EARLY TUBERCULOSIS IMMUNODETECTION USING GENOMIC ANTIGENS AND INDUCEMENT OF T-CELLS IN HIV+TB SUBJECTS IN LAGOS, NIGERIA.

A thesis submitted to the School of Postgraduate Studies, University of Lagos, Nigeria fulfillment of the requirements for the award of Doctor of Philosophy (Ph.D.) Degree in Clinical Pathology.

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Certification

This is to certify that the thesis:

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DOCTOR OF PHILOSOPHY (Ph.D),

is a record of original research work carried out

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DEDICATION

This thesis is dedicated to God Almighty, Jehovah. The author and finisher of my faith, and pillar of my life. It is also dedicated to my late parents Mr & Mrs Jimoh Olayiwola Adeiga. My wife, Adetutu Adeiga and my children, Oludolapo, Adeniyi, and Oluwasegun Adeiga and all humanity.

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ABSTRACT

The probability of developing tuberculosis (TB) disease is higher in immune compromised subjects such as HIV/AIDS patients. Conventional methods of diagnosing TB which include sputum smear microscopy, culture and chest X-ray have drawbacks and established limitations. The limitations are pronounced in immuno-compromised subjects. The consequence of poor diagnosis of TB especially in resources limited environment like Nigeria contributes to the rising incidence of TB. Introducing immuno detection method using genomic antigens is priming for early detection of TB. This study was designed to evaluate the available genomic TB antigens for T-cell stimulation and to explore possibility of improving diagnostic performance in immuno-compromised patient by inducing T-cells. The study population includes subjects with active TB only, HIV+TB, HIV only, subjects with TB contacts and apparently healthy controls. Smear microscopy and culture were performed on sputum samples obtained. Whole blood cells from suspected TB infected subjects and TB contacts were incubated with RPMI 1640 and glutamine to provide enriched medium of protein and vitamins for the T-cells in the blood and fetal calf serum (FCS) to induce T-cells during incubation so as to make the T-cells respond better when stimulated during incubation. Low dosage of 2.5 µg/ml of TB antigens (ESAT-6,CFP-10 and ELLI the new TB antigen) obtained from LIONEX Inc. Germany was used to stimulate the T-cells in the whole blood during the 72 hour incubation. Sera obtained after stimulation were used for Interferon Gamma Release Assay (IGRA) to assess the IFN-γ released during T-cell stimulation. The result of smear microscopy and culture revealed that even though a high percentage of positivity was obtained from the result of smear microscopy and culture, some of the positives (20.83%) smear microscopy were negative for culture. These were part of the drawbacks of high sensitivity and low specificity observed. Result of using TB antigens for T-cell stimulation showed that ELLI the new antigen was the most immunogenic of the antigens used when compared with existing ESAT-6 and CFP-10. T-cells performed optimally when induced with 1% FCS and stimulated with low dose antigen of ELLI and the combined ESAT-6+CFP-10. The inducement of T-cells improved the diagnosis of TB in HIV subjects that were asymptomatic of TB and individuals at risk especially

the previous TB contacts. Sensitivity and specificity of IGRA increased above 80% with the inducement of T-cells. In conclusion, the study attempts to improve TB diagnosis using genomic antigens relative to the conventional non genomic antigens methods. This approach has the potential to improve detection of TB subjects using immunodetection technique.

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Tuberculosis (TB) is a common deadly infectious disease caused by *Mycobacterium tuberculosis* in man. The organism was discovered by Robert Koch on 24th March 1882. It is an aerobic non-motile bacillus. The pathogen is an obligatory aerobic intracellular organism that has predilection site for the lung tissue that is rich in oxygen supply. It can also spread or affects other parts of the body such as the central nervous system, lymphatic, circulatory, genitourinary, gastro intestinal systems, the bones, joints and even the skin in the active case of the disease. The disease is spread by people with active TB when they cough, sneeze, speak, sing or spit. They expel infectious aerosol droplets of 0.5 to 5.0 µm in diameter. A single sneeze can release up to 40,000 droplets. Each droplet may transmit the disease since the infectious dose of TB is very small (Nicas *et al.*, 2005). People with prolonged frequent close contact with person with TB are at high risk of becoming infected with an estimated 22 % infection rate (Ahmed and Hasnain, 2011). Most infections are initially latent, but about 10% eventually progress to active TB disease which if left untreated can kill the person. Seventy five percent (75%) of TB cases are pulmonary, featuring the signs such as chest pain, coughing up blood and prolonged, productive cough for more than three weeks. Systemic symptoms include fever, chills, input sweats, loss of appetite, weight loss, pallor and easy fatigue (WHO, 2006a).

