.

The aim of this study was to isolate and identify *Mycobacterium tuberculosis* complex organisms to determine the frequency of multi-drug resistance among patients with pulmonary tuberculosis in Khartoum State, Sudan.

Out of 100 AFB positive sputum samples 83 isolates of MTC were obtained, 10% were considered rapidly growers and 7% samples showed no growth. From the epidemiological findings in this study it was found that number of TB infected males was significantly higher than that of the females, 67% versus 33%, similar results were reported by El Eragi (2004) who found that 66.7% of cases were males and 33.3% were females, and also Mogahid (2007) who found that 73.3% were males and 27.7% were females. This findings are in accordance with that of Bellamy, *et al*., (2002) who suggested that an X chromosome susceptibility gene contributes to high susceptibility of males to TB than females as observed in many different populations The gene in the X chromosome becomes dominant when joined with the Y chromosome and recessive when joined with the X chromosome. Moreover, according to job type, males are more exposed to the source of infection than females.

Regarding the correlation between the age of patients and infection rate, it was found that young patient between 21–50 years were the most affected (72%) similar results were obtained by El Eragi (2004) and Mogahid (2007). Murnay, Styblo and Rouillon (1990) postulated that almost 80% of TB patients were living in developing countries and most of these were with an age less than 50 years.

This study showed that TB is distributed among different occupation group with high prevalence in labours (29%), followed by people with marginal jobs (18%) , housewifes (12%), students (11%), officers (10%), farmers (9%), unemployed (7%) and teachers (4%). These findings agree with the statement that economic status plays a major role in TB morbidity, Other factors such as overcrowding of poor, ill-nourishment people, racial differences and in availability of health care (Lowell, 1976).

The ethnic study play a role that TB infection is distributed among different tribes with different rates Most patients were from outside of Khartoum State and ethnic study showed correlation between patients and geographical areas. Patients from Deinka tribe constituted 27% of cases, followed by Nuba (20%) and Foar (15%), where as patients from other tribes were fewer in number (5%). The number of patients from tribes of Western Sudan and Southern Sudan were the highest. This may be attributed to the poor socioeconomic status in these area. Nearly similar results was reported by El Eragi (2004) found that most cases were from economically poor areas, mainly from Deinka tribe (12.9%), Gaalieen (12.7%) and Nuba 9 (10.9%), but close results were obtained by Mogahid (2007) who found that patients from Deinka tribe constituted 20%, followed by Nuba (16%) and Foar (9%).

All 83 isolates of *Mycobacterium tuberculosis* complex subjected to catalase, nitrate, PNB, TCH testing gave results nearly similar to WHO criteria (1998). The result of antibiotics susceptibility testing revealed that (36 %) of the isolates were sensitive to the four drugs used (INH, RIF, STM AND ETH), 38% of the isolates were resistant to INH, 24 % were resistant to RIF, 37 % were resistant to STM and 29 % were resistant to ETH . Three percent of The isolates were resistant to two drugs, INH and RIF,There was resistance s to different combination of three drugs, INH + RIF + STM (4%), INH + RIF + ETH (1%), INH + STM + ETH (2%), RIF + STM + ETH (1%) and 22% were resistant to the four drugs. This result was agreed with Ariel Pablos, *et al*., 1998 who found 7.3% resistant to INH, 1.8% resistant to RIF, 6.5% resistant to STM, 1.0% resistant to ETH and 13% resistant to the four drugs. The median rate of drug resistant reported by David, *et al*., (1997) who found that 10.6% resistant to (INH), 2.4% resistant to RIF, 4.9% resistant to STM, 1.8% resistant to ETH. The highest resistance was against INH in both studies, followed by STM (4.9%), RIF (2.4%), then ETH 0.8% in David study, and in Ariel Pablos study (1998) 6.5% resistant to STM, 1.8% resistant to RIF, 1.0% resistant to ETH, but in present study followed by STM 37%, ETH 29%, then RIF 24%. In generally the difference between these findings and previous studies may be due to the relative small sample size and environmental variations .

