

CHAPTER ONE

1. Introduction and Objectives

1.1 Introduction

Tuberculosis (TB) is a chronic contagious disease which has a major impact on global public health problem. The disease is caused by an obligate aerobic intracellular bacillus called, *Mycobacterium tuberculosis* (MTB).

Tuberculosis kills ~2 millions people each year, and in 1989, the World Health Organization (WHO) estimated that ~300,000 children under 15 years of age die of tuberculosis per year worldwide (WHO–WHO/TUB, 2003). Pediatric tuberculosis diagnosis is impeded by difficulty obtaining sputum samples from children and the paucibacillary nature of their disease that often necessitates invasive procedures such as gastric aspiration or bronchoscopy (WHO, 1995). Recently, it has been shown that induced sputum has comparable diagnostic sensitivity to gastric aspirate in HIV-positive and -negative children, whereas some other studies have shown less promising results for induced sputum (Zar *et al.*, 2005). Although less invasive than gastric aspirates, induced sputum is still unpleasant and requires precautions to prevent airborne tuberculosis transmission to staff and other patients (Schoch *et al.*, 2007). The important issues and challenges in pediatric tuberculosis differ markedly between developed and developing countries. In more developed countries, rates of tuberculin skin test (TST) reactivity in the general population are low, so the tuberculin skin test is a useful diagnostic test for tuberculosis. In contrast, in many developing countries, interpretation of the tuberculin skin test for diagnosis of pulmonary tuberculosis is less reliable. The diagnostic yield of gastric aspiration ranges from 20% to 40% (Getchell *et al.*, 1992).

The diagnosis of childhood TB is complicated by the absence of a practical gold standard, as bacteriologic confirmation is rarely achieved due to the predominantly paucibacillary nature of childhood TB. Sputum microscopy, often the only test available in endemic areas, is positive in less than 10-15% of children with probable TB and culture yields are usually low (30-40%) (Marais *et al.*, 2006).

Specifically regarding the diagnosis of TB in children, this relies on a careful and thorough assessment of all the evidence derived from a careful history, clinical examination and relevant investigations, e.g., tuberculin skin test, chest radiograph and sputum smear microscopy. Although bacteriological confirmation of TB is not always possible, it should be sought whenever possible, e.g., by sputum microscopy in children with suspected pulmonary TB who are old enough to produce a sputum sample. A trial of treatment with TB medications is not generally recommended as a method to diagnose TB in children. New, improved diagnostic tests are urgently needed (WHO, 2006).

The diagnosis of childhood tuberculosis is often problematic and depends on a combination of epidemiological factors, clinical and radiological features, and/or bacteriologic confirmation. Bacteriologic confirmation is not routinely attempted in children with possible TB and specimens for mycobacterial culture are collected only in children admitted to hospital (Graham *et al.*, 2004).

Rapid diagnosis and treatment are important for preventing transmission of *Mycobacterium tuberculosis*. However, the diagnosis of tuberculosis continues to pose serious problems, mainly because of difficulties in differentiating between patients with active tuberculosis and those with healed lesions, normal *Mycobacterium bovis* BCG (Bacillus Calmette Guerin) vaccinated individuals, and unvaccinated Mantoux positives. Physicians still rely on conventional methods such as Ziehl-Neelsen (ZN) staining, fluorochrome staining, sputum culture,

gastric lavage, and other non-traditional methods. Although the tuberculin skin test has aided in the diagnosis of tuberculosis for more than 85 years, its interpretation is difficult because sensitization with non-tuberculous mycobacteria leads to false-positive tests (Garg *et al.*, 2003).

1.2 Rationale

The diagnosis of pulmonary tuberculosis presents challenges in children, because symptoms are nonspecific, sputa are not accessible, and *Mycobacterium tuberculosis* cultures and smears often are negative.

This study aimed help in detection of childhood tuberculosis cases by using Polymerase Chain Reaction (PCR), the vast majority of them have smear-negative disease by using conventional techniques used in diagnosis of childhood tuberculosis.

Few studies have reported as high as 33% bacteriological positivity of childhood tuberculosis even in primary disease such as hilar lymphadenopathy. This contradicts the concept of primary tuberculosis, which we understand till date as being difficult to diagnose by demonstration of AFB due to its paucibacillary nature, and the fact that Ziehl-Neelson stain can reveal AFB only if the sample contains >10,000 bacilli per mL (Working Group on Tuberculosis, 2010).

Although some previous studies demonstrated the role of molecular diagnosis of tuberculosis, there has been no previous study reported showing the role of molecular diagnosis of childhood tuberculosis by detection of specific gene in gastric aspirate in Sudan.

1.3 Objectives

1.3.1 General Objective

To improve detection of *M. tuberculosis* infection among children under eighteen years old in Khartoum State using IS 6110.

1.3.2 Specific Objectives

1. To identify *Mycobacterium tuberculosis* as acid fast organism using ZN stain.
2. To recognize *Mycobacterium tuberculosis* using Auramine stain as the best staining technique for detection of TB.
3. To isolate *Mycobacterium tuberculosis* from gastric lavage and sputum samples.
4. To determine the best method for extraction of DNA from gastric lavage samples.
5. To characterize *Mycobacterium tuberculosis* in gastric lavage samples using PCR technique.

CHAPTER TWO

2. Literature Review

2.1 Historical Background

Mycobacterium tuberculosis (*M. tuberculosis*) is the causative agent of tuberculosis (TB), a disease whose history has been traced back as far as the Egyptians in 2400 BCE. For many centuries this tiny invader killed people worldwide without any insight into its causative agent and pathogenesis. In 1720 Dr. Benjamin Marten first proposed that TB could be caused by “wonderfully minute living creatures”, a truly revolutionary thought at the time. Then in 1882 Dr Robert Koch described these bacteria and developed a successful staining technique, allowing him to visualize *M. tuberculosis* for the first time. In 1921 *Mycobacterium bovis* Calmette -Guerin (*M. bovis* BCG) was administered as a vaccine for the first time. By the 1940s chemotherapeutics were being developed and utilized against *M. tuberculosis*, ushering in a new era in the fight against TB (Wang and Xing, 2002).

The history of tuberculosis mixtures with the history of humanity since TB is one of the oldest infectious diseases affecting mankind. Bone TB was identified in 4000 years old skeletons, from Europe and Middle East, as the cause of death, showing that this disease was already a widespread health problem back then. In recorded history, Hippocrates writes about patients with wasting away associated with chest pain and coughing, frequently with blood in sputum. These symptoms allowed Hippocrates to diagnose TB, which at that time was called “consumption”. The frequency of descriptions of patients with these symptoms indicated that the disease was already well entrenched in ancient times (Luisa and Otilia, 2011).

2.2 Epidemiology

More than 2 billion people (about one-third of the world population) are estimated to be infected with *Mycobacterium tuberculosis* (Lonnroth and Raviglione, 2008).

According to World Health Organization, tuberculosis remains the second leading cause of death worldwide, killing nearly 2 million people each year (WHO, 2005).

The global tuberculosis caseload appears to be growing slowly. If tuberculosis hits first the Third World (around 95% of the cases, mainly in sub-Saharan Africa and South East Asia), there has been a significant increase in countries of the former Soviet Union and in Eastern Europe these last years (WHO, 2005; Frieden *et al.*, 2003). Since the mid 80's tuberculosis is decreasing less rapidly than expected in the majority of other industrialized countries (Dolin *et al.*, 1994).

Different factors contribute to the resurgence of this infectious disease in industrialized countries: degradation of socio-economic conditions, increasing immigration from countries with high tuberculosis prevalence, dismantling of tuberculosis sanitary structures from the period where the disease was decreasing, and high sensitivity of people infected with HIV (Porter and Mc Adam, 1994). This outburst is also correlated with the increasing development of species of the *M. tuberculosis* complex resistant to first line anti-tuberculous drugs. Late diagnosis, inadequate patient's treatment, unadapted safety equipment and premises, contribute to the disease transmission (Castro, 1995).

Childhood tuberculosis is responsible for approximately 15-20% of the total TB burden in South Africa and other low income countries (Nelson and Wells, 2004).

Children less than 15 years of age constitute approximately 15% of the total TB case load in many developing communities (Nelson and Wells, 2004). Children are mainly infected by adult pulmonary TB source cases and childhood TB therefore reflects the intensity of ongoing transmission of *Mycobacterium tuberculosis* within a community (Donald, 2002).

2.2.1 Tuberculosis in Sudan

Sudan is a large country with a diverse population and history of civil conflict. Poverty levels are high with a gross national income per capita very low comparing to other countries in the area. The country has been severely affected by war, famine and flood in recent decades and has a large population of internally displaced persons (El-Sony *et al.*, 2002). It has high burden of tuberculosis with a prevalence of 209 cases per 100,000 of the population and 50,000 incident cases during 2009 (WHO, 2010). The estimated adult HIV prevalence of 1.5% remains lower than that of its African neighbors to the south and a report from 2002 suggested 4% of tuberculosis patients were co-infected with HIV (El-Sony *et al.*, 2002). Tuberculosis care and treatment is provided by the National Tuberculosis Control Program under the auspices of the Ministry of Health and by a number of non-governmental organizations (NGOs) who provide care to displaced persons, including those living in refugee camps (El-Sony *et al.*, 2002). Treatment is also provided by the private sector (Maalaoui, 2008). Few studies have been undertaken on TB in Sudan and the prevalence of drug resistant disease is not known (Ghada *et al.*, 2011). The first published reports about TB in Sudan were carried out by Cummins (1908 – 1911), who studied TB in Egyptian army. He noted that the incidence of TB among Sudanese soldiers was 3.7/1000 which is higher than Egyptian soldiers (1.5/1000). The first tuberculin survey was conducted in 1925 in the Blue Nile province on 700 school boys, soldiers and hospital patients. The positive result ranged from 7 – 27% in different groups (Bloom and Murray., 1992). A survey carried out of two groups rural and urban school children in Khartoum province in 1932 showed slightly higher positive rate in rural (40%) than in urban areas (37%) (WHO, 1982).

2.3 Susceptibility of Children to Tuberculosis

Pediatric and adult TB differs markedly in epidemiological features, clinical appearance, and pathogenesis. Before the advent and use of prophylactic anti-TB medication, there were higher rates of disease progression in young children than in older children and adults infected with *M. tuberculosis*. One study in Norway observed 73% of TB disease occurred in children less than 6 years of age (Gedde, 1952). Children younger than one year of age with reactive tuberculin skin tests, pulmonary lesions developed in 43% and 15 to 20% had meningitis or miliary disease. Even today in regions where access to health care and medication is often limited, a little more than half of the disease burden is carried by children less than three years of age (Marais and Pai, 2006). Overall, the lifetime risk of progression from infection to active disease with *M. tuberculosis* is 5 to 10% for immunocompetent older children and adults and 40 to 50% for children in the first 2 years of life (Marais *et al.*, 2004). Adolescents have a slightly higher risk of disease progression than adults (Comstock *et al.*, 1974).

Not only is the risk of progression higher in young children, but also tuberculosis disease in this age group is often more severe and is already disseminated on clinical presentation. In children less than five years of age, early hematogenous and lymphatic spread of primary infection cause extrapulmonary manifestations such as miliary and meningitic disease (Alcais *et al.*, 2005). Tuberculosis meningitis developed in 15 to 20% of children younger than a year old, whereas central nervous system (CNS) disease was not observed in children more than four years of age. In a large Puerto Rican cohort miliary TB or meningitis developed in 10% of children less than 6 years of age, 1% between ages 7 and 12 years, and 0.4% in the adolescent group (Comstock *et al.*, 1974). Overall, disseminated TB occurs in 40% of active TB cases in children less than one year of age and in less than 1% in adults with active TB (Lewinsohn *et al.*, 2004). Young children with

severe and complicated disease have a much higher mortality rate than older children and adults. Some studies report a mortality rate exceeding 50% in children less than one year of age who have not received anti-TB medication (Lincoln, 1951).

2.4 Classification and Taxonomy of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis belongs to the Kingdom: Bacteria, Phylum: Actinobacteria, Class: Actinobacteridae, Order: Actinomycetales, Suborder: Corynebacterineae, Family: Mycobacteriaceae, Genus: *Mycobacterium*, Species: *Mycobacterium tuberculosis*. It is one of the most clinically important and extensively studied of bacterial taxa. They bear a taxonomical relationship to actinomycetes such as the antibiotic producing Streptomycetes because of the high guanine cytosine (GC) content in their DNA (Gey van *et al.*, 2001). When the last American Thoracic Society (ATS) statement about *Mycobacterium* was prepared in 1997, there were approximately 50 *Mycobacterium* species that had been identified. Currently, more than 125 *Mycobacterium* species have been cataloged (McNabb *et al.*, 2004). There has been a dramatic recent increase not only in the total number of mycobacterial species but also in the number of clinically significant species. The increase relates to improved microbiologic techniques for isolating *Mycobacterium* from clinical specimens and, more importantly, to advances in molecular techniques with the development and acceptance of 16S rRNA gene sequencing as a standard for defining new species (Tortoli, 2003).

The genus *Mycobacterium* comprises a wide range of organisms, including obligate parasites causing serious human and animal diseases, opportunistic pathogens, and saprophytic species found in nature (Kim *et al.*, 1999).

For the most part, *Mycobacteria* can be divided into two major groups based on fundamental differences in epidemiology and association with disease: those

belonging to the *Mycobacterium tuberculosis* complex, and those referred to as Nontuberculous Mycobacteria (NTM) (Forbes *et al.*, 2007).

The term complex is frequently used to describe two or more species whose distinction is complicated and of little or no medical importance (Shinnick and Good, 1994).

Mycobacterium tuberculosis complex includes *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. pinnipedii* and *M. microti* (Wayne, 1984; Cousins *et al.*, 2003). The four first species are human pathogens, *M. microti* infecting voles, guinea-pigs, rabbits and sometimes bovines. *M. pinnipideii* is responsible for tuberculosis in seals (Cousins *et al.*, 2003). *M. bovis* is responsible for pulmonary disease in bovine and sometimes to mammary lesions with passage of tubercle bacilli in milk. Both *M. bovis* and *M. pinnipedeii* are responsible for zoonosis. *M. bovis* is responsible for extra-pulmonary infections in human following ingestion of contaminated milk or milk products, but also pulmonary infections by inhalation of infected droplets through direct contact with infected animals. *M. africanum* is responsible for 20 to 80 % of human tuberculosis in sub-Saharan Africa, but also for some tuberculosis cases diagnosed outside this continent. In most of the occidental countries, tuberculosis cases are mainly caused by *M. tuberculosis*. Some domestic animals, in contact with people suffering from tuberculosis, are able to develop tuberculosis and become themselves a source of infection (Cousins *et al.*, 2003; Grange, 1990). Early taxonomic studies compared up to 100 growth and biochemical tests of large numbers of strains in multiple collaborative laboratories. Work focused around the International Working Group on Mycobacterial Taxonomy. New species were defined on the ability to phenotypically separate the new taxon from established species. This work was time and labor intensive and not adequate for separating many NTM species. Subsequently, species were identified by comparisons of genomic DNA; new species had similarity (homology) of less than 70% on DNA–

DNA pairing experiments with established species. This type of comparison was highly technical, highly labor intensive, and required comparison of possible new species to all established related species. By its very nature, this technique limited identification of new species (McNabb *et al.*, 2004).

