CHAPTER ONE

INTRODUCTION

AND

OBJECTIVES

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1.1 Introduction

Tuberculosis (TB) is a chronic infectious bacterial disease that threatens public health and responsible for killing nearly 2 million infected individuals a year together with a further figure of about 9 million freshly emerged active cases worldwide as estimated by the World Health Organization (WHO, 2009;2013). It is caused by *Mycobacterium tuberculosis* (Mtb) which belongs to *Mycobacterium tuberculosis* complex (MTBC) (Koch, 1882; Niemann *et al*., 2000). Other members of the complex include; *M. bovis*, *M. africanum*; *M. microti*, *M. pinnipedii, M. caprae,* and *M. canetti* (Ahmad *et al*., 2011). These members have distinguished phenotypic properties and wide host range; though they are genetically and closely related species. Genomic sequence analysis of Mtb in comparison with *M. bovis* has shown minor differences mounting only to <0.05% (Garnier *et al*., 2003). Although the latter species is primarily an animal pathogen, it also infects humans (Ahmad, 2011).

Transmission of TB occurs by inhalation of infected droplet aerosols released by an infected person through coughing or sneezing to a susceptible one in close contact. Following infection with Mtb, only a small proportion of the population gets the disease (Russell, 2006). While the remaining majority incubates infection in a latent granulomatous stage which reactivates under appropriate conditions (Russell., *et al* 2006). TB is mainly pulmonary affecting the lungs, and accounts for >85% (Russell., *et al* 2006). But, an extra-pulmonary form exists as exemplified by lymphadenopathy (inflammatory disease of lymph nodes) or TB meningitis (TBM) (Cooke *et al.,* 2006). This extra-pulmonary form occurs due to weakening of immune response to clear infection or failure of the host to suppress bacterial dissemination to different parts of the body (Grange, 1996; Cooke *et al.,* 2006). Active TB is clinically exhibited by symptoms of fever, persistent cough with or without blood, weight loss and night sweating.

Increased incidence of TB has been reported in various countries primarily in sub-Saharan Africa, Asia and several pockets across the globe including Eastern Europe (WHO, 2009; Zager and McNerney, 2008). Such resurgence of the disease is associated with emergence of human immune-deficiency virus (HIV) and multiple TB drug resistance, low standard of living accompanied by poor hygiene and delivery of health services (Zager and McNerney, 2008).

Mycobacterium tuberculosis is a facaltitave intracellular pathogen and cellular immune response is quite crucial for the host to combat and limit infection (Awomoyi *et al*., 2004). Interferon gamma (IFN-γ) plays a great role in this process as it activates macrophages to release antimycobacterial toxic products such as nitric oxide to kill invaders (Newport *et al*., 1996., Awomoyi *et al*., 2004). The cytokine is a dimeric cell surface glycoprotein encoded by a gene which expresses the cytokine upon stimulation of lymphocytes with various antigens (Gray and Goeddel, 1982; Liu, 2005). It is comprised of two transmembrane protein receptor subunits; IFN-γR1 ligand-binding chain essential for phosphorylation and IFN-γR2 chain responsible for transducing signal pathways between immune cells and conferred by the intracellular membrane (Dorman and Holland, 1998). Binding of IFN-γ with IFN-γR1 results in oligomerization which enhances a Janus kinase attached to each receptor unit causing phosphorylation of IFN-γR1. The phosphorylated receptor subsequently binds with signal transducer and activator of transcription (Stat1), and the complex induces transcription of IFN-γ genes in the nucleus (Newport *et al*., 1996). After being released, the cytokine permits signaling between the host immune cells and stimulates their bactericidal killing power to eliminate invaders (Liu, 2005).

Defective familial genetic factors and IFN-γ signalling pathway have been reported to affect host immune response and trigger TB severity (Newport *et al*., 1996). In this context, genetic abnormalities within IFN-γ receptors (1 and 2) are implicated in increased susceptibility to mycobacterial infection (Dorman and Holland, 1998). Hence, affected children with mutation in IFN-γ receptor ligand are predisposed to susceptibility with mycobacterial infection and ultimately, they may be at risk of developing mycobacterial dissemination leading to death (Dorman and Holland, 1998). Mutations in *IFN-γ* gene and its subunits; IFN-γR1 and IFN-γR2 have been reported in association with increased susceptibility to TBin different populations (Lio *et al*., 2002; Lopez-Maderuelo *et al*., 2003; Rossouw *et al*., 2003). Of these genetic polymorphisms which were found in correlation with the risk of developing TB, is the one at position +874 within *IFN-γ* gene (Lio *et al*., 2002). Several other genetic polymorphisms at positions -56, -1616, and +3234 within the promoter region of *IFN-γ*R1 have also been shown to predispose susceptibility of TB (Cooke *et al*., 2006). By contrast, Cooke *et al*., (2006) who have studied one of these polymorphisms at position +874 of the IFN-γR1 gene did not find any association of the polymorphism with the disease.

In the undergoing project, we have investigated the role of a number of genetic polymorphisms and mutational alleles within genomic *IFN-γ*R1 gene extracted from blood immune cells of infected TB patients and their effect in developing pulmonary TB among Sudanese patients. This was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of extracted genomic *IFN-γ*R1 DNAusing *Eco*47**Щ** enzyme to verify identity of product bands when they were run on agarose gel electrophoresis. Other laboratory tests were conducted to support the findings.

1.2 Rationale

Pulmonary tuberculosis is an infectious chronic bacterial disease that cripple's humans' health causing increased morbidity and mortality across the globe. Sudan is among the top 22 countries with high TB burden. Several risk factors including familial genetic make-up predispose susceptibility to the disease. IFN-γR1 protein release has a central key role in potentiating host immune responses against TB. A number of single-gene mutation within IFN-γR1 signaling pathway or changes in the promoter region have been identified and reported to associate with severity of mycobacterial disease. Yet, relevance of genetic polymorphisms panning such genes to the common phenotypic clinical characteristics of TB has remained uncertain.

In the Sudan and with the exception of one scientific publication in which the authors linked a single IFN-γR1 mutation to increased resistance of TB (Attalla *et al*., 2011). there isn't any research work to date that associates any genetic defects in the development of pulmonary TB. The undergoing study was designed to investigate the effect of IFN-γR1 gene polymorphism or mutation in predisposing active pulmonary TB among Sudanese patients attending hospitals within Khartoum State. Additionally, other laboratory tests were carried out to permit support or early detection of IFN-γR1 genetic defects in order to prevent further development of any complications.

1.3 Objectives of the Study

1.3.1 General objective

This project aimed to investigate the role of IFN-γR1 gene polymorphisms in the development of pulmonary TB among Sudanese patients attending several hospitals in Sudan.

1.3.2 Specific objectives

1. To demonstrate acid fast bacillus (AFB) on Ziehl-Neelsen (ZN) sputum-stained smears collected from active pulmonary cases and culture of positive specimens to isolate causative agents.
2. To identify the causative agent employing cultural and biochemical conventional methods following standard procedures.
3. To extract and isolate genomic DNA from collected blood immune cells of confirmed TB patients and from healthy controls.
4. To amplify IFN-γR1 gene from extracted D by PCR-assay combined with restriction fragment length polymorphism of DNA products using *Eco*47**Щ** enzyme in order to determine the presence and role of three genetic polymorphisms within the gene; one at position +95, the second at position -56 and the third one at position +295 deletion 12 in precipitating development of pulmonary TB.
5. To collect demographic data and risk factors of diseased patients and assess their association with mentioned genetic polymorphisms in IFN-γR1 gene, and see whether there is any significant role in predisposing risk of developing TB.

CHAPTER TWO

LITERTURE REVIEW

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LITERATURE REVIEW

2.1 Historical Background

Far back in history dated to the ancient Egyptian times (~ 5000 BC.), human TB existed and recognized by various names such as Phthisis (Greek term for TB), Consumption Disease and White Plague (Palomino*.,* 2007). Robert Koch, (1882), a German Microbiologist, was the first who discovered the tubercle bacillus or Koch's bacillus; named *M. tuberculosis* as a causative agent of the disease. To prove his hypothesis right, Koch carried out several guinea pig experiments and managed to reproduce the disease features after inoculation with various pathological TB suspected materials (Palomino*.,* 2007). In 1905, Koch was honoured a Nobel Prize for his remarkable achievement. At the earliest times of disease discovery, the only available advice for a consumptive patient who contracted infection was to take a rest and consume nutritious food (Palomino*.,* 2007). However, subsequent prominent work carried out by scientists and researchers have paved the way to nearly consider TB a disease of the past. Such revolutionary period, along with later great discoveries initially marked by tuberculin test in 1890 facilitating early diagnosis of the disease followed by development of Bacillus-Calmette Guerin (BCG) vaccine in 1908 and use of anti-tuberculosis drugs in 1943, offered hope for eradication of a major fatal disease and limitingits spread (Palomino *et al.,* 2007; WHO report, 2014).

2.2 Taxonomy and Characteristics Features of Mycobacteria

Mycobacterium tuberculosis belongs to the Genus, *Mycobacterium*, Family, Mycobacteriaceae, Order, Actinomycetales and Class Actinomycetes (van Soolingen *et al*., 1997; Godreuil *et al*., 2007). Mtb is a Gram positive with slightly curved rod-shaped appearance, aerobic or facultative anaerobic bacterium, non-motile, non-capsulated, non-spore forming, and shows slow growth rate (Godreuil *et al*., 2007). The organism's genomic DNA has high content of guanine plus cytosine (G+C) ranging from 61 to 71% (Palomino*.,* 2007). The bacillus shares similar phenotypic and genotypic characteristics with *Corynebacterium*, *Nocardia* and *Rhodococcus* (Palomino*.,* 2007). Mycobacteria exhibit distinct tropism to the lung tissues probably due to plentiful amount of available oxygen. They are recognized as intracellular pathogens infecting

macrophages and residing within phagosomes from where they gradually attack and spread to various host tissues. The bacillus is also distinguished by thick hydrophobic cell wall rich with mycolic acids and lipid content utilized as energy source. It further possesses a biosynthetic ability to synthesise most of nutrients substantial for survival (Godreuil *et al*., 2007). Under microscopic examination with stained-smears using Ziehl-Neelsen (ZN), Mtb displays red bacilli embedded on the background of counter stain methylene blue (Gutierrez *et al*., 2005; Godreuil *et al*., 2007).

There are more than 80 species of mycobacteria which exist naturally as saprophytes while a few become pathogenic and cause disease (Palomino., 2007). On the basis of epidemiology, growth rate and ability to cause disease, members of the genus *Mycobacterium* are split into two main groups; the slow growers including members of *Mycobacterium tuberculosis* complex (MTBC) (*M. tuberculosis*, *M. bovis* and *M. africanum*) which can exert serious chronic TB infections, and rapid growers belonging to environmental non-tuberculous mycobacteria (NTM) (Forbes *et al.,* 2007). The fast-growing bacteria produce visible colony growth within 7 days under favourable conditions. They are exemplified by a number of species like *M. fortuitum* and *M. abscessus* inducing decreased pathogenicity and presenting atypical clinical signs in human and animal hosts (Forbes *et al.,* 2007).

2.3 Evolution of *M.tuberculosis* Complex

Evolutionary studies are vital in molecular epidemiology and have been applied to unravel population genetics (Alland *et al*., 2003). At the moment, *M. tuberculosis* complex (MTBC) is comprised from seven species; *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canettii*, *M. microti*, *M. pinnipedii* and *M. caprae* (Ernst *et al*., 2007). Members of the group have a high degree of genetic similarity with 99.9% nucleotide sequence and identical 16S rRNA sequence (Smith *et al*., 2006). There is also little or no significant evidence of genetic exchange among strains (Baker *et al*., 2004). Moreover, sequence analysis of a couple of housekeeping genes namely; *katG*(encodes catalase-peroxidase enzyme)**,** *gyrB*(encodes the subunit B protein of DNA gyrase), *gyrA*(encodes the subunit A protein of DNA gyrase), *rpoB* (encodes the β subunit of bacterial RNA polymerase), *hsp65* (encodes heat shock protein 65) and *soda* (encodes superoxide dismutase enzyme) displayed that isolates of *M. canettii* recovered from humans in East Africa substitute progenies of ancestral species from which MTBC emerged (Gutierrez *et al*., 2005).

Based on those previous studies, it is assumed that strains of the complex are relatively young pathogens of a clonal progeny diverged from ancestral *M. tuberculosis* strains that were subjected to an evolutionary bottleneck dating back 20,000 - 35,000 years (Brosch *et al*., 2002).The common ancestor is believed to resemble *M.prototuberculosis* species or *M. canettii* which coevolved with hominids living in the Horn of East Africa about 3 million years ago from where they gradually and globally disseminated (Gutierrez *et al*., 2005; Ernst *et al*., 2007).A number of studies have supported the present evolutionary scheme of *M. tuberculosis* bacilli. Brosch *et al*. (2002) investigated the pattern of deletions in Mtb genome as compared with that of *M. canettii* and proposed the latter organism diverged from the rest of MTBC. Further analysis using single nucleotide polymorphisms (SNPs) and spoligotyping (direct spacer repeats patterns) have added more support in deciphering phylogeny of *M. tuberculosis* complex (Ernst *et al*., 2007).

Loss of *M. tuberculosis* specific deletion 1 (TbD1) distinguishes modern strains of the bacillus, while successive deletions of several regions of difference (RD) contributes to emergence of various lineages (Brosch *et al*., 2002, Ernst *et al*., 2007). The successive accumulative losses of RDs from the common ancestor are shown flowing in a stepwise order initially branching in *M. africanum*, then more host specific species before they ended in *M. bovis*(Appendix1). The latter species has acquired the greatest chromosomal deletions indicating its recent emergence (Brosch *et al*., 2002, Fabre *et al*., 2004; Ernst *et al*., 2007) (Appendix 1). Consequently, these findings have disproved earlier claims suggesting that *M. tuberculosis* evolved from *M. bovis* as an ancient progenitor and the animal pathogen passed to humans in whom it became adapted following domestication of cattle (Stead *etal*., 1995).

2.4 Epidemiology of the Disease

Tuberculosis is a devastating infectious disease of grave public health consequences infecting one-third of the global population, and considered second to human immunodeficiency virus (HIV) in causing death in majority parts of the world (Sulis *et al*., 2014; Fogel, 2015). Despite global decrease in reported TB incidence, prevalence and mortality during the last 10 years, eradication of the disease has not yet been achieved, and great efforts must be launched to make it possible (Sulis *et al*., 2014). In 2013, estimated TB incidents cases were accounted for 9 million fresh cases with 1.5 million deaths each year worldwide (WHO, 2014). Of the new TB

cases, those co-infected with HIV formed 12%, while deaths from such combination were 24% (Trinh *et al*. 2015).

The geographic incidence of infection and disease impact extremely varies. Regions notified with high disease burden occurred in Asia (55%) followed by Africa 30% (WHO report, 2014). Currently, the greatest global TB threat is posed by 22 countries such as India, China and Indonesia in Asia, and Nigeria, Ethiopia, South Africa and Uganda are in Africa (WHO report, 2014). These countries share greater than 80% of global TB burden (WHO reports, 2010; 2014). About one million prevalent cases were reported in Eastern Mediterranean Regional office of the World Health Organisation (EMRO). This union provides services for 22 countries and territories in the Middle East, North Africa, the Horn of Africa and Central Asia. As stated by the WHO, 95% of TB burden in the region is contributed by nine countries namely; Afghanistan, Pakistan, Islamic republic of Iran, Iraq, Egypt, Morocco, Sudan, Somalia and Yemen (WHO, 2014). In 2010, incidence rate in the same region formed 7% of the global trend (WHO report, 2010).

Several epidemiological risk factors, socio-economic and socio-demographic characteristics attribute to TB existence and emergence. These associated risk factors include; poverty, malnutrition, overcrowding, tobacco smoking, gender, age, emergence of MDR-TB, migration and alcoholism (Bates *et al*., 2007; Martineau *et al*., 2007; Trinh *et al*., 2015). In poor-resourced countries which lack a reasonable health infrastructure and facilities for early diagnosis and treatment programmes, TB flourishes (Bates *et al*., 2007). Other factors such as vitamin D deficiency and co-infection with HIV can also aid in reactivation and resurgence of the disease (Martineau *et al*., 2007). In TB patients co-infected with HIV, treatment efficacy decreased while case fatality rate increased (Sulis *et al*., 2014). With regard to gender, 2.9 million infected women were reported out from about 9 million cases in 2012 (WHO report, 2012). Development of multiple drug resistance tuberculosis (MDR-TB, resistance to least isoniazid and rifampacin) was associated with 450,000 new cases and 170,000 deaths (WHO, 2013).

Sudan is not an exception from other African countries in constituting an area with high TB prevalence estimated as 209 cases/100,000 population, while a figure of 50,000 incident cases were reported (Sharaf Eldin *et al*., 2011). Following an investigation of emergent MDR-TB in the Eastern region of Sudan, the same co-authors detected a higher rate of 24% in patients who previously received anti-tuberculosis drugs as opposed to 5% rate in freshlytreated TB cases.

They further identified and concluded that the Eastern region of Sudan as a geographic risk factor for inducing emergent drug-resistant cases. A relatively recent Sudanese study spanning the role of epidemiological and socio-demographic risk factors in predisposing TB in the mentioned region showed that illiteracy and male gender were the most probable attributes associated with the disease (Abdulla and Ali, 2012). However, both studies did not tackle the role of genetic background in increasing risk of developing TB.

2.5 Host Genetic Susceptibility to Tuberculosis

Before the advent of Pasteur's microbial theory of disease in 1860, it was believed that infectious diseases at a certain stage were intrinsic, and as such TB reflects host genetic susceptibility (Puffer, 1944; Stead *et al*., 1990). To support such belief, clinical and epidemiological surveys have been carried out. Increased infection with *M. tuberculosis* and manifested clinical TB were observed in homozygotic twins (twins derived from one ovum); less so in case of dizygotic twins (twins derived from two separate ova) but more so in association with people and their relatives originating from areas with no previous history of the disease (Kallmann and Reisner, 1943;). High incidence and prevalence of clinical TB among the black ethnicity during disease-outbreaks were reported as twice as that among the whites (Stead *et al*., 1990). A number of genes have been implicated in TB susceptibility. An example of these susceptibility genes is the solute carrier family 11A member 1(SLC11A1) known previously as natural resistance associated macrophage protein 1 (NRAMP1) occurring mainly among certain African and Asian families (Bellamy, 1999; Hoal *et al*., 2004, Hsu *et al*., 2006).

In an endemic area with high exposure to *M. tuberculosis* infection, about 20% of individuals have never contracted infection (Stewart *et al*., 2003). By contrast, the remaining infected group and depending on inter-individual genetic variation and age, infection with the bacillus will further progress eliciting various forms of TB (Abel *et al*., 2014; Stewart *et al*., 2003). It is well documented that the primary and acute disseminated forms mostly imply childhood TB; whereas development of latent form of infection and chronic pulmonary form display adult TB (Abel *et al*., 2014; Marais *et al*., 2006). The disseminated bacilli at a certain stage in a susceptible child may spread to the meninges and nervous system; while in the majority of people infection will be restricted to the lungs. Such host differences in response to infection with *M. tuberculosis* appealing for genuine scientific explanation and more reasoning to the role of genetic factors in predisposing increased susceptibility of the disease.

Pioneer molecular studies have indicated that congenital defects of immunity affecting the adaptive and innate immune systems can predispose to severe form of TB (Abel and Casanova, 2000). As such, many primary immunodeficiency diseases (PIDs), a group of potentially serious disorders in which inherited defects influence the immune system, have been found to confer severe susceptibility to opportunistic infections, and to less degree with other pathogens (Abel and Casanova, 2000; Reichenbach *et al*., 2001). These PIDs are characterized by a single mutation in a rare gene but with high penetrance leading to a group of genetic disorders including; hyper-immunoglobulin (Ig)E and hyper-IgM syndromes, chronic granulomatous disease, anhidrotic ectodermal dysplasia with immunodeficiency, severe combined immunodeficiency and Mendelian susceptibility to mycobacterialdisease (MSMD). It was reported that 50% of these rare PIDs have been found in many countries mainly in the Middle East due to habitual customs which encourage marriage between very close relatives (Al-Muhsen, 2008). Further advanced genetic investigations involving MSMD indicated association of a set of single gene defects in causing selective susceptibility to weakly virulent infections such as live BCG vaccine, less so to *Salmonella* and least so to *M. tuberculosis* (Mimouni, 1951; Casanova *et al*., 1996; Alcais *et al*., 2005; Casanova and Abel, 2007). Nonetheless, affected patients with this congenital syndrome seem to develop high resistance to a wide range of infectious agents (viral, bacterial and fungal) (Casanova *et al*., 1996).

In the event of mycobacterial and *Salmonella* infections, macrophages are stimulated with the release of TNF-α and IL12, promotion and activation of NK cells and Th1 cells respectively to generate IFN-γ (Jouanguy *et al*., 1996; Ramirez-Alejo and Santos-Argumedo, 2014). The latter cytokine in turn binds to IFN-γR1 on macrophages resulting in production of TNF-α and mycobactericidal toxic products such as nitric oxide synthase to eliminate the pathogen (Jouanguy *et al*., 1996; Ramirez-Alejo and Santos-Argumedo, 2014) (Fig. 2).