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

On the basis of the results obtained,in the present study conclud the
: following

- All age groups, occupations, both sexes, and different tribes were found to be affected with different rates.
- The age between 21–50 years, male patients and people from poor economic and health status are at high risk.
- Failure to control the infection of patients with multi-drugs resistance lead to high mortality and ominous implication for the public health.
- only (36%), isolate were sensitive to the four drugs,
- (38%) isolates were resistant to INH, (24%) isolates were resistant to RIF, (37%) isolates were resistant to STM and (29%) isolates were resistant to ETH,
- (3%) isolates were resistant to INH and RIF.
- Resistant to INH + RIF + STM (4%) , INH + RIF + ETH (1%) , INH + STM + ETH (2%) , RIF + STM + ETH (1%) .
- (22%) isolates were resistant to the four drugs.

6.2. Recommendations

- Culture method is more sensitive and specific than smear method in diagnosis tuberculosis identification, so it is recommended culture should be made for all positive acid fast bacilli samples beside the smear method.
- The rate of tuberculosis increases in patients with HIV, so that HIV infected patients should be counseled to avoid exposure to patient with tuberculosis.

- Due to the increased resistance pattern early diagnosis and detection of resistant strain is so vital.
- Patients with tuberculosis should be isolated from community and the treatment should be followed up to ensure the compliance of medical regimens.
- Drug-resistant case, each year should consider additional measure to prevent transmission, including more stringent administrative measures, periodic tuberculin testing, improved ventilation.

REFERENCES

Ananthanayanan, R., Picker, J.C-K(1990). Text Book of Microbiology 4th ed. Orient, Longman, India p. 341-360.

Bellamy, R., Betyers, N., McAdam, K., Ruwende, C., Gile, Samaai, P., Bester D., Meyers, M, Corrah T, Collin, M., Camidge, D., Willkinson, D., Hoal-va Helden, E., Whittle, H., Amos, W., van Helven,P., and Hill, A. (2002). Genetic susceptibility of tuberculosis in Africans: a genome – wide scan. J. Proc.Natl.Acad.Sci.97: p.8005-8009

Betty A.F., Daniel F.S., and Alice S.W. (1998). Diagnostic microbiology, 10th ed. Mosby, London p. 715 – 718.

Bloom B.R., and Murray J.L. (1992). Tuberculosis: commentary on an emergent killer. Science 257: p. 1055 – 1064.

Brooks G.F., Butel J.S., Morse S.A. (2004). Medical microbiology. 23rd edition, McGraw-Hill Companies, inc p . 326 – 329.

Carter G.R., Claus G.W., and Rikihisa Y. (1986). Mycobacteria. In: Essentials of veterinary bacteriology and mycology, 3rd edition, Lea and Febiger, Philadelphia p . 185 – 192.

David L.C, Flavia B., Mario C.R. (1997). Drug-resistant tuberculosis: Review of the worldwide situation and the WHO/IUATLD Global surveillance project, the University of Chicago Press, 24.

El-Eragi A.M. (2004). Molecular characterization and drug resistance patients of Mycobacterium tuberculosis isolated from Khartoum State. PhD. Thesis.

Elizabeth L. Corbett, Catherie W. N., Denmotmaher B.G., Williams M.C, Raviglione C.D . (2003). The growing burden of tuberculosis, global tends and interaction with the HIV epidemic archives of internal medicine. 163 (9).

Gibbons N.E., and Buchanan R.E. (1989). Medical bacteriology, 9th edition, Williams and Wilkin Company p. 172 – 173.

Grange J.M. (2002). *Mycobacterium*. In: David, Greenwood, Richard C.B., Slack John F.P. (editors). Medical microbiology, 16th ed., Churchill Livingstone.