2.5 Characteristics of *Mycobacterium tuberculosis*

2.5.1 Definition

Mycobacteria are typically rod-shaped aerobic. However, variable morphology can be observed when grown on solid media and some species exist as shorter cocci-bacilli or curved rods on artificial media (Belisle and Brennan, 1989). Although Mycobacteria are considered obligate aerobes, metabolic pathways exist to support adaptation to environments with reduced to no oxygen for extended periods of time while maintaining viability (Boshoff and Barry, 2005; Wayne and Sohaskey, 2001). The ability to adapt extends to numerous environmental stresses, a likely survival tool for their natural habitat mainly soil, water and host cells (in the case of facultative pathogenic species). There are more than 70 distinct *Mycobacterium* species (Goodfellow and Magie, 1998). Analysis of 16S rRNA, highly conserved ribosomal (r) RNA also defines similarities to other families such as *Corynebacteriaceae* and *Nocardiaceae* establishing the CMN branch within the *Actinomycete* genera (Lee *et al.*, 1996). Within this group of organisms many structural and biosynthetically complex systems are related. Mycobacteria bear several notable distinctions, however: (i) resistance to decolorization by acid alcohol following basic fuchsin staining (acid fast bacilli) (De Voss *et al.*, 1999); (ii) production of two different siderophores, iron scavengers (exochelins and mycobactins) (Raghu and Sarma, 1993); (iii) complexity of mycobacterial mycolates (cell wall components) (Brennan, 1995); (iv) extremely slow division compared to other bacteria (Goodfellow and Magie, 1998).

With the exception of *M. leprae* which cannot be cultivated in vitro, *Mycobacteria* are assigned to two groups based primarily on the relative growth rates of the individual species. Few phenotypic properties have been found to distinguish between fast and slow growing *Mycobacteria* but there are differences that are distinctive and have been included in the classification process; neutral red staining, composition of lipid structures and sensitivity to high salt concentrations (Soto Carlos *et al.*, 2002).

2.5.2 Cell Wall Structure

Much of the early structural definition of the cell wall of *Mycobacterium* spp. was initiated in the 1960s and 1970s. There was a long period of inactivity, but more recent developments in analytical techniques combined with definition of the *M. tuberculosis* genome have resulted in a thorough understanding, not only of the structure of the mycobacterial cell wall and its lipids but also the basic genetics and biosynthesis (Vilkas *et al.*, 1973). *Mycobacteria* with unusual cell walls that are resistant to digestion, being waxy, very hydrophobic and rich in lipid, especially esterified mycolic acids (Adam *et al.*, 1969). The cell wall is composed of two segments, upper and lower. Beyond the membrane is peptidoglycan (PG) in covalent attachment to arabinogalactan (AG), which in turn is attached to the mycolic acids with their long meromycolate and short α -chains. This is termed the cell wall core-the mycolyl arabinogalactan-peptidoglycan (mAGP) complex. The upper segment is composed of free lipids, some with longer fatty acids complementing the shorter α -chains, and some with shorter fatty acids complementing the longer chains. Interspersed somehow are the cell-wall proteins, the phosphatidylinositol mannosides (PIMs), the phthiocerol-containing lipids, lipomannan (LM), and lipoarabinomannan (LAM). When cell walls are disrupted, for instance extracted with various solvents, the free lipids, proteins, LAM, and PIMs are solubilized, and the mycolic acid-arabinogalactan-peptidoglycan

complex remains as the insoluble residue (Lederer *et al.*, 1975). In simplistic terms, it can be considered that these lipids, proteins, and lipoglycans are the signaling, effector molecules in the disease process, whereas the insoluble core is essential for the viability of the cell and should be addressed in the context of new drug development (McNeil *et al.*, 1990).

2.5.3 Acid Fastness

Unlike Gram-negative bacteria, *Mycobacteria* do not have an additional membrane in the outer layers of the cell wall. They are structurally more closely related to Gram-positive bacteria. However, *Mycobacteria* do not fit into the Gram-positive category as the molecules attached to the cell wall are distinctively lipids rather than proteins or polysaccharides. Frequently, they do not retain the crystal violet and appear as “ghosts” after Gram staining. The waxy cell wall of *Mycobacteria* is impermeable to aniline and other commonly used dyes unless these are combined with phenol (Barrera, 2007).

To discover the causative agent of TB, Robert Koch had to develop a specific staining process using alkaline dyes. Soon after, Ehrlich discovered the acid fastness of the tubercle bacillus, which has been the prominent characteristic of *Mycobacteria* up until now. The expression “acid-fastness” describes the resistance of certain microorganisms to decolorization with acid-alcohol solutions after staining with arylmethane dyes such as carbol fuchsin. This feature is of utmost practical importance in identifying the tubercle bacillus, particularly in pathological specimens (Barrera, 2007).

In spite of being a hallmark, the wall permeability to alkaline dyes and the mechanisms preventing their removal by acids are still not totally understood in molecular terms. Most of the current knowledge on this phenomenon was disclosed in pioneer experiments. The beading observed inside the cells was interpreted as accumulation of free dye rather than staining of particular structures,

which led to the early hypothesis that alkaline stains are retained in the cytoplasm (Yegian and Kurung, 1947). Later, evidence was provided sustaining the role of lipids in trapping the dyes. Indeed, there is a parallelism between the increasing degree of acid fastness displayed by microorganisms in the genera *Corynebacterium*, *Nocardia*, and *Mycobacterium*, and the increasing length of mycolic acid chains in their walls. This correspondence suggests that the chemical binding of the dye to these molecules might be a determinant for acid fastness (Barrera, 2007).

2.5.4 Growth Requirements

The tubercle bacillus is prototrophic (i.e. it can build all its components from basic carbon and nitrogen sources) and heterotrophic (i.e. it uses already synthesized organic compounds as a source of carbon and energy) (Palomino *et al.*, 2007). The microorganism macromolecular structure and physiological (metabolic) capabilities result in high adaptation to the specific environment. In turn, the nutritional quality of the environment determines the bacillus life style and limitations, either in the natural habitat or in culture media, as do various physical conditions such as oxygen availability, temperature, pH and salinity (Palomino *et al.*, 2007).

As the environment changes, the bacillus is able to bring into play different physiological pathways in order to survive even in harsh conditions. This is a highly resourceful strategy, not only for pathogenicity but also for species persistence (Palomino *et al.*, 2007).

It has been shown that, during the course of infection in mice, *M. tuberculosis* metabolism may shift from an aerobic, carbohydrate-metabolizing mode to one that is more micro-aerophilic and utilizes lipids (Segal and Bloch, 1956). These demonstrations, which were reported a long time ago, were supported in recent times by the complete sequencing of the *M. tuberculosis* genome in which an

unusually high number of genes putatively involved in fatty acid metabolism were identified. This phenomenon may be related to the ability of the pathogen to grow or persist in host tissues where fatty acids may be the major carbon source (Neyrolles, 2006).

In vitro, the members of the *M. tuberculosis* complex are not fastidious unless damaged by some noxious agents (Wayne, 1982). In fact, the medium used by Koch to cultivate *M. tuberculosis* was simply sterile coagulated blood serum (Wayne, 1982). The tubercle bacilli can also grow in salt solutions using glycerol as a carbon source, ammonium ions and asparagine as nitrogen sources, and micronutrients (Wayne, 1982). *M. tuberculosis* is able to metabolize glycerol into pyruvate, whereas *M. bovis* is not (Wayne, 1982). Indeed, the genome sequence analysis confirmed that all the genes required for the formation of pyruvate are non-functional in *M. bovis*. Being defective in this metabolic process, *M. bovis* grows much better in the presence of a pyruvate salt as a source of carbon (Wayne, 1982). Albumin, which is normally provided by adding eggs or bovine serum albumin to the culture media, promotes the growth of these microorganisms. Other subsidiary media components may be used, such as Tween 80, a detergent that disperses the bacilli in liquid media. It was postulated that bovine serum albumin may bind the excess of oleate that can be released from the detergent up to toxic amounts (Wayne, 1982). Biotin and catalase have been incorporated to the Middlebrook series media to stimulate the revival of damaged bacilli in clinical specimens (Wayne, 1982).

Trace elements found by the microorganism in the water, inorganic ions, small molecules, and macromolecules have either a structural or a functional role in the cell. Magnesium and iron are essential for life. A deficiency in these elements frequently reduces the virulence of bacterial pathogens, including the tubercle bacillus (De Voss, 2000).

The tubercle bacillus requires oxygen as a final electron acceptor in aerobic respiration. Molecular oxygen is reduced to water in the last step of the electron transport system (Palomino *et al.*, 2007). In nature, the bacillus grows most successfully in tissues with high oxygen partial tension, such as the lungs, particularly the well-aerated upper lobes (Palomino *et al.*, 2007).

Carbon dioxide is essential and may be taken from the atmosphere and also from carbonates or bicarbonates. In the laboratory, an atmosphere of 5 to 10 % carbon dioxide favors culture growth, at least during the early stage of incubation. On the other hand, *M. bovis* is microaerophilic, i.e. it grows preferentially at a reduced oxygen tension (Palomino *et al.*, 2007). *M. tuberculosis* is mesophile and neutrophile as its multiplication is restricted to conditions offered by warm-blooded animals: about 37°C and a neutral pH. The temperature and hydrogen ion concentration ranges, in which the bacillus is able to multiply, are relatively narrow (Palomino *et al.*, 2007). High saline concentration such as that found in media containing 5 % sodium chloride, inhibits the growth of the microorganism (Palomino *et al.*, 2007).

2.5.5 Generation Time

Under favorable laboratory conditions, *M. tuberculosis* divides every 12 to 24 hours. This pace is extremely slow compared to that of most cultivable bacteria, which duplicate at regular intervals ranging from about 15 minutes to one hour (Chauhan *et al.*, 2006). Recently, the low multiplication rate of the tubercle bacillus was nicely exposed by Chauhan *et al.* These authors demonstrated the small proportion of cells initiating the separation process prior to division among tubercle bacilli growing either in broth or inside macrophages (Chauhan *et al.*, 2006).

The slow growth rate might be partially determined by the cell wall impermeability that limits nutrient uptake. However, only a minimal stimulus to bacterial

multiplication is achieved when the permeability is increased through treatment with some compounds that interact with the cell envelope (Palomino *et al.*, 2007). Harshey and Ramakrishnan identified ribonucleic acid (RNA) synthesis to be a major factor associated with the long generation time of the tubercle bacillus. They demonstrated that both the ratio of RNA to DNA and the RNA chain elongation rate are ten-fold lower in *M. tuberculosis* compared to *E. coli* (Harshey and Ramakrishnan, 1977). Another unusual feature is the existence of a unique operon commanding RNA synthesis. Furthermore, when the tubercle bacillus switches from the stationary to the active multiplying phase, its total RNA content increases only two-fold. Consequently, the protein synthesis must be retarded (Verma *et al.*, 1999). The influence of nutrient availability on the ribosome synthesis rate, which is a proxy of metabolic activity, remains controversial (Hampshire, 2004).

The low multiplication rate explains the typically sub-acute to chronic evolution of the disease and the long time required to attain visible growth *in vitro*. Numerous experiences using different nutrients and culture conditions have demonstrated that some factors may abrogate a lag in adaptation of the bacilli in culture media but, once growth is initiated, the replication cycle will still take not less than 12 hours. This limitation in accelerating the tubercle bacillus growth could not be overcome (Palomino *et al.*, 2007). Instead, the main achievements for diagnosis have been made through the use of tools that enable the detection of a minimal quantity of bacilli in the media. First, transparent agar medium allowing the detection of tiny colonies were introduced; more recently, the addition of biosensors has been adopted to detect redox changes produced by the bacilli metabolism (Barrera, 2007).

2.5.6 Susceptibility to Physical and Chemical Agents

The high lipid content of the cell wall confers to the *Mycobacteria* a great resistance to physical and chemical agents; it is able to survive for weeks to

months on inanimate objective if protected from sunlight. Members of the *M. tuberculosis* complex, for instance, survive for several months on surface or in soil or cow dung, from which other animals may be infected. *Mycobacteria* are easily killed by heat (>65°C for at least 30 min) and by UV sun light but not by freezing or desiccation. The bacilli are generally more resistant to chemical disinfection than other vegetative bacteria. Their resistance to disinfectants is considered intermediate between other non-sporulating bacteria and spores (Kunz and Gundermann, 1982). The acquired multidrug resistance does not seem to modify the resistance to disinfectants (Sattar *et al.*, 1995). Quaternary ammoniums inhibit tubercle bacilli but do not kill them. *M. tuberculosis* is also resistant to acids and alkali. Mercurial compounds are considered to be ineffective against the *Mycobacteria*. Efficient disinfectants are 5% phenol, 5% formaldehyde for at least ten minutes, 2% glutaraldehyde for 30 minutes exposure or sodium hypochlorite 5% for one minute. Ethyl and isopropyl alcohols in high concentrations are generally accepted to be excellent mycobactericidal agents. 70% ethyl alcohol can be used as surface disinfectant. Formaldehyde vapours can be used to disinfect biosafety cabinets (BSC's) and facilities. Iodine and ionophores are considered to be effective against *Mycobacteria* and are generally used in combination with ethyl alcohol (Rubin, 1991).

2.6 Transmission of Tuberculosis

Person-to-person transmission of tuberculosis occurs via inhalation of droplet nuclei (airborne particles 1 to 5 microns in diameter). Coughing and singing facilitate formation of droplet nuclei (Bates *et al.*, 1965). Normal air currents can keep infectious particles airborne for prolonged periods and spread them throughout a room or a building (Gupta and Atreja, 2006). Individuals with active untreated pulmonary or laryngeal disease are contagious, particularly when cavitory disease is present or when the sputum is AFB smear positive. Patients

with sputum smear-negative, culture-positive pulmonary TB can also transmit infection (Tostmann *et al.*, 2008).

The essential factors that determine the risk of transmission of tubercle bacilli to a healthy subject were two: the concentration of the infecting droplets suspended in the air, and the period of time during which the exposed individual breathes this contaminated air (Khaled and Enarson, 2003).

2.7 Size of Infectious Particle

Infection with *M. tuberculosis* can occur when an individual exposed to an infectious case of TB inhales particles (<5 µm in size) containing the tubercle bacilli (Patel *et al.*, 2009). When only fine particles or “droplet nuclei” inhaled, containing 1–3 tubercle bacilli are capable of starting the infection, because they remain suspended in the air stream that enters the alveolar spaces. The heavier droplet nuclei containing more bacilli and/or bits of caseous material impinge upon the mucosal surfaces of both the nasopharynx and the bronchial tree. The bacillary particles are moved up the bronchial tree by cilia and eventually swallowed. The mucosal surfaces of the respiratory and gastrointestinal systems are not easily infected by tubercle bacilli since very large numbers of bacilli are required to do so (Manabe *et al.*, 2006).