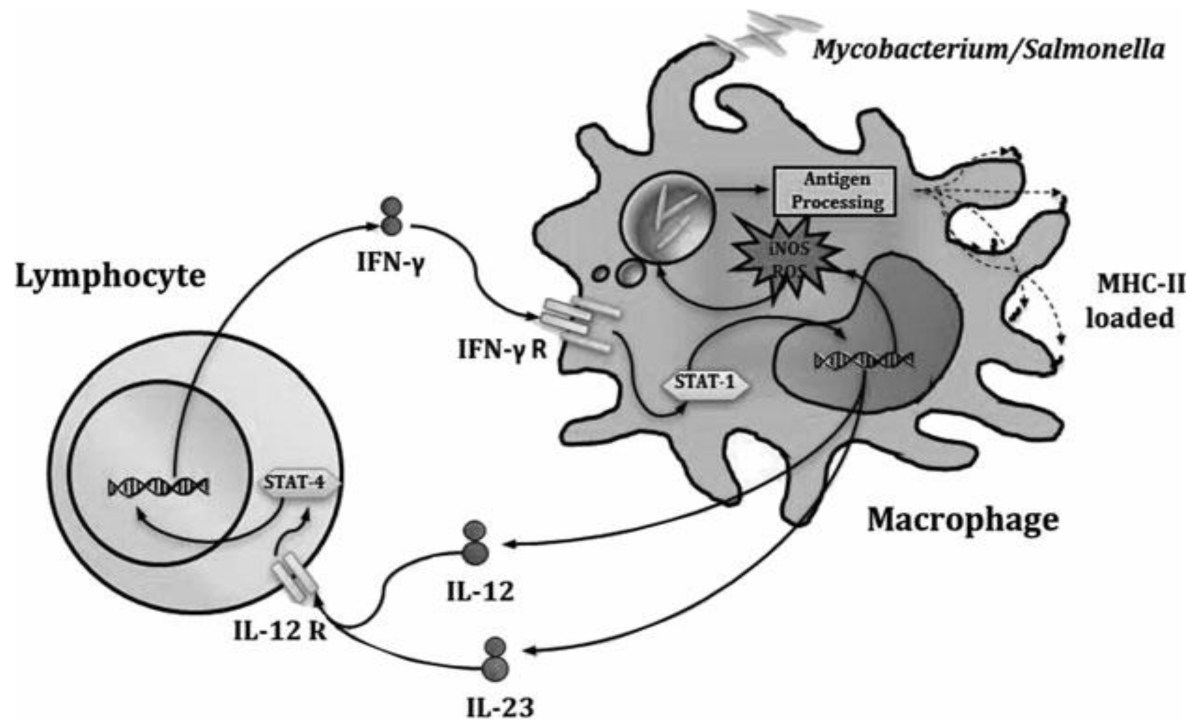


Figure1: Interplay between human innate immune response and T lymphocytes upon infection with mycobacteria and *Salmonella*

This interplay leads subsequentto release of IFN-γ and activation of macrophages anti-bactericidal toxic products and other factors. Adapted from Ramirez-Alejo and Santos-Argumedo, (2014).

The disease-causing mutations of MSMD implicate seven autosomal genes and two X-linked chromosomal genes (Abel *et al*., 2014; Al- Muhsen, 2008). The identified mutated autosomal genes comprise; IFN-g receptor 1 (IFNGR1*),* IFN-g receptor 2 (IFNGR2), signal transducer and activator of transcription 1 [*STAT1*], IL-12 p40 subunit (L12P40) and IL-12 receptor B-subunit (IL12RB1), interferon regulatory factor 8 (IRF8), and IFN stimulated gene 15 (ISG15) (Abel *et al*., 2014; Al- Muhsen, 2008). On the other hand, the two X-linked chromosomal genes are; nuclear factor-kB-essential modulator (NEMO) and cytochrome b-245, beta subunit (CYBB) (Bustamante *et al*., 2011; Casanova and Abel, 2013). Mutation in NEMO gene impairs production of IFN-γ and IL-12 cytokines reducing immunity; while mutation in CYBB gene contributes to reduced ability of IFN-γ in inducing macrophage anti-mycobactericidal damaging activities (Bustamante *et al*., 2011; Casanova and Abel, 2013).

The protein products expressed by genes causing MSMD are associated in conferring IL12/IL23 dependent pathway of IFN-γ immunity (Alcais *et al*., 2005). Frequent mutations occurring in loci of the above-mentioned genes account for great allelic heterogeneity leading to 17 different

genetic disorders attenuating host immunity (Abel *et al*., 2014). In a study involving a closely-related parents' family, complete mutation of IFNGR1gene in a child inoculated with attenuated BCG strain vaccine resulted in disseminated infection and death (Jouanguy *et al*., 1996). Partial IFNGR1 deficiency due to missense allele mutation at position 260 (T/C) of the gene/codon (I87T) changing isoleucine (I) at position 87 to thrionine (T) in the two homozygous siblings attributed to making one child susceptible to disseminated infection with BCG vaccine which was curable; while in the other non-vaccinated one it initiated severe tuberculosis (Jouanguy *et al*., 1997; Ramirez-Alejo and Santos-Argumedo, 2014). Accordingly, a mature granuloma revealing differentiated cells and clearly demarcated capsule was observed in the child with partially defective receptor harbouring a mutated IFNGR1 gene in comparison with a poorly immature BCG granuloma in the one with complete IFNGR1 deficiency(Jouanguy *et al*., 1996; 1997).It was also noticeable that both siblings with partial defects in IFNGR1did not contract NTM infection perhaps due to stimulation of residual immunity response mediating IFN-γproductionwhich was sufficient to safeguard against these weakly virulent pathogens (Jouanguy *et al*., 1997).

Dorman and Holland (1998) showed genetic nucleotide alterations within IFNGR2 gene in a non-BCG vaccinated child contributing to reading frame shift and premature stop codon in the extracellular portion of the receptor and subsequent lacking of receptor. This genetic defect affected IFN-γ signalling and was responsible in this particular patient for disseminated infection with *M. fortuitum* and *M. avium* (Dorman and Holland, 1998). An investigation of a French woman with a widespread infection with BCG in her infancy and remained prone to recurrent viral infections concluded that it was due to mutation in STAT1 gene disrupting IFN receptor signalling (Dupuis *et al*., 2001) (Fig. 2). (Altare *et al*., 2001) linked susceptibility of mycobacterial infections in two homozygous siblings (a boy and girl) born to first-cousin Moroccan parent to missense mutation in IL12RB1 DNA. The inherited defect caused conformational changes in IL12RB1 affecting expression on T lymphocytes leading to weakenedIL12Rβ1-dependent/IFN-γ mediated immunity (Altare *et al*., 1998; 2001). Hence, the first IL12Rβ1 deficient sibling (a boy) included in the authors'study contracted disseminated *M. bovis* vaccine but survived it and resisted NTM infection (Altare *et al*., 2001). His sister on the other hand, resisted both infections yet she instead caught Mtb infection manifested by severe abdominal TB at the age of 18 years (Altare *et al*., 2001). Administration of recombinant IFN-γ

coupled with antimycobactericidal medications was shown to be effective in managing patients with IL12Rβ1 deficiency (Alangari *et al*., 2011). In connection with IL12, mutation in the gene encoding IL12-p40 was reported to express a non-functional p40 and receptor which interfere with signalling pathway of IFN-γand thus predispose infection of patients with mycobacteria and *Salmonella* (Filipe-Santos *et al*., 2006; Ramirez-Alejo and Santos-Argumedo, 2014).

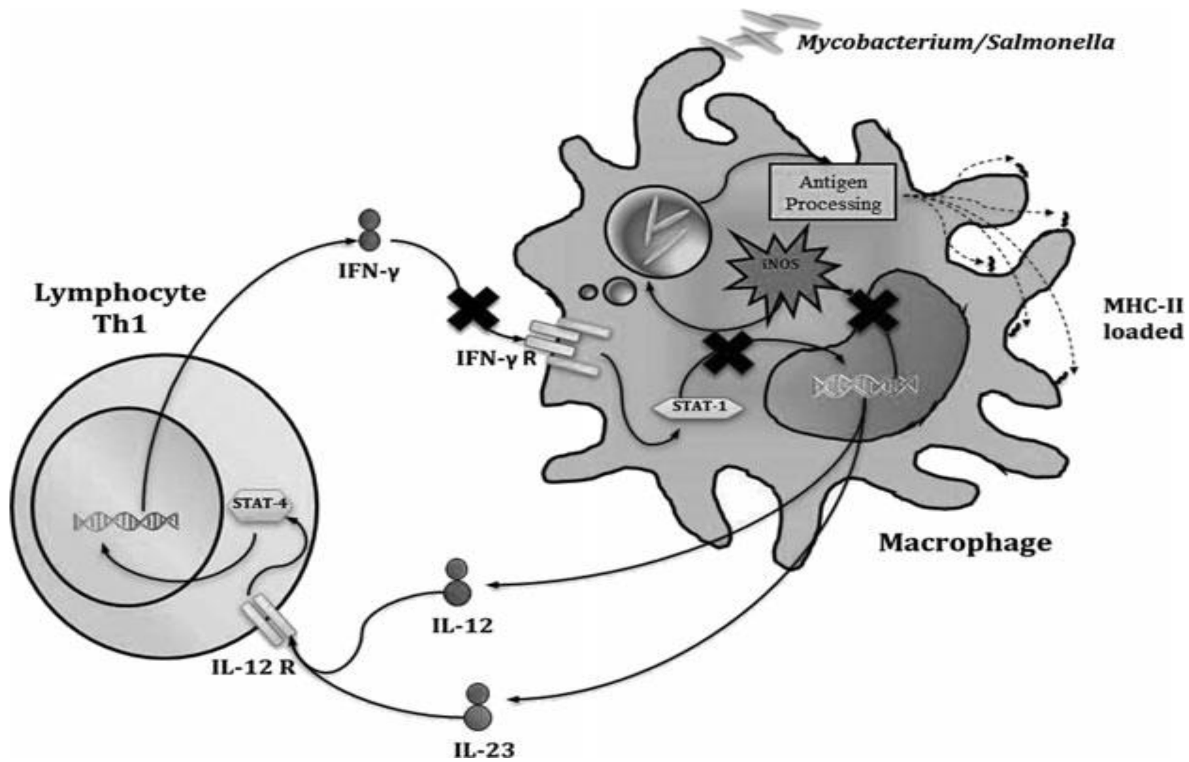


Figure 2: detection of genetic defects affecting IFN-γand its various components including receptors (IFN-gR1 and 2) and signal transducer and activator of transcription (STAT1)
This genetic defect affects cytokine secretion with failure to potentiate antimicrobial mechanisms in macrophages. This figure was Adapted from Ramirez-Alejo and Santos-Argumedo (2014).

2.6 Pathogenesis and Host Immune Response

Natural human infection with *M. tuberculosis* occurs mainly through inhalation of infectious aerosolized droplet nuclei released by an active person and implanted in alveolar passages of uninfected victim (Smith, 2003). Once these bacilli reach the lung, their cell wall components interact with mannose-capped lipoarabinomannan (ManLAM) and/or complement receptors and a variety of receptors on the surface of macrophages, and initiate innate immunity with generation of cytokines and chemokines (Schluger, 2005). Toll-like receptors (TLR)-transmembrane proteins, particularly TLR2 and TLR4 are examples in this direction (Schluger, 2005). Similarly, specific recognition of the pathogens and modulation of immune response is further carried out by other receptors found on phagocytic cell surfaces involving surfactant protein A and cholesterol, nucleotide-binding oligomers like-receptors, and dendritic adhesion molecules (El-Etr and Cirillo, 2001; Ahmad, 2011). Ultimately, the bacilli can be phagocytosed by active resident alveolar macrophages and subsequently internalized by the receptors (Kang *et al.*, 2005).

Othe rcomponents of the organism's cell wall including mixtures of lipids and polysaccharides content which form many surface ligands such as the lipomannan (LM) and ManLAM interact with the phagocytic TLRs (Noss *et al.*, 2001). This interaction leads to activation of macrophage and generates signals inducing a cascade of proinflammatory immune response involving IFN-γ, TNF-α and IL-12 (Lykouras *et al.*, 2008). This also induces recruitment of immune cells like monocytes and neutrophils to the affected area but they become less able to clear infection (Chen *et al.*, 2006). The cytokines-derived immune responses further stimulate macrophages to release antimicrobial products to kill intracellular invaders (Lykouras *et al.*, 2008). Following the cytokineseffect, Mtb may be immediately eliminated by alveolar macrophages and in such situation infection will never be established (van Crevel *et al.*, 2002).

A few virulent bacteria may resist or escape initial phagocytic destruction and remain alive within macrophages. Then, they migrate to phagosomes aided by ManLAM ligand on the bacterial surface which inhibits phagosome maturation and phagosome-lysosome fusion (Nigou *et al.*, 2001; Kang *et al.*, 2005). The ligand also interacts with mannose receptors initiating an anti-inflammatory response since ManLAM blocks mannose receptor-dependant IL-12 production (Nigou *et al.*, 2001; kang *et al.*, 2005). Such inhibition favours bacterial evasion, promotes infection and subsequent survival in resident cells (Kang *et al.*, 2005). Once they get

access to the new cellular resident home, bacteria continue to replicate and may disseminate to alveolar lymph nodes, and other potential sites (Bermudez *et al*., 2002). Such invading bacteria may continue multiplying inside phagosomes, the homing cells rupture with extracellular release of pathogens which can further infect more newphagocytic cells and the process goes oninfecting nearby cells (Chen *et al*., 2006). Extensive tissue damage may cause necrosis (complete lysis of infected macrophages involving disruption of plasma membrane) and induce local inflammation (Birkness *et al*., 2007; Behar *et al*., 2011).

Besides, virulent *M. tuberculosis* possesses a genomic region called region of difference (RD1) which is absent from the attenuated BCG vaccine strain of *M. bovis* (Ernst *et al*, 2007). The region forms a part of the pathogen's novel protein secretory system (ESX-1) encompassing genes coding for cytotoxic effector molecules; early secreted antigenic target-6 kDa (ESAT-6) and culture filtrate protein-10 (CFP-10) (Ernst *et al*, 2007). These protein molecules particularly ESAT-6 contribute to the pathogenesis of the disease by damaging cell membranes causing lysis of infected-macrophages, inhibit apoptosis (a form of death of infected macrophages but the plasma membrane remains intact) induce necrosis and enable bacterial spread from one cell to another (Behar *et al*., 2011). Deletion of RD1 or alteration in the gene coding for ESAT-6 resulted in reduced virulence in mice (Guinn *et al*., 2004). By contrast, phagocytic cells fight back and release toxic products involving reactive oxygen species (ROS) such as superoxide radicals, and reactive nitrogen intermediates (RNI) like inducible nitrous oxide synthase (iNOS) that kill the pathogens (Schluger, 2005). Escalating levels of iNOS gene product were expressed by alveolar macrophages in patients with active pulmonary TB (Nicholson *et al*., 1996). Experimental mice subjected to disruption in the gene coding for iNOS had vulnerable and severe mycobacterial infection (Chan *et al.,* 1995; Scanga *et al*., 2001; Russell-Goldman *et al*., 2006).

Several other strategies were further evolved by the bacillus to avoid elimination by the host immune system and enhance persistence (Tufarlello *et al*., 2003). Firstly, bacillus tubercle utilizes tyrosine receptors to prevent the action of TLRs and cytokines-driven proinflammatory immune responses (Noss *et al*., 2001). Secondly, it was shown that continuous signalling of TLR2 interacting with the pathogen's lipoproteins such as 19 kDa could adversely affect expression of major histocompatibility complex (MHC)-class II/or human leukocyte antigens (HLA), bacterial antigen processing within macrophages and subsequently presentation of the

antigens to $CD4^+$ T-cells (Fulton *et al*., 2004; Pai *et al*., 2004). This effect may in turn impair host immunity and increase chances of intracellular survival of invader (Noss *et al*., 2001). Accordingly, polymorphism targeting TLR was reported to attenuate host immune response and increase susceptibility to TB disease (Hawn *et al*., 2007).

At the local inflamed area in the lung, another group of bacilli is engulfed by resident antigen presenting cells like dendritic cells become primed; migrate via lymphatic circulation to regional and peripheral lymph nodes to sensitize CD4 and CD8 T-cells with the processed antigens (Smith, 2003). Such presentation of antigens occurred through major histocompatibility complex (MHC) class II and I molecules (Houde *et al*., 2003). Specific immune response provokes the primed T-cell subsets to proliferate, expand and travel back guided by chemokines to the inflammatory area where they activate macrophages to destroy bacilli (Lykouras *et al*., 2008). Enlargement of the local inflamed lesion is enhanced by release of tumour necrosis factor alpha (TNF-α) and chemokines such as interleukin-8 (IL-8) deriving more aggregates of different immune cell types in addition to macrophages and T cells including B cells, dentritic cells, endothelial cells, fibroblasts and stromal cells culminating in granuloma formation as an initial focus of infection, limiting bacterial spread (Tufarlello *et al*., 2003; Russell, 2007). The development of granuloma also marks a pathological feature of TB disease permitting persistence (Tufarlello *et al*., 2003; Cardona, 2009). In this context, about 90% of infected people do not develop the disease; remain clinically asymptomatic and non-infectious throughout their lifetimes although tuberculin skin test (TST) is positive (Ehlers, 2009). In contrast, infection progresses to active disease in a minority of about 5-10% of individuals (Tufarlello *et al*., 2003).

Within the granuloma microenvironment, tubercle bacillus may be confronted with inhospitable and stressful conditions (Ulrichs and Kaufmann, 2006). These implicate hypoxic conditions, low pH, released nitric oxide and associated RNI toxicity, presence of carbon monoxide, and nutrient starvation (Rustad *et al*., 2009; Ahmad, 2011). To counteract these challenges, *M. tuberculosis* has evolved a resistance mechanism in which it expresses several gene products involving generation of alkyl hydroperoxide reductase subunit C, superoxide dismutase (SOD) enzyme and catalase to detoxify the effects of ROS and RNI produced by activated macrophages, and render such toxic products ineffective (Bryk *et al*., 2002; Ohno *et al*., 2003; Miller *et al*., 2004; Kumar *et al*., 2008). In addition, it has been reported that some mycobacteria such as *M. bovis* BCG under severe harsh conditions can form spore-like structures contributing to dormancyof the

pathogens (Ghosh *et al*., 2009). These dormant bacilli may exist in the granuloma of host for lifetime but they can revive and wake up when the host immune system wanes down for example, due to co-infection with HIV (Hett *et al*., 2007; Martineau *et al*., 2007; Kana *et al*., 2008; Shiloh and DiGiuseppe Champion, 2010).

Fate of the bacillus during latent infection is not fully clear (Tufarlello *et al*., 2003). It has been reported that the bacteria persist in a dormant non-replicated phase residing host macrophages in necrotic debris in the centre of a mature granuloma (Voskuil *et al*., 2003**;** Cosma *et al*., 2004). The formed debris may also play a role in the process of apoptosis during which the pathogen undergoes reduced viability (Behar *et al*., 2011). Although necrosis represents a focal source for the organism to evade the host immune system and disseminate, apoptosis augments innate immunity and lately it also stimulates adaptive immunity with the processed antigens to suppress bacterial invasion (Behar *et al*., 2011). A number of factors are considered as inducers of apoptosis including 19-KiloDalton (kDa) glycoprotein and ESAT-6 kDa protein (Derrick and Morris, 2007; Sanchez *et al*., 2012; Stenger, 2005). On the other hand, the organism releases some antiapoptotic factors such as ManLAM to inhibit apoptosis (Rojas *et al*., 2000). Yet, some studies have questioned the role of apoptosis in controlling Mtb since a few researchers think that it enhances bacterial replication and spread (D'Avila *et al*., 2008; Davis and Ramakrishnan, 2009). Several genes associated with apoptosis were shown to be highly expressed in patients with active TB (Abebe *et al*., 2010).

The cell-mediated immunity has been reported to be quite crucial for restriction of infection with Mtb (Flynn and Chan, 2001, Rook *et al*., 2001). The granuloma micro environment creates interplay between T cells, macrophages and cytokines. Great emphasis is directed towards the role of $CD4^{+}$T cells in protection against TB since depletion of these cells in mouse experiments showed impaired immunity manifested by increased virulence of bacilli terminating in death (Tufarlello *et al*., 2003). In addition, $CD4^{+}$T cells perform other important secondary functions as they contribute to the process of apoptosis of infected macrophages by interaction with Fas/Fas ligand on the pathogen’s surface, thus boosting host immune response and restricting intracellular survival (Cella *et al*., 1996; Chan and Flynn, 2004; Behar *et al*., 2011; Oddo *et al*., 1998). Similar effect and lysis of infected cells occurs when $CD4^{+}$cells trigger secretion of several cytokines such as IL-2, IL-12 and IL-15 (Cooper, 2009). $CD4^{+}$ T cells further release IFN-γ cytokine which recognizes infected macrophages and initiates signalling cascade, and

together with TNF-α stimulate these cells to generate RNS to get rid of invaders (Schluger, 2005; Wolf *et al*., 2008).

Interferon gamma is also secreted by other immune cells such as $CD8^+$ T cells and NK cells and subsequently it activates macrophages to eliminate the bacteria (Tufarlello *et al*., 2003). Moreover, activated $CD8^+$ T cells lyse infected macrophages by secreting toxic granulysin (Grotzke and Lewinsohn, 2005). Importantly, IFN-γhas a critical influence on transcription and upregulation of expression comprising several genes within macrophages such as MHC-class II molecules and follow-up presentation to effector cells (Chan and Flynn, 2004; Cooper, 2009). Furthermore, the cytokine induces macrophages to release antimicrobial toxic agents including oxygen radicals and nitric oxide (Scanga *et al*., 2001). Further significant evidence in support of the role IFN-γ was demonstrated by clinical improvement in patients who were suffering from refractory multiple drug resistant pulmonary TB following aerosol administration of the cytokine (Condos *et al*., 1997). As such, any defect in expression of IFN-γ gene product or cytokine receptor genes may affect the cellular signalling response and render an individual prone to bacterial infection (Flynn *et al*., 1993). By contrast, the pathogen tends to down-regulate impact of the cytokine effects by releasing certain potential virulence factors like 19 kDa lipoprotein that can suppress transcription of the cytokine gene and eventually weaken macrophage response (Pai *et al*., 2003).