Grange J.M., Greenwood D.G., Slack R.C., and Peuther J.F. (1992). Mycobacterium in medical microbiology. A guide to microbial infections: pathogenesis, immunity, laboratory diagnosis and control. 14th edition. Churchill Livingstone, New York p . 241 – 252.

Greenwood D.G., Slack R.C., and Peuthere J.F. (2002). Medical microbiology, 6th edition, Elsevier inc p . 200 – 201.

Greenwood D.G., Slack R.C., Peuther J.F. (2002). Medical microbiology, 6th edition. Elsevier, inc 2002 (part I) p. 2000 – 2001.

International Union Against Tuberculosis and Lung Disease (1998). Guideline for surveillance of drug resistance in tuberculosis. WHO, Geneva/IUATID Pairs.int.j.tuberc.lung.,Dis 2: p. 72-89.

Kenneth J.R ., John C.S., Geonge C.R, James J.P., Lawrence C., John S., Murnay R.R. (1984). Medical microbiology an introduction to infectious disease. Clearance Center, inc p . 291 – 294.

Kubica T., Rusch-Gendes S. and Niemann S. (2003). *Mycobacterium bovis* subsp. Caprae cause one-third of human *M. bovis* –associated tuberculosis cases report in Germany between 1999 – 2001. J. Clin. Microbiol., 41: p. 3070-3077.

Levinson W.N., and Jawetz E.T. (2002). Medical microbiology and immunology. 7th edition, McGraw-Hill Companies, inc p . 144 – 145.

Liebana E., Aranaz A., Francis B. and Cousin S.D. (1996). Assessment of genetic markers for species differentiation within the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol., 34: p . 433 – 938.

Lowell A.M. (1976). Tuberculosis in world. DHEW publication. No. DC 76-8317. Washington, Dc, US Government Printing Office. 3 -27.

Mackie and McCantrey (1996). Practical medical microbiology, 14th edition. Churchill Livingstone p . 329 – 337.

Metchock B.G., Nolte F.S., and Wallace R.J. (2003). Mycobacterium. In: Murray P.R, Baron E.J., Jorgensen D.H., Pfaller M.A., and Yolken R.H. (ed.). Manual of Clinical microbiology, 18th edition p . 532 – 584.

Mogahid M.E. (2007). Homologous bacterial *Proteinsim nocardia*: A proteonic study of *Nocardia Africana*, *Nocardia africanica* and *Nocardia asteroids*. PhD thesis.

Montgomery G.L. (1965). Textbook of pathology, 3rd edition. E and S Livingstone LTD p . 33 -36.

Murray C.J., Styblo K., and Rouillon A. (1990). Tuberculosis in developing countries: burden, intervention and cost. Bulletin of IUATLD . 62: p .6 -24.

Murray P.R., Rosenthal K.S., and Pfallen M.A. (2002). Medical microbiology. 5th edition, Elsevier inc p. 296 – 297.

Niemann S., Harmsen D., Rusch G., Endes S., and Richard E. (2000). Differentiation of clinical *Mycobacterium tuberculosis* complex isoales by gynB DNA sequence polymorphism analysis. J. Clin. Microbiol., 38 :p . 3231 – 3234.

Niemann S., Kubica T., Bange F.C., Adjei O. E., Chinbuah M.A., Diel R., Gyapong J., Horstmann R.D, Jobaa and Rusch-Gender S. (2004).

The species *Mycobacterium africanum* in the light of new molecular markers. J. Clin. Microbiol, 42: p.3958 – 3962.