2.8 Risk Factors

Anyone can get tuberculosis, but certain factors can increase the risk of the disease. These factors include:

2.8.1 HIV/AIDS

HIV-related immune compromise is one of the main risk factors that increases the vulnerability to developing active TB following infection, which explains why sub-Saharan Africa, the region worst affected by HIV, reports the highest TB incidence rate in the world (Corbett, 2003). HIV infection has contributed to a significant increase in the worldwide incidence of TB (Raviglione *et al.*, 1992). In HIV

infection, defective macrophages function in response to TB infection, which may in part increase susceptibility to TB disease (Patel *et al.*, 2009). Once infection does occur, however, the risk of rapid progression is much greater among persons with HIV infection, because HIV impairs the host's ability to contain new TB infection. Immunocompetent individuals infected with *M. tuberculosis* have approximately a 10% lifetime risk of developing TB, with half of the risk occurring in the first 1-2 years after infection. In contrast, HIV-infected individuals with latent TB are approximately 20-30 times more likely to develop TB disease than those who are HIV uninfected, at a rate of 8-10% per year (Hopewell and Bloom, 2000). HIV co-infection also increases the risk of progression of recently acquired infection to active disease (Whalen *et al.*, 2011). In several outbreak settings, 35-40% of HIV-infected patients exposed to TB in health care or residential settings developed active TB disease within 60-100 days of exposure (Daley *et al.*, 1992). Although TB can be a relatively early manifestation of HIV infection, it is important to note that the risk of developing TB, and of disseminated infection, increases as the CD₄ cell count decreases. Even with effective immune reconstitution with Antiretroviral Therapy (ART), the risk of TB generally remains elevated in HIV-infected patients above the background risk of the general population, even at high CD₄ cell counts (Moore *et al.*, 2007).

2.8.2 Malnutrition

Malnutrition and tuberculosis are both problems of considerable magnitude in most of the underdeveloped regions of the world. It is important to consider, how these two problems tend to interact with each other. The term consumption has been virtually synonymous with tuberculosis throughout the history (Rubin, 1995) and the link between tuberculosis and malnutrition has long been recognized; malnutrition may predispose people to the development of clinical disease and

tuberculosis can contribute to malnutrition. Nutritional status is one of the most important determinants of resistance to infection (Macallan, 1999).

It is well established that nutritional deficiency is associated with impaired immune functions (Perronne, 1999). While malnutrition limits cell mediated immunity and increases susceptibility to infection, infection can lead to nutritional stress and weight loss, thereby weakening immune function and nutritional status (Chandra, 1991).

The oral traditions of medicine and public health have it that malnutrition is an important risk factor for the development of tuberculosis. Malnutrition profoundly affects cell-mediated immunity (CMI), and CMI is the principle host defense against TB (Cegielski and McMurray, 2004). It has long been known that there is an association between TB and malnutrition, malnutrition enhances the development of active TB, and active TB makes malnutrition worse (Van *et al.*, 2004). It has been suggested that generalized malnutrition—by reducing the expression of gamma interferon, tumor necrosis factor alpha, and other mycobactericidal substances—may selectively compromise portions of the cell-mediated response that are important for containing and restricting TB (Perronne, 1999).

2.8.3 Contact to Infected Individuals

Since the discovery of the tubercle bacillus by Robert Koch in 1882, the study of household contacts of infectious tuberculosis cases has contributed substantially to the current understanding of tuberculosis and its transmission. In the era before effective anti-tuberculous chemotherapy, studies of household contacts established that tuberculosis cases were most infectious when acid-fast bacilli were present in sputum (Rose *et al.*, 1979). Tuberculosis outbreaks often occur in overcrowded, poorly ventilated facilities when there is prolonged close exposure to an infectious person. Outbreaks of TB have been reported in a variety of crowded settings,

including bars, hospitals, ships, commercial aircraft and school buses (Kenyon *et al.*, 1996). Instances of widespread infection from a source case are not rare; indeed, that is what might be expected in homes where there is a person with active TB. Unfortunately, infected individuals are often lost in the crowd, because primary TB is usually asymptomatic and, without contact investigation, few people become aware that they have it. Numerous studies have found contact investigation to be a very efficient method to identify TB cases (Ferebee and Mount, 1962).

2.9 Immunology of Tuberculosis Infection

Infection with *M. tuberculosis* starts with phagocytosis of the bacilli by phagocytic antigen-presenting cells in the lung including alveolar macrophages and dendritic cells (Akira *et al.*, 2006). The alveolar macrophages, after entry of *M. tuberculosis*, produce inflammatory cytokines and chemokines that serve as a signal for infection. The monocytes, neutrophils, and lymphocytes migrate to the focal site of infection, but they are unable to kill the bacteria efficiently. During this time, the bacilli resist the bactericidal mechanisms of the macrophage (phagolysosome) by preventing phagosome-lysosome fusion, multiply in the phagosome, and cause macrophage necrosis (Chen *et al.*, 2006). The released bacilli multiply extracellularly, are phagocytosed by another macrophage that also fails to control the growth of *M. tuberculosis*, and likewise are destroyed. In the meantime, dendritic cells with engulfed bacilli mature, migrate to the regional lymph node, and prime T cells (both CD4+ and CD8+) against mycobacterial antigens (Bodnar *et al.*, 2001). The specific immune response produces primed T cells which migrate back to the focus of infection, guided by the chemokines produced by the infected cells. The accumulation of macrophages, T cells, and other host cells (dendritic cells, fibroblasts, endothelial cells, and stromal cells) leads to the formation of granuloma at the site of infection (Gonzalez *et al.*, 2001).

The granuloma formation walls off tubercle bacilli from the rest of the lung tissue, limits bacterial spread, and provide microenvironment for interactions among macrophages and other cells of the immune system and the cytokines produced by these cells. The CD4⁺ T cells producing interferon- γ (IFN- γ) recognize infected macrophages presenting antigens from *M. tuberculosis* and kill them (Wolf *et al.*, 2008). Recent studies have shown that differences exist in the immunological response mounted by different individuals that lead to the formation of physiologically distinct granulomatous lesions in individuals exposed to *M. tuberculosis*. Some of these lesions suppress (sterilizing immunity) while others promote the persistence of viable *M. tuberculosis* in the microenvironment (Young *et al.*, 2009).

2.10 Pathogenesis

M. tuberculosis usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is thought be with resident macrophages, but it is also possible that bacteria can be initially ingested by alveolar epithelial type II pneumocytes. This cell type is found in greater numbers than macrophages in alveoli, and *M. tuberculosis* can infect and grow in these pneumocytes *ex vivo* (Mehta *et al.*, 1996).

The tubercle bacilli have ability to survive with in macrophage rather than to production of toxic substance .The cell-mediated immune response depend on the type of T helper cells involved, which may either lead to protective immunity and resolution of the disease or tissue –destroying hypersensitivity reaction and progression of the disease process. The nature of the immune responses following infection changes with time so that human tuberculosis divisible in to primary and post –primary form with quite different pathological features (Greenwood *et al.*, 1997).

2.10.1 Primary tuberculosis

Primary infection occurs when a susceptible person inhales droplet nuclei, which in turn deposit most commonly in the middle and lower lobes of the lung (Haque, 1990). Once in the alveoli, *M. tuberculosis* is ingested by alveolar macrophages. If these cannot destroy the offending organisms, bacilli multiply in this intracellular environment until the macrophages burst and release them, being in turn, ingested by other macrophages. During this period of rapid growth, *M. tuberculosis* spread through the lymphatic channels to hilar and mediastinal lymph nodes and through the bloodstream to other sites of the body (Leung, 1999). This is arrested with the development of cell-mediated immunity and delayed-type hypersensitivity at 4–10 weeks after the initial infection. At this time, the tuberculin reaction becomes positive (Van Dyck *et al.*, 2003). The macroscopic hallmark of hypersensitivity is the development of caseous necrosis in the involved lymph nodes and the pulmonary parenchymal focus, the Ghon focus (Agrons *et al.*, 1993), which together with the enlarged draining lymph nodes, constitutes the primary complex, also known as the Ranke or Ghon complex (Van Dyck *et al.*, 2003). In the immunocompetent individuals, development of specific immunity is generally adequate to limit multiplication of the bacilli; the host remains asymptomatic and the lesions heal (Bass *et al.*, 1990), with resorption of caseous necrosis, fibrosis and calcification. The pulmonary focus and the lymph nodes become calcified and minimal haematogenous dissemination may originate calcifications in lung apices (Simon's foci) and in extra-pulmonary locations. Some bacilli in these healed lesions remain dormant and viable, maintaining continuous hypersensitivity to tuberculous antigen, and in situations of immunodepression, they can reactivate. In immunocompromised individuals (HIV-positives, alcoholics, diabetics, drug addicts, elderly and patients with chronic renal failure, malignancy or undergoing immunosuppressive medication), more widespread lymphogenic and

haematogenous dissemination occurs, resulting in lymphadenopathy and more peripheral locations, respectively (VanDyck *et al.*, 2003). If immunity is inadequate, active disease often develops within 5 years after initial infection, the so-called progressive primary TB, which occurs in about 5% of infected patients (Park *et al.*, 1996). In the patients with little or no host response, disseminated (miliary) TB occurs (Miller *et al.*, 1993).

2.10.2 Post Primary Tuberculosis

Post primary disease can result from endogenous reactivation of dormant bacilli in residual foci in the lung apices (VanDyck *et al.*, 2003). Haematogenous spread and reactivation occurs preferentially in the upper lung zones, due to the higher oxygen tension and impaired lymphatic drainage in those areas. After reactivation, the apical foci reach confluence, liquefy and excavate (Goodwin and DesPrez, 1983).

Perforation of a lymph node into a bronchus may cause a tuberculous bronchitis with bronchial ulceration, and aspiration of intraluminal bacilli can cause bronchogenic dissemination; a classic finding is an infiltrate in the subapical infraclavicular region. Postprimary disease can also occur, although less frequently, from exogenous reinfection, particularly in countries with low infection risk (VanDyck *et al.*, 2003). Age may often determinate the presentation of the disease: whereas neonates and children develop primary disease, adults present with postprimary TB. This picture, however, is altered by the changing epidemiology, with atypical and “mixed” radioclinical patterns occurring in adults, especially in immunocompromised patients, with a consequent fading of the age-related distinction between primary and postprimary TB (Stead, 1998).

2.10.3 Miliary Tuberculosis

The term miliary tuberculosis, first used to describe the resemblance of the pathologic lesions to millet seeds, now describes any progressive disseminated hematogenous TB. In children, the illness is acute or sub-acute, with high

intermittent fevers, night sweats, and occasional rigors. Pleural effusion, peritonitis or meningitis occurs in as many as two thirds of patients. The illness in young adults is usually more chronic and initially less severe. Miliary TB also is considered an unusual cause of acute respiratory distress syndrome (Huseby and Hudson, 1976).

Miliary TB still remains a perplexing disease that continues to elude the most erudite and experienced clinicians and is a diagnostic and therapeutic challenge.

Mortality from this disease has remained high despite effective therapy being available. The myriad clinical manifestations, atypical radiographic findings and difficulties in establishing TB as the aetiological diagnosis, among others, are challenges in diagnosis and treatment of miliary TB (Surendra *et al.*, 2011).

2.11 Diagnostic Methods

2.11.1 Tuberculin Skin Test

For over 100 years the standard diagnostic test for *M. tuberculosis* infection has been the Tuberculin Skin Test (TST). Tuberculin is a glycerol extract of the tubercle bacillus. Purified protein derivative (PPD) tuberculin is a precipitate of non-species-specific molecules obtained from filtrates of sterilized, concentrated cultures. It was first described by Robert Koch in 1890. The test is named after Charles Mantoux, a French physician who developed on the work of Koch and Clemens von Pirquet to create his test in 1907. In 1882 Robert Koch boiled the culture of tubercle bacilli and injected it into people as a means to treat tuberculosis. This experiment failed, as overwhelming inflammatory responses developed and resulted in several deaths. However, what emerged from this experience was a definitive means to identify *M. tuberculosis* infection. In 1934, an American scientist, Dr. Florence Siebert, developed a method of purifying the tuberculin and made a simple protein precipitate (purified protein derivative PPD), a solution of antigens produced by the metabolic activity of *M. tuberculosis*. Today

the definitive TST uses five tuberculin units of PPD injected intradermally with the Mantoux technique. Multiple puncture tests are not as reliable as the Mantoux method and are not recommended for diagnosis. A wheal of fluid measuring 6 to 10 mm in diameter is raised immediately when the tuberculin is injected properly. A delayed hypersensitivity reaction to the Mantoux skin test peaks in infected individuals at 48 to 72 hours after injection. In some individuals the reaction may occur after 72 hours and is considered a positive result. The diameter of induration, not erythema, is measured and recorded in millimeters. Occasionally, an allergic or Arthus-like reaction to skin test components may cause erythema and induration, peaking at 24 hours, and usually waning by 48 hours (Nash and Douglass, 1980).

2.11.2 Specimen Collection

In children, TB is often paucibacillary and extra-pulmonary and it is therefore challenging to obtain adequate specimen for culture. Sterile leak-proof containers without fixative agents should be used for collection of specimen and quickly transported to the laboratory where examination should be performed on the day of receipt. Different types of collected specimens have been used for confirmation of pulmonary TB including gastric aspirates, induced sputum, bronchoalveolar lavage specimens, or specimens collected using the string test (Somu *et al.*, 1995).

In young children (less than 7–8 years of age), the routine specimens collected are two or three fasting gastric aspirates. The collection of two or three fasting, early morning gastric aspirate specimens is cumbersome and usually requires hospitalization. The collection of a single hypertonic saline-induced sputum specimen seems to provide the same yield as three gastric aspirate specimens (Zar *et al.*, 2005).

2.11.3 Transport and Preservation of the Specimen

Sputum sample should be transported at 2-4°C and processed within 24-48 hrs of collection (Bobadilla *et al.*, 2003). Few laboratories are performing culture and

drug susceptibility in addition to molecular biology detection tests; therefore, the sputum specimens have to be sent to central mycobacteriology laboratories for culture and drug susceptibility tests and there is usually a delay in the transporting and processing of the specimens for about a week. This leads to an increased contamination rate and loss of positive cultures (Paramasivan *et al.*, 1983). Therefore, a simple inexpensive storage method for transport of sputum specimens that preserves the viability of tubercle bacilli in sputum specimen up to 8 days will be useful. Cetylpyridinium chloride (CPC) is a quaternary ammonium compound, when added to sputum specimen at final concentration of 0.5%, does not kill tubercle bacilli for 14 days (Tazir *et al.*, 1979).

2.11.4 Microscopic Techniques

The bacilli in the sputum can be detected microscopically by ZN stain and fluorochrome stain. In fluorochroming, a direct chemical interaction occurs between the fluorescent dye and a component of the bacterial cell; this interaction is the same as occurs with the stains used in light microscopy (Forbes *et al.*, 1998). But, a significant difference in the sensitivity of the two methods has been reported (Kumar *et al.*, 1998).

2.11.5 Ziehl-Neelsen Method

In 1882 Robert Koch reported the discovery of the tubercle bacillus and described the appearance of the bacilli resulting from a complex staining procedure. During the same time period several other researchers (Ehrlich, Ziehl, Rindfleisch, and Neelsen), intending to improve on Koch's method, introduced modifications to the reagents and the procedure. Franz Ziehl was the first to use carbolic acid (phenol) as the mordant. Friedrich Neelsen kept Ziehl's mordant, but changed the primary stain to the basic fuchsin (first used by Ehrlich in 1882). This method became known as the Ziehl-Neelsen method in the early to mid-1890s (Bishop and Neuman, 1970).

Ziehl-Neelsen method is one of the easiest, most inexpensive and rapid method for demonstrating the presence of *Mycobacteria* in clinical specimens and cultures. Fluid and solid specimens that have been liquefied can also be stained. Stain is used to penetrate the bacilli's cell wall. Once stains are applied, they are difficult to remove, even when mineral acids are applied, hence the name "acid-fast". ZN stained smears are examined with a Bright field microscope under oil immersion. AFB appears as red and rod-shaped filaments against a blue background (Steingart *et al.*, 2007).