Other important cytokines such as TNF-α and IL-12 are required to augment the role of IFN-γ in protection against mycobacterial infection. TNF-α is mainly secreted by macrophages, dendritic cells and T cells (Keane, 2005). Synergy exists between TNF-α and IFN-γ to potentiate the antimycobacterial ability of macrophages (Scanga *et al*., 2001). TNF-α also has multiple functions and a vital role in recruitment of various immune cell types particularly blood monocytes, neutrophils and lymphocytes to the site of infection, and arrangement into organized granulomas (Birkness *et al*., 2007). Disruption of TNF-α gene or its receptor in mice contributes to increased susceptibility of the pathogen (Bean *et al*., 1999). The cytokine stimulates expression of chemokines such as IL-8 that are responsible for cell migration to the infected area (Algood *et al*., 2003). Indeed, the reported hypervirulence shown by W-Beijing family strain of *M. tuberculosis* is due to the presence of phenolic glycolipid in its cell wall which blocks generation of proinflammatory cytokines including TNF-α by macrophages (Reed *et al*., 2004). Similarly, interleukin-12 is produced by phagocytic cells such as macrophages (Berrington and

Hawn, 2007). Through its receptors (IL-12Rβ1 and IL-12Rβ2) signalling, IL-12 stimulates T cells to produce IFN-γ and TNF-α (Berrington and Hawn, 2007). Any defect surrounding the IL-12 cytokine or associated receptors make an individual highly susceptible to active disease (Lichtenauer-Kaligis *et al*., 2003). However, these genetic defects constitute small proportion among the community; while transmission and dynamic of infection have progressed, and TB remains as a great public health threat with deaths and million fresh cases emerging each year.

2.7 Diagnosis of Tuberculosis

2.7.1 Diagnosis of Active Tuberculosis

Accurate and rapid diagnosis is a prerequisite for proper treatment of patients and control of TB. Active TB cases are diagnosed on the basis of clinical presentations, response to treatment, chest radiographs, sputum smear staining with Ziehl-Neelsen stain for demonstration of acid fast bacillus, and culturing of pathological specimens s with isolation of the causative agent (Brodie and Schluger, 2005). In addition, recent molecular diagnosis tests i.e., nucleic acid amplification (NAA) assays which can be applied directly on the pathological specimens are used (Brodie and Schluger, 2005). Although one must consider the symptoms of TB disease and take them into account during diagnosis, the forms of the disease can resemble several other forms of other diseases and such clinical judgement may be misleading (Godreuil *et al*., 2007). The chest radiography on the other hand has a high sensitivity but gives poor specificity and increased overall cost, while conclusive findings can be overestimated (Brodie and Schluger, 2005; Dasgupta and Menzies, 2005).

Microscopic examination of sputum-stained smears to visualize the presence of bacillus is easy to perform, cheap and can be afforded by poor-resourced countries (Perkins, 2000). But, the test is presumptive with low sensitivity ranging from 43%-55% (Wilkinson *et al*., 1997). Besides, smear-negative results were quite high during co-infection of pulmonary TB with HIV (Colebunders and Bastian, 2000). Culturing of suspicious pathological specimens and isolation of causative is confirmative (Brodie and Schluger, 2005). In addition, culturing method is highly sensitive when liquid media are used for recovery of the organism resulting in shortening the time taken for isolation (Morgan *et al*., 1983; Godreuil *et al*., 2007). Moreover, it permits drug-susceptibility testing, genotypic identification of the pathogen, and facilitates molecular epidemiological tracing (Godreuil *et al*., 2007). But, the culturing method is costly and time-consuming (Brodie and Schluger, 2005).

Although conventional methods are considered as standard gold to use, they require several weeks to conduct before any conclusive diagnostic report can be submitted. Accordingly, molecular diagnostic tools such as polymerase chain reaction (PCR) and DNA probes are shown to be more rapid and sensitive (Godreuil *et al.*, 2007). For instance, Mtb can be diagnosed by amplification of heat shock protein 65 kDa) (hsp-65) gene using specific primers (Palomino, 2007). In another study, culture-negative results in 15-20% of sputum specimens obtained from suspected TB cases were shown to be positive after evaluation using genotypic assays (Brodie and Schluger, 2005). However, these genotypic tests are expensive and poor-resourced countries cannot affordto purchase them (Kaul, 2001).

Another recent molecular test used for rapid diagnosis of TB disease is the Xpert MTB/RIF Assay (Steingart *et al.*, 2014). It is a nucleic acid amplification (NAA) test which can be performed on an automatic GeneXpert machine following specific guidelines. The test can simultaneously identify the organism and resistance to rifampicin (RIF) in about 2 hours. But, the test cannot replace the conventional methods such as culturing of suspected pathological material (Steingart *et al.*, 2014).

2.7.2 Diagnosis of Latent Infections

Latent TB cases do not present any symptoms to assist in diagnosis. But, these cases constitute potential reservoirs due to chances of reactivation to active disease thenbacteria can spread and infect healthy individuals, and eventually they stand as obstacles for controllingthe disease (Brodie and Schluger, 2005). Previously, these cases were detected using TST or Mantoux test (Dasgupta and Menzies, 2005). The test involves intradermal injectionof 0.1 ml purified protein derivative (PPD) antigen of *Mycobacterium* resulting in the development in erythema and induration of >10 mm in size after 48-72 hours indicating a positive reaction (Anderson *et al.*, 2005). In practice, TST shows high sensitivity and low cost, butit cross-reacts with *M. bovis* BCG vaccine and environmental non-tuberculous mycobacteria (NTM) (Barry *et al.*, 2009). Another advantage of TST is its use to diagnose active TB patients especially if the skin lesion measures over 20 mm (Ahmad, 2011). However, a negative skin result in this context may denote state of anergy (inability of cellular immunity to mount a robust response against the pathogen) or the skin test is not administered correctly (Ahmad, 2011).

Due to the above-mentioned drawbacks, IFN-γreleased assay (QuantiFERON-TB) has been developed to diagnose latent infections (Pai *et al.*, 2004; Barry *et al.*, 2009). Basically, the test is

an in vitro assay designed to measure T-cell responses and quantify the released IFN-γ following sensitization with Mtb specific ESAT-6 and CFP-10 antigens, and incubation for overnight (Barry *et al*., 2009). Quantification of the cytokine is evaluated by enzyme-linked immuno-absorbent assay (ELISA). The utilized Mtb antigens in this assay differentiate *M. tuberculosis* from other mycobacterial species (*M. bovis* BCG vaccine strain and environmental mycobacteria) since the genes encoding these protein antigens in the mentioned species are deleted (Ahmad, 2011).

2.8 Challenges for Controlling Tuberculosis Infection

Tuberculosis is one of the most ancient infectious bacterial diseases that co-evolved with mankind causing great losses among people each year. The essential target for TB control is to eliminate the disease via reducing transmission,and by conducting accurate and rapid identification of infectious cases succeeded by effective treatment (Clancy *et al*., 1991).There are many challenges which act as barriers for control of TB worldwide including; existence of poverty in many regions, flourished illiteracy among communities, social stigma of patients to seek treatment, poor adherence to treatment, lack of sufficient food, drug abuse, heavy consumptionof alcohol and overcrowding in prisons (Sulis *et al*., 2014). There are also other vulnerable groups which may impede control of the disease such as patients with Multi-Drug Resistant TB (MDR-TB) (is defined as resistance to at least, isoniazid and rifampicin among the first-line drugs), those co-infected with HIV and immigrants with latent infection flowing from countries with high TB prevalence to countries with low incidence rate (Sulis *et al*., 2014).

Earlier and pioneer attempts for control of TB were launched by the World Health Assembly (WHA) resolution in 1991 aiming to accomplish two targets. The first target was to detect 70% of new smear-positive cases whereas the second one was to treat 85% of these cases by the year 2000 (WHO, 1994). In 1994, the WHO initiated another control strategy known as directly observed short course therapy (DOTS) topromote efforts of the former WHA control plan. Thenew control strategy is based on five key elements including; government political willingness to financially support TB control programme, case detection of patients presented with overt clinical symptoms among the community in a quality-assured bacteriological laboratory, standardized short-course therapy under supervision and proper case management to, at least, all confirmed sputum smear-positive cases,effective and maintained supply of drugs, and regular monitoring programme for supervision and evaluation (WHO, 1994). Adoptionand

implementation of DOTS strategy had expanded in covering as many as 182 countries in which successful TB control programmes were achieved in most of them with the exception of two regions where incidence and prevalenceof disease still remain high(WHO, 2006).Theseregions include; the sub-Saharan Africa due to emergence of HIV epidemic besides presence of poor health care facilities; and the Eastern Europe region where people suffer from poverty aggravated by low standard of living and treatment failure associated with MDR-TB (Migliori *et al*., 2006; WHO, 2006).

Directly observed short course therapy (DOTS) policy has continued as planned, and by 2004 the global TB incidence and mortality declined (WHO, 2006). In addition, more than 20 million cases were treated with 16 million cases cured, and the policy had achieved its target for the year 2003.This was witnessed in 2005 by treating 83% of patients with a detected rate of 53% of cases, just a bit less than the required goal (WHO, 2005). Based on relative successful achievements of DOTS, thorough revision of the strategy was carried out by the WHO and co-partners. Accordingly, they decided to introduce an extended New Stop TB Strategy from the year 2006 to 2015as a further support to strengthen the DOTS strategy (WHO, 2006). Establishment of the new strategy was aimed to counteract new challenges relative to socioeconomic problems and health changes (WHO, 2006; Zignol *et al*., 2007). This strategy is launched to address the poor health system and care, and make treatment and supervision accessible to vulnerable groups such as the poorer, refugees, homeless and people who are hard to reach (WHO, 2006). Importantly, this strategy addresses interaction of TB/HIV, and cooperation between the two programmes,and tacklesand controlsemergence of MDR-TB and Extensively Drug Resistant TB (XDR-TB) (defined as resistance to MDR-TB plus resistance to at least, any of fluoroquinolones (i.e.,ciprofloxacin), and any one of three second-line injectable drugs (i.e. capreomycin, kanamycin and amikacin) (Raviglione and Smith, 2007; Sulis *et al*., 2014).However, emergence of XDR-TBhas worsenedthe burden of disease and complicated further progress for control (Raviglione and Smith, 2007).

In addition, the New Stop TB Strategy calls for more health professionals and health care providers to participate and cooperate with the private sector to provide sufficient and sustained extra funding and other resources for establishing efficient health care infrastructure and proper services that can promptly meet peoples'care and other needs (WHO, 2006). Moreover, the strategy has been planned to achieve several issues such as promoting global TB research in

order to explore more effective drugs, develop new efficacious vaccines as well as rapid diagnostic tools (WHO, 2006). To support these achievements, the strategy planners called for establishment of highly-equipped laboratory applying advanced techniques and managed by a qualified staff. Furthermore, the strategy intends to educate people in various communities about the public health hazards and danger of the disease. By fulfilling the mentioned issues, the proposed strategy will eventually reduce stigmatization, improve care and encourage the government in power to increase financial and political commitment (Raviglione and Uplekar, 2006; WHO, 2006). However, an efficient management system is crucially needed to monitor and supervise implementation strategy.

More recently, the WHO has further proposed a post-2015 strategy which addresses ambitious goals and targets to eliminate the worldwide TB epidemic by 2035 (WHO, 2013). The strategy calls for reducing TB deaths by 95% and incidence by 90%, while it avoids putting any financial burden on families (Sulis *et al*., 2014). The expanded strategy also targets promoting rapid detection tools for drug resistance and development of new effective therapies. In this context, recent discovery of anti-tuberculosis drugs; Bedaquiline and Delamanid may facilitate controlling MDR-TB and limit transmission, and subsequently may contribute to implementationof the post-2015 strategy (Sulis *et al*., 2014).

In Sudan, the National TB Programme (NTP) has adopted fully the DOTS strategy since 2002 and according to WHO proposed criteria. In all heath centres, TB treatment regimen follows a period of 8-monthsincluding the first 2 months are performed under medical supervision.

CHAPTER THREE

MATERIALS

AND

METHODS

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Design

A cross-sectional labrotary based study was employed in this project.

3.2 Collecion of Data and Ethical Clearance

Demographic data involving; age, sex gender, tribe, history of BCG vaccination and other variables in conjunction with risk factors such as smoking, alcoholism, associated disease and socioeconomic status were collected through a questionnaire and filled in a Structured Form under medical supervision (Table 4).

The undergoing TB research project also investigated the role of genetic variation or mutation in *IFN-γR1*gene that might induce pulmonary TB among patients attending different hospitals, Khartoum State, Sudan. Participants were randomly selected and found to originate from 26 tribes (Table 4). Majority of participant patients belonged to seven main tribes including; Galiyeen (19), Tama (11), Nuba (10), Zagawah (8), Hadndawah (6), Fur (6), and Bargo (5). In addition, a few patients were assigned to other tribes as shown (Table 4).

A confidential written consent was obtained from every TB participant. Approval to conduct this research project was sought from the Ethical Committee of Federal Ministry of Health, Sudan.

3.3 Study Area, Population and Duration

One hundred human participants exhibiting clinical features of active pulmonary TB attending various central hospitals in Khartoum State in Sudan (from 2015 to 2016) were randomly selected to be enrolled as a target population for this study. 50 healthy individuals or a group with other respiratory infections other than TB were selected as controls.

3.4 Dignostic scheme

3.4.1 Conventional method for diagnosis

3.4.1.1 Collection of Pathological Specimen

Only suspected TB participants who were presented with clinical signs of active pulmonary disease and those with ZN-stained sputum smears showed acid fast bacillus (AFB) were included in this study. TB participants who were presented with clinical signs of active pulmonary disease but ZN-stained sputum smears did not show any bacilli were excluded. 100 sputum and 100

whole blood specimens were collected before starting chemotherapy from TB suspects with active pulmonary disease for further testing. For comparison, 50 sputum specemens and 50 whole blood specemens were simultaneously collected from healthy controls.

3.4.1.2 Processing of Specimen

Each patient was provided with a clean, disposable, screw capped plastic sputum container and instructed to produce sputum specimen by deep coughing in a well-ventilated area. The specimens were immediately transferred to the National Tuberculosis Reference Laboratory, Khartoum for further investigation.

Provisionally, ZN-stained sputum smears were prepared. Direct sputum smear was made on slide, fixed by gentle heating and stained by ZN method. The method involves covering the smear with filtered 1% carbol fuchsin stain, heated to steaming using a Bunsen burner, left to stand for 5 minutes and rinsed with tap water. Then, smears were flooded with sulphuric acid (H_2SO_4) for complete destaining, washed with tap water and finally drained. The slide was flooded with methylene blue as a counter stain for 1 minute, washed with water and air dried. Finally, smears were microscopically examined for the presence of acid AFB under oil immersion objective lens Hans *et al* (2007).

3.4.1.3 Decontamination of Sputum Specimen

Positive sputum smear specimens were decontaminated for further processing. The specimens were transferred to 50ml disposable plastic tube and an equal amount of 4% of sodium hydroxide was added, vortexed and allowed to stand at room temperature for 20 minutes. The specimen was neutralized with 1NHCL containing 0.1% phenol red as an indicator. The prepared specimens were inoculated on three Lowenstein-Jensen medium slope media; two containing glycerol and the third one was supplemented with pyruvate. Media were prepared as Hans *et al* (2007).

3.4.1.4 Identification of *M. tuberculosis* Isolates

3.4.2 Cultural Characteristics and Growth Rate Identification

All cultural procedures were carried under biological safety cabinet and chemical agents were prepared following standard methods (Kent and Kubica,1985). Decontaminated sputum specimens

were further processed for inoculation on appropriate (LJ) medium agar slope medium to isolate M. *tuberculosis* and identify the causative agent following standard procedures (Kent and Kubica,1985). Cultures were also inoculated and incubated in slope position for three days in tubes with loosen caps to evaporate the excess fluid. Then, bottle caps were tightly closed to prevent drying of media, left on upright position and lately examined for contamination.

Re-examination of grown cultures was performed in the first week to detect rapid growers and then every week until the eighth week for detection of slow growers. The result was recorded as negative if no growth appeared, exact number of colonies was recorded as such, (+) if the number of colonies is less than 20, (++) if the number of colonies is 20 to 100or, (+++) if the number of colonies is100 to 200. Positive cultures were maintained through alternative sub-culturing on LJ medium. Pure cultures were stored in20% glycerol in distilled water at -20°C for future processing Hans *et al* (2007).

Bacterial isolates were firstly identified depending on growth rate and colonial morphology as described by Kent and Kubica (1985). 0.1ml of 10^{-4} and 10^{-2} bacillary suspension of each grower's isolate was inoculated on LJ slope media, incubated at 37°C and examined after 5 to7days to identify the rapid growers by appearance of grossly visible colonies.

3.4.2.1 Pigments Production

Mycobacteria synthesize carotenoid pigments in various amounts and kinds, depending upon the species and exposure to the light. Using these variations in pigment production, *Mycobacterium* are categorized into three groups: photochromogenic, scotochromogenicand non-photochromogenic. Four slopes of LJ medium were inoculated with 10^{-4} bacillary suspension; two of them were covered with aluminium foil, incubated at 37°C. Growth was examined for pigment formation in the unshielded slopes. Shields were removed and media were examined for appearance of pigmentation after exposure to light for 5 hours. Results were then recorded.

3.4.3 Biochemical Tests

3.4.3.1 Catalase Test

Catalase is an intracellular enzyme capable of splitting hydrogen peroxide into water and oxygen. Formation of oxygen bubbles within the reaction mixture is an indicative of catalase activity. Virtually all mycobacteria possess catalase enzymes, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis* Hans *et al* (2007).

3.4.3.2 Detection of Heat-Labile Catalase Test at 68°C/pH7

Some bacteria lose catalase activity when suspended in a buffer with pH7and heated to 68°C for 20 minutes. Included in such group are *M. tuberculosis, M. bovis, M. gastri and M. haemophilum.* This hot catalase test is valuable in identifying strains of *M. tuberculosis* which give weakly positive or negative niacin test. All slow-growing non-chromogens mycobacteria were subjected to this test. Suspected isolates were suspended in 0.5 ml of 0.067 M phosphate buffer saline with pH7 and then incubated in water bath at 68°C for 20 minutes. Contents were cooled down to room temperature and 0.5 ml of Tween-peroxide mixture was added. Presence or absence of air bubbles formation was detected Hans *et al* (2007).

3.4.3.3 Nitrate Reduction Test

The ability of some mycobacteria to reduce nitrate has proven valuable in differential identification of some mycobacteria that possess similar characteristics. It has been observed that mycobacteria differquantitatively in their ability to reduce nitrate (Virtanen,1960). This reaction is influenced by the age of the culture, temperature, enzyme inhibitors, and hydrogen ion concentration. Isolates were emulsified in 2ml $NaNO_3$substrate and broth was incubated in upright position for two hours in water bath at 37°C. Contents removed, Lame reagent added (Appendix) and reaction was immediately examined for development of pink to red colour Hans *et al* (2007).

3.4.3.4 Para-Nitrobenzoic Acid (PNB) Susceptibility Test

Two hundred µg of para-Nitrobenzoic acid (PNB) were dissolved in 15ml sterile distilled water. 2ml of 1 N NaOH solution neutralized with1N HCL containing phenol red as an indicator were added. End point was determined and volume was completed to 20ml. Each 5 ml of the drug was added to 100ml of LJ medium, dispensed, and bottles were inspissated in slope position.100 µl of 10^{-4} and 10^{-2} bacillary suspension were inoculated onto two PNB (500 mg/L) containing LJmedia; while two other drug-free LJ slope media were left as controls. The test is important to differentiate Mtb from non-tuberculous mycobacteria (NTM) which are resistant to the test whereas Mtb are sensitive to it (Sharma *et al.*, 2010). Contents were incubated at 37°C for 4 weeks and proportion of growth was assessed Hans *et al* (2007).

3.4.3.5 Thiophen-2-Carboxylic Acid Hydrazide (TCH) Susceptibility Test

Twenty-five mg of TCH were dissolved in 20ml distilled water and placed in water bath at 45°C for 30 minutes and each 4 ml of drug was added to 1000 ml LJ medium, dispensed and inspissated in slope position. 100 µl of 10^{-4} and 10^{-2} bacillary suspension were inoculated onto two slopes of drug containing LJ media and another two slopes of drug-free media as controls, incubated at 37°C for 4 weeks and proportion of growth was calculated.

This test is valuable for distinguishing *M. bovis* from *M. tuberculosis* and other non-chromogenic slow growing mycobacteria. Only *M. bovis* is susceptible to low concentration (1 to 5ug/ml) of TCH. However, isoniazid-resistant strains of *M. bovis* may be resistant to TCH. *M. tuberculosis* and other mycobacteria on the hand are usually resistant and not inhibited by TCH Hans *et al* (2007).

3.4.4 Preparation of McFarland Turbidity Solution

One percent solution of anhydrous barium chloride ($BaCL_2$) and a 1% (v/v) cold solution of pure sulphuric acid (H_2SO_4) were prepared. 0.1 ml of 1% $BaCL_2$ was added to 9.9 ml of 1% $H_2SO_4$to prepare McFarland tube standard No. 1. Hans *et al* (2007).

3.4.5 Preparation of Bacillary Suspension

Bacillary suspension was derived from colonies taken from subcultures of 1 to 2 weeks, placed into a McCartney bottle containing 1ml of sterile distilled water and 6 to 8 glass beads using an inoculating loop. The mixture was homogenized on a vortex mixer for up to 1 minute and opacity of the suspension was adjusted by addition of sterile distilled water to match that of McFarlandtube standard No. 1 turbidity Hans *et al* (2007).

3.4.6 Preparation of Drug-Containing Media

Five ml of isoniazid (INH)stock solution was added to500 ml of the LJ medium to provide 0.2 ug/ml media critical drug concentration; while 2.5 ml of rifampicin (RIF)stock solution were added to 500 ml of LJ medium to provide 40 ug/ml media concentration. For preparation of streptomycin (SM)stock solution, 5 ml of the drug were added to 500 ml of LJ medium to provide 8 ug/ml media concentration. Similarly, 5 ml of ethambutol (EMB) stock solution was added to 500 ml LJ medium to provide 2 ug/ml media concentration. Prepared media were

inspissated at 85°C for 15 minutes, checked for contamination and stored in a refrigerator at 4°C. These media were used to carry out drug susceptibility testing Hans *et al* (2007).