Ocepek M., Pate M., Zolnin-Dave M., and Poljak M. (2005). Transmission of *Mycobacterium tuberculosis* from human to cattle. J. Clin. .Microbiol p . 3555 – 3557

Smith R.M., Drobniewski F., Gibson A., Montague J.D., Logan M.N., Hunt D., Hewinson G., Salmon R.L. and O'Neill B. (2004). Mycobacterium bovis infection united Kingdom. Emerg. Infect. Dis. .10:p . 539 – 541

Sommers H.M. (1987). The identification of mycobacteria. Lab. Med., .9(2):34

Stephen E.W., Philip C.S., Francis X.B., Barbara K, Mary N., Burgis G.M, Enriqueta G., Brian H.F (1992). The effect of directly observed therapy on the rates of drug resistance and relapse in tuberculosis. The New England Journal of Medicine, 330:p. 1179 – **.**1184

Vincet V.,Brawn Eillib., Jostk C.,Wallace R.g.(2003). Mycobacterium phenotypic and genotypic identification .In Manual of clinical microbiology 8th edition. Washing Dc p.560-840

Walt B., Rayner A., Harris G. (1993). Modern methods in .mycobacteriology. Review in Medical Microbial. 4: p. 97 – 105

World Health Organization (1996). Report of the tuberculosis epidemic. .World health Organization, Geneva, Switzerland

World Health Organization (1998). Culture examination and identification. .In Laboratory services in tuberculosis control. WHO 196-200

Appendix (I)

Staining (Ziehl- Neelsen's stain)

(A) Reagent:

Solution (A): Formula for basic stain solution.

Solution (A): saturated a alcoholic solution of fuchsin.

- Basic fuchsin 3 g.
- Ethanol, 96% 100 ml.

Solution (B): phenol solution, 50g/L (5%), aqueous:

- Phenol crystals 10 g.
- Distilled water 200 ml.

Then: - Solution A 10 ml.

- Solution B 90 ml.

Formula for decolorizing agent:

- Ethanol 96% 970 ml.
- Hydochloric 30 ml.

Formula for counter staining solution:

- Methylene blue 0.3 g.
- Distilled water 100 ml.

(B) Procedure:

Thin smears from primary and pure colonies were prepared, air-dry and fixed by gentle heat. Smears were flooded with carbol fuchsin and were placed over a beaker of water on hot plate. The preparations were allowed to steam for 5 minutes. Then slides were washed with tap water and decolorized with 3% acid alcohol for 2-3 minutes and washed again with tap water. Finally, methylene blue was added for 30 seconds as counter stain and the smears were then examined under oil immersion lens (X100) for presence of AFB.

Appendix (II)

Media for isolation: (Lowenstein Jensen medium)

(A) Components **g/L**

- Potassium dihydrogen phosphate anhydrous	2.40 g
- Mangnisium sulphate	0.24 g
- Magnisium citrate	0.60 g
- L.Asparagine	3.60 g
- Glycerol	12 ml
- Distilled water	600 ml

B) Procedure:

All the solid ingredients above were added at one time and dissolved in distilled water ad then autoclaved for 15 minutes at 121°C, cooled at room temperature, and then 600 ml of this salt were mixed with 100 ml sterile whole-egg homogenate containing 20 ml 1% malachite green to give a homogenous mixture. Then 7 ml of the mixture were dispensed into steril glass universal bottles, and inspissated in a slant position at 85°C for 45 minutes.

Appendix (III)

Decontamination of sputum specimens

Decontamination and concentration of sputum:

(A) Reagent:

- HcL (IN)	8.3 ml.
- Distilled water	91.7 ml.

- NaOH (IN)	4 g/100 ml.
- Phenol red	1 g.
- Distilled water	50 ml.

B) Procedure:

The specimen was transferred into a sterile 50 ml conical tube, vortex with an equal volume of IN sodium hydroxide (NaOH 4%) and kept at room temperature for 20 minutes. IN hydrochloric acid with phenol red was added to neutralize the alkaline reaction until the solution become yellow to clear. The sediment was used for microscopy and culture.