All *Mycobacteria* exhibit various degrees of acid fastness so ZN stain cannot be used to determine the individual species of *Mycobacteria* including *M. tuberculosis*. Despite this, staining has significant advantages because it remains the most rapid technique and is of value in identifying the most infectious patients for hospital and community infection control. The burden of bacterial infection is usually reflected by the number of organisms seen on microscopic examination of stained smears. As a general rule, a positive sputum smear examination indicates a concentration of at least 10^4 bacteria/ml. Higher grading of results (1+ to 3+) indicates greater concentration of bacteria (Starke, 2003).

However, its limited sensitivity (detection limit: 1000 bacilli/ ml of sputum) and specificity

(identifies only acid fast bacilli) (Mustapha *et al.*, 1999) make it less dependable than other methods, especially in cases of poor sputum quality and low mycobacteria content (Elliott *et al.*, 1993).

Given the paucibacillary nature of pediatric TB, most children are smear-negative. Less than 10 to 15% of children with proven TB will have sputum or gastric aspirate stain positive for AFB. The rates of positive smears from other specimen sources are even lower. Adolescents, however, often develop adult type disease and have higher bacteriological yield. Further, microscopy evaluation in low-

income settings is often performed on unconcentrated sputum and the sensitivity of this method for detecting *Mycobacteria* is limited (Lipsky *et al.*, 1984).

2.11.6 Auramine Staining Method

Fluorescence microscopy can increase sensitivity and decrease the time required for sputum smear examination compared to Ziehl-Neelsen staining and light microscopy (Steingart *et al.*, 2006). It has been shown in numerous studies to be at least 10% more sensitive than traditional light microscopy (Falkinham, 1996). Thus, fluorescent stains are of paramount importance, not only in confirming the presence of *Mycobacteria* in a given specimen, but also in providing an estimated quantification of organisms (Steingart *et al.*, 2006).

2.11.7 Conventional Culture Methods

Culture is more sensitive than microscopy and can detect as few as 10 to 100 bacteria/mL of material, but is positive in less than 50% of children with active disease. There are two major forms of media which are routinely used in mycobacteriology laboratories, solid and liquid media (Yeager *et al.*, 1967).

2.11.7.1 Solid Media

Solid media may be egg-based or agar-based. Egg-based media, of which Löwenstein-Jensen (LJ) is the most commonly used, support the growth of *M. tuberculosis* well, it contains malachite green that suppresses growth of contaminating bacteria and fungi and is used for both detection and susceptibility testing and have a long shelf life. Agar-based media have the advantage of being transparent, allowing earlier detection of mycobacterial colonies (Middlebrooks agar). They are more expensive than LJ, have a shorter shelf life (Tenover *et al.*, 1993).

Solid media should be inspected regularly and kept for eight weeks. Cultures from smear-positive specimens that have no growth at eight weeks should be kept for a further six to eight weeks. Detection of colonies on solid media offers several

advantages over growth in broth. Colonial morphology and pigment production may provide clues for identification and mixed cultures can be detected.

At least one solid medium must be used for each mycobacterial culture (Salfinger and Morris, 1994).

2.11.7.2 Liquid-Based Culture Systems

Liquid media support growth of the *M. tuberculosis* complex better than solid media with an increased recovery of positive cultures (Hanna, 2004), even in cases in which a child was started on anti-TB medication before obtaining cultures (Stager *et al.*, 1991). An important advantage of the liquid media is the shorter time to detection of positive cultures than in solid media. Isolation and susceptibility testing of *M. tuberculosis* in liquid media can be reported on the average of 2 and 4 weeks, respectively (Morgan *et al.*, 1983). The average time to recovery for smear-positive specimens is only 8 days compared with 18 days for solid conventional media (Roberts *et al.*, 1983). The first broth-based mycobacterial detection system was the BACTEC 460, which uses modified Middlebrook broth and a novel radiometric detection scheme. Mycobacterial growth can be periodically ascertained by the liberation of $^{14}\text{CO}_2$ as metabolized by the *Mycobacteria* and detected by the BACTEC instrument. Most recently, non-radiometric liquid culture systems have been developed to minimize the handling and disposal of radioactive waste (Hanna, 2004).

2.11.8 Methods of Identification

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 presence of light, scotochromogens produce pigmented colonies when grown in the dark, while

nonchromogens are nonpigmented neither in light nor in dark place, but only have light tan or buff-colored colonies (Geo *et al.*, 2001).

Pigmented *Mycobacteria* are classified as nontuberculous mycobacteria (NTM) because *M. tuberculosis* does not pigment producer (Ve'ronique, 2003).

Growth rate, colony morphology, and biochemical tests are other important differentiating factors among *Mycobacteria* species. Some *Mycobacteria* species grow in less than 14 days, which called rapid growers, while others need more than 14 days to produce visible colonies, and those called slow growers. Examination of the morphology of colonies is important especially in mixed cultures. After 15 days growth, *M. tuberculosis* produces thin, nonpigmented, rough colonies (Ve'ronique, 2003).

Traditionally, the identification of *M. tuberculosis* complex has relied on acid-fastness, niacin production, nitrate reduction, and inactivation of catalase at 68°C.

Unlike most mycobacterial species, *M. tuberculosis* complex lacks the enzyme to convert free niacin-to-niacin ribonucleotide, and niacin accumulates in the medium resulting in a positive niacin test result (Manabe, 2006).

Mycobacterium tuberculosis complex possesses nitro reductase and yields a positive result using nitrate reduction testing. Among *Mycobacteria*, the quantity of catalase produced and its stability at 68°C is species dependent.

Mycobacterium tuberculosis complex produces a column measuring less than 50 mm in the quantitative catalase production test performed with L-J slants. This organism also produces heat-labile catalase that is inactivated after 20 minutes of exposure to 68°C (Manabe, 2006).

2.11.9 Advance Diagnostic Methods

2.11.9.1 Nucleic Acid Amplification Techniques (NAAT)

NAATs are diagnostic methods based on the amplification of *M. tuberculosis* DNA. During the past decade NAAT techniques have been developed for a more

rapid identification of *M. tuberculosis* on direct or cultured specimens, for genotypic drug-resistance testing, and for molecular typing used in epidemic investigations (Piersimon and Scarparo, 2003). However, NAATs have not been routinely applied to clinical specimens due to variations in methodology, limited accuracy, and the high cost of running the assays. The DNA sequence most frequently used to detect *M. tuberculosis* has been the insertion element IS6110. Primers amplify a target fragment of 200 base pairs from the insertion-like *M. tuberculosis* sequences element IS6110. Various NAATs are commercially available, including polymerase chain reaction (PCR), transcription-mediated amplification, strand displacement amplification and ligase chain reaction. These tests offer high specificity for *M. tuberculosis*, usually ranging between 80 and 95% (Gomez, 2002). Recently, two commercial molecular assays have used conventional PCR followed by amplicon hybridization onto a series of oligonucleotide probes on nitrocellulose strips to detect rifampin resistance with a 1-day turnaround time. A number of trials have shown good correlation with conventional rifampin susceptibility testing (Somoskovi *et al.*, 2006).

2.11.9.1.1 Polymerase Chain Reaction (PCR)

Diagnostic PCR is a technique of DNA amplification that uses specific DNA sequences as markers for microorganisms (Schluger and Rom, 1994). In theory, this technique can detect a single organism in a specimen such as sputum, gastric aspirate, pleural fluid, cerebrospinal fluid, or blood. Recent publications show that various PCR techniques, most using the mycobacterial insertion element IS6110 as the DNA marker for *M. tuberculosis*-complex organisms, have a sensitivity and specificity greater than 90% for detecting pulmonary TB in adults (Eisenach *et al.*, 1991). However, these tests are not performed correctly in all clinical laboratories and may offer little advantage over high-quality microscopic examination of sputum. The cost involved and the need for sophisticated equipment and

scrupulous technique to avoid cross contamination of specimens preclude the use of PCR techniques in many developing countries. PCR may have a special role in the diagnosis of extra-pulmonary TB and pulmonary TB in children since sputum smears are usually unrevealing in these cases (Noordhock *et al.*, 1994).

Overall, PCR-based tests have not lived up to their early promise, but efforts are under way to simplify testing protocols and increase their accuracy. However, PCR-based tests have definite value in routine species identification (confirming the presence of *M. tuberculosis* complex), molecular epidemiology and the rapid detection of mutations associated with drug resistance.

With increased awareness of the emergent drug resistant tuberculosis epidemic, the use of PCR to rapidly detect drug-resistant specimens may offer the most relevant application to date (Pai *et al.*, 2003).

2.11.9.1.1 Insertion Sequence IS 6110

IS 6110 is a 1191-bp repetitive insertion sequence that is usually present 6-20 times in the *M. tuberculosis* complex genome although as few as one copy has been observed (Eisenach *et al.*, 1990).

2.11.9.2 INF- γ - Releasing Assays (IGRAs)

A new generation of immune-based rapid blood tests for the diagnosis of latent TB infection (LTBI), called IGRAs, offers particular advantages over the century-old TST. These tests rely on the host response to *M. tuberculosis* infection by measuring the IFN- γ produced by T-cell responses to *M. tuberculosis* -specific antigens called early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10. These antigens are transcribed from the region of difference-1, which is a region on the mycobacterium genome specific for *M. tuberculosis* and absent in BCG and most other *Mycobacteria*. Two IGRAs are available commercially, the Quantiferon TB Gold assay which is based on a whole-blood enzyme-linked immunosorbent assay (ELISA) and the T-Spot test which is based on the ex vivo

overnight enzyme-linked immunospot assay. The T spot enumerates individual T-cells producing IFN- γ after antigenic stimulation, while the QFT measures the level of IFN- γ in the supernatant of the stimulated whole blood (Diel *et al.*, 2008). IGRAs have several important advantages over the TST. Testing requires only one patient visit and these assays are *ex vivo* tests that eliminate the potential of boosting when testing is repeated. Results can be obtained within a day of blood draw. However, IGRAs do not distinguish between latent and active disease. It has been suggested by scientists that very high or rising levels of IFN- γ in IGRAs may be able to predict the asymptomatic individual with LTBI that is at highest risk of progressing to disease, but long-term prospective studies are needed to investigate this interesting finding. A significant limiting factor of IGRAs in resource-challenged regions is the high cost of IGRAs and trained personnel needed to run the assays (Andersen *et al.*, 2007).

2.12 Treatment

The history of tuberculosis changed dramatically after the introduction of anti-mycobacterial agents. Drug treatment is fundamental for controlling TB, promoting the cure of the patients and breaking the chain of transmission when the anti-tuberculosis drug regimen is completely and correctly followed. Anti-tuberculosis drug treatment started in 1944, when streptomycin (SM) and para-aminosalicylic acid (PAS) were discovered. In 1950, the first trial was performed comparing the efficacy of SM and PAS both as monotherapy or combined. The study demonstrated that combined therapy was more effective and resulted in the first multidrug anti tuberculosis treatment that consisted of a long course of both drugs. In 1952, a third drug, isoniazid (INH), was added to the previous combination, greatly improving the efficacy of treatment, but which still had to be administered for 18-24 months. In 1960, ethambutol (EMB) substituted PAS, and the treatment course was reduced to 18 months. In the '70s, with the introduction

of rifampicin (RIF) into the combination, treatment was shortened to just nine months. Finally, in 1980, pyrazinamide (PZA) was introduced into the anti-tuberculosis treatment, which could be reduced further to only six months. Two biological features explain why combined drug therapy is more effective at curing TB than monotherapy. One is that treatment of active TB with a single drug results in the selection of drug resistant bacilli and failure to eliminate the disease. The other is that different populations of tubercle bacilli – each of them showing a distinct pattern of susceptibility for anti-tuberculosis drugs – may co-exist in a TB Patient (Shamputa *et al.*, 2006).

The two main objectives of Anti-tuberculosis treatment First, there is a need to rapidly kill those bacilli living extracellularly in lung cavities, which are metabolically active and are dividing continuously; this is required in order to attain the negativization of sputum and therefore to prevent further transmission of the disease. Second, it is necessary to achieve complete sterilization and elimination of those bacilli replicating less actively in acidic and anoxic closed lesions, and to kill semi-dormant bacilli living intracellularly in other host tissues, otherwise these bacilli may persist and will be responsible for subsequent TB relapses. INH is the drug with the highest activity against rapidly dividing bacilli, whereas RIF and PZA have the greatest sterilizing activity against bacteria that are not dividing. These reasons, along with the prevention of drug resistance, support the use of a combination therapy for the treatment of TB (Onyebujoh *et al.*, 2005).

Drugs for treating TB are usually classified as first- and second-line drugs. Traditionally, there are five first-line drugs: INH, RIF, PZA, EMB, and SM. Second-line drugs include the aminoglycosides kanamycin and amikacin, the polypeptide capreomycin, PAS, cycloserine, the thioamides ethionamide and prothionamide and several fluoroquinolones such as moxifloxacin, levofloxacin and gatifloxacin. Some reports, however, include SM among the second-line drugs,

since its use has declined in recent years, due to the high rates of resistance, and also, because other more effective drugs have been incorporated into the anti-tuberculosis treatment. Similarly, new drugs such as the rifamycin derivatives rifapentine and rifabutin can be considered among the first-line drugs, and in the near future, it is quite likely that some fluoroquinolones could be incorporated into the standard anti-tuberculosis thus being considered as first-line drugs. The current short-course treatment for the complete elimination of active and dormant bacilli involves two phases:

Initial phase: three or more drugs (usually isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin) are used for two months, and allow a rapid killing of actively dividing bacteria, resulting in the negativization of sputum (Crofton, 1997).

Continuation phase: fewer drugs (usually isoniazid and rifampicin) are used for 4 to 7 months, aimed at killing any remaining or dormant bacilli and preventing recurrence (Crofton, 1997).

2.12.1 Drug Resistance

The incidence of tuberculosis has increased; there has been a corresponding rise in the incidence of drug-resistant strains of *M. tuberculosis* because of the prolonged infectivity which increases the risk of transmission (Doustdar *et al.*, 2008).

TB can be resistant to one or more chemotherapeutic agents. Multidrug-Resistant (MDR TB) is defined as resistance to at least the first-line drugs isoniazid and rifampin. Extensively Drug-Resistant (XDR TB) is defined as MDR plus resistance to any fluoroquinolone, and at least one of the second line injectable drugs, capreomycin, kanamycin or amikacin. Such extensive resistance markedly limits treatment options because few effective and reasonably well tolerated alternative drugs are available. Resistance to drugs currently used to treat TB is rapidly becoming a global public health emergency (Gandhi *et al.*, 2006).

The appearance of drug-resistant TB is not a recent occurrence, but rather, an unfortunate and expected consequence of the adaptation of *M. tuberculosis* to the use of antibiotics. Progressive development of drug resistance can be expected if we fail to improve treatment and control measures for TB. Recently, the first cases of completely drug-resistant TB were reported from Italy, where two HIV-negative patients were diagnosed with TB that was resistant to all known anti-TB drugs (Migliori *et al.*, 2007).

2.13 Prevention and Control

The most effective way to interrupt the transmission of *M. tuberculosis* is to identify and treat the individuals who have active tuberculosis. However *M. tuberculosis* continues to be transmitted to others largely because most transmission occurs before diagnosis and initiation of therapy (Daley, 2005).