3.4.7 Drug Susceptibility Testing (DST)

Hundred µl of $10^{-2}$and 10^{-4} bacillary suspension were inoculated separately onto two LJ slope media and left as controls assigned as number 1 and 2 respectively. Suspension of mycobacterial isolate cultures containing 10^{-2} bacilli were also inoculated onto two LJ slope media containing separately; SM, RIF, INH and EMB. Cultures were examined after six weeks. If the percentage of colonies on drug-containing media were less than 1% compared with growth on control 2, the isolates were considered resistant. But if the proportion of growth was equal to or greater than 1%compared with growth on control 1, the isolates were considered sensitive (Hans *et al*,2007).

3.4.8 Quality Control of Prepared Media for Drug Susceptibility Testing

Every new batch of drug containing media was inoculated with *M. tuberculosis* H37Rv sensitive strain (ATCCCCH)to ensure the critical concentration of the drug contained in the media.

3.4.2 Molecular Investigation

In this study, three genetic polymorphisms namely; a single nucleotide polymorphism located at position +95 in IFN-γR1, -56 in exon 1 (an exon is a portion of DNA that is converted into mature messenger RNA (mRNA) to form a protein) and 295deletions 12 polymorphisms of the gene were screened for the presence of potential polymorphisms that might trigger susceptibility to TB among the diseased participants using PCR - Restriction Fragment Length Polymorphism (PCR-RFLP) with *Eco*47**Щ** restriction enzyme as compared to healthy controls.However, amplification of the polymorphism at position 295 deletion 12 of IFNγR1 two primer pairs (4 primers) were confronted using PCR- Confronting Two-Pairs Primers (PCR-CTPP)**.**

3.4.2.1 DNA Extraction from Culture

DNA extraction protocols was done by (Jena Bioscience GmbH, Jena, Germany).

3.4.2.2 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′)

3.4.2.3 Screening of IFN-γR1 Polymorphisms (PCR-RFLP)

When a mutation creates or abolishes a restriction site for a specific enzyme, the cleavage with the enzyme can be used as verification for the presence or absence of that mutation, sometimes a PCR mutagenesis around the polymorphic site of the mutation, using a primer carrying a single nucleotide mismatch, together with one of two allelic forms of the mutation, can be used to create a recognition site for specific restriction enzyme which can be used to type the polymorphism. Two single nucleotide polymorphisms of IFN-γR1 in exon 1 of chromosome six at position +95T→ C at the junction of first exon and intron, and the other at position -56 T→ C of the gene were characterized and selected for screening in ongoing studyinvolving 100 human subjects and 50 controls.

3.4.2.4 Screening of IFNγR1 +95 T→C Polymorphisms by (RFLP)

A170 bp DNA fragment was PCR-amplified using 100 ng of genomic DNA in a total reaction volume of 30µl using the following primer pair:

IFNγR1 E1F (5`-CGGGGTTGGAGCCAGCGAC-`3)

IFNγR1 E1R (5`-CCTCCCTCCCTCTCGTCC-`3)

The PCR conditions were programmed as initial temperature 95°C for 5 minutes, followed by 35 cycles of 94°C as melting temperature for one minute, annealing temperature at 60°Cfor one minute and 72°C as an extension temperature, and a final prolongation step at 72°C for 5 minutes.

3.4.2.5 Screening of IFNγR1 -56 T→ C Polymorphisms by (RFLP)

A DNA fragment 285 bp was PCR amplified using 100ng of genomic DNA in a total PCR reaction volume of 30µl using the primer pair below:

IFNγR-56 T→ C F: (5`-GGGCGTGGGCGGGGTCAA-`3)

IFNγR -56 T→ CR: (5`-CCTCCCTCCCTCTCGTCC-`3)

refrence conditions are as initial temperature 95°C for 5 minutes, followed by 35 cycles of 94°C as melting temperature for one minute, 66°C as annealing temperature for one minute and 72°C as extension temperature, then final prolongation step at 72°C for 5 minutes.

3.4.2.6 Screening of IFNγR1 295 Deletions 12 Polymorphisms by (PCR-CTPP)

The principle of PCR-CTPP is based on the fact that the efficiency of PCR amplification depends on the complete matching of primer sequence at 3`end. Thus, any mismatch at this end of the primer will affect the PCR amplification system. Accordingly, PCR-CTPP genotyping system needs four primers; two common primers flanking the polymorphic site; one at each side, but with different length from the polymorphic site. The other two primers were allele specific primers. Each primer should be designed to be absolutely complementary to one allele sequence specially, at the 3` end of the primer. PCR-CTPP is an accurate method for genotyping of single nucleotide polymorphisms (SNPs). It is time-saving and inexpensive to perform and can be used for genotyping of all types of mutations (Hamajima, 2001).This system was used for genotyping of IFNγR1 295 del. 12. Four primers were designed by (Ahmad *et al*, 2011) for amplifying IFN-γR1at position 295 del. 12 PCR-CTPP.

IFNγR295 CP: 5'CTCTGCTCTTTCTACCGCTTT 3'

IFNγR295 12: 5'AACCCTGGCTTTAACTCTGACC 3'

IFNγR295del12:5' CCATCAAATTCTCTTAAAGCCAGG 3'

IFNγR295del CP:5' CTAATAAAAGCAAACATACAGAAGAC 3'

In the absence of polymorphism 295 del 12, the primer IFNγ-R1 295 del. 12 will work to amplify with the primer IFN-γR1 295 CP to give a PCR fragment of 232bp while in the presence of the 295del. 12, the primer IFN-γR1 295 del. 12 may work to amplify with the primer IFN-γR1 295 del CP to give a PCR fragment of 160bp. A third PCR product of 365bp may be obtained as a result of the IFN-γR1 295 CP and IFN-γR1 295del. CP PCR product was electrophoresed in 2% agarose gel. Then, gels were stained in 1μg/ml Ethidium Bromide solution for 10-15 minutes and later visualized under UV (ultra violet) light using GDS.

Genotypes were assigned according to the obtained profile. Two bands of 365 and 232bp will be assigned as a homozygote wild type, three bands 365bp, 232bp and 160bp as heterozygote wild type 295del. 12 while two bands of 365bp and 160bp will be assigned as homozygote mutant type 295del. 12.

3.4.2.6 DNA Prepration

3.4.2.6.1 Preparation of Host Immune Cells Lysate for Extraction of Genomic IFN-γR1 DNA

Nine hundred µl Red Blood Cells (RBCs) Lysis Solution was pipetted into 1.5 ml microtube following instructions in the Kit (Jena Bioscience GmbH, Jena, Germany). 300 µl of the whole blood was added and mixed by inverting the tube 10 times. Contents in the tubes were incubated at room temperature with occasional inversion for 3 minutes (min.). The tubes were then centrifuged at 15,000 g revolution for 30 seconds (sec.). The supernatants were removed with pipettes leaving behind visible cell pellet representing host white immune cells. Then, each tube was vigorously vortexed for 10 sec. to resuspend the white immune cells in the residual liquid. The immune cells pellet in each tube was completely resuspended. 300 µl Cell Lysis Solution were added to the re-suspended cells to extract genomic IFN-γR1 DNA.

3.4.2.6.2 Protein Precipitation in Cells Lysate

Hundred µl protein precipitation Solution were added to the prepared cell lysate. Then,contents were thoroughly and vigorously vortexed for 20 sec. The tube was centrifuged as before for 1 min. The supernatant containing extracted IFN-γR1DNA was pipetted and collected in a clean tube; while the precipitated sediment discarded.

3.4.2.6.3 Precipitation of Extracted IFN-γR1 DNA

Three hundred µl isopropanol were pipetted into a 1.5 ml microtube and the supernatant containing extracted DNA containing extracted DNA was added. The specimens was gently mixed by inverting the tube for 1 min. Then,contents were centrifuged at 15,000 g for 1 min. The supernatant was discarded and tube containing the precipitated DNA was briefly drained on a clean absorbent paper. To wash the pelleted DNA, 500 µl washing buffer were added and the tube was inverted several times, and washing buffer discarded.

3.4.2.6.4 DNA Hydration

Fifty µl DNA Hydration Solution were added to the DNA pellet in the tube. Contents in the tube were vortexed and gently mixed at a medium speed for 5 sec. specimens containing the DNA were incubated at 65°C for 30 min. to accelerate rehydration. Purity of isolated genomic IFN-γR1DNA was checked by agarose gel electrophoresis and its concentration was determined by spectrophotometer.

3.4.4 Polymerase Chain Reaction for Detection IFN-γR1

Polymerase chain reaction (PCR) involved preparation of PCR-reaction mixture as described (Table 1). The technique was performed on all DNA specimens extracted from human immune cells as templates to amplify IFN-γR1 gene at different polymorphic locations (Table 1). Different primers were included to amplify the gene (Table 2). PCR amplification was done using a thermal gene cycler (Biometra T Professional Gene) Thermocycler TPersonal combi while optimal conditions of temperatures and cycling were performed as shown (Table 3).

Table 1: PCR Master Mix for Amplifying Genomic IFN-γR1

Material	Amount used
DNA template	1µl
Forward primer (2.5µM)	1µl
Reverse primer (2.5µM)	1µl
GoTaq PCR Master Mix	12.5 µl
PCR grade water (Nuclease free)	14.5 µl

Table 2: Primers list for Amplification of Various Polymorphic Regions Along IFN-γR1

Polymorphism	**Forward primer**	**Reverse primer**
IFN-γR1 +95 T→C	IFN-γR1 E1F (5`-CGGGGTTGGAGCCAGCGAC-`3)	IFN-γR1 E1R (5`-CCTCCCTCCCTCTCGTCC-`3)
IFN-γR1 -56 T→ C	IFN-γR-56 T→ C F (5`-GGGCGTGGGCGGGGTCAA-`3)	IFN-γR1-56 T→ C R (5`-CCTCCCTCCCTCTCGTCC-`3)
IFN-γR1 295 deletions 12	IFN-γR295 CP: CTCTGCTCTTTCTACCGCTTT IFN-γR295 12: AACCCTGGCTTTAACTCTGACC IFN-γR295del12: CCATCAAATTCTCTTAAAGCCAGG IFN-γR295del CP: CTAATAAAAGCAAACATACAGAAGAC	

Table 3: Gene Cycling Conditions for PCR Amplification of IFN-γR1

Polymorphism	Initial denaturation	Denaturation	Primer annealing	Extension	Final extension	Number of cycles
IFN-γR1 +95 T→C	95ºC/5 min(s).	94ºC/1 min.	60ºC/1 min.	72ºC/1 min.	72ºC/5 min(s).	35
IFN-γR1-56 T→ C	95ºC/5 min(s).	94ºC/1 min.	66ºC/1 min.	72ºC/1 min.	72ºC/5 min(s).	35
IFN-γR1 295 del 12	95ºC/5 min(s).	94ºC/1 min.	62ºC/1 min.	72ºC/1 min.	72ºC/5 min(s).	35

3.4.3.1 Procedure for Preparation and Running of Agarose Gel Electrophoresis

Polymerase chain reaction of IFN-γR1 DNA products were analysed on an agarose gel with a concentration of 1.5%. Then, agarose was dissolved in 1X TAE buffer and boiled under fire until the solution became clear. The agarose dissolution was left to become warm during which 2 µl of (10mg/ml) Ethidium Bromide (Bio-Rad) was added. The mixture was poured into a sealed gel caster, a comb was loaded into the dissolved agarose and contents were left to solidify. Following complete agarose solidification, the seal and comb were removed and gel was placed into an electrophoresis tank filled with 1X TAE buffer. Specific amounts of tested IFN-γR1DNA specimen were gently mixed with certain amounts of loading dye and contents were loaded into the formed wells.

Electrodes of gel apparatus were then connected to the power pack and run at a constant rate of 100 V until the dye reached about two thirdsof gel. IFN-γR1 DNA bands were visualized under ultraviolet (UV) trans-illuminator and gel was documented using DNA Gel Documentation System (GDS) Accuris E3000 UV Transilluminator.

3.4.4 Preparation of Reagents Used for Detection of Interferon Gamma (IFN-γ) in human Sera

3.4.4.1 Preparation of Serum Specimens

A 10-fold dilution of the test specimens (sera) whether collected from TB patients or controls were prepared by adding 50 μL of specimen to 450 μl of diluent.

3.4.4.2 Preparation of Wash Buffer

The entire contents of the 30X Wash Buffer (50ml) bottle were added to a container and diluted to a final volume of 1.5 L with ultrapure water.

3.4.4.3 Preparation of standard human IFN-γ (PharmPak) cytokine

Six tubes were labelled, one for each standard curve point; 1000 pg/ml, 400 pg/ml, 160 pg/ml, 64 pg/ml, 25.6 pg/ml and 0 pg/ml (Fig. 3). Then 1:2.5 serial dilutions were prepared for the standard curve as follows: 240 μl of appropriate diluent were pipetted into each tube. 160 μl of the reconstituted standard were pipetted into the first tube labelled 1000 pg/ml and gently mixed. Then, 160μL from this dilution were removed and pipetted into the second tube labelled 400pg/ml and mixed well. Three more times of serial dilutions using 160 μl were performed to complete the standard curve points (Fig. 5).

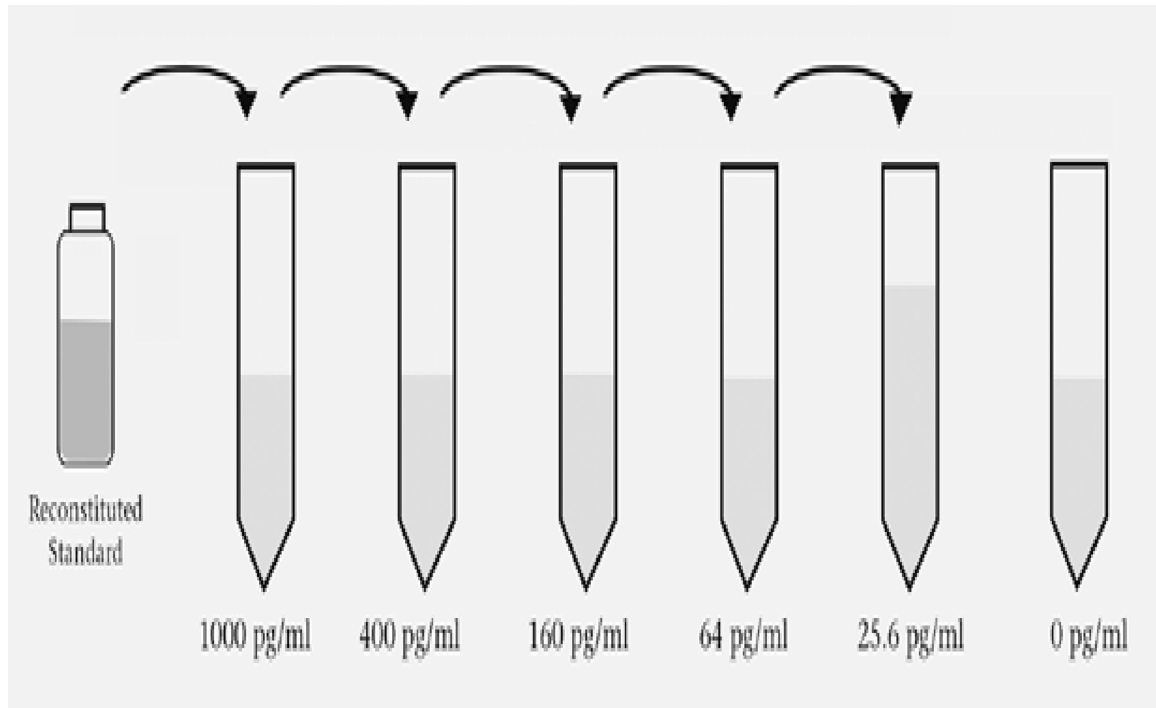


Fig. 3 Serial Dilutions of Standard Human Gamma Interferon (IFN-γ)Using 160µl Volumes

3.4.4.4 Evaluation of (IFN-γ) Cytokine in Sera of Infected Patients and Healthy Controls Using ELISA

Fifty µl of Biotinylated Antibody Reagent were added to each of certain wells of 96-microplate wells. 50µl of reconstituted standard or test specimens were addedin duplicates to each well. To another separate set in the same plate, 50 µl of Standard Diluent were added to all wells that did not contain standards or specimens. A squirt bottle was used to vigorously fill each well completely with Wash Buffer. The procedure was repeated two additional times for a total of three washes. Then, the plate washings were discarded. Any unbound substances were washed out. The plate was blotted onto paper towels to dry out.

After that, 50 µl of streptavidin-horseradish peroxidase (HRP) conjugate solution was added to the wells. The plate was incubated for 30 minutes at room temperature and plate was washed as described previously. To assist testing enzymatic reaction, 100 µl of 3, 3', 5, 5'-tetramethyl benzidine (TMB) Chromogenic Substrate buffer were added into each tested well. Colour reaction was allowed to develop after incubation at room temperature in the dark for 30 minutes. Development of colour was proportional to the amount of boundIFN-γ. 100 µl of Stop Solution were added to each well to stop colour development. Finally, intensity of colour development was measured by determining absorbance on an ELISA plate reader set at 450n.

3.4.5 Statistical Analysis of Collected Data

To analyse collected data, statistical package of social sciences (SPSS) was used. Chi-square (χ^2) test was used to evaluate the collected data to detect the significant of result.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

RESULTS

4.1 Demographic Data/Risk Factors for Enrolled Participants

In relation to demographic and personal data, 75% of TB participants were males while the remainder 25% were females (Table 5). The average age of cases was 34 years old in comparison with 33 years old for controls.When infected cases were grouped, the frequency of distribution was greater in the range from 20-30 years old accounting for 50% (Table 5). In relation to frequency of BCG-vaccination against TB, were more in males than females (Table 6). But, the correlation between vaccination and gender was statistically non-significant (*P*= 0.580). Non-significant correlation was also seen with associated disease and alcoholism; while a significant association (*P*=0.033) was observed in smoking.

Table 4: Demographic Data and Questionnaire for TB Infected-Patients

Pt.#	**Age**	**G**	**Tribe**	**S E**	**BCG Vaccina-ion**	**Associated disease**	**Alcoholism**	**Smoking**	**Pregnancy**	**Family (h)**
1	40	M	Galiyee-n	L	Yes	NO	NO	YES	-	NO
2	20	M	Zagawa -h	L	NO	NO	NO	NO	-	NO
3	35	M	Flatah	L	NO	NO	YES	YES	-	NO
4	31	M	Mosaw-at	L	NO	NO	YES	YES	-	NO
5	25	M	Tama	L	NO	NO	NO	NO	-	NO
6	22	M	Jamoey ah	L	NO	NO	NO	YES	-	NO
7	24	M	Saloha-b	L	YES	NO	NO	NO	-	NO
8	30	M	Hadnda-wah	M D	NO	NO	NO	NO	-	NO
9	37	M	Nuba	L	NO	NO	NO	NO	-	NO
10	37	F	Zagawa h	M	NO	NO	NO	NO	Y	NO
11	28	M	Nuba	L	NO	NO	NO	NO		NO
12	30	M	Salama-t	M	NO	NO	YES	YES	-	NO
13	22	F	Hawsa	M	NO	NO	NO	NO	-	NO
14	30	F	Galiyee-n	L	NO	NO	NO	NO	-	NO

15	35	M	Galiyeen	L	NO	NO	NO	NO	-	NO
16	21	F	Galiyee-n	L	NO	NO	NO	NO	-	NO
17	33	M	Tama	G	NO	NO	NO	NO	-	NO
18	36	M	Fur	M	NO	NO	NO	NO	-	NO
19	37	F	Fur	L	NO	NO	NO	NO	-	YES
20	34	F	Galiyee-n	L	NO	NO	NO	NO	-	NO
21	40	M	Bedeari-yah	M	NO	NO	NO	NO	-	NO
22	25	M	Nuba	L	NO	NO	YES	NO	-	NO
23	53	M	Bedeari-ya	L	NO	NO	NO	NO	-	NO
24	31	M	Fur	L	NO	NO	NO	NO	-	NO
25	29	M	Fur	L	NO	NO	NO	NO	-	NO
26	32	M	Nuba	L	YES	NO	NO	NO	-	NO
27	45	M	Hawsa	L	NO	NO	NO	NO	-	NO
28	23	M	Zagawa-h	M	NO	NO	NO	NO	-	NO
29	22	M	Bedeari-ya	L	NO	NO	NO	NO	-	NO
30	24	M	Galiyee-n	L	NO	NO	NO	YES	-	NO
31	21	F	Hamar	L	NO	NO	NO	NO	-	NO
32	31	M	Sholo k	M	NO	NO	NO	NO	-	NO
33	22	M	Zagawa-h	L	NO	NO	NO	NO	-	NO
34	55	M	Galiyee-n	G	NO	NO	NO	NO	-	NO
35	30	M	Galiyee-n	M	NO	NO	NO	NO	-	NO
36	23	F	Nuba	M	NO	NO	NO	NO	-	NO
37	33	M	Tama	L	NO	NO	NO	NO	-	NO
38	40	M	Fur	L	NO	NO	NO	NO	-	NO
39	27	M	Galiyee-n	M	YES	NO	NO	NO	-	NO
40	36	M	Barno	L	NO	NO	NO	YES	-	NO
41	27	F	Nuba	L	NO	NO	NO	NO	Y	NO
42	34	F	Zagawa-h	L	YES	NO	NO	NO	-	NO
43	30	M	Fur	L	YES	NO	NO	NO	-	NO