Inoculation on to Lowenstein-Jensen (LJ) medium:

Two tubes of LJ were inoculated with a 4 drops Pasteur pipette of the neutralized sputum sample. One of the two tubes contained glycerol, while the other one contained pyrvate to isolate *M. tuberculosis* and *M. bovis*. Inoculated tubes were incubated aerobically at 37°C for up to 8 weeks before being discarded. Growth was monitored daily during the first week to observe the presence of rapidly growing Mycobacteria, which can show growth within 7 days, and then the growth was observed weekly up to the 8^{th} week. Characteristics of different colony types found were observed and recorded and smear for microscopy were made. Enough growth of *M. tuberculosis* complex members mostly appeared after 6 weeks.

Growth rate:

The organism was noted as rapid-grower if it produced visible colonies on subculture within 5 days post incubation (usually after 2-3 days) and as slow grower if it produced visible colonies on subculture after 2-6 weeks post inoculation.

Colonial morphology:

Cultures were identified from their morphology as eugenic or dysgenic, smooth or rough.

Morphology of the acid-fast organism:

Colonies were examined for acid-fastness by the zielh-Neelsen staining method and morphology characteristics such as shape (rods or bacilli) were recorded.

Appendix (IV)
Biochemical tests

Catalase test:

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen. The bubbles in the reaction mixture to

medicate catalase activity. Catalase activity can be demonstrated by using the 68C at pH 7 (indicates loss of catalase activity due to heat).

(A) Reagent:

0.067 M phosphate buffer solution, pH 7.0

Solution 1: 0.067 M solution

- $Na_2 HPO_4$ anhydrous	9.47 g
- Distilled water	1000 ml.

Solution 2: 0.067 M solution

- $KH_2 PO_4$	9.07 g
- Distilled water	1000 ml.

Solution 1 and solution 2 were added to each other to provide 0.067 M.

Phosphate buffer solution, pH 7.0

- Hydrogen peroxide	30%
- Tween 80	10%
- Tween 80	10 ml
- Distilled water	90 ml

Tween 80 was mixed with distilled water and autoclaved at 121 C for 10 minutes. Complete catalase reagent (Tween peroxide mixture) immediately before use, equal parts of 10% tween 80 and 30% hydrogen peroxide were mixed.

B) Procedure:

Point five milliliter of 0.067M phosphate buffer solution, pH 7.0 was added aseptically, with a sterile pipette to screw-capped tubes. Several loopfull of each test culture were suspended in the buffer solutions using sterile loops. Tubes containing the emulsified culture were placed in water bath at 68°C for 20 minute. Tubes were removed from heat and cooled to room temperature. 0.5 ml of freshly prepared tween peroxide mixture was added to each tube and cap replaced loosely. Positive results was recognized by formation of bubbles on the surface of the liquid, don’t shake the tubes, because tween 80 also may

form bubbles, when shaken resulting false positive. Negative tubes were hold for 20 minutes before discarding.

Result and interpretation:

Positive: bubbles.

Negative: no bubbles.

Nitrate reduction test:

The test was done to demonstrate the enzyme nitrate reductase which causes the reduction of nitrate in the presence of suitable electron donor to nitrite.

(A) Reagent:

- Sodium nitrate $NaNO_3$	0.01 M (0.085%)
- Distilled water	1000 ml.

Sodium nitrate was dissolved in distilled water and autoclaved at 121°C for 10 minutes.

LAMP Reagent:

- N-1-naphtyle diaminedihydrochloride	1 g
- Sulphanilic acid	1g
- Tartaric acid	10 g

The base components were mixed and shaking vigorously. The mixture is stable for at 6 months if stored in dark bottle.

(B) Procedure:

A heavy loop full of a recently grown culture on solid LJ glycerol, LJ pyrvate was transferred to tube containing 0.5 ml of 0.01 M (0.085%) solution

of sodium nitrate, the suspension was incubated for 3 hours at 37C, small quantity of LAMP reagent was added (quantity was not critical).

Result and interpretation:

Positive red colour which vary from pink to very deep red.

Faint pink: +/-

Clear pink: 1+

Deep pink: 2+.