Prophylactic chemotherapy, usually with isoniazid alone, is now used in situations in which known or suspected primary tuberculous infection poses the risk of clinical disease. Isoniazid can be used alone in prophylaxis because the load of tubercle bacilli in a subclinical primary lesion is small in relation to that in reactivation tuberculosis, and experience has shown that the development of subsequent clinical disease from isoniazid-resistant strains selected by prophylaxis can be discounted. Unfortunately, isoniazid may cause a form of hepatitis, and the risk increases progressively after 20 years of age. Its use in older subjects involves balancing risk against potential benefit and requires monitoring with liver function tests (Ryan and Ray, 2004).

2.13.1 Vaccination

Neither dead vaccines of tubercle bacilli nor tuberculin will stimulate immunity against tuberculosis. Immunity can, however, be produced by the use of living vaccine, consisting of strain of bovine bacilli, Bacille Calmette-Guerin (BCG) attenuated by prolonged culture on a bile-containing medium. The BCG strains are

not completely virulent. It will grow to a limited extent in tissues; in fact its immunizing effectiveness appears to depend on its being able to do so. It is, however attenuated to the extent that it does not normally initiate a progressive infection. The BCG vaccine strains currently employed differ considerably in their degree of attenuation. Consequently, their immunizing potency must be kept continually under review; particularly to ensure that they do not become over-attenuated. When BCG vaccine was introduced in 1920s it was widely used in France where it was given orally. Although vaccination was popular, there were no statistically controlled trails and many considered the procedure unsafe. In 1930 BCG receive a setback (as does practically every new vaccine) with the Lubeck disaster in which 73 infant (27percent) who had been fed with vaccine died. It was apparent that the children had accidentally been fed with a virulent strain of *M .tuberculosis* which had been kept in the same laboratory as the stock BCG strain. This disaster led to regulations controlling the production of BCG to ensure exclusion of all other strains. The current (BCG) vaccine, an attenuated strain of *Mycobacterium bovis* is a core component of the expanded program for immunization (EPI) (Fruth and Young, 2004). The WHO recommends that infants should be immunized a soon after birth as possible with a single intradermal dose of BCG in all countries with a high risk of TB. Although a consensus is developing that BCG protects children efficiently against the early manifestation of TB, estimates of protection against adults pulmonary TB range from 0-80% based on a large, well controlled field trails (Andersen and Doherty, 2005).

CHAPTER THREE

3. Materials and Methods

3.1 Type of the Study

3.1.1 Study Approach

The study is a qualitative study, aimed to highlight the importance of using (molecular technique) in the detection of childhood tuberculosis.

3.1.2 Study Design

Cross sectional laboratory based study was conducted by collection of gastric aspirate and sputum samples from children with clinical manifestations of pulmonary tuberculosis during six months from 25 May 2011 through 25 December 2011.

3.1.3 Study Area

Five TB clinics located in Khartoum State were included in the study. Two of these TB clinics provide services to children only (Elbolok Hospital and Jafar Ibn owf Hospital), while the other three clinics (Elasha'ab Teaching Hospital, Soba University Hospital and Academic Charity Hospital) provide services for both adult and children.

3.1.4 Study Population

One hundred and ninety seven children less than eighteen years old suspected of tuberculosis who attended to above mentioned hospitals were included in the study.

3.2.1 Ethical clearance

Proposal of this study was submitted to the Federal Ministry of Health as well as the Collage Medical Laboratory Sciences in Sudan University for ethical approval. Form of consent was taken from parents of children participating in the study.

3.2.2 Data Collection

Data were collected by using a standard data questionnaire eliciting basic information on age, sex, symptoms and signs of tuberculosis at presentation, history of exposure to risk factors, previous vaccination. Additional information captured included patient demographics, social status, history of previous tuberculosis infection, and tuberculosis treatment (if any) (Appendix I).

3.3 Diagnostic Methods

3.3.1 Tuberculin Skin Test

Tuberculin skin test (TST) was performed on all children using the Mantoux test with intradermal injection of 5 tuberculin units of Tuberculin purified protein derivative (Statens Serum Institute (SSI) tuberculin RT23 in 0.1 ml solution for injection) into the volar aspect of the forearm using a 27-gauge needle. Results were read at 48–72 hrs and recorded as the transverse diameter of palpable induration.

3.3.2.1 Sample Collection

Collecting an adequate sample presents a significant challenge, particularly in small children who cannot produce a good sputum specimen (Ben and Madhukar, 2007). In young children (less than six years of age), early morning gastric lavage samples were collected by a nurse or physician who had been previously trained in the protocol for collecting gastric aspirates. A silastic nasogastric tube was placed, usually without sedation, before the child had taken anything by mouth. At least 5 mL of the patient's gastric secretions were collected by aspiration. When the initial attempt to aspirate was unsuccessful, 10 to 20 mL of sterile distilled water were instilled and aspirated. Specimens were delivered immediately to the laboratory in sealed biohazard bags in a safely closed container.

Sputum samples were collected from children more than seven years of age in clean, wide mouthed, and leak proof specimen containers. Each patient was given a

new sputum container and clearly instructed on: how to produce an adequate sputum specimen, the importance of sputum examination for diagnosis of TB, how to open and close the containers and the need for collecting real sputum, not saliva.

3.3.2.2 Preservation of Specimens

All specimens were transported to the reference tuberculosis laboratory within three hours from collection except specimens that collected in Soba University Hospital and Elbolok Hospital transport after three days of collection, so were preserved by adding equal amount of 1% cetyl pyridinium chloride (CPC) in 2 % sodium chloride solution and mixed thoroughly.

3.3.2.3 Preparation of Culture Media

Lowenstein Jensen (LJ) medium is the most widely used for tuberculosis culture the modification of the International Union Against Tuberculosis and lung Disease (IUATLD) and Reference Tuberculosis Laboratory is recommended LJ medium containing glycerol favors the growth of *M. tuberculosis* while LJ medium with pyruvate encourage the growth of *M. bovis*.

3.3.2.3.1 Preparation of Lowenstein Jensen Medium with Glycerol

3.3.2.3.2 Preparation of Mineral Salts Solution

Potassium dihydrogen phosphate anhydrous (KH_2PO_4) was dissolved with 0.24 g Magnesium Sulphate, 0.6 g magnesium citrate, and 3.6 asparagine in 600 ml distilled water, 12 ml glycerol were also added and then autoclaved at 121°C for 15 minutes after the preparation was left to cool down to 50°C (Appendix II).

3.3.2.3.3 Preparation of Malachite Green Solution 2%

The dye was dissolved in sterile distilled water and then the solution placed in the incubator for 1 hour (Appendix III).

3.3.2.3.4 Preparation of Homogenized Whole Eggs

Fresh hen's eggs, not more than 4-5 days old, were cleaned by scrubbing thoroughly with a hard brush in warm water and plain alkaline soap. The eggs were soaked for 15-30 minutes in the soap solution. The eggs rinsed thoroughly in running water and soaked in 70% ethanol for 15 min, the hands were scrubbed and washed before handling the clean dry eggs, the eggs cracked with sterile knife into sterile blender.

3.3.2.3.5 Preparation of Complete Medium

Six hundred ml from mineral salt solution pooled aseptically in sterile flask and mixed well with 20 ml of malachite green and 1000 ml of homogenized eggs. The complete medium distributed in 6-8 ml volumes in sterile universal container and the tops was securely fastened. To quicken the build-up of the temperature the inspissator heated to 80° C before loading. Then the bottles placed in a stand position in the inspissator and the medium coagulated for 45 min at 80° C. After inspissator the whole media batch incubated at 35°C - 37° C for 24 hours as a sterility check (Appendix IV).

3.3.2.3.6 Preparation of (LJ) Medium with Sodium Pyruvate

As pervious but glycerol was omitted and 8.0g Sodium pyruvate added to the mineral solution. Thus LJ medium was enriched with 0.5% Sodium pyruvate.

3.3.2.4 Decontamination of Samples

Samples were decontaminated using modified Petroff's method by transferring to 50-ml disposable plastic screw-capped tube and equal amount of 4% of sodium hydroxide (Appendix V) was added for decontamination, vortexed and allowed stand at room temperature for 20 minutes. The specimen then centrifuged in cold centrifuge at 3000 rpm for 15min, the supernatant was discarded and the deposit was washed with sterile distilled water two times, and inoculated in three slopes of LJ medium. The specimen that treated with CPC reagent was transferred in 50-ml

screw-capped centrifuge tubes; sterile distilled water was filled till brim of the tube, capped and centrifuged at 3000 rpm for 20 min. Two or three drops of the sediment were inoculated onto the three LJ slopes according to (Smithwick *et al.*, 1975).

Two slides smears were prepared from the sediment for Ziehl Neelsen stain and Auramin stain, the remaining used for extraction of DNA and molecular diagnosis.

3.3.2.5 Culture Method

Three tubes of Lowenstein Jensen medium were labeled with patient number and date, and then inoculated with 20 μ l of the sediment that was obtained from the digestion and decontamination of the sputum sample. Two of the three tubes contain glycerol while the third tube contains pyruvic acid to isolate the *M. bovis* species if present. All cultures were incubated at 37°C in slope position for three days with loosen caps to evaporate the excess fluid then caps were closed tightly to prevent drying of media, bottles were placed on upright position and examined for contamination. Growth was monitored daily during the first week to observe the presence of rapid growers which if present will show growth within 7 days and finally every week to detect slow growers up to the eighth week.

3.3.2.6 Identification of the Isolates

Isolates were firstly identified according to methods described by Kent and Kubica (1985).

3.3.2.7 Growth Rate

A suspension of culture under test was inoculated onto LJ slope, the organism is either slow grower, if it produce visible colonies on subculture usually after 2-6weeks incubation, or rapid grower if it produce visible colonies on subculture with 5 days incubation (usually after 2-3 days).

3.3.2.8 Catalase Test

In screw capped tube, several loopful of test culture were suspended in the buffer solution. Tubes containing the emulsified cultures were placed in a previously heated water bath at 68°C for 20 minutes (time and temperature is critical). Tubes were removed from heat and allowed to cool to room temperature, 0.5 ml of the freshly prepared Tween – pyroxide mixture (Appendix VI) was added to each and caps were placed loosely. Formation of bubbles was observed appearing on the surface of the liquid, the tubes should not be shaking because Tween 80 also may form bubbles when shaken resulting in false positive results. Negative tubes were hold for 20 minutes before discarding.

3.3.2.9 Nitrate Reduction Test

A heavy loopful of a recently grown culture on L.J media was transferred to tube containing solution of sodium nitrate (Appendix VII). The suspension of sodium nitrate was incubated for 3 hours at 37°C. One dropperfull of sulfanilic acid and one dropperfull of α -naphthylamine (LAMP reagent) (Appendix VIII) were added to each bottle. A color changed to red indicates a positive nitrate reduction test.

3.3.3 Ziehl Neelsen Stain

3.3.3.1 Preparation of Reagents and Staining Procedure

Carbol fuchsin (1%) was prepared from 10 g of basic fuchsin (Hi-Media) dissolved in 100 ml of methanol and 50 ml of melted phenol in a flask maintained at 60°C in a water bath. This solution was made up to 1,000 ml with distilled water. Carbol fuchsin (0.3%) was prepared from 33 ml of the above solution diluted to 100 ml with distilled water before use. Sulfuric acid (25%) was prepared from 250 ml of concentrated sulfuric acid slowly added to 750 ml of distilled water. Methylene blue (0.1%) was prepared from 1 g of methylene blue dissolved in 1,000 ml of distilled water (Appendix IX).

Two drops from the sediment of decontaminated sputum were placed on clean and dry microscope slide and spread. Smear was left to dry completely and fixed by passing through the flame three times carefully. The proper temperature for flame fixation was checked by touching the slide to the back of hand immediately after removing it from the flame. Smear was covered with carbol fuchsin and heat gently until vapour raised, the heating stain allowed for 5 minutes and washed off with clean water. Decolourization was done by 25% Sulfuric acid for few seconds. Washed and lastly, methylene blue was added for 2 minutes. Washed, dried and examined under microscope. Positive result show red bacilli with blue background, while negative result show blue background with no red bacilli.

3.3.4 Auramine Stain

3.3.4.1 Preparation of Reagents and Staining Procedure

0.1 g of Auramine powder dissolved in 10 ml of 95% ethanol. 3.0 g of phenol crystals dissolved in 87 ml of distilled water. Then the two reagents were mixed and transferred to one bottle and filtered before used.

Acid Alcohol was prepared carefully by adding 0.5 ml of concentrated hydrochloric acid to 100 ml of 70% ethanol.

0.5 g of potassium permanganate (KMnO_4) dissolved in 100 ml of distilled water.

Smear was prepared as mentioned in ZN stain and fixed by heat.

Smear was flooded with Auramine solution and allows staining for 10 minutes. Then smear was rinsed and drained, acid alcohol was used to de-stain for 2 minutes, smear Rinsed again in distilled water and drained, finally smear was flooded with potassium permanganate for 10 seconds, followed by several rinses with distilled water, back of the slide was wiped to clean and placed in a draining rack for drying.

Smears were examined systematically for AFB by fluorescent microscope (Olympus BX51) using the 40X objective. Positive results show fluorescent apple green rods glowing against a dark background.

3.3.5 Molecular Identification

Nucleic Acid Amplification Techniques (NAAT) are diagnostic methods based on the amplification of *M. tuberculosis* DNA. Polymerase Chain Reaction (PCR) was used as a rapid and accurate diagnostic tool for detection of pulmonary tuberculosis in children.

3.3.5.1 DNA Extraction

Three DNA extraction protocols were tested for their efficacy of detection of mycobacterial DNA in gastric wash and sputum samples by PCR assay. Chelex method, Isopropanol Extraction Method and Phenol Chloroform method were performed and the quantity of extracted DNA yielded from each protocol was estimated using Eppendorf photometer machine and compared with other protocols.

3.3.5.1.1 Chelex Extraction Method

The decontaminated pellet was washed with TE-Triton X-100 buffer and re-suspended in 100 μ L of Chelex 10%/Triton X-100 1% TE. The samples were boiled for 30 minutes, cooled at -20°C for 10 minutes, and centrifuged at 6,000 *rpm* for 5 minutes, and the 3- μ L supernatant was used for PCR (Montenegro *et al.*, 2003).

3.3.5.1.2 Isopropanol Extraction Method

100 μ l of decontaminated sample was mixed with 400 μ l of lysis buffer and 300 μ l of isopropanol, centrifuged at 12000 *rpm* for 10 minutes, the supernatant was decanted by gently inverting the tube, and then the pellet was washed with 75% ethanol and re-centrifuged at 12000 *rpm* for 5 minutes. The previous step was

repeated three times, the final pellet was re-suspended in 50µl Tri EDTA (Mogahid *et al.*, 2010).

3.3.5.1.3 Phenol Chloroform Method

According to (Jain *et al.*, 2002) 200µl of decontaminated sample was placed in boiling water bath at 100°C for 10 min. It was followed by incubation at 56°C for 3 hours after addition of equal amount of lysis buffer (Tris 10mM, EDTA 2mM, NaCl 0.4M and Triton X-100 0.5%) (pH 8.0) and 10µl of Proteinase K (10mg/ml). The sample was then vortexed and boiled at 100°C for 10 minutes to inactivate proteinase K. DNA purification was done by addition of equal volume of Phenol: Chloroform (24:1) followed by chloroform only. The aqueous phase was finally transferred in 2.5 volume of chilled ethanol and sodium acetate (0.3M final concentration.) was added. Tubes were kept at -20°C overnight. The sample was centrifuged at 10,000 rpm for 10 minutes and the DNA pellet was washed with 70% chilled ethanol by centrifugation. The pellet was allowed to air dry and finally suspended into 25µl of D.W. (sterile) for PCR analysis.