44	28	M	Nuba	L	NO	NO	NO	NO	-	NO
45	39	M	Habani-yah	M	NO	NO	NO	NO	-	NO
46	37	F	Barno	L	NO	NO	NO	NO	-	NO
47	38	M	Tama	L	NO	NO	NO	NO	-	NO
48	22	M	Galiyee-n	L	NO	NO	NO	NO	-	NO
49	25	F	Habani-yah	L	NO	NO	NO	NO	-	NO
50	60	F	Galiyee-n	L	NO	NO	NO	NO	-	NO
51	27	M	Hadnda wah	L	NO	NO	NO	NO	-	NO
52	28	M	Flatah	L	NO	NO	NO	YES	-	NO
53	50	M	Bargo	L	NO	YES	NO	NO	-	NO
54	50	M	Tama	L	NO	NO	NO	YES	-	NO
55	48	F	Galiyee-n	M	NO	NO	NO	NO	Y	NO
56	50	M	Galiyee-n	M	NO	NO	NO	NO	-	NO
57	26	M	Bargo	L	YES	NO	NO	NO	-	NO
58	60	F	Zagawa-h	L	NO	NO	NO	NO	-	NO
59	37	F	Tama	L	NO	NO	NO	NO	-	NO
60	18	F	Galiyee n	G	NO	NO	NO	NO	-	NO
61	24	F	Habani-yah	M	NO	NO	NO	NO	-	NO
62	22	F	Rashyd-ah	M	YES	NO	NO	NO	-	NO
63	24	F	Shukriy-ah	M	YES	NO	NO	NO	-	NO
64	27	F	Tama	L	NO	NO	NO	NO	-	NO
65	55	F	Zagawa-h	L	NO	NO	NO	NO	-	NO
66	35	M	Miseari-yah	L	NO	NO	NO	NO	-	NO
67	30	M	Nuba	L	NO	NO	NO	NO	-	NO
68	30	M	Denka	L	NO	NO	NO	NO	-	NO
69	34	M	Hamar	L	YES	NO	NO	NO	-	NO
70	30	M	Kawahl-ah	L	NO	NO	NO	NO	-	NO

71	45	F	Nuba	L	NO	NO	NO	NO	-	NO
72	51	M	Tama	L	NO	NO	NO	NO	-	NO
73	24	M	Galiyee-n	L	YES	NO	NO	NO	-	NO
74	36	M	Hadnda wah	L	YES	NO	NO	NO	-	NO
75	70		Bargo	L	NO	NO	NO	NO	-	NO
76	49	M	Jamoay-ah	L	NO	NO	NO	NO	-	NO
77	32	F	Denka	L	NO	NO	NO	NO-	-	NO
78	21	M	Nuba	L	NO	NO	NO	NO	-	NO
79	50	M	Hadnda-wah	L	NO	NO	NO	NO	-	NO
80	25	M	Shukriy-ah	L	NO	NO	NO	NO	-	NO
81	27	M	Hadnd-wah	L	NO	NO	NO	YES		NO
82	30	M	Hadnd-wah	L	NO	NO	NO	NO		NO
83	22	F	Niwear	L	NO	NO	NO	NO	-	NO
84	56	M	Tama	L	NO	NO	NO	NO	-	NO
85	40	M	Kawahl-ah	L	NO	NO	NO	NO	-	NO
86	2 4	M	Galiyee-n	L	NO	NO	NO	NO	-	NO
87	25	M	Shygiy-ah	L	NO	NO	NO	NO	-	NO
88	27	M	Nuba	L	NO	NO	NO	NO	-	NO
89	26	M	Danagl-ah	L	NO	NO	NO	NO	-	NO
90	27	M	Zagawa-h	L	NO	NO	NO	NO	-	NO
91	45	M	Galiyee-n	L	NO	NO	NO	NO	-	NO
92	63	M	Miseari yah	L	NO	NO	NO	NO	-	NO
93	45	M	Kawahl-ah	M	NO	NO	NO	NO	-	NO
94	80	M	Misrari-yah	M	NO	YES	NO	NO	-	NO
95	50	M	Kawahl-ah	L	NO	NO	YES	YES	-	NO
96	33	M	Galiyee n	L	NO	YES	NO	YES	-	NO
97	22	M	Galiyee-n	M	YES	NO	NO	NO	-	NO
98	42	M	Shygiy-ah	M	YES	NO	NO	YES	-	NO
99	50	M	Tama	L	NO	YES	NO	NO	-	NO
100	25	M	Taish-ah	M	YES	NO	NO	YES	-	NO

Pt.#, patient number; G, gender; M, male gender; F, female gender; SE, socioeconomic status; L, Low income; MD, moderate income; h, history; BCG, Bacillus Calmette-Guerin vaccine.

Table 5 Frequency Distribution of Infected Cases According to Age Grouping

	Variables	Frequency	Percent (%)
Age	20-30	50	50
	31-40	28	28
	41-50	10	10
Gender	>50	12	12
	Males	75	75
	Females	25	25

Table 6 Frequency Distribution of BCG Vaccinated Cases According to Gender

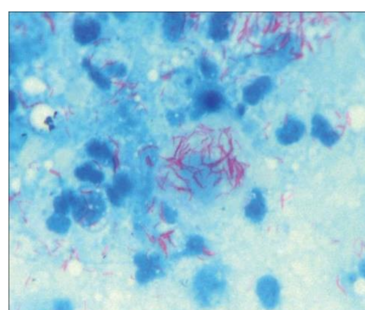
Gender	**BCG vaccination**			
	Yes		No	
	N	%	N	%
Males	9	81.8	66	74.2
Females	2	18.2	23	25.8
Total	11	100	89	100
P-value	0.580			

BCG. Bacillus-Calmette Guerin; N, number of participants.

4.2 Conventional Identification of *M. tuberculosis*

4.2.1 Microscopic Examinationof *M. tuberculosis* from Sputum and Culture Smears

Stained positive sputum and culture smears with ZN stain and microscopic examination



ZN smear

4.2.2 Culturing of Smear-Positive Sputum Specimens

Sputum smear positive specimens taken from active pulmonary TB patients were decontaminated and cultured onto LJ slope media. Following incubation and tracing of growth for about four to six weeks non-pigmented, dried and rough colonies were seen.

Culture in (LJ) media

4.2.3 Biochemical Analysis

4.2.3.1 Catalase Assay

When cultures were subjected to biochemical tests, the suspected organisms reacted positively to catalase enzyme with formation of air bubbles but negative results and absence of bubbles were obtained when the enzyme was heated at 68°C under pH7 as its enzyme is heat-labile.

4.2.3.2 Nitrate Reduction Assay

Upon inoculation of nitrate broth with loopful of cultures, development of pink colour was noticed due to reduction of nitrate to nitrite.

4.2.3.3 Para-Nitrobenzoic (PNB) Acid Susceptibility Test

Inoculation of culture isolates considered as M. *tuberculosis* onto LJ slope media containing PNB, showed inability of tubercle bacillus to grow in the presence of the drug in comparison to its growth on media without having it.

4.2.3.4 Thiophen 2-Carboxylic Acid Hydrazide (TCH) Susceptibility Test

When suspension of diluted culture isolates were inoculated onto LJ slope media containing TCH and after 4 weeks of incubation, the organism was observed to grow in the presence of the drug and its growth was similar to that of culture media kept as controls.

4.2.4 Drug Susceptibility Testing

Diluted bacterial suspensions identified as Mtb were inoculated on LJ media. Following 6 weeks of incubation, the growth containing EMB and SM appeared morphologically similar to that of controls lacking the drugs. However, scarce growth of organisms was observed on media containing either RIF or INH indicating resistance.

4.3 Genotyping of Human Genomic IFN-γR1 Gene

PCR - RFLP was performed to screen any mutations at positions +95 and -56 in exon 1separately within the extracted DNA from TB patients and healthy participants following digestion with *Eco*47**Щ** restriction enzyme and subsequent running of electrophoresis. Another genotype at position 295 deletion 12 was screened in both groups utilizing the CTPP method.

4.3.1 PCR Results

All of the 100-sputum specimen that were collected from TB patients were present harbouring the target sequence (123 bp). At the same time all negative controls were appeared negative for this sequence

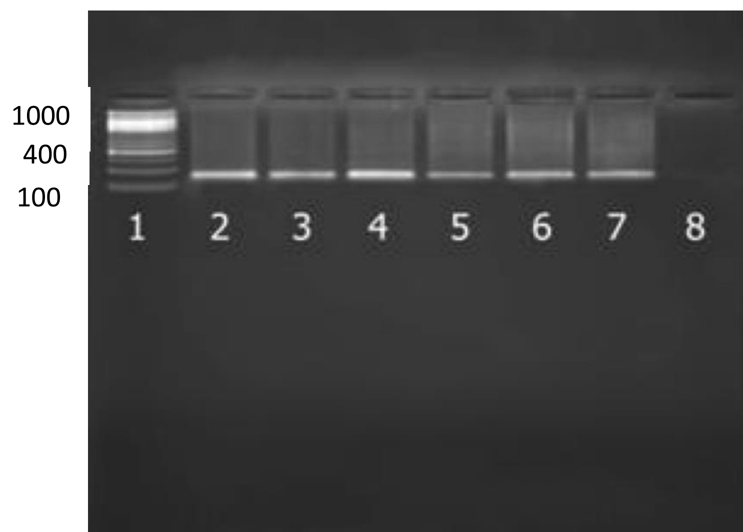


Figure 4: 1.5% Agarose gel with positive sputum specimens (2-7), while specimens 8 appeared free from IS6110 with 123 bp.

4.3.2 Genotyping of PCR-Amplified IFNγR1 DNA Products

An amount of 100 ng genomic IFNγR1DNA extracted from immune cells of 100 infected patients and 50 healthy controls was PCR-amplified to screen any mutations at position +95 within the gene using the following primers:

IFN-γR1E1F (5`-CGGGGTTGGAGCCAGCGAC-`3)

IFN-γR1E1R (5`-CCTCCCTCCCTCTCGTCC-`3).

When IFNγR1 DNA products at position +95T→Cwere enzymatically-cleavaged with *Eco*47**Щ** restriction enzyme, DNA fragments were released at molecular sizes of 170bp resembling wild type, IFN-γR1 (Fig. 5).The frequency distribution of genotype +95TT at position +95 of gene promoter was 100% for both infected and healthy groups (Fig. 5, Tables 7; 8).However, IFN-γR1 gene at the mentioned position lacked the presence of any polymorphic alleles representing +95T/C or +95C/C alleles with zero variant allele frequency for both TB patients and healthy controls (Fig. 5; Tables 7; 8). Significant difference (*P*=0.033) was shown between wild type +95TT and 95T/C polymorphism, and groups (Fig. 6; Appendix 5). Therefore, +95TT genotype was significantly associated with risk factor for development of TB among Sudanese patients.

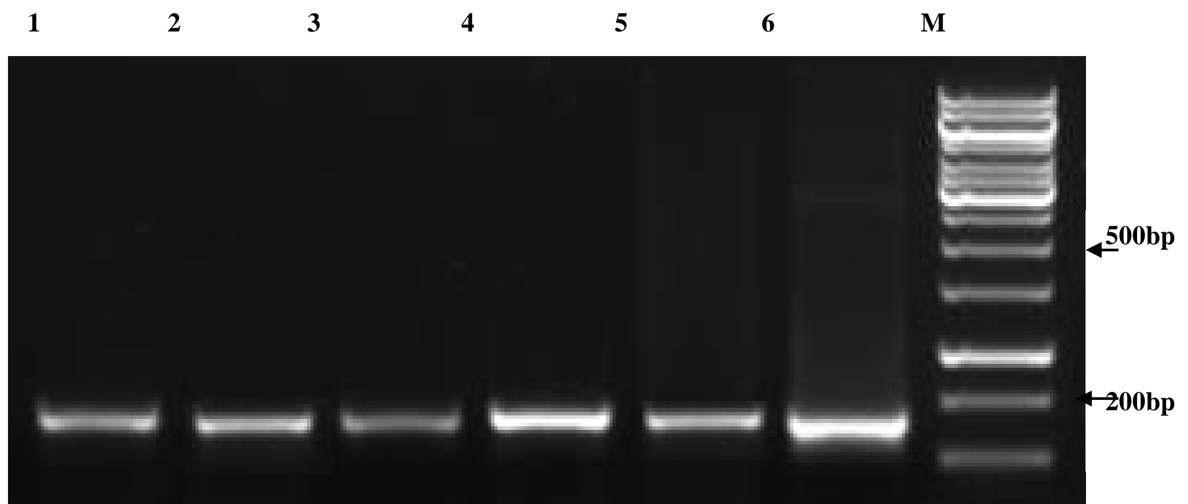


Figure 5 Agarose gel electrophoresis showing absolute absence of digestion of 170bp amplicon at position +95 promoter of IFN-γR1 gene extracted from immune cells of infected patients and healthy controls and amplified by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method.
From lane1 to lane 6 170bp there is no any mutation

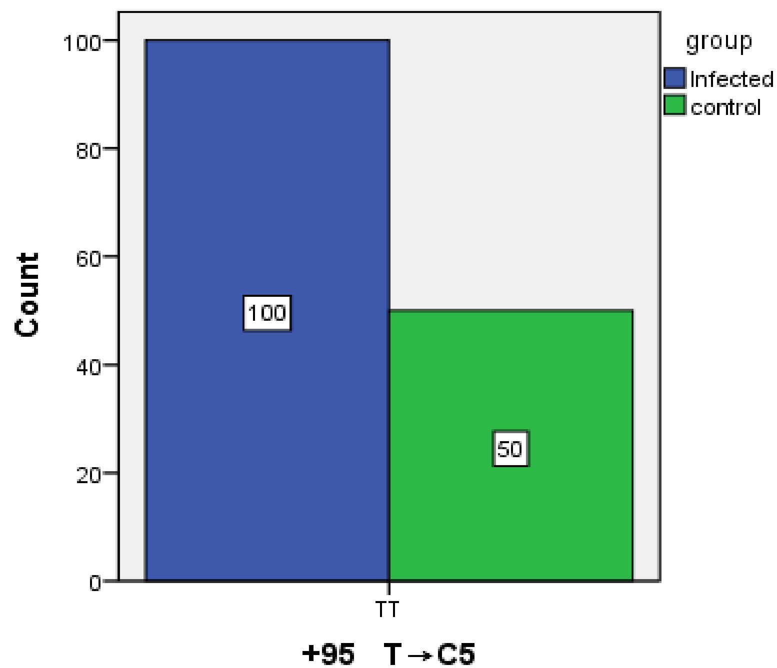


Figure 6: Significant Differences Between +95TT and +95T→C, and the infected and control Groups

Upon analysis of PCR-amplified IFN-γR1 DNA at position -56 of promoter region using the underneath forward and backward primers respectively: IFN-γR1-56T→C F(Forward) 5`-

GGGCGTGGGCGGGGTCAA-`3) and IFN-γR1 -56T→C R (Reverse) (5`-CCTCCCTCCCTCTCGTCC-`3), and followed by enzyme restriction of PCR products, two DNA fragments at molecular levels of 193bp and 92bp were observed (Fig. 7).Such characteristics impliedto a mutant with a homozygous CC genotype profile as compared with a non-restricted 285bp DNA fragments representing IFN-γR1 wild type with TTgenotype (Fig. 7, Tables 7; 8). The latter TT genotype had 77% frequency of distribution within the gene in TB patients versus 82% distribution in healthy controls (Appendix 6; Tables 7; 8).

However, incomplete digestion of genomic IFN-γR1 DNA at position -56 yielded three DNA fragments at molecular sizes of 285bp, 193bp and 92bp characteristics of -56T/C heterozygote (Fig. 7). The latter polymorphic -56T/C alleles were harboured by 16 TB patients with 16% frequency of distribution(Fig. 7; Tables 7; 8). By contrast, polymorphic -56T/C alleles were carried by 6 individuals of healthy controls with 12% frequency of distribution (Tables 7; 8).

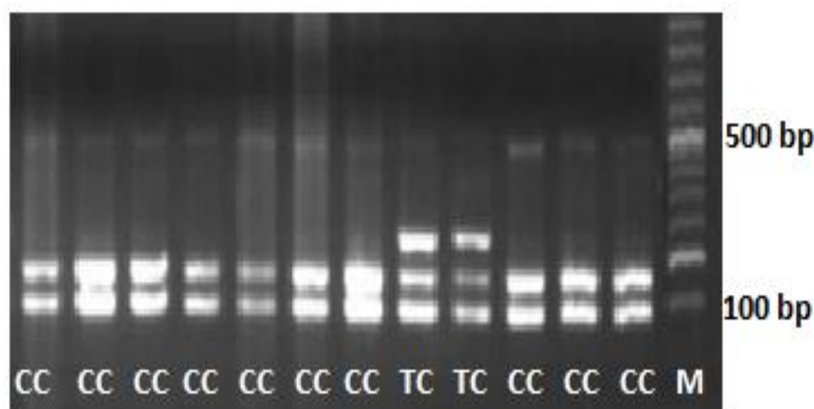


Figure 7 Detection of Allelic Polymorphism at Position -56 of *IFNγR1*Extracted from Immune Cells of Infected Patients and Healthy Controls (PCR-RFLP) Method

With reference to polymorphic -56 C alleles, they were carried by 7 TB patients with th 7% frequency of distribution in comparison with only 3 individuals of healthy control group bearing the same alleles, and having 6% frequency of distribution (Fig. 8; Tables 7; 8). Therefore, frequency distribution of -56C alleles was more in TB cases than those in healthy controls. But, the difference in occurrence between the two groups was statistically non-significant (*P*-value =0.771) (Appendix 6). Again, distribution of -56C alleles in TB patients was more than -56T/C alleles; while distribution of -56C alleles was less than T/C alleles in

the healthy. When both allelic variants i.e., -56T/C and -56CC were added together they had frequency of distribution of 23% among infected patients (Appendix 6). The same variants when added together, they had also more frequency of distribution than in healthy controls.

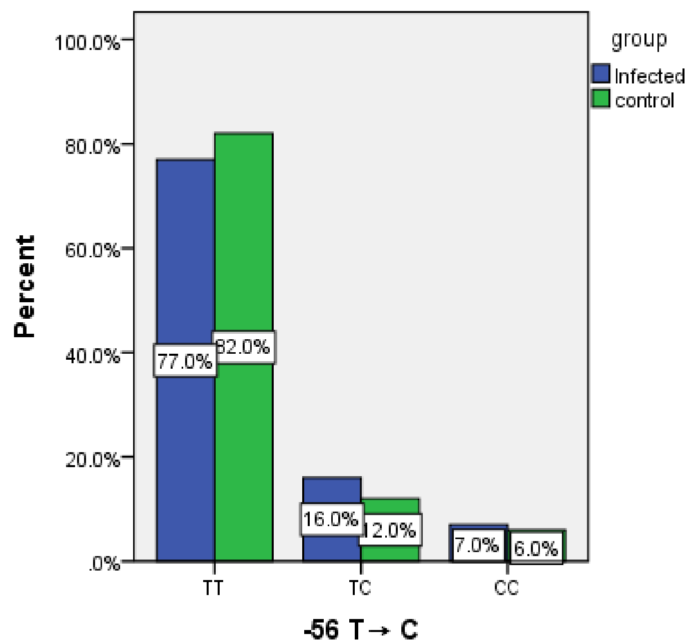


Figure 8: Non-Significant Differences of Frequency Distribution Between -56TT Genotype, -56TC and -56CC, and the Groups

With regard to genotypying of IFN-γR1 at position +295 deletion 12 within the gene, CTPP method was performed using the four primers namely; IFN-γR1 295 CP, 5'CTCTGCTCTTTCTACCGCTTT 3', IFN-γR1 295 del.12: 5'AACCCTGGCTTTAACTCTGACC 3', IFN-γR1 295 del.12 5' CCATCAAATTCTCTTAAAGCCAGG 3' and IFN-γR1 295del. CP 5' CTAATAAAAGCAAACATACAGAAGAC 3'.

The PCR-amplified products showed different DNA patterns after running electrophoresis. The pattern showed two DNA bands at molecular sizes of 365bp and 232bp representing WTtype IFN-γR1 homozygotic +295TT genotype at position of +295 del. 12 (Fig. 9). On the other hand, formation of two DNA bands at molecular levels of 365bp and 160bp represented homozygote polymorphic type +295CC alleles (Fig. 9; Tables 7; 8). Appearance of three DNA bands at molecular sizes of 365 bp, 232bp and 160 bp represented heterozygote +295T/C alleles at position 295 del. 12 (Fig. 9; Tables 7; 8).

Genotyping analysis of IFN-γR1 gene at position +295 deletion 12, 90 TB cases exhibited +295TT genotypes with 90% frequency of distribution in comparison with the same percentage of distribution in healthy controls (Fig. 10; Tables 7; 8). Genetic variation of homogenous +295C/C alleles showed frequency distribution of 6% among TB patients as opposed to 2% distribution for healthy controls. Accordingly, +295C alleles at deletion 12 (6 cases) harbouring these polymorphisms were more than their counterparts in the healthy controls (Tables7; 8). This difference was statistically non-significant (*P*-value =0.343).

In relation to heterogeneous +295T/C allelic changes, they had 4% frequency of distribution across the gene in comparison with 80% occurrence in healthy controls (Fig. 10; Tables 7; 8). Similarly, frequency of distribution after addition of both +295C and +295T/C alleles was 10% in TB cases as well in healthy participants.