Red: 3+.

Deep red: 4+

Purple red: 5+

Only 3+ to 5+ was considered positive.

Sensitivity to para-nitrobenzoic acid (PNB):

L.J media slope with PNB.

(A) Reagent:

- PNB	0.2 g
- Distilled water	15 ml
Na OH (1 mol/L)	2 ml

(B) Procedure:

A loop full of homogenized culture suspension was inoculated on to a slope of medium containing PNB 500 mg/L and then incubated at 37°C for 4 week. The presence of growth indicated that the organism was resistant to PNB and the result was considered positive.

Sensitivity to thiophen-2-carboxylic acid hydrazid (TCH):

LJ media slope with TCH

(A) Reagent:

- TCH	25 mg
- Distilled water	20 ml

(B) Procedure:

A loop full of homogenized culture suspension was inoculated on to slope of medium containing TCH 5 mg/L and then incubated at 37C for 4 weeks. The presence of growth indicated that the organism was resistant to TCH and the result was consider positive.

Appendix (V)
Drugs susceptibility testing

Preparation of antituberculosis-drug media:

(A) Reagent:

- Isoniazid	20.0 mg
- Distilled water	40 ml
- Rifampicin	80.0 mg
- Methanol	5.0 ml
- Streptomycin	4.0 mg
- Distilled water	50 ml
- Ethambutol	20 mg
- Distilled water	100 ml (200.0 mg/ml

(B) Procedure:

Lowenstein Jensen Proportion method:

Susceptibility testing was performed by LJ proportion method, fresh growth of the test organism on Lowenstein Jensen medium was used as the source of inoculum. Sufficient number of colonies was picked up to make a suspension (original suspension). The standard stain *M. tuberculosis*, H37 was used to test each batch of the medium. Two dilutions were made from the original suspension, 10^{-2} and 10^{-4} using the calibrated inoculating loop and sterile glass bottle containing 1 ml of distilled water. Two slopes of control medium and two slopes of each drug containing medium were inoculated with a loop full (0.01 ml) of each dilution. The bottles were incubated at 37°C for 6 weeks after the incubation period, presence of resistant bacilli was determined by expression the resistant portion as a percentage of total population used.

Appendix (VI)

Biochemoical tests of *Mycobacterium tuberculosis* complex isolates

Strain no	Acid fastness	Growth rate	Catalase test		Nitrate reduction test	TCH	PNB	Species
			at RT	at 68°C				
1	+	Rapid	+	-	-	-	-	MOTT
2	+	Slow	+	-	+	+	-	MTC
3	+	Slow	+	-	+	-	-	MTC
4	+	Slow	+	-	+	+	-	MTC
5	+	Rapid	+	+	+	-	-	MOTT
6	+	Slow	+	-	+	+	-	MTC
7	+	Rapid	-	-	+	+	-	MOTT
8	+	Rapid	+	-	-	-	-	MOTT
9	+	Slow	+	-	+	+	+	MTC
10	+	Slow	Weak	-	+	-	-	MTC
11	+	Slow	+	-	+	+	-	MTC
12	+	Slow	Weak	-	-	+	-	MTC
13	+	Rapid	+	-	-	+	-	MOTT
14	+	Slow	+	-	+	+	-	MTC
15	+	Slow	-	-	+	+	-	MTC
16	+	Rapid	+	-	-	+	-	MOTT
17	+	Rapid	Weak	-	-	-	-	MOTT
18	+	Slow	+	-	+	-	-	MTC
19	+	Slow	+	-	+	+	-	MTC

20	+	Slow	-	-	+	+	-	MTC
21	+	Slow	+	-	+	+	-	MTC
22	+	Slow	+	-	-	+	-	MTC
23	+	Rapid	+	-	+	-	-	MOTT
24	+	Slow	+	-	+	-	-	MTC
25	+	Slow	+	-	+	-	-	MTC
26	+	Slow	weak	-	-	-	-	MTC
27	+	Slow	+	-	+	+	-	MTC