Phenol Chloroform method were found to be the best method as it gave higher quantity of DNA after reading by (Eppendorf BioPhotometer plus), so all specimens were treated by Phenol Chloroform method.

3.3.5.2 Primers of Insertion Sequence *IS6110*

The DNA sequence most frequently used to detect *M. tuberculosis* has been the insertion element IS6110 (Eisenach *et al.*, 1990). Primers amplify a target fragment of 123 base pairs from the insertion-like *M. tuberculosis* sequences element IS6110, having the following sequence:

IS6110-F (5'-CTCGTCCAGCGCCGCTTCGG-3')

IS6110-R (5'-CCTGCGAGCGTAGGCGTCGG -3')

3.3.5.3 Preparation of Master Mix

Before starting master mix preparation, hood was disinfected using 70% ethanol before and after preparation of each batch, then sterilized further by turning on the UV light for at least 20 min.

22 μ l of master mix was prepared for one reaction using VIVANTIS kit (VIVANTIS Co., Ltd., Selangor Darul Ehsan, Malaysia) according to (David *et al.*, 2006), as follow:

2.5 μ l of 10x buffer was placed in sterile eppendorf tube (1x), 0.3 μ l from 10mM forward primer was added (0.12mM), 0.1 μ l from each dNTP 50 mM (0.2 mM), 1.5 μ l of 25mM MgCl₂ (1.5mM), 0.125 μ l of 500 units at 5U/ μ l Taq polymerase (2.5 units), 0.3 μ l from 10mM reverse primer (0.12mM), the volume was completed to 22 μ l by adding 16.875 μ l of sterile distilled water, the contents of master mix was vortexed after addition of each item and lastly 3 μ l of template DNA was added.

In negative control 3 μ l of sterile distilled water was added, while DNA extracted from *M. tuberculosis* strain H37R was used as positive control.

3.3.5.4 PCR Amplification

The reaction mixtures were then placed in the thermal cycler (CONVERGYS® ltd Peltier Thermal Cycle) that carried out the following PCR program: initial 5 minutes denaturation step at 94°C for one cycle followed by repeating cycles of denaturation (30 seconds at 94°C), annealing (45 seconds at 58°C) and extension (40 seconds at 72°C) for 35 cycles, followed by a 5 minutes final extension step at 72°C (Peres *et al.*, 2009).

3.3.5.5 Prepare of Agarose Gel

500 ml of 1X Tris/borate/EDTA (TBE) was prepared to prepare the gel and to fill the electrophoresis tank, 1.5 gram of agarose powder (AppliChem) was added to 100 ml of electrophoresis buffer (TBE) in an Erlenmeyer flask for preparation of

1.5% agarose, the agarose solution was heated in hot plate to allow all of the grains of agarose to dissolve after covering the flask with aluminum foil to prevent evaporation, then the solution was cooled down to 60°C and 5 µl of 0.5 µg/ml ethidium bromide (Et Br) was added, 1.0 mm comb positioned above the gel casting tray before pouring the liquid agarose gel to permit complete well formation when the agarose solidify, after solidification comb was gently removed and enough electrophoresis buffer was added to the tank to cover the gel (about 1 mm of depth), the top of the wells were submerged.

3.3.5.6 Loading of Samples and Electrophoresis

8 µl of PCR product from each sample were mixed with 2 µl of loading dye and then the mixtures were delivered into the well. 7 µl of DNA ladder (marker) length 100 bp ladder with fragments ranging from 100 bp to 1000 bp were added to one well in each run to estimate the size of tested DNA sequence. The gel electrophoresis apparatus was connected to a power pack (Serva BluePower 500, Germany). The electrophoresis was performed at 50 V for 30 minutes.

3.3.5.7 Visualization of PCR Product

After electrophoresis period the gel tray was removed from the electrophoresis apparatus and the buffer was discarded. One hundred twenty-three base pair (bp) target DNA fragments specific for *M. tuberculosis* complex were viewed under ultraviolet transilluminator (SYNGENE, UK). Lastly the gel was transferred to gel documentation system (Olympus) for photography documentation.

CHAPTER FOUR

4. Results

4.1 Epidemiological Finding

4.1.1 Gender

During the six months study period, 197 children of both sexes with clinical manifestation of tuberculosis were enrolled in the study, 71 (36%) were females and 126 (64%) males giving an average sex ratio of 1:1.8. Fig (1) below shows the gender distribution of the number of children registered at the five participating hospitals during the study period.

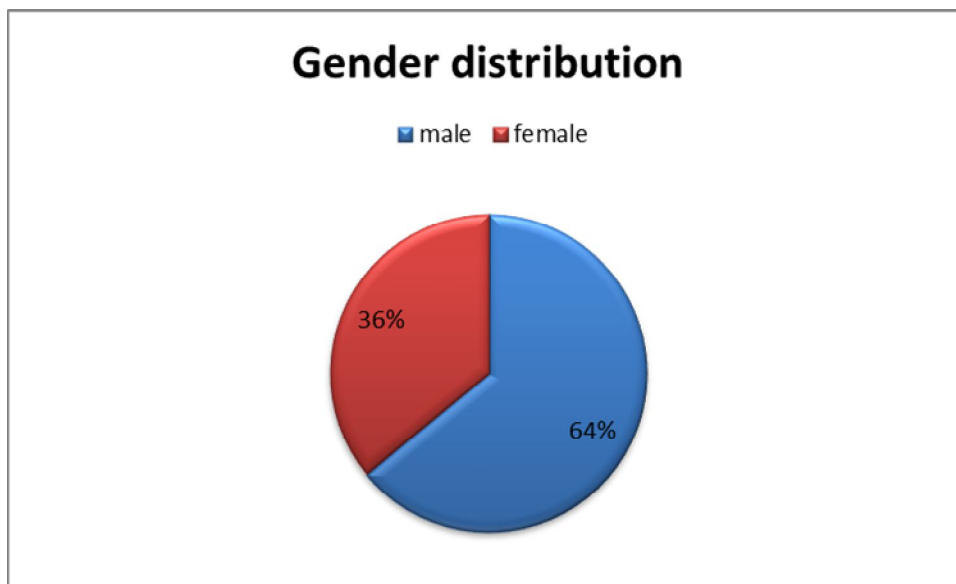


Figure 1. Distribution of children participating in the study according to the gender.

4.1.2 Age Group

The median age at investigation was 8 years. Study population divided into three age groups according to ability to expectorate sputum sample. 86 (43.7%) child less than six years old, who cannot give good sputum sample, so gastric aspirate collected from them. 63 (32%) child between 7 and 12 years old, some of them

produced sputum sample, and gastric aspirate collected from the rest. Last group 48 (24.3%) child, all of them gave good sputum sample. Of the 197 samples, there were 89 (45.2%) gastric aspirate, while 108 (54.8%) were sputum samples.

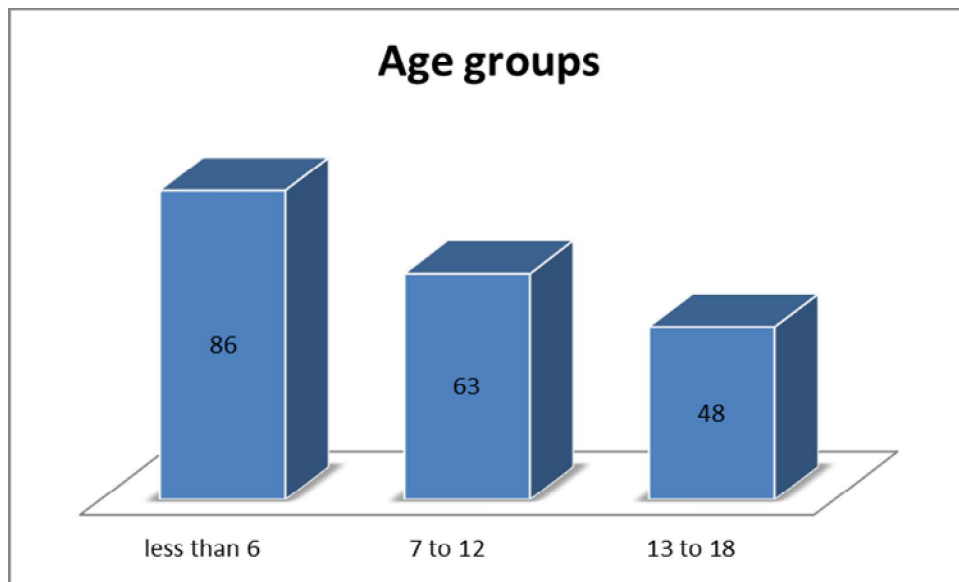


Figure 2.Number of children with in each age group.

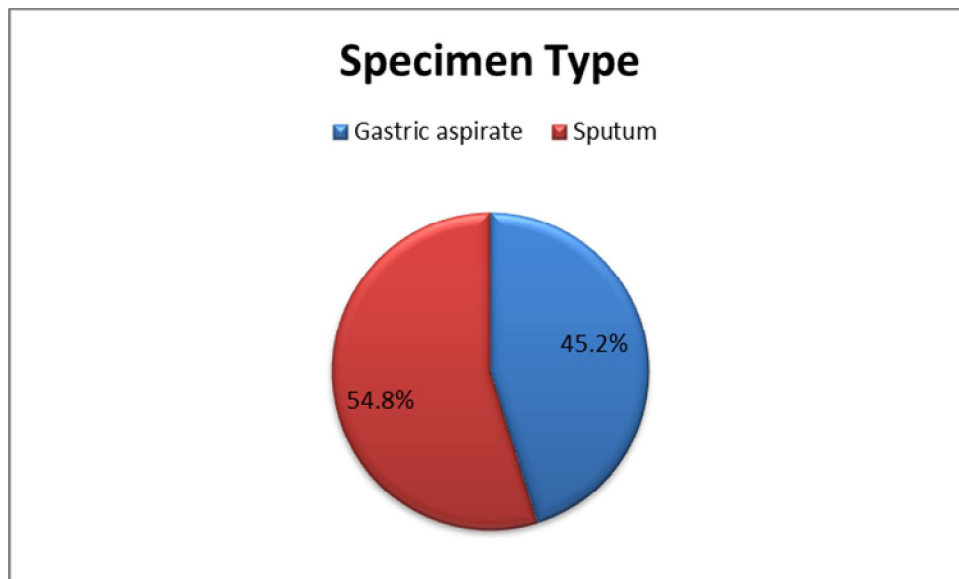


Figure 3. Distribution of specimen type.

4.1.3 Exposure to Risk Factor and Disease Manifestations

Contact with an adult with tuberculosis was reported for 61 children (31.0%), and 173 children (87.8%) had cough lasting more than two weeks. Weight was recorded as being less than 40% of expected weight-for-age according to standard of (Ministry of health - Primary Health Care) in 166 (84.3%). Of the 197 children studied, there were 174 (86.6%) patient suffering from fever. Among the participating children there were no HIV positive cases.

4.2 Laboratory Findings

4.2.1 TST

Tuberculin skin test was performed for 201 children, 4 children didn't return after two days for result reading and excluded from the study, 197 children were enrolled in the study. Of 197 children enrolled in the study, 86 (43.7%) tested positive for TST. Fig 4.



Figure 4. Palpable induration of positive Mantoux test.

4.2.2 ZN Stain

Slide smear prepared from all specimens, of the 197 sample studied, 16 (8.1%) tested positive for ZN stain. All positive results were found to be from sputum samples.

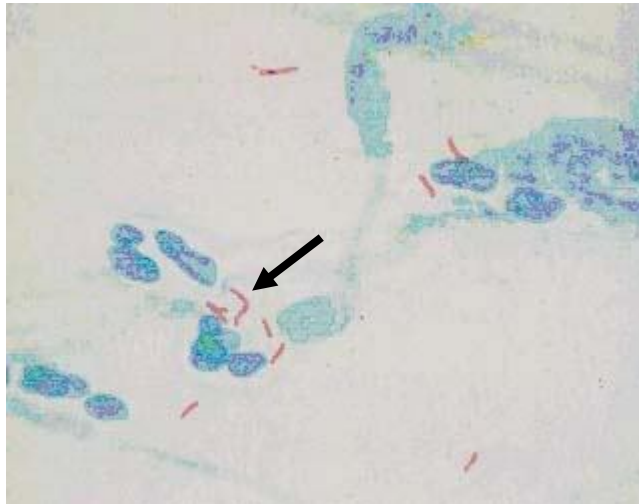


Figure 5. Red acid fast bacilli against blue back ground.

4.2.3 Auramine Stain

Of the 197 specimens studied, 22 (11.2%) tested positive for Auramin stain. All positive results were found to be from sputum samples.

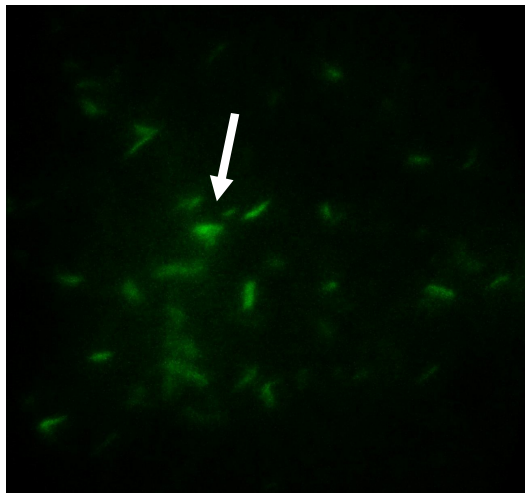


Figure 6. Green-yellow rods glowing against a dark background.

Table 1. Correlation of the fluorescent method using Auramine stain with the conventional ZN method.

			ZN		Total
			positive	negative	
Auramine	positive	Count	14	8	22
		% of Total	7.1%	4.1%	11.2%
	negative	Count	2	173	175
		% of Total	1.0%	87.8%	88.8%
Total	Count	16	181	197	
	% of Total	8.1%	91.9%	100.0%	

4.2.4 Isolation

During the period of the study, 204 specimens were subjected from the above mentioned hospitals. All of these samples were decontaminated and cultured on LJ media. Out of these, 7 specimens found to be contaminated. 197 specimens of results were used in the study. Out of 197 specimens, 32 (16.2%) showed slow growth MTC-like colonies, 2 (1.01%) show rapid growth and were considered as negative for MTB. Of 32 slow growth MTC-like colonies, there were 30 (93.8%) organisms isolated from sputum samples, while two (6.2%) were isolated from gastric aspirate. The MTC-like colonies were confirmed by conventional methods.

4.2.4.1 Growth Rate

The growth rate of the isolates ranged between 3 days and 6 weeks. Most of the isolates showed visible growth after 3 weeks. Two out of 197 isolates were identified as rapid growers of Mycobacteria, while the growth rate of 32 isolates ranged between four and six weeks and they were identified as slow growers and

tentatively considered belonging to MTC species. The rest of culture 163 (83%) show no growth. Fig 7.