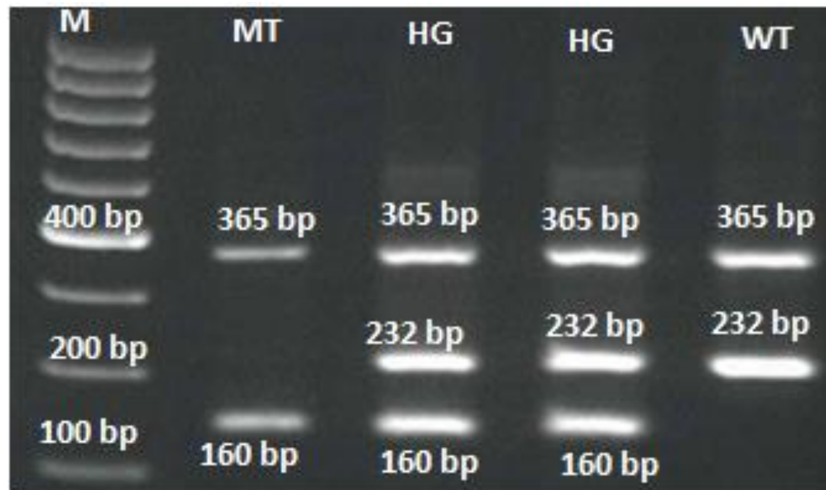


Figure 9: Detection of allelic polymorphismat position+295 deletion 12 within *IFNγR1* extracted from immune cells of infected patients and healthy controlsand amplified by Polymerase Chain Reaction–Confronting Two-Pair primers (PCR-CTPP) method

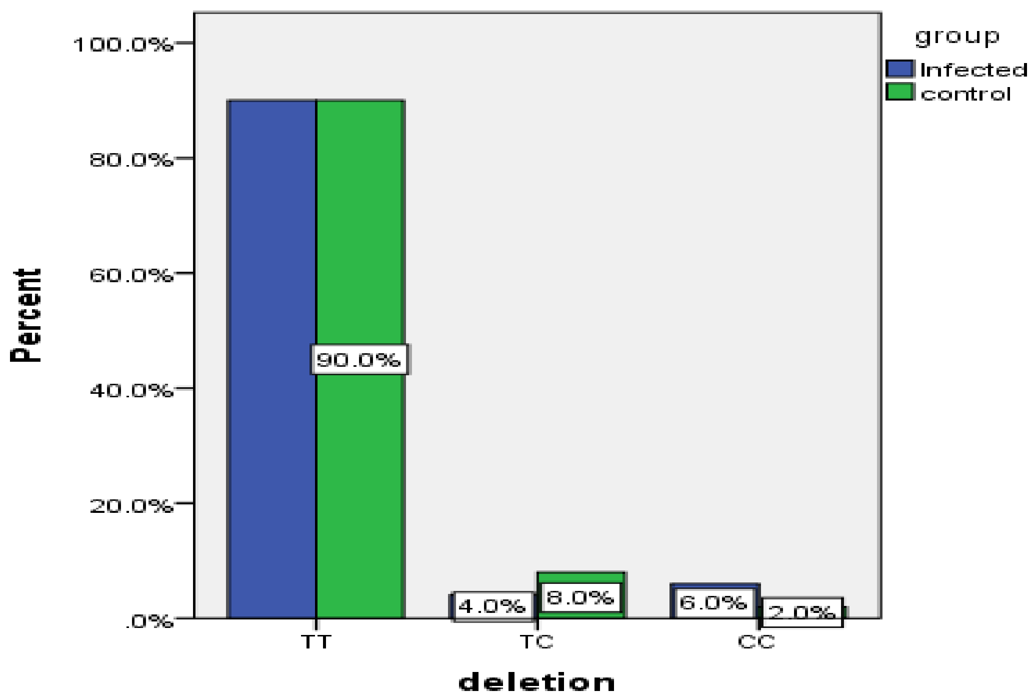


Figure 10: This graph Showing Non-Significant Differences Between +295 Deletion 12, and the Groups.

4.4 Association of Risk Factors with Genetic Variations in TB Confirmed Cases

Out of the 7 TB cases carrying genetic variations identified as -56CC genotype positioned within the promoter region of IFN-γR1 gene, 2 cases originated from each of Zagawah tribe and Galiyeen tribe; while one case for each of Tama, Miseariyah and Rashydah tribe. With reference to -56T/C allele, was observed in TB cases belonged to Tama (5 cases) tribe, followed by Nuba (4 cases), Zagawah (2 cases) tribes, and one case for each of Barno, Bedaeriyah, Galiyeen and Sholock tribes. Therefore, TB cases carrying genetic polymorphisms -56T/C in certain tribes were more frequently identified than -56CC genotype.

Similarly, out of 6 TB cases carrying genetic variation identified as +295CC genotype located in the promoter of gene, 2 cases were originated from Fur tribe, while each of the remainder cases belonged to Tama, Galiyeen, Shygiyah and Hadndawah tribes. With respect to +295T/C polymorphism, TB cases carrying this variation were belonged to each of Fur, Falata, Hawsa and Habaniyah.

Smoking, alcoholism and low income were shown as dominant risk factors linked to TB cases with tendency for certain tribes. Smoking and alcoholism were identified as risk factors in relation with TB cases belonged to Salamat, Mosawat, Kawahla and Falata tribes. The risk of low income was identified as a factor among cases mostly originated from Galiyeen tribe (14 cases) followed by cases belonged to Tama tribe (11 cases), Nuba tribe (10 cases) and Zagawah tribe (7 cases).

Table 7: Genotyping of *IFN-γR1* Gene Extracted from Immune Cells of Healthy Human Controls at Genomic Positions (+95, and -56) Using PCR-RFLP or at Position +295 Deletion 12 Employing PCR-CTPP Method

Sample ID	Group	+95 T→C			-56 T→ C			295 deletion 12		
		TT	TC	CC	TT	TC	CC	WT (TT)	HG (TC)	MT (CC)
C1	Control	+			+			+		
C2	Control	+			+			+		
C3	Control	+				+		+		
C4	Control	+			+			+		
C5	Control	+			+			+		
C6	Control	+			+			+		
C7	Control	+			+			+		
C8	Control	+					+	+		
C9	Control	+			+			+		
C10	Control	+			+			+		
C11	Control	+			+			+		
C12	Control	+			+			+		
C13	Control	+			+			+		
C14	Control	+			+				+	
C15	Control	+					+	+		
C16	Control	+			+			+		
C17	Control	+			+			+		
C18	Control	+				+		+		
C19	Control	+			+			+		
C20	Control	+			+			+		
C21	Control	+			+			+		
C22	Control	+			+			+		
C23	Control	+			+			+		
C24	Control	+			+			+		
C25	Control	+					+	+		
C26	Control	+			+			+		
C27	Control	+			+			+		
C28	Control	+			+			+		
C29	Control	+				+		+		
C30	Control	+			+					+
C31	Control	+			+			+		
C32	Control	+			+			+		
C33	Control	+			+			+		
C34	Control	+			+			+		
C35	Control	+			+			+		
C36	Control	+				+		+		
C37	Control	+			+			+		
C38	Control	+			+			+		
C39	Control	+			+			+		
C40	Control	+			+				+	
C41	Control	+			+			+		
C42	Control	+			+			+		
C43	Control	+			+			+		
C44	Control	+				+		+		
C45	Control	+			+			+		

C46	Control	+			+				+	
C47	Control	+			+			+		
C48	Control	+				+		+		
C49	Control	+			+			+		
C50	Control	+			+				+	
Total		50	0	0	41	6	3	45	4	1
Variant allele freq:		0			0.12			0.05		

WT, wild type; MT, homogenous mutant; HG, heterozygous mutant; freq.; frequency.

Table 8: Genotyping of *IFN-γR1* Gene Extracted from Immune Cells of Infected TB Patients at Genomic Positions (+95, and -56) Using PCR-RFLP or at Position +295 Deletion 12 Employing PCR-CTPP Method

Sample ID	Group	+95 T→C			-56 T→ C			295 deletion 12		
		TT	TC	CC	TT	TC	CC	WT (TT)	HG (TC)	MT (CC)
I1	Infected	+			+			+		
I2	Infected	+					+	+		
I3	Infected	+			+				+	
I4	Infected	+			+			+		
I5	Infected	+			+			+		
I6	Infected	+			+			+		
I7	Infected	+			+			+		
I8	Infected	+			+					+
I9	Infected	+				+		+		
I10	Infected	+			+			+		
I11	Infected	+				+		+		
I12	Infected	+			+			+		
I13	Infected	+			+				+	
I14	Infected	+			+			+		
I15	Infected	+			+			+		
I16	Infected	+			+			+		
I17	Infected	+				+		+		
I18	Infected	+			+					+
I19	Infected	+			+			+		
I20	Infected	+				+		+		
I21	Infected	+			+			+		
I22	Infected	+			+			+		
I23	Infected	+				+		+		
I24	Infected	+			+				+	
I25	Infected	+			+			+		
I26	Infected	+			+			+		
I27	Infected	+			+			+		
I28	Infected	+					+	+		
I29	Infected	+			+			+		
I30	Infected	+			+			+		
I31	Infected	+			+			+		
I32	Infected	+				+		+		
I33	Infected	+			+			+		
I34	Infected	+			+			+		
I35	Infected	+			+			+		
I36	Infected	+			+			+		
I37	Infected	+				+		+		
I38	Infected	+			+					+
I39	Infected	+			+			+		
I40	Infected	+				+		+		
I41	Infected	+			+			+		
I42	Infected	+			+			+		
I43	Infected	+			+			+		

I44	Infected	+			+			+		
I45	Infected	+				+		+		
I46	Infected	+			+			+		
I47	Infected	+			+			+		
I48	Infected	+					+	+		
I49	Infected	+			+			+		
I50	Infected	+			+			+		
I51	Infected	+			+			+		
I52	Infected	+			+			+		
I53	Infected	+			+			+		
I54	Infected	+				+				+
I55	Infected	+			+			+		
I56	Infected	+			+			+		
I57	Infected	+			+			+		
I58	Infected	+				+		+		
I59	Infected	+			+			+		
I60	Infected	+			+					+
I61	Infected	+			+			+		
I62	Infected	+					+	+		
I63	Infected	+			+			+		
I64	Infected	+				+		+		
I65	Infected	+			+			+		
I66	Infected	+			+			+		
I67	Infected	+				+		+		
I68	Infected	+			+			+		
I69	Infected	+			+			+		
I70	Infected	+			+			+		
I71	Infected	+			+			+		
I72	Infected	+					+	+		
I73	Infected	+			+			+		
I74	Infected	+			+			+		
I75	Infected	+			+			+		
I76	Infected	+			+			+		
I77	Infected	+			+			+		
I78	Infected	+				+		+		
I79	Infected	+			+			+		
I80	Infected	+			+			+		
I81	Infected	+			+			+		
I82	Infected	+			+			+		
I83	Infected	+			+			+		
I84	Infected	+			+			+		
I85	Infected	+			+			+		
I86	Infected	+			+			+		
I87	Infected	+			+			+		
I88	Infected	+			+			+		
I89	Infected	+			+			+		
I90	Infected	+				+		+		
I91	Infected	+			+			+		
I92	Infected	+			+			+		
I93	Infected	+			+			+		
I94	Infected	+					+		+	

I95	Infected	+			+			+		
I96	Infected	+					+	+		
I97	Infected	+			+			+		
I98	Infected	+			+					+
I99	Infected	+				+		+		
I100	Infected	+			+			+		
Total		100	0	0	77	16	7	90	4	6
Variant allele freq:		0			0.15			0.05		

I, infected; WT, wild type; MT, homogenous mutant; HG, heterozygous mutant; freq.; frequency.

4.5 Evaluation of Gamma Interferon (IFN-γ) Cytokine in Sera of Infected TB Patients and Healthy Controls Using ELISA

Absorbance readings of bound cytokine were as shown (Table 9). Although there were some differences in absorbance between test specimens and controls, these differences were statistically insignificant (Fig. 11). Thus, immunological findings did not link identified genetic alterationsin IFN-γR1 gene with development of pulmonary TB in this study.

Table 9: Determination of Gamma Interferon (IFN-γ) Levels in the Sera of TB-Infected Patients as Compared to Control Subjects

Sample ID	IFN-γ pg/mL	Sample ID	IFN-γ pg/mL	Sample ID	IFN-γ pg/mL
C1	6.8	I1	9.8	I51	10.0
C2	12.1	I2	7.1	I52	6.8
C3	10.9	I3	12.8	I53	11.3
C4	7.9	I4	8.6	I54	6.5
C5	9.3	I5	12.5	I55	11.9
C6	10.5	I6	8.7	I56	11.5
C7	8.1	I7	11.4	I57	10.8
C8	12.2	I8	10.2	I58	8.8
C9	8.0	I9	6.3	I59	12.9
C10	12.5	I10	7.2	I60	13.3
C11	7.0	I11	11.0	I61	6.4
C12	10.7	I12	6.2	I62	7.3
C13	9.9	I13	10.1	I63	8.4
C14	7.8	I14	12.1	I64	9.9
C15	8.5	I15	7.3	I65	12.4
C16	10.6	I16	6.2	I66	7.8
C17	11.5	I17	10.9	I67	9.8
C18	7.3	I18	11.1	I68	12.0
C19	7.1	I19	8.9	I69	10.9
C20	12.6	I20	12.6	I70	6.3
C21	8.9	I21	11.3	I71	12.5
C22	9.6	I22	7.0	I72	11.1
C23	13.3	I23	6.6	I73	8.2
C24	9.2	I24	9.8	I74	6.9
C25	10.2	I25	11.2	I75	11.0
C26	10.9	I26	8.3	I76	6.1
C27	7.5	I27	7.4	I77	12.5
C28	10.9	I28	10.5	I78	10.7
C29	8.5	I29	12.1	I79	9.8
C30	10.8	I30	8.5	I80	7.2
C31	11.1	I31	8.1	I81	8.1
C32	8.3	I32	13.4	I82	6.9
C33	13.5	I33	13.0	I83	11.6
C34	10.1	I34	7.4	I84	6.1
C35	9.8	I35	12.2	I85	11.7
C36	12.4	I36	7.6	I86	7.8
C37	11.3	I37	10.3	I87	7.5
C38	8.2	I38	6.7	I88	12.9
C39	12.8	I39	7.1	I89	8.2
C40	13.3	I40	13.4	I90	12.3
C41	10.0	I41	13.1	I91	8.4
C42	9.8	I42	8.0	I92	9.8
C43	10.3	I43	11.8	I93	10.4
C44	9.0	I44	10.6	I94	11.6

C45	10.1	I45	7.8	I95	6.9
C46	6.9	I46	6.4	I96	6.5
C47	10.2	I47	7.7	I97	7.9
C48	7.3	I48	6.3	I98	7.5
C49	11.2	I49	13.3	I99	11.3
C50	6.8	I50	10.7	I100	13.2
Mean±SD	9.87±1.91	Mean±SD	9.56±2.31		

C, refers to control of healthy participants; ID, identification; I, represents infectedTB patient; SD, standard deviation.

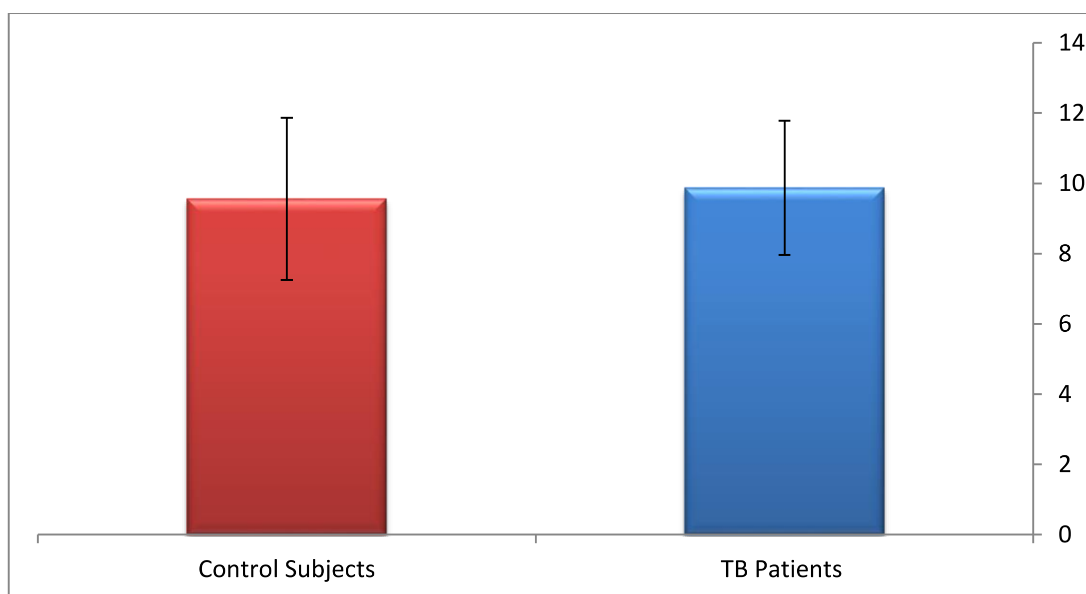


Figure 11: Determination of Gamma Interferon (IFN-γ) Levels in Sera of Patients group with Pulmonary Tuberculosis and in sera of control group.

CHAPTER FIVE

DISCUSSION

CHAPTER FIVE
DISCUSSION

Many studies have reported a link between host genotypes and risk of disease. In this context, SNPs in IFN-γR1gene were detected to explore any potential mutation that might predispose for TB in participant cases as compared to healthy controls. With annual occurrence of nearly 2 million deaths and around 9 million fresh emergent cases, TB remains the major infectious bacterial disease that threatens humans'public health (WHO, 2013). Patients with active disease and who show sputum smear-positive indicative of pulmonary TB constitute the main focus for the spread of infection.It has been reported that only a minority proportion of people with waned immune system might get *M.tuberculosis* infection upon exposure and could present overt clinical signs of TB; while a few people would never develop the disease (Abel *et al*., 2014).In several studies, TB has been documented as a disease with host genetic susceptibility and familial inheritance (Al-Muhsen and Casanova,2008). This belief is evidenced by increased infection with *M.tuberculosis* in homozygotic twins than dizygotic twins, and in close marriages than in distant relationships (Kallmann and Reisner, 1943; Al-Muhsen and Casanova,2008; Abel *et al*., 2014).

Being a T-helper cytokine 1, IFN-γgene and through its signalling pathway is responsible for activating macrophages to release bactericidal toxic products and combat progression of Mtb infection and other pathogens (Collins and Kaufmann, 2001; Cooke *et al*., 2006). Specific mutations in IFN-γ receptor lgene ligand binding chain were involved in Mendelian susceptibility to bacillary infection (Awomoyi *et al*., 2004). Mice with genetic alterations in host *IFN-γR1*gene failed to yield toxic reactive nitrogen intermediates and control wild pathogen after challenge (Cooper *et al*., 1993). Similarly, children with inherited genetic disorder of partial or complete deficiency of *IFN-γR1*were highly prone to infection with atypical mycobacteria (Casanova and Abel, 2004). Moreover, a quite number of single-nucleotide polymorphisms (SNPs) in the *IFN-γ*such as +874 A/A genotype have been demonstrated in association with susceptibility of developing TB (Tso *et al*., 2005; Cooke *et al*., 2006).

In the current reserch, pathological materials taken from diseased and healthy participants were examined by microscopy and cultured to isolate and recover the causative agent. Genotyping analysis was performed on three potential polymorphic alleles located across the promoter region of IFN-γR1 gene at positions; -56, +95 and +295 deletion 12. PCR assay

combined with RFLP analysis by specific restriction enzyme or CTPP of genomic *IFN-γR1*DNA isolated from immune cells of enrolled participants were used as tools to achieve the target.

Microscopic examination of stained sputum smears coupled with culturing of pathological materials as well as biochemical testing have confirmed that the enrolled participants who showed clinical active TB signs were infected with *M. tuberculosis*. Some of genotypic findings of the three potential polymorphisms IFN-γR1 gene exploited in this project were consistent with those of others who have previously identified and explored them in different regions and populations worldwide (Awomoyi *et al*., 2004; Tso *et al*., 2005; Cooke *et al*., (2006). As such, we have attempted to investigate their genetic defects and subsequent effects in triggering active TB disease among the Sudanese populations.

The data from the present study demonstrated that PCR-amplification of human genomic IFN-γR1 gene extracted from affected host immune cells followed by RFLP analysis has successfully identified DNA products resembling the three polymorphic alleles at positions; +95, -56, and +295 deletions 12 of the gene.

The lack of polymorphic *IFN-γ*R1 +95C/C and T/C alleles was surprising and difficult to explain (Fig. 5, Tables 7; 8). Frequency of +95TT genotype found in the promoter region of *IFN-γ*R1 was overrepresented in both TB patients and controls with 100% frequency of distribution, and significantly (*P*=0.033) indicative of susceptibility to the disease (Fig. 6, Appendix 5). In addition, +95CC genotype; and +95C/C and +95T/C variants have previously been demonstrated in association with PTB in several African populations (Awomoyi *et al*., 2004; Cooke *et al*., (2006).

Distribution of -56CC genotypes was more in TB cases than those in healthy human subjects. Hence, distribution of -56C>Twas seen in TB cases while -56C<T allele swas observed in the control subjects (Tables 7; 8). However, this difference between the two groups was statistically insignificant (*P*-value =0.771) (Fig. 8; Appendix 6).Accordingly, -56C allele is probably associated with risk of developing TB. These findings were in concordance with Cooke *et al*., (2006) who showed that the -56CC genotype was linked with risk of developing TB. The obtained statistical bias in the present project could probably be related to the small size of collected specimens. Conversely, these findings were not in agreement with others who reported that polymorphism within *IFN-γ*R1 at -56C >T allele was significantly associated with twice fold reduction in the risk of susceptibility to TB in a similar population (Attalla *et al*., 2011).

Distribution of +295C allele deletion 12 within *IFN-γ*R1 was found to be more represented in TB patients (6) than the healthy controls (only one)(Fig. 10; Tables 7; 8). Therefore, polymorphic +295C allele was more than T/C allele (+295C>T) suggesting non-significant (*P*-value=0.343)indicating increased chances of developing TB. However, we could not find any evidence in literature to support these findings.

Evidence from earlier studies invitro cell cultures which involved transformation of a construct harbouring -56C allele into cells and expression of IFN-γR1-56C resulted in low transcription and expression of the receptor on cell surfaces (Cooke *et al*., 2006). Subsequently, this low immune response may be accompanied by less damage with reduced effects of immunopathology and providing more chances of protection against PTB (Cooke *et al*., 2006). Undoubtedly, polymorphism in the promoter region of IFN-γR1 is greatly associated with TB but the host immune response and other genetic factors including production of IFN-γ may modify this pathway leading to susceptibility to TB or protection against it (Cooke *et al*., 2006).