Strain	**Acid fastness**	**Growth rate**	**Catalase test**		**Nitrate reduction test**	**TCH**	**PNB**	**Species**
			at RT	**at 68°C**				
28	+	Slow	+	-	-	-	-	MTC
29	+	Slow	+	-	+	+	-	MTC
30	+	Slow	+	-	-	+	-	MTC
31	+	Slow	+	-	-	-	-	MTC
32	+	Slow	+	-	+	+	-	MTC
33	+	Slow	+	-	+	+	-	MTC
34	+	Slow	+	-	+	+	-	MTC
35	+	Slow	+	-	-	-	-	MTC
36	+	Rapid	+	-	-	+	-	MOTT
37	+	Slow	+	-	+	+	-	MTC
38	+	Slow	weak	-	+	-	+	MTC
39	+	Slow	+	-	+	+	-	MTC
40	+	Slow	+	-	+	+	-	MTC
41	+	Slow	+	-	+	-	-	MTC
42	+	Slow	+	-	+	+	-	MTC
43	+	Slow	+	-	-	+	-	MTC
44	+	Slow	+	-	+	+	-	MTC
45	+	Slow	+	-	+	+	-	MTC
46	+	Slow	+	-	+	+	-	MTC
47	+	Slow	-	-	+	+	-	MTC
48	+	Rapid	+	-	-	-	-	MOTT
49	+	Slow	+	-	+	+	-	MTC
50	+	Slow	+	-	+	+	-	MTC
51	+	Slow	+	-	+	+	-	MTC
52	+	Slow	+	-	+	+	-	MTC
53	+	Slow	+	-	+	+	-	MTC
54	+	Slow	+	-	+	+	-	MTC

Strain	**Acid fastness**	**Growth rate**	**Catalase test**		**Nitrate reduction test**	**TCH**	**PNB**	**Species**
			at RT	**at 68°C**				
55	+	Slow	+	-	+	+	-	MTC
56	+	Slow	+	-	+	+	-	MTC

57	+	Slow	+	-	+	+	-	MTC
58	+	Slow	+	-	+	+	-	MTC
59	+	Slow	+	-	+	+	-	MTC
60	+	Slow	+	-	+	+	-	MTC
61	+	Slow	+	-	+	+	-	MTC
62	+	Slow	+	-	+	+	-	MTC
63	+	Slow	+	-	+	+	-	MTC
64	+	Slow	+	-	+	+	-	MTC
65	+	Slow	+	-	+	+	-	MTC
66	+	Slow	+	-	+	+	-	MTC
67	+	Slow	+	-	+	+	-	MTC
68	+	Slow	+	-	+	+	-	MTC
69	+	Slow	+	-	+	+	-	MTC
70	+	Slow	+	-	+	+	-	MTC
71	+	Slow	+	-	+	+	-	MTC
72	+	Slow	+	-	+	+	-	MTC
73	+	Slow	+	-	+	+	-	MTC
74	+	Slow	+	-	+	+	-	MTC
75	+	Slow	+	-	+	+	-	MTC
76	+	Slow	+	-	+	+	-	MTC
77	+	Slow	+	-	+	+	-	MTC
78	+	Slow	+	-	+	+	-	MTC
79	+	Slow	+	-	+	+	-	MTC
80	+	Slow	+	-	+	+	-	MTC
81	+	Slow	+	-	+	+	-	MTC
82	+	Slow	+	-	+	+	-	MTC
83	+	Slow	+	-	+	+	-	MTC

Appendix (VII)

Structural Questionnaire

Serial No. .....

Patient's name: .....

Age (in years): .....

Sex (M/F): .....

Tribe: .....

Occupation .....

Treatment status:

- **New case:** .....
- **Previously treated case:** .....
- **Relapse:** .....
- **Treatment failure:** .....