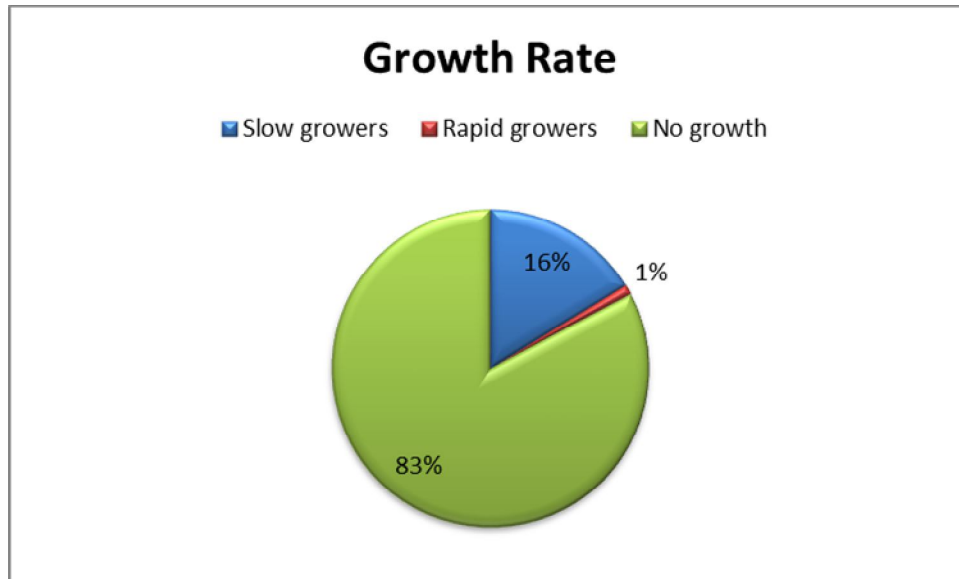


Figure 7. Illustrate the difference in percentage of organism growth rate.

4.2.4.2 Cultural Characteristics

Cultural properties of all 32 slow growing isolates of *Mycobacterium tuberculosis* complex on Lowenstein Jensen medium at 37°C were almost the same and all colonies were found dry, rough and (buff) coloured as shown in Figure 8.

No *M. bovis* was isolated from these samples.

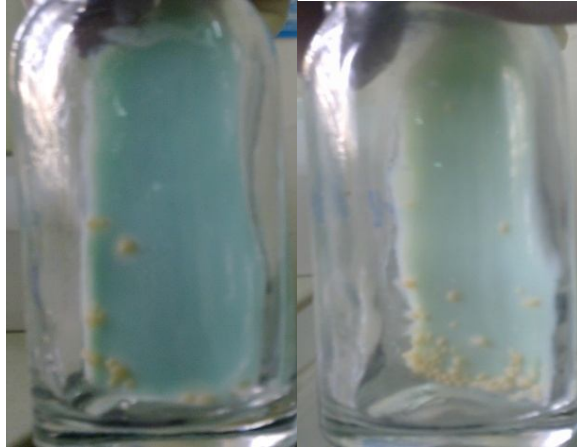


Figure 8. Growth of *M. tuberculosis* on LJ medium after 3 weeks incubation at 37°C showing cream yellow, tough, rough, raised, and buff colonies.

4.2.4.3 Biochemical Tests

4.2.4.3.1 Nitrate Reduction

All of 32 isolates of MTC organisms (slow growers), were Positive for nitrate reduction (reduce nitrate to nitrite) with different intensity of colour different positive grade. The grades of positive result ranged between one and three crosses. 24/32 (75%) were three crosses, 5/32 (15.6%) were two crosses and 3/32 (9.4%) were one cross. **Appendix VII.**

4.2.4.3.2 Catalase Test

All isolated organisms were found to be negative for catalase test at 68°C and positive at room temperature. **Appendix VI.**

4.2.5 Polymerase Chain Reaction

From the 197 specimens which were directly subjected to PCR. 35/197(17.8%) showed a band typical in size (123 bp) to the target gene (IS 6110) as indicated by the standard DNA marker, as shown in (Figure 9), 162/197 (82.2%) samples were negative. Out of 35 positive results, 4 (11.4%) were yielded from gastric aspirate samples, while 31 (88.6%) were yielded from sputum samples.

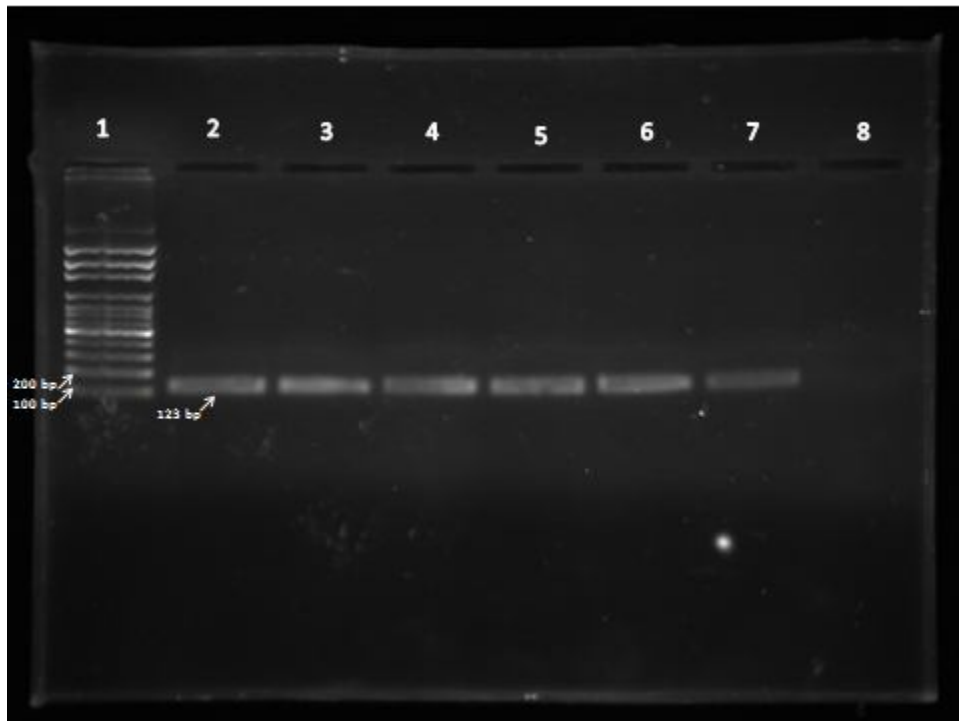


Figure 9. Agarose gel electrophoresis of IS 6110 based polymerase chain reaction for detection of *M. tuberculosis* from clinical specimens. Lanes: 1 = 100 bp molecular marker; 2 = positive control (123 bp); 3,4, 5, 6, & 7 = positive from direct PCR using clinical specimens; 8 = negative control.

4.3 Correlation of PCR with Other Investigations

The correlation between the PCR and other diagnostic methods is shown in figure 12, 13, 14 and 15.

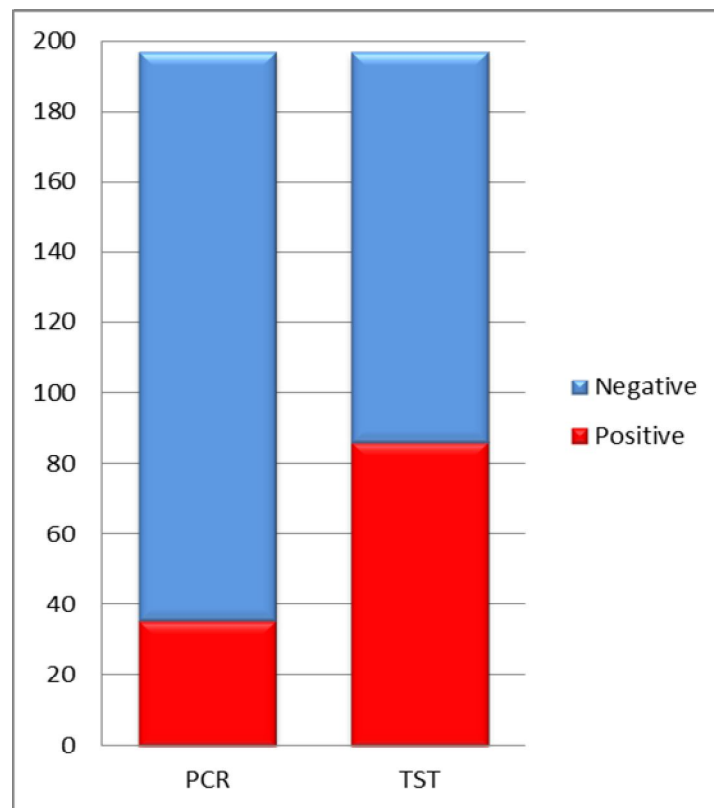


Figure 10. Comparison between PCR and Tuberculin skin test results.

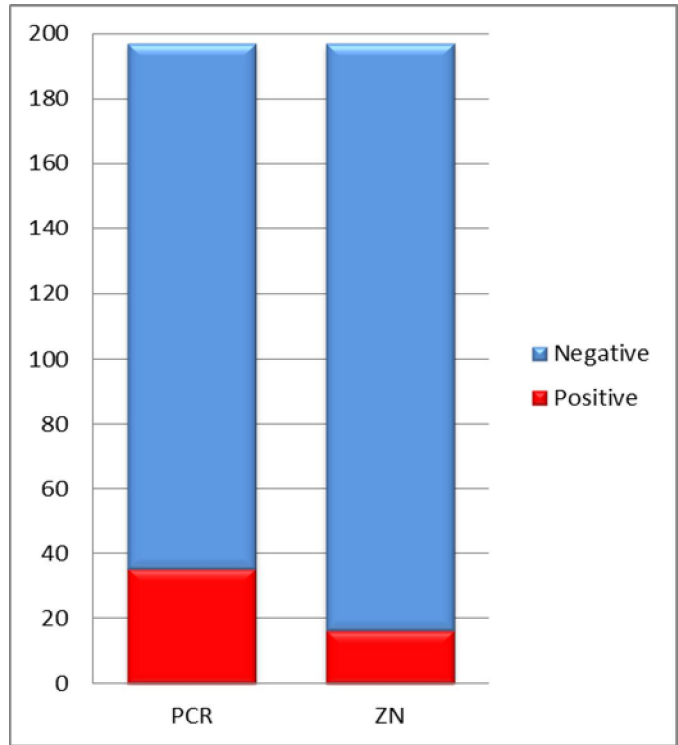


Figure 11. Comparison between PCR and ZN results.

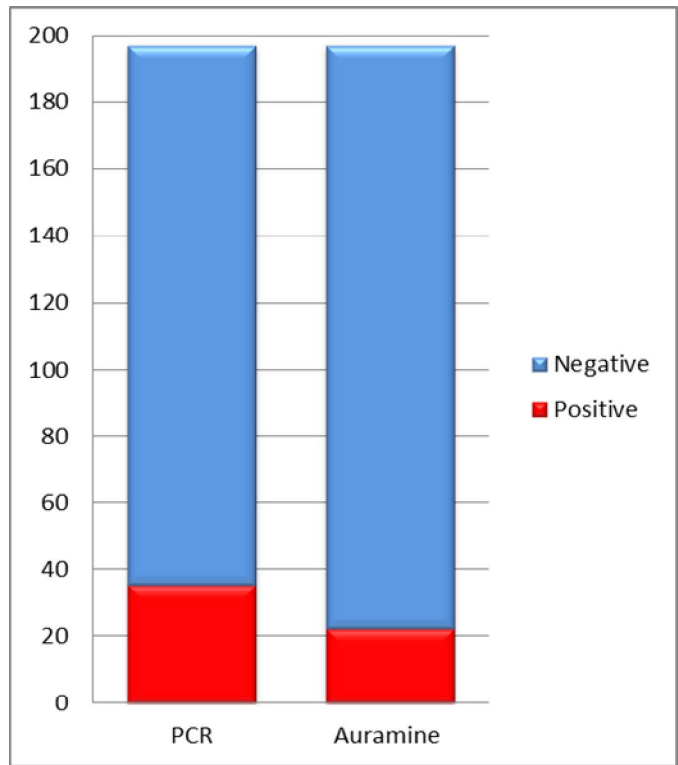


Figure 12. Comparison between PCR and Auramine results.

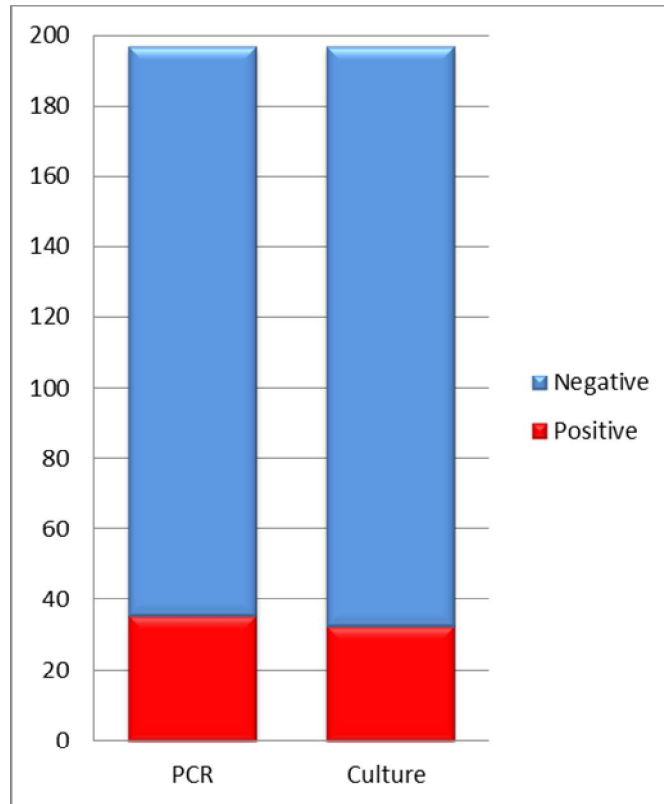


Figure 13. Comparison between PCR and culture results.

4.4 Sensitivity and specificity

The sensitivity and specificity of TST, microscopic techniques and PCR based on results of culture as gold standard technique for diagnosis of tuberculosis were as follow; sensitive were 100%, 59.38%, 43.75% and 100% for TST Figure 12, Auramine, ZN and PCR test respectively. While specificity were 67.27%, 98.18%, 89.79% and 98.18%. Positive Predictive Value (PPV) was 37.23% for TST, 86.36% for Auramine and 87.50% for ZN stain and 91.43 for PCR. Negative Predictive Value (NPV) was 100%, 92.57%, 90.06% and 100% respectively.

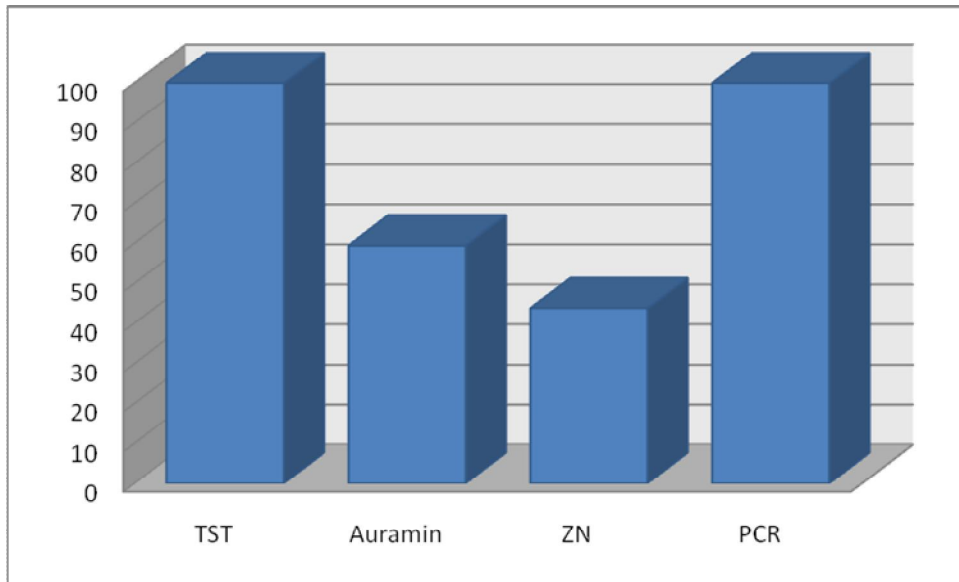


Fig. 12 Sensitivity of TST, Auramin, ZN and PCR based on culture as gold standard method.