Collected demographic characteristics and genotyping data in this study showed that the Tama, Nuba, Zagawah and Fur tribes were in the top of lead in recording the highest number of positive cases linked with polymorphic alleles; -56 T/C and -56CC at position -56 of IFN-γR1gene probably triggering pulmonary TB cases (Table 8). Yet, it was not significant when compared with healthy controls (Tables 7 and 8). At position +295 deletion 12, cases originated in Tama and Fur tribes had more mutations in association with positive TB cases. Increased prevalence of polymorphism in these tribes could be attributed to traditional habit of close marriage practised by most families within the tribe. The act of close ancestral marriage was reported to trigger and precipitate IFN-*γ*R1 genetic disorders in association with increased susceptibility to pulmonary TB (Al-Muhsen and Casanova,2008).

To our knowledge, this is the first in home molecular epidemiological project which implicated an investigation of three potential genetic polymorphisms within IFN-γR1gene for their role in triggering development of TB among the Sudanese populations. However, the current study project has encountered several limitations. Firstly, collected specimens specimens for further processing were quite small which has complicated statistical analysis and the findings. Secondly, most of molecular epidemiological work was performed outside the country as facilities including advanced molecular techniques and expertise are not available. Due to this, the investigator was forced to travel and pay unforeseen extra costs. Thirdly, a couple of valuable laboratory tests were cancelled as it was impossible to obtain fresh specimens from enrolled participants for further processing. Fourthly, some crucial

molecular assays like sequencing of *IFN-γ*R1 DNA products, which can verify our results could not be carried out.Lastly, some obtained biased results were not unexpected since the current work relied wholly on the PCR-RFLP analysis which was probably insensitive method to apply.

Part of demographic risk factors such as cigarette smoking and air pollution, both indoor and outdoor, pose major threats to lung health as they reduce local defences and increase chances of TB disease (Bates *et al*., 2007; Martineau *et al*., 2007; Trinh *et al*., 2015).

There are many factors increase infected by tuberculosis. To identify the factors gender, socio economic status, BCG vaccination and tobacco, we conducted analysis by used ssps analysis programme.

In many countries TB registered double in as more in men than women. Male and female different susceptibility to infect by PTB according to many reasons behaver and the immune response in this study percentage was 3:1 male: female (Austin, J *etal*.2004)

The male was beoame more infected than women according to many reasons and differences in immune response biological differences in male and female susceptibility to infect by PTB

The people who had been vaccinated with BCG vaccine low infected with pulmonary tuberculosis than the other who didn't the result was significant (p-value =0.000) as we found in other countries the BCG protects against infectious by TB (Sachin *etal*.,2004)

Data revealed that most risk factors for TB was poverty which increase the prevalent among the poor people more than in the wealthier one (Olivia *etal*.,2012) as such we have.

The proportion of tobacco smokers was significantly higher in TB patients in (p-value =0.000) Which is the same in result Part of demographic risk factors such as cigarette smoking and air pollution, both indoor and outdoor, pose major threats to lung health as they reduce local defences and increase chances of TB disease (Bates *et al*., 2007; Trinh *et al*., 2015)

CHAPTER SIX

COCLUSIONS

AND

RECOMMENDATIONS

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

in conclusion, this study project was designed to investigate using PCR- RFLP analysis the role of three genetic polymorphisms located in the promoter region of IFN-γR1 gene in triggering development of TB among Sudanese patients. Collected data showed that the tested polymorphisms have potential link in increasing risk of developing tb among Sudanese patients, Demographic characteristics in relation to the identified polymorphisms were also sought out.

6.2 Recommendations

1. It is important to collect a large-sized specimens to extract DNA and be stored for further molecular analysis.
2. Advanced laboratory equipments and molecular tools should be available to aid in detection of potential mutations.
3. Dedicated staff equipped with knowledge and experience in molecular genetics should be available to analyze and interpret obtained molecular data.
4. Sequencing of amplified products should be performed to verify presence of various polymorphisms.

REFERENCES

Abebe, M., Doherty, T. M., Wassie, L., *et al* (2010) Expression of apoptosis-related genes in an Ethiopian cohort study correlates with tuberculosis clinical status. **40**:291–301.

Abel, L., and Casanova, J.L. (2000) Genetic predisposition to clinical tuberculosis: bridging the gap between simple and complex inheritance. *Am. J. Hum. Genet.*, **67**:274–277.

Abel, L., El-Baghdadi, J., Bousfiha, A. A., Casanova, J.-L. and Schurr, E. (2014) Human genetics of tuberculosis: a long and winding road. 369:20130428.

Abdalla, T. M and Ali, A. A. A. (2012) Epidemiology of tuberculosis in Eastern Sudan. **2**: 999-1001.

Ahmad, S. (2011) Pathogenesis, Immunology, and Diagnosis of Latent *Mycobacterium tuberculosis*Infection. Clinical and Developmental Immunology.

Alangari, A. A., Al-Zamil, F., Al-Mazrou, A *et al* (2011) Treatment of disseminated mycobacterial infection with high-dose IFN-gamma in a patient with IL-12R beta1 deficiency.**2011**:1-5.

Alcais, A., Fieschi, C., Abel, L., *et al* (2005). Tuberculosis in children and adults: two distinct genetic diseases. **202**:1617-21.

Algood, H. M. S., Chan, J. and Flynn, J. L. (2003) "Chemokines and tuberculosis," *Cytokine and Growth Factor Reviews*, **14**:467–477.

Al-Muhsen, S., and Casanova, J.-L. (2008) The genetic heterogeneity of mendelian susceptibility to mycobacterial diseases. **122**:1043-51.

Alland, D., Whittam, T. S., Murray, M. B., *et al.* (2003) Modeling bacterial evolution with comparative genome-based marker systems: application to *Mycobacterium tuberculosis* evolution and pathogenesis. **185**: 3392-9.

Altare, F., Durandy, A., Lammas, D., *et al* (1998) Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. **280**:1432-5.

Altare, F., Ensser, A., Breiman, A., *et al* (2001) Interleukin-12 receptor beta1 deficiency in a patient with abdominal tuberculosis. **184**:231–236.

Andersen, P., Munk, M. E., Pollock, J. M., *et al* (2000) Specific immune-based diagnosis of tuberculosis. **356**:1099-1104.

Attalla, M.a., Mogahid, M.E., Nagla, G. M., The *et al* (2011) role of Interferon- γ Receptor-1 Gene (-56 T < C) polymorphism indevelopment of susceptibility to pulmonary Tuberculosis in Central Sudan. 3(1):31–35

Austin, F., Dick, M. and Zwarenstein, M. (2004) Gender disparity amongst TB suspects and new TB patients according to data recorded at the South African Institute of Medical Research laboratory for the Western Cape Region of South Africa, Volume 8, Number 4, April 2004, pp. 435-439(5).

Awomoyi, A. A., Nejentsev, Richardson, S., *et al* (2004) No association between interferon-c receptor-1 gene polymorphism and pulmonary tuberculosis in a Gambian population sample. **59**: 291–294. doi: 10.1136/thx.2003.013029.

Baker, I., Brown, T., Maiden, M. C., *et al* (2004) Silent Nucleotide Polymorphisms and a Phylogeny for *Mycobacterium tuberculosis*. Emerg. Infect. Dis., **10**:1568-1577.

Barry, C. E. III, Boshoff, H., *et al*., (2009) “The spectrum of latent tuberculosis: rethinking the biology and intervention strategies,” *Nature Reviews Microbiology*, **7**:845–855.

Bates, M.N., Asheena K., Madhukar P., *et al* (2007) Risk of Tuberculosis From Exposure to Tobacco SmokeA Systematic Review and Meta-analysis.Arch. **167**:335-342.

Bean, D., Roach, D. R., Briscoe, H. *et al*., (1999) “Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin,” *The Journal of Immunology*, **162**:3504–3511.

Behar, **S. M., Martin**, **C. J., Booty**, **M. G., *et al*** (2011) Apoptosis is an innate defence function of macrophages against *Mycobacterium tuberculosis*. **4**:279–287.

Bellamy R. (1999) The natural resistance-associated macrophage protein and susceptibility to intracellular pathogens. **1**: 23–27.

Bermudez, L. E., Sangari, F. J, Kolonoski, P., *et al* (2002) “The efficiency of the translocation of *Mycobacterium tuberculosis* across a bilayer of epithelial and endothelial cells as a model of the alveolar wall is a consequence of transport within mononuclear phagocytes and invasion of alveolar epithelial cells, **70**:140–146.

Berrington, W. R. and Hawn, T. R. (2007) *Mycobacterium tuberculosis,* macrophages, and the innate immune response: does common variation matter? **219**:167–186.

Birkness, K. A., Guarner, J., Sable, S. B., *et al*., (2007) An in vitro model of the leukocyte interactions associated with granuloma formation in *Mycobacterium tuberculosis* infection. **85**:160-8.

Brodie, D., Schluger, N. W. (2005) The diagnosis of tuberculosis.**26**:247-71.

Brosch, R., S. V. Gordon., M. Marmiesse., *et al* (2002) A newevolutionary scenario for the *Mycobacterium tuberculosis* complex **99:**3684–3689.

Bryk, R., Lima, C. D., Erdjument-Bromage, H., *et al* (2002) "Metabolic enzymes of mycobacteria linked to antioxidant defense by a thioredoxin-like protein," **295**:1073–1077.

Bustamante J., Arias A. A.,Vogt, G., *et al* (2011) Germline *CYBB* mutations that selectively affect macrophages in kindreds with X-linked predisposition to tuberculous mycobacterial disease. **12**:213–221.

Cardona, J., (2009) "A dynamic re-infection hypothesis of latent tuberculosis infection," *Infection*, **37**: 80–86.

Casanova, J. L., Blanche, S., Emile, J. F., *et al* (1996) Idiopathic disseminated Bacillus Calmette-Guerin infection: a French national retrospective study **98**:774–778.

Casanova, J. L., and Abel, L. (2013) The genetic theory of infectious diseases: a brief history and selected illustrations. Genomics Hum. Genet., **14**, 215–243.

Casanova and Abel (2007) Human genetics of infectious diseases: a unified theory. **26**, 915–922.

Casanova, J. L., Abel, L., (2004) The human model: a genetic dissection of immunity to infection in natural conditions. Immunol.; **4**: 55–66.

Cella M.; Scheidegger D, Palmer L., *et al* (1996) "Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation," **184**:747–752.

Chan, J. and Flynn, J. (2004) "The immunological aspects of latency in tuberculosis,", **110**:2–12.

Chan, J., Tanaka, K., Carroll, D., *et al* (1995) Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. **63**:736–740.

Chen, M., Gan, H. and Remold, H. G. (2006) "A mechanism of virulence: virulent *Mycobacterium tuberculosis* strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis," **176**:3707–3716.

Clancy, L., Rieder, H. L., Enarson, D. A., *et al* (1991) Tuberculosis elimination in the countries of Europe and other industrialized countries. **4**:1288-1295.

Colebunders, R. and Bastian, I. A., (2000) A review of the diagnosis and treatment of smear-negative pulmonary tuberculosis. **4**:97-107.

Collins, H. L., Kaufmann, S. H. (2011) The many faces of host responses to tuberculosis. **103**: 1–9.

Condos, R, Rom, W. N., Schluger, N.W. (1997) Treatment of multidrug-resistant pulmonary tuberculosis with interferon-gamma via aerosol. **349**:1513–1515.

Cooke, G. S., Campbell, S. J., Sillah, J. *et al.* (2006) Polymorphism within the interferon-gamma⁄receptor complex is associated with pulmonary tuberculosis. **174**:339–343.

Cooper, A. M., (2009) "Cell-mediated immune responses in tuberculosis," **27**:393–422.

Cooper, A. M , Dalton, D. K., Stewart, T. A., *et al* (1993) Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med.; **178**: 2243–2247.

Cosma, C. L., Humbert, O., Ramakrishnan, L. (2004) Superinfecting mycobacteria home to established tuberculous granulomas. **5**: 828-835.

Dasgupta, K. and Menzies, D. (2005) Cost-effectiveness of tuberculosis control strategies among immigrants and refugees. **25**:1107-1116.

D'Avila, H., Roque. N. R., Cardoso, R. M., *et al* (2008) Neutrophils recruited to the site of *Mycobacterium bovis* BCG infection undergo apoptosis and modulate lipid body biogenesis and prostaglandin E production by macrophages. **10**:2589e604.

Davis, J. M and Ramakrishnan, L. (2009) The role of the granuloma in expansion and dissemination of early tuberculous infection. **136**:37e49.

Derrick, S. C and Morris, S. L. (2007) The ESAT6 protein of *Mycobacterium tuberculosis* induces apoptosis of macrophages by activating caspase expression. **9**:1547e55. **Dick, G. (1978).**

Dorman, S. E and Holland, S. M. (1998). Mutation in the signal-transducing chain of the interferon gamma receptor and susceptibility to mycobacterial infection.**101**:2364–2369.

Dupuis, S., Dargemont, C., Fieschi, C., *et al* (2001). Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. **293**:300–303.

Ehlers, S. (2009) Lazy, Dynamic or Minimally Recrudescent? On the Elusive Nature and Location of the *Mycobacterium* Responsible for Latent Tuberculosis. **37**:87-95.

El-Etr, S. H. and Cirillo, J. D., (2001) "Entry mechanisms of mycobacteria," *Front Biosci*., **6**:737–747,

Ernst, J. D., Trevejo-Nuñez, G., and Banaiee, N. (2007) Genomics and the evolution, pathogenesis, and diagnosis of tuberculosis **117**:1738–1745.

Fabre M, Koeck JL, Le Fleche P, *et al.* (2004) High genetic diversity revealed by variable-number tandem repeat genotyping and analysis of hsp65 gene polymorphism in a large collection of ''Mycobacterium canettii'' **42**: 3248–3255.

Filipe-Santos, O., Bustamante, J., Chapgier, A., *et al* (2006). Inborn errors of IL-12/23 and IFN-g mediated immunity: molecular, cellular, and clinical features. **18**:347–361.

Flynn, J. L., Chan, J., Triebold, K. J., *et al* (1993) "An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection," **178**:2249–2254.

Flynn, J. L., and Chan, J. (2001) Immunology of tuberculosis.**19**:93–129.

Fogel, N., (2015) Tuberculosis: A disease without boundaries. **95**:527e531.

Forbes, B. A, Sahm, D. F., Weissfeld, A. S. (2007) Mycobacteria in: Baily & Scott's Diagnostic Microbiology. 12th. pp. 497-503.

Fulton, S. A., Reba, S. M. Pai, R. K. *et al*., (2004) "Inhibition of major histocompatibility complex II expression and antigen processing in murine alveolar macrophages by *Mycobacterium bovis* BCG and the 19-kilodalton mycobacterial lipoprotein, **72**:2101–2110.

Garnier, T., K., Eiglmeier, J.-C., Camus *et al*., (2003) "The complete genome sequence of *Mycobacterium bovis*," *Proceedings of theNational Academy of Sciences of the United States of America*, **100**:7877–7882.

Ghosh, J., Larsson, P., Singh, B. *et al*., (2009) "Sporulation in mycobacteria, "*Proceedings of the National Academy of Sciences of the United States of America*, **106**: 10781–10786.

Godreuil, S., Torrea, G., Terru, D., *et al*., (2007) First Molecular epidemiology study of *Mycobacterium tuberculosis* in Burkina Faso. **45**:921-7.

Grange J. (1996) Mycobacteria and human disease. [Oxford University Press, Inc, 198 Madison Avenue, New York, NY10016].

Gray, P. W. and Goeddel, D. V. (1982) Cloning and expression of murine immune interferon cDNA. **80**:5842-5846.

Grotzke, J. E. and Lewinsohn, D. M., (2005) "Role of $CD8^+$ T lymphocytes in control of *Mycobacterium tuberculosis* infection. **7**:776–788.

Guinn, K. M., Hickey, M. J., Mathur, S. K. *et al*., (2004) "Individual RD1-region genes are required for export of ESAT-6/CFP-10 and for virulence of *Mycobacterium tuberculosis*," **51**:359–370.

Gutierrez, M. C., Brisse, S., Brosch, R., *et al* (2005). Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. **1**: e5.

Hamajima, N. (2001) PCR-CTPP: a new genotyping technique in the era of genetic epidemiology. **1**:119–123.

Hans L.R., Armand V.D., Kai M. K, *etal* (2007) Priorities for tuberculosis bacteriology services in low lncome countries **2.**

Hawn, T. R., Misch, E. A., Dunstan, S. J. *et al*., (2007) "A common human TLR1 polymorphism regulates the innate immune response to lipopeptides, **37**:2280–2289.

Hett, E. C., Chao, M. C., Steyn, A. J., *et al* (2007) "A partner for the resuscitation promoting factors of *Mycobacterium tuberculosis*," **66**: 658–668.

Hoal, E. G., Lewis, L-A., Jamieson, S. E., *et al* (2004) SLC11A1 (NRAMP1) but not SLC11A2 (NRAMP2) polymorphisms are associated with susceptibility to tuberculosis in a high-incidence community in South Africa. **8**:1464–1471.

Houde, M., Bertholet, S., Gagnon, E. *et al*., (2003) “Phagosomes are competent organelles for antigen cross-presentation. **425**:402–406.

Hsu, Y. H., Chen, C. W., Sun, H. S., *et al* (2006) Association of NRAMP 1 gene polymorphism with susceptibility to tuberculosis in Taiwanese aboriginals **105**:363e9.

Jouanguy, E., Altare, F., Lamhamedi, S., *et al* (1996) Interferon-*g*-Receptor Deficiency in an Infant with Fatal Bacille Calmette–Guérin Infection. **335**:1956-1961.

Kallmann F. J., and Reisner D. (1943) Twin studies on the significance of genetic factors in tuberculosis. **47**:549–574.

Kana, B. D., Gordhan, B. G., Downing, K. J. *et al*., (2008) “The resuscitation- promoting factors of *Mycobacterium tuberculosis* are required for virulence and resuscitation from dormancy but are collectively dispensable for growth in vitro.**67**: 672–684.

Kang, P. B., Azad, A. K., Torrelles, J.B., *et al* (2005) The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. **202**:987-999.

Kaul, K. L. (2001) Molecular detection of *Mycobacterium tuberculosis*: impact on patient care. **47**:1553-8.

Keane, J. (2005) TNF-blocking agents and tuberculosis: new drugs illuminate an old topic. **44**:714–720.

Kent, P.T and Kubica G.P. (1985). Public Health Mycobacteriology: A Guide for the Level III Laboratory. US Department of Health and Human Services. CDC, Atlanta, Georgia.

Koch, R. (1882). Classics in infectious diseases. The etiology of tuberculosis **4:** 1270-4

Kumar, A., Deshane, J. S., Crossman, D. K., *et al*., (2008) “Heme oxygenase-1-derived carbon monoxide induces the *Mycobacteriumtuberculosis* dormancy regulon18032–18039.

Lichtenauer-Kaligis, E. G. R., de Boer, T. F., Verreck, A.W. *et al*., (2003) “Severe *Mycobacterium bovis* BCG infections in a large series of novel IL-12 receptor β1 deficient patients and evidence for the existence of partial IL-12 receptor β1 deficiency, **33**: 59–69.

Lio, D., Marino, V., Serauto, A., *et al* (2002) Genotype frequencies of the _874T→A single nucleotide polymorphism in the first intron of the interferon-gamma gene in a sample of Sicilian patients affected by tuberculosis. **29**:371–374.

Liu, X. Q., Dosanjh, D., Varia, H., *et al* (2004) Evaluation of T-cell responses to novel RD1- and RD2-encoded *Mycobacterium tuberculosis* gene products for specific detection of human tuberculosis infection. Infect Immun., **72**:2574–2581.

Lopez-Maderuelo, D., Arnalich, F., Serantes, R., *et al* (2003) Interferon-gamma and interleukin- 10 gene polymorphisms in pulmonary tuberculosis. **167**:970–975.

Lykouras, D., Sampsonas, F., Kaparianos, A., *et al* (2008) Human genes in TB infection: their role in immune response. **69**:24-31.

Marais B. J, Gie R. P, Schaaf H. S, *et al* (2006) Childhood pulmonary tuberculosis: old wisdom and new challenges. **173**:1078–1090.

Martineau, A. R., Wilkinson, R. J., Wilkinson, K. A., *et al* (2007) A single Dose of Vitamin D Enhances Immunity to Mycobacteria **176**:208-213.

Migliori, G. B., Hopewell, P. C., Spanevello, *et al* (2006) Improving the TB case management **28**:687-690.

Miller, B. H., Fratti, R. A., Poschet, J. F. *et al*., (2004) "Mycobacteria inhibit nitric oxide synthase recruitment to phagosomes Clinical and Developmental Immunology during macrophage infection. **72**:2872–2878.

Mimouni, J. (1951) [Our experiences in three years of BCG vaccination at the center of the O.P.H.S. **55**:1138–1147.

Morgan, M. A., Horstmeier, C. D., De Young, D. R., *et al* (1983) Comparison of a radiometric method (BACTEC) and conventional culture media for recovery of mycobacteria from smear-negative specimens. **18**:384-8.

Newport, M. J., Huxley, C. M., Huston, S., *et al* (1996) A mutation in the interferon - gamma– receptor gene and susceptibilityto mycobacterial infection. **335**:1941-9.

Nicholson, S., Bonecini-Almeida M. d. G., Lapa e Silva, J. R., *et al* (1996) Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. **183**:2293–2302.

Niemann, S., Richter, E., Rusch-Gerdes, S., (2000) Differentiation between members of *Mycobacterium tuberculosis* complex by molecular and biochemical features: evidence for two pyrazinamide-susceptible subtypes of *M. bovis*. **38**:152-7.

Nigou, J., Zelle-Rieser, C., Gilleron, *et al* (2001) "Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor,**166**:7477–7485.

Noss, E. H., Pai, R. K., Sellati T. J. *et al* (2001) "Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. **167**:910–918.