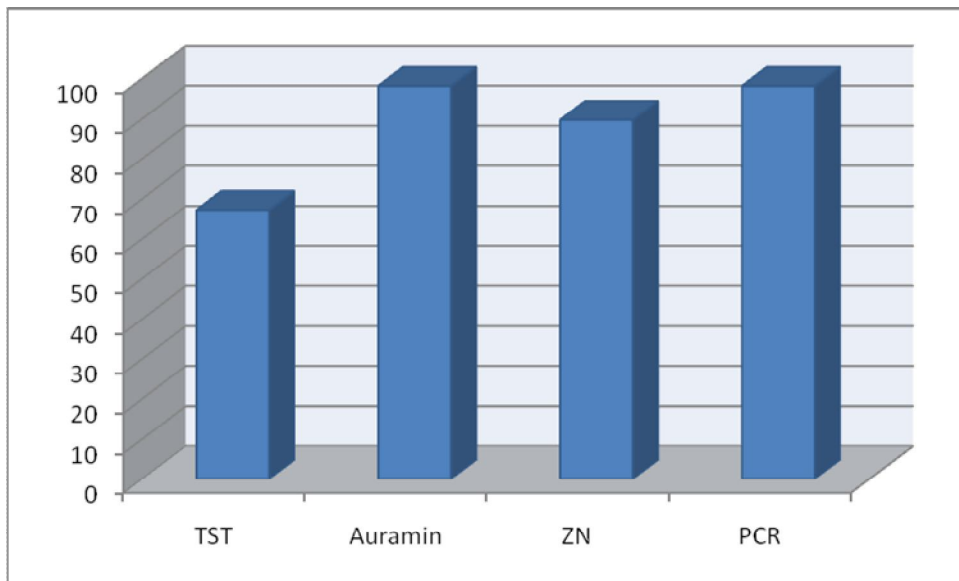


Fig. 13 Specificity of TST, Auramin, ZN and PCR based on culture as gold standard method.

CHAPTER FIVE

5. Discussion

Childhood TB is often regarded as unimportant in the epidemiology of TB because >95% of children with TB are sputum smear negative and therefore do not contribute to the immediate spread of the disease. Childhood TB, however, is a marker for ongoing transmission of infection within a community and infected children represent the pool from which a large proportion of future cases of adult TB will arise(Starke, 1993).

Furthermore, TB is an important cause of morbidity and mortality in children. The World Health Organization estimated that 1.3 million cases of TB and 450,000 deaths from TB occur annually among children in developing countries (Kochi, 1991).

This study showed that the frequency of childhood tuberculosis using PCR technique is 17.8% and 16.2 using culture technique which is similar to the results of published studies in near countries Ethiopia 16.1%, Nigeria 12.4%, Tanzania 16.1% (Clydette, 2004)

Many promising advances have been made in the development of novel tools to diagnose tuberculosis in adults (Brock *et al.*, 2006) but none of these tests are currently in position to replace microscopy or culture (Perkins *et al.*, 2006). Few of these novel approaches have been tested in children, the group in whom the diagnostic dilemma is most pronounced. At present, the use of adequately validated symptom-based diagnostic approaches and improved access to chest radiography and anti-tuberculosis treatment seem to offer the most immediate benefit to children in tuberculosis-endemic countries with limited resources (Marais *et al.*, 2006).

Most recently developed sensitive and specific diagnostic tests have not found a place in the routine evaluation of children with suspected TB. Clinical criteria, particularly skin-test results, radiographic changes, and documented exposure to an infectious adult remain standard diagnostic methods.

Molecular diagnosis for *M. tuberculosis* in children is mainly done at referral laboratories and their value as a diagnostic tool is often debated, especially in resource-limited settings.

The main aim of this study was to determine the extent to which molecular technology characterized *Mycobacterium tuberculosis* in pediatric cases by detection of specific gene directly from gastric aspirate and sputum samples. The performance of PCR test was compared with other conventional methods used in diagnosis of tuberculosis TST, ZN, Auramin and culture on LJ media (gold standard method).

Despite the low specificity of TST particularly in children, the test is widely used in diagnosis of childhood tuberculosis. In this study interpretation of TST results is depended on the Centers for Disease Control and Prevention (CDC) recommendations regarding the size of the induration created by the TST that is considered a positive result and indicative of disease. The TST is interpreted on the basis of three cutoff points; 5 mm or more is considered a positive TST result for children having close contact with known or suspected contagious cases of the disease, including those with household contacts with active TB whose treatment cannot be verified before exposure. 10 mm or more is considered a positive TST result for children who are at a higher risk of dissemination of TB disease, including those younger than 5 years or those who are immunosuppressed because of conditions such as malnutrition. Induration of 15 mm or more is considered a positive TST result in children aged 5 years or older without any risk factors for the disease.

TST was found to be highest positive result 86/197 (43.7%) among all diagnostic method used in the study. There are two possible explanations for the high TST results compared with other investigation. First, false-positive TST reactions due to BCG vaccination in young children, the vast majority of children enrolled in the study were less than ten years old. In a review of many published studies regarding the effect of BCG vaccination on TST, only 1% of subjects vaccinated as infants were TST-positive if tested ≥ 10 years after BCG (Farhat *et al.*, 2006). Second, false-positive due to infection by environmental non-TB mycobacteria (due to cross-reactivity).

Previous studies showed the success of microscopy is highly variable from 22% to 96% and most authors rate it at round 60% (Querol *et al.*, 1995). ZN stain is routinely used in all tuberculosis diagnostic centers in Sudan, while Auramine fluorochrome method is only used in the National Reference Laboratory (NRL). Different smear microscopy results were achieved by Jain *et al.*, (2003) ZN 32.7%, Auramine 41.6%, (Githui *et al.*, 1993) ZN 65%, Auramine 80%, (Ulukanligil *et al.*, 2000) ZN 67.6%, Auramine 85.7%, (Prasanthi and Kumari, 2005) ZN 50%, Auramine 69%. It was evident that Auramine method results scored higher than that of ZN method in all these studies as was the case in this study (ZN 8.1%, Auramine 11.2%). In this study, Auramine was found to be 3.1% more effective than ZN staining. This shows that fluorochrome staining of sputum smears in comparison to that of ZN staining is a better method of microscopy.

The poor performance of conventional *M. tuberculosis* detection techniques, based on microscopic examination of Ziehl-Neelsen stained, culture of *M. tuberculosis* on LJ Medium and TST are still in widespread use for diagnostic purposes, still though they fail to provide the required sensitivity and specificity. The PCR test would be particularly useful in the diagnosis of childhood tuberculosis where

conventional microbiological techniques for *M. tuberculosis* are showing poor performance of sensitivity and specificity.

This study shown that microscopic techniques were positive in 22 (11.2%) specimens, whereas IS6110 PCR showed that 35 (17.8%) specimens were positive for *M. tuberculosis*. The difference was found that to be statistical significant ($p < 0.05$).

On the other hand, culture technique detected 32 positive out of 35 positive which detected by PCR, with different only three samples less than PCR.

One of the major achievements of this study was the efficiency and success of PCR technique in characterization of *M. tuberculosis* from gastric aspiration samples (the sample which is mainly collected for detection of childhood tuberculosis for children under six years old) which detect 5 out of 89 samples, better than culture technique which detected only two gastric samples.

In addition to advantage of PCR which is rapid (needs only one day), while culture method was time consuming (4-8 weeks) and has restricted growth conditions.

There are a few limitations in this study. First, only one gastric aspirate sample collected from non-admitted children (under six years) which minimize the chance of detection positive cases, whilst two samples collected from hospital admitted children. Second, many children didn't return back after 72 hours for reading of tuberculin skin test result, which led to exclusion of them from the study.

Conclusion and Recommendations

5.1 Conclusions

PCR technique is more efficient over conventional methods in diagnosis of childhood tuberculosis, especially from gastric aspirate samples.

IS6110 PCR test for DNA specific *M. tuberculosis* may be hopes of a rapid and accurate diagnostic test for childhood tuberculosis and it will help where conventional diagnosis fails and provisional diagnosis of childhood tuberculosis is made on the basis of clinical presentation without evidence of AFB. IS6110 PCR may be great potential to improve the clinician vision for the early diagnosis, treatment and prevention of childhood tuberculosis.

The specificity, sensitivity and speed of PCR test in diagnosis of *M. tuberculosis* infection shown in this study should encourage the use of this method in routine diagnosis of pediatric tuberculosis.

Auramine stain performs better than ZN stain in detecting TB bacilli in sputum, particularly the paucibacillary cases. Since screening is done under lower power of magnification (40x), fluorescence microscopy has been found to be less time consuming as compared to ZN method (100x) in the diagnosis of tuberculosis. Hence, it has been advocated to be a method of choice where a large number of sputum smears are to be examined. The fluorescing bacilli are easily identifiable and cause less eye-strain.

Tuberculin skin test is highly sensitive method, but specificity affected by recently BCG vaccination, whereas majority of participants in this study were young children.

5.2 Recommendations

1. Further systematic surveillance is needed in order to reliably determine the prevalence of childhood tuberculosis in Sudan.
2. An effort should be made to obtain several specimens in any child being evaluated for active TB, as the paucibacillary nature of pediatric TB limits the rates of positive AFB smear, culture and PCR.
3. The study suggest that TB reference laboratory in countries such as Sudan with high TB burdens should consider the use of PCR in combination with smear microscopy to enhance prompt detection of *M. tuberculosis* in gastric aspirate and in other samples with low Mycobacteria content.
4. It is recommended to use Nested PCR in further studies to increase quantity and specificity detecting gene, especially from gastric aspirate samples which has low DNA contents.
5. Further studies must consider Spoligotyping to differentiate between types of *Mycobacterium* species which cause infections in children, although they recently received BCG vaccine.

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Appendices

Appendix I

Questionnaire

Patient's Name:

No:

Age:yrs

date:

Weight:kg

Social status Student Worker specify:

Gender: Male Female

Type of investigation: Diagnosis Follow up

History and symptoms:

		Yes	No	Don't know
1	Have you ever had a BCG injection?			
2	Do you have a cough that has lasted longer than 3 weeks?			
3	Have you had fever, chills, or night sweats?			
4	Have you ever had an abnormal chest x-ray?			
5	Have you ever been told you had TB?			
6	Have you ever taken medicine for TB?			

Exposure to risk factor:

		Yes	No	Don't know
1	Have you ever lived with or had close contact with someone who had TB?			
2	Have you ever had a HIV test?			Result()
3	Are you suffering from weight loss (malnutrition)?			

Medical center:

Doctor's name:

Sign:

Appendix II

Mineral salt solution

- Potassium dihydrogen phosphate anhydrous(KH_2PO_4).....2.4g
- Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$).....0.24g
- Magnesium citrate0.6g
- Asparagine.....3.6g
- Glycerol.....12ml
- Distilled water.....600ml

Appendix III

Malachite green solution 2%

- Malachite green dye.....2.0g
- Sterile distilled water.....100ml

Appendix IV

Preparation of media (Lowenstein-Jensen Egg medium)

- Fresh, antibiotic free eggs were cleaned by scrubbing with a hand brush in a soap solution, rinsed through running water and soaked in ethanol 70% for 15 minutes. Hands were washed and scrubbed well before processing. Eggs were broken and transferred in to sterile flask, blindered in sterile mug and filtered through four layers of sterile gauze into graduated cylinder. A mineral salts solution was prepared by dissolving:-
- Monopotassium phosphate (anhydrous).....2.4g
- Magnesium sulfate.....24g
- Magnesium citrate.....0.6g
- Asparagine3.6g
- Sterile distilled water600ml

- The solution was dissolved in water bath 100°C for 45 minutes, cooled to room temperature .Then:-
- Malachite green (2%).....20 ml
- Glycerol.....12ml
- Homogenized whole eggs.....1000ml

Were added, the complete medium was poured into sterile funnel and 6to8ml was dispensed into sterile universal bottles, slanted, coagulated by inspissation at 85⁰C for 45 minutes .A random sample of each patch was incubated at 37°C for 48hour for sterility checking.

APPENDIX V

Decontamination of Sputum

Materials and equipment

- 4% NaOH (w/v) autoclaved at 121°C for 15 min.
- Distilled water autoclaved at 121°C for 15 min.
- HCl 8.3 + 1.7 D.W + 0.1 phenolized indicator 100 ml HCl / 1 N
- Screw-capped plastic sterile containers.
- Vortex

Appendix VI

Freshly prepared tween – pyroxide mixture

- Hydrogen peroxide 30 %
- Tween80..... 10 %
- Tween80 10 ml
- Distilled water..... 90 ml
- Mix and autoclave at 121°C for 10 min
- Complete catalase reagent: (tween – peroxide-mixture)

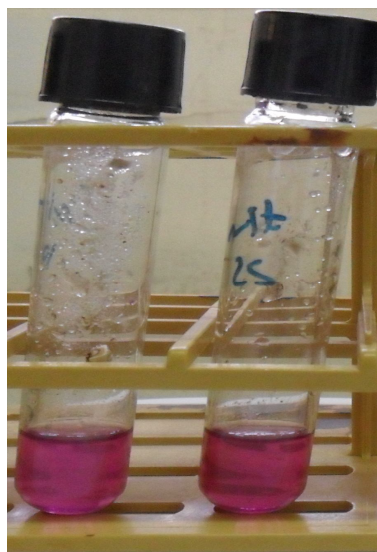


Results of catalase test for *M. tuberculosis* strains. Positive at room temperature to the left side which is indicated by formation of air bubbles and negative at 68°C on the right side.

Appendix VII

Nitrate Reduction Test

- sodium nitrate NaNO_3 0.01 M (0.085) %
- distilled water100 ml
- Mixed and autoclaved at 121°C for 10 min.



Positive results three crosses of nitrate reduction test for *M. tuberculosis*.

Appendix VIII

LAMP reagent

- N.I. naphthyle. Diamine dihydrochloride 1g
- Sulphanilic acid 1g
- Tartaric acid10g

Appendix IX

Z.N. staining

1. Staining solution

Stock Solution A

- L.O.C. High Suds (Amway)0.6 ml
- Distilled water100 ml

Stock Solution B

- Basic fuchsin1 g
- Absolute ethyl alcohol10 ml

The two solutions can be kept as stock solution and mixed before use

2. Working Solution (stable for 1 month)

Mix 50ml of A with 5 ml of B.

3. 3% hydrochloric acid in 95% ethyl alcohol

- Absolute ethyl alcohol 95ml
- Distilled water 2 ml
- Concentrated hydrochloric acid 3 ml

Make up the alcohol solution then add the concentrated acid. Use extreme care when handling concentrated acid.

4. 0.25% methylene blue in 1% acetic acid

- Methylene blue 0.25 g
- Distilled water 99 ml