Oddo, M., Renno, T., Attinger, A., *et al* (1998) "Fas ligand-induced apoptosis of infected human macrophages reduces the viability of intracellular *Mycobacterium tuberculosis*.**160**:5448–5454.

Ohno, H., Zhu, G., Mohan, V. P. *et al*., (2003) "The effects of reactive nitrogen intermediates on gene expression in *Mycobacteriumtuberculosis*," *Cellular Microbiology*, **5**: 637–648.

Olivia, O and Megan, M., (2012) Tuberculosis and Poverty**:** Why Are the Poor at Greater Risk in India? **7**(11); 2012 PMC3501509

Onyebujoh, P., Zumla, A., and Ribeiro, I. (2005). Treatment of tuberculosis: present status and future prospects. **83**: 857-65.

Pai, R. K., Convery, M., Hamilton, T. A., ***et al*** (2003) "Inhibition of IFN-*γ*-induced class II transactivator expression by a 19-kDa lipoprotein from *Mycobacteriumtuberculosis*: a potential mechanism for immune evasion.**171**:175–184.

Pai, R. K., Pennini, M. E., Tobian, A. A. R., ***et al*** (2004) "Prolonged toll-like receptor signaling by*Mycobacterium tuberculosis* and its 19-kilodalton lipoprotein inhibits gamma interferon-induced regulation of selected genes in macrophages. **72**:6603–6614.

Palomino J.C. (2007). Non-conventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. **26**: 1-12.

Perkins, M. D., (2000) New diagnostic tools for tuberculosis. Int. J. Tuberc. **4** S182-8.

Puffer, R. (1944) Familial susceptibility to tuberculosis; its importance as a public health problem, Cambridge.106.

Ramirez-Alejo, N. and Santos-Argumedo, L. (2014) Innate Defects of the IL-12/IFN-g Axis in Susceptibility to Infections by Mycobacteria and *Salmonella*. **34**:307-317.

Raviglione, M. C. and Uplekar, M. W. (2006) WHO'S New Strategy, **367:**952-955.

Raviglione, M. C. and Smith, I. M. (2007) XDR Tuberculosis–Implications for Global Public Health. **356**:656-659.

Reed, M. B., Domenech, Manca, P., C. ***et al.*****,** (2004) "A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. **430**:84–87.

Reichenbach, J., Rosenzweig, S., Doffinger, R., ***et al*** (2001) Mycobacterial diseases in primary immunodeficiencies. 1:503–511.

Rojas, M., L. Garcia, F., Nigou, J., ***et al*** (2000) "Mannosylated lipoarabinomannan antagonizes *Mycobacteriumtuberculosis*- induced macrophage apoptosis by altering CA+2-depen Garcia dent cell signalling. **182**:240–251.

Rook, G. A. W., Seah, G., Ustianowski, A. M. (2001) Tuberculosis: immunology and vaccination. **17**: 537-557.

Rossouw, M., Nel, H. J., Cooke, G. S., ***et al*** (2003) Associationbetween tuberculosis and a polymorphic NFkappaB binding site inthe interferon gamma gene. 361:1871–1872.

Russell-Goldman, E., Xu, J., Wang, X., ***et al*** **(**2006) "A *Mycobacterium tuberculosis* Rpf double-knockout strain exhibits profound defects in reactivation from chronic tuberculosis and innate immunity phenotypes. **76**: 4269–4281.

Rustad, T. R., Sherrid, A. M, Minch, K. J. *et al* (2009) "Hypoxia: a window into *Mycobacterium tuberculosis* latency. **11**:1151–1159

Sanchez, A., Espinosa, P., Garcia, T., *et al* (2012) The 19 kDa Mycobacterium tuberculosis lipoprotein (LpqH) induces macrophage apoptosis through extrinsic and intrinsic pathways: a role for the mitochondrial apoptosis-inducing factor. **2012**:950503.

Scanga, C. A., Mohan, V. P., Tanaka, K., *et al* (2001) "The inducible nitric oxide synthaselocus confers protection against aerogenic challenge of bothclinical and laboratory strains of *Mycobacterium tuberculosis*in mice. **69**:7711–7717.

Sachin, R, Abhay, K, Sudhakar.M., *et al* (2004) Cultural concepts of tuberculosis and gender among the general population without tuberculosis in rural Maharashtra, India. 1365-3156.2004.01321

Schluger, N. W., (2005) The Pathogenesis of Tuberculosis. **32**:251–256

Sharaf Eldin, G. S., **Fadl-Elmula**, **I.**, **Ali**, **M., S.,** ***et al*** **(2011)** Tuberculosis in Sudan: a study of*Mycobacterium tuberculosis*strain genotype and susceptibility to anti-tuberculosis drugs. **11**:219.

Sharma, B., Pal, N., Mahorta, B., *et al* (2010) Evaluation of a Rapid Differentiation Test for*Mycobacterium Tuberculosis*from other Mycobacteria by Selective Inhibition with p-nitrobenzoic Acid using MGIT 960. **2**: 89-92.

Shiloh, M. U. and DiGiuseppe Champion, P. A.(2010) "To catch a killer. What can mycobacterial models teach us about *Mycobacterium tuberculosis* pathogenesis **13**: 86–92.

Smith, I., (2003) *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clinical Microbiology Reviews, **16**, 463–496.

Smith, N. H., (2006) Ecotypes of the *Mycobacterium tuberculosis* complex..**239**:220–225.

Stead, W. W., Senner, J., W., Reddick, W. T., *et al* (1990) Racial differences in susceptibility to infection by Mycobacterium tuberculosis. **322**:422-427

Stead, W., Eisenach, K., Cave, M., *et al* (1995). When did *Mycobacterium tuberculosis* infection first occur in the New World? An important question with public health implications. **151**:1267–1268.

Steingart, K. R., Schiller, I., Horne, D, J., *et al* (2014) Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. **1**: 1-166.

Stenger, S.,(2005) Immunological control of tuberculosis: role of tumour necrosis factor and more. **64**: iv24–iv28.

Stewart, G. R., Robertson, B. D.and Young, D. B. (2003)Tuberculosis: a problem with persistence. **1**: 97-105.

Sulis, G., Roggi, A., Matteelli, A. *et al* (2014). Tuberculosis: Epidemiology and Control.

Trinh, Q.M., H.L. Nguyen, V.N. Nguyen, T.V.A. *et al* (2015) Tuberculosis and HIV co-infection-focus on the Asia-Pacific region. **32**:170–178.

Tso, H. W., Ip, W. K., Chong, W. P., *et al* (2005) Association of interferon gamma and interleukin 10 genes with tuberculosis in Hong Kong Chinese. **6**: 358-363.

Tufariello, J. M., Chan, J., and Flynn, J. L. (2003) Latent tuberculosis: mechanisms of host and bacillus that contributes to persistent infection. **3**:578-590.

Ulrichs, T. and Kaufmann, S. H. E. (2006). "New insights into the function of granulomas in human tuberculosis. **208**: 261–269.

Van Crevel, R., Ottenhoff, T. H. M., *et al* (2002) Innate immunity to *Mycobacterium tuberculosis*. **15**:294-309.

Van Soolingen, D., Hoogenboezem, T., de Haas, P. E., *et al* (1997) A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterisation of an exceptional isolate from Africa. **47**:1236-45.

Virtanen, J. A. (1960) Study of nitrate reduction by mycobacteria. **48:** 1-5.

Voskuil, M. I., Schnappinger, D., Visconti, K. C. *et al*., (2003) "Inhibition of respiration by nitric oxide induces a *Mycobacteriumtuberculosis* dormancy program," *Journal of ExperimentalMedicine*, **198**: 705–713.

Wilkinson, D., Pillay, M., Crump, J., Lombard, C., Davies, G. R., Sturm, A. W. (1997) Africa. **2**:747-53.

World Tuberculosis Programme (1994) Framework for effective tuberculosis control. Geneva, World Health Organization, (WHO/TB/94.179).

World Health Organization (2005) Global tuberculosis control: surveillance, planning, financing. WHO/HTM/TB/2005.349, World Health Organization, Geneva

World Health Organization: WHO report (2006) Global Tuberculosis Control Surveillance, planning, Financing. WHO/HTM/TB/2010. Geneva, Switzerland: WHO.

Wolf, A. J., Desvignes, L., Linas, B. *et al*., (2008) "Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. **205**:105–115.

World Health Organization (WHO), (2009) Global Tuberculosis Control: Surveillance, Planning, Financing. Geneva:. (WHO/HTM/TB/2005.349.

World Health Organization and Stop TB Partnership (2010) The global plan to stop TB 2011/2015.GenevaWorlHealthOrganization.Available:http://www.stoptb.org/assets/documents/global/plan/TB_GlobalPlanToStopTB201**.**

World Health Organization (WHO), (2012) Global Tuberculosis report. WHO/HTM/TB/2012.6.

World Health Organization: WHO report (2013): Global Tuberculosis Control, Geneva: (http://www.who.int/tb/data).

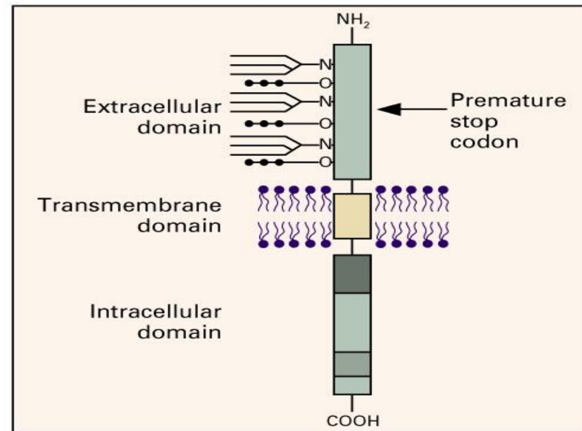
World Health Organization (WHO) report (2014). Global Tuberculosis Control, WHO Report. Geneva: (cited 2014 November 11). Available at: http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf.

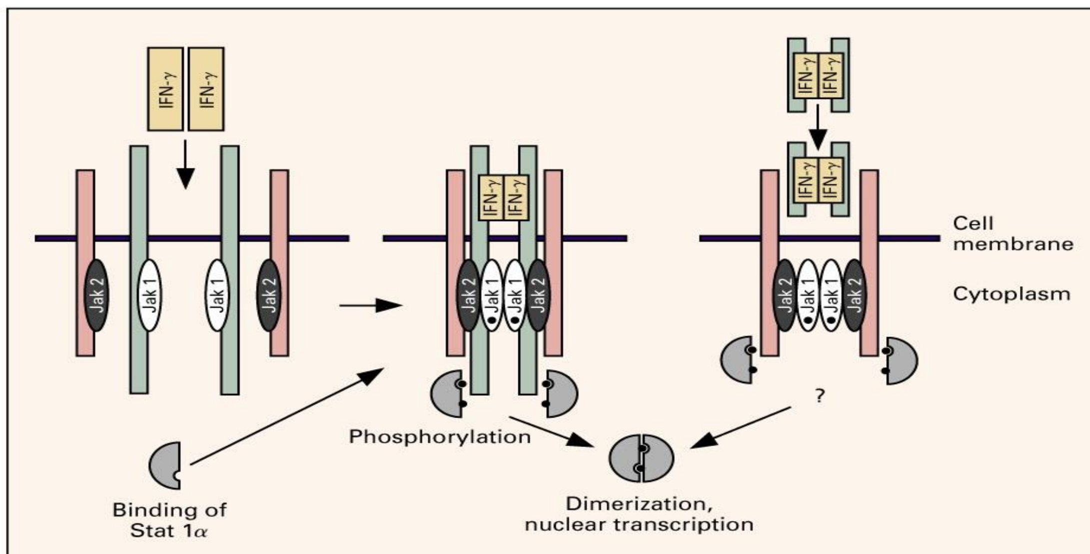
Zager, E. M., McNerney, R., (2008) Multidrug-resistant tuberculosis. BMC. **8**:10.

Zignol, M., Wright, A., Jaramillo, E., Nunn, P., Raviglione, M. C. (2007) Patients with previously Treated Tuberculosis No longer Neglected. **44**: 61-64.

APPENDICES

Appendix 1:

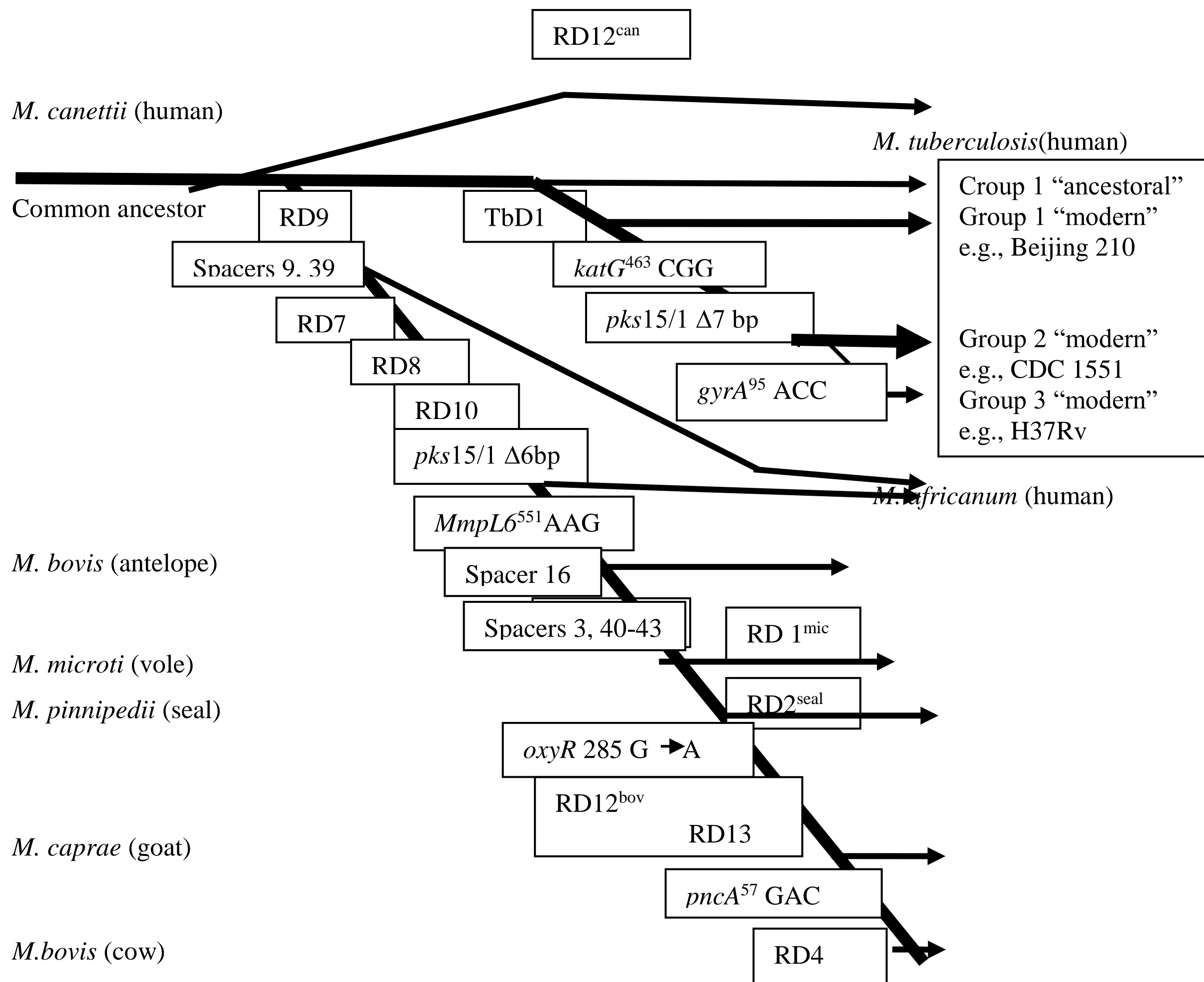




Appendix 1: structure of interferon gamma receptor 1 protein involving location of a premature stop codon due to mutation (Panel A) and its receptor complex (Panel B)

Panel B,demonstrating interferon gamma receptor complex comprising receptors 1 (green bar) and 2 (red bar). Combination of receptors through protein kinases (Jak 1 and 2) resultsin phosphorylation and signal transduction mediated by signal transducer and activator of transcription 1 (STAT 1α) with signal induction.The black spots represent phosphorylation after binding with STAT 1α.The diagram was sourced from Newport *et al*., 1996).

Appendix 2



Appendix 2 schematic flow pathway of the *M. tuberculosis* bacilli showing stepwise loss of DNA defining certain lineages (adapted from Ernst *etal.* 2007).

RD, region of difference; Tb 1, tuberculosis deletion 1; *pks*, polykinase synthase; *gyrA*, gene coding for gyrase enzyme.

Appendix 3: Preparation of Ziehl-Neelsen Staining and Procedure

a. 13% Fuchsin

Basic Fuchsin……………………………………3.0 g

a) 95% Ethanol……………………………… 100ml of solution1

b. Phenol

Phenol crystals………………………………… 5.0g

c) Distilled water………………………………100ml solution

Gentle heating may be required.

a. Preparation of the Decolorizing Agent, 25% Sulphuric Acid

i) Concentrated sulphuric acid ………………………25 ml

Distilled water……………………………………100 ml

The concentrated sulphuric acid was carefully added to water, stored at room temperature for 6-12 months before use.

b. Preparation of 0.3g Counter Stain Methylene Blue

Methylene blue…………………………………0.3g

Distilled water…………………………………..100 ml

The prepared solution was stored in an amber bottle at room temperature (RT) for 6 to 12 months.

c. Procedure for Direct Ziehl-Neelsen Staining Method

Direct smear was made, fixed by heating, stained by ZN and microscopically examined for acid fast bacilli. Smears were covered withfiltered 1% carbol-fuchsin stain.The mixture was heated with Bunsen burneruntil steam rose up, left to stand for 5 minutes after which it was rinsed with tap water. Then, smears were flooded with sulphuric acid (H_2SO_4) to allow for complete destaining, washed with tap water and drained out. Finally, the slide was counterstainedby floodingwith methylene blue stain for 1 minute, washed with water, air dried and microscopically examined under oil immersion for presence of causative agent.

Appendix 4 Preparation of Culturing Media

Lowenstein-Jansen medium (LJ) containing glycerol and LJ supplemented with pyruvate were prepared according to (Kent and Kubica, 1985).

A) Preparation of Lowenstein-Jensen (LJ) 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 70% ethanol for 15 minutes. Hands were washed and scrubbed well before processing. Eggs were broken and transferred into a sterile flask, blended in sterile mug and filtered through four layers of sterile gauze into graduated cylinder. Mineral salts solution was prepared by dissolving the following:-

Monopotassium phosphate (anhydrous)……………………………..2.4g

Magnesium sulfate………………………………………………… 24g

Magnesium citrate……………………………………………….....0.6g

Asparagine ………………………………………………………..3.6g

Sterile distilled water ………………………………………………600ml

The solution was dissolved in water bath at 100°C for 45 minutes, cooled down to room temperature .Then, the following ingredients were added:

Malachite green (2%)………………………………………………20 ml

Glycerol……………………………………………………………12ml

Homogenized whole eggs……………………………………..1000ml

The complete medium was poured into sterile funnel and dispensed in 6to8ml volumes into sterile universal bottles. Then, bottles were laid in slanted positionand coagulated by inspissation at 85°C for 45 minutes.Sterility testing for prepared media was carried out by taking a random specimens from each patch and incubated at 37°C for 48hours.

Appendix 5 Preparation of Working Solutions

a. Phosphate Buffer Solution (PBS) at pH7

9.47g of anhydrous disodium phosphate (Na_2HPO_4) was dissolved in 1000 ml of distilled water to make 0.067M Na_2HPO_4 (solution 1). 9.07g of monopotassium phosphate (KH_2PO_4)was dissolved in 1000 ml distilled water to make 0.067M KH_2PO_4 (solution 2). To prepare a buffer solution at pH6.8, 50 ml of solution 1 was mixed with 50 ml of solution 2 and checked on a pH meter. If the final buffer requires adjustment, solution 1 was added to raise the pH otherwise solution 2 is needed to lower it.

b. NaOH Solution

Four g of NaOH was dissolved in100 ml of distilled water and sterilized by autoclaving.

c. N HCL Solution

110ml of distilled water was added to 10ml of HCL 37 %(12N) precaution was followed, autoclaved at 121°C for 15 minutes, and then stored at room temperature.

d. Preparation of Tween 80 Solution

Forpreparation of10% Tween 80: 10 ml of Tween 80 was mixed with 90 ml distilled water and autoclaved at 121°C for 10 minutes. Immediately, the solution was swirled after autoclaving and during cooling to re-solubilize Tween 80, and then it was stored at 5°C.

e. Nitrate Substrate Solution

The following materials; 0.085g NaNO3, 0.117g KH2PO4, 0.485g Na2HPO4 were dissolved in 100ml of distilled water to prepare 0.01 M sodium nitrate in 0.002 M PBS, pH 7.0 and sterilized by autoclaving.

f. Lame Reagent

One part of N-1-naphthylethylene-diamine dihydrochloride, one part of sulphanilic acid and ten part of tartaric acid

Appendix 6: Significant Frequency of Distribution of Genetic Polymorphism +95T→C According to Infected and Control Groups

Genetic polymorphism +95T→C	**Group**			
	Infected		Control	
	N	%	N	%
TT	100	100	50	100
TC	0	0	0	0
CC	0	0	0	0
Total	100	100	50	100
P-value	0.033			

N; number of participants

Appendix7: Frequency Distribution of -56T→C According to Groups

-56T→C	**Group**			
	Infected		Control	
	N	%	N	%
TT	77	77	41	82
TC	16	16	6	12
CC	6	6	3	6
Total	100	100	50	100
P-value	0.771			

Appendix 8: Frequency Distribution of +295 Deletion 12According to Groups

295 deletion 12	**Group**			
	Infected		Control	
	N	%	N	%
TT	90	90	45	90
TC	4	4	4	8
CC	6	6	1	2
Total	100	100	50	100
p-value	0.343			