In 15-25% of active cases, the infection moves from the lungs to other parts of the body to cause extra pulmonary infection (Jindal, 2011; WHO, 2013). This movement is common in children and the immune compromised. This movement is about 50% in cases of HIV-TB co-infection. The extra pulmonary infection sites include the pleura, central nervous system in meningitis, the genitourinary system in urogenital tuberculosis, bones and joints in Pott's disease of the spine.

The disease is one of the major causes of morbidity and mortality in the world. Tuberculosis cases detected in 2012 were estimated at 8.6 million with 940,000 deaths (WHO, 2013). M*ycobacterium tuberculosis* infects one third of the world population with 27% in Africa and 58% in Asia (WHO, 2013).

In the course of HIV+TB co-infection, the problem of TB became globally endemic. From the 8.6 million TB cases detected in 2012, 1.1million (12.8%) were HIV positive. An estimate of 320,000 of the 940,000 deaths occurring in TB were HIV positives (WHO, 2013), making TB the most common cause of death among people living with HIV/AIDS. Nigeria, as at 2012, had an incidence of 180,000 for all forms of TB cases. Out of these TB cases, 52,901 were smear positives (WHO, 2013). From the 97,853 TB cases registered in 2012, 90,305 (92.3%) were new cases and 2,513 (2.57%) were relapsed cases (WHO, 2013). Among these new cases, 52,901(58.58%) were sputum smear positive, 32,972 (36.5%) were smear negatives while 4,432 (4.91%) were extra pulmonary (WHO, 2013). Nigeria also recorded 180,000 incidence of TB with 27,000 deaths in 2012 (WHO, 2013). This was a decrease in incidence compared with 460,000 cases of TB in 2009 (WHO, 2013). Although there is decline in incidence of TB, poor and late diagnosis plus poverty state of the subjects had led to the emergence of drug resistance TB in Nigeria. The notified cases of drug-resistant TB in Nigeria enrolled for treatment was 95 cases (WHO, 2012). HIV poses the greatest risk factor for the progression of latent or recent TB infection to active TB disease. It had been estimated that about 10% of HIV negative people with latent infection of TB would progress to active TB disease in their life time while, 10% of people with latent TB infection and HIV positive would develop active TB disease in a year. (WHO, 2005a).

Tuberculosis ranks high among the most important causes of morbidity and mortality in people living with HIV/AIDS (PLWHA). Autopsy studies have found disseminated TB in 40 to 45% of HIV-infected people in HIV prevalent countries, many of whom were undiagnosed prior to death (WHO, 2005b).

Late and poor diagnosis using conventional methods adopted at local levels especially in developing countries contributed immensely to the rise in incidence of TB globally. The most widely used

conventional methods employed for TB diagnosis are smear microscopy, culture and chest x-ray of which the first two can only be applied to patients who can produce sputum. The two methods have significant limitations in their performances, the smear microscopy's sensitivity is grossly compromised when bacterial load is less than 10,000 organisms/ml of sputum sample and culture, though specific, can also become insensitive when contaminated. Aside from issue of insensitivity, children or adults with latent TB or cavitated state of the lung tissue and disseminated TB cannot produce sputum (Tomans, 2004a).

Chest radiography is not specific for TB diagnosis, because of the subjectivity of the reading of the x-ray films. Molecular techniques such as the use of polymerase chain reaction (PCR) technique in TB diagnosis using the sputum is highly specific and is useful in rapid diagnosis and species identification. However, the infrastructural requirements to provide the diagnosis is expensive and are often out of the scope of most diagnostic facilities that offer TB diagnostics to communities especially in the developing countries (Keeler *et al.,* 2006)

The tuberculin skin test (TST), a cell mediated immune response to purified protein derivative (PPD) when inoculated subcutaneously to cause measurable induration that occurs within 24 to 72 hours, is one of the oldest methods of screening TB. It is however not specific because the content of PPD used contains more than 200 antigens widely shared by environmental mycobacteria (Fine *et al.,* 1999) and it is a hypersensitivity reaction that involves lymphocytes which makes it unsuitable for HIV patients due to their state of immune suppression (Stavri *et al.,* 2009).

Specific antigens obtained from the genome of *M. tuberculosis* have also been used in TB diagnosis which led to the production of Early Secretory Antigen Target 6 (ESAT-6) and Cultured Filtrate Protein 10 (CFP-10) antigens derived from Region of Difference 1 (RD1) of *M. tuberculosis* genome. These are found to be specific in diagnosing TB caused by *M. tuberculosis* (Ravin *et al.,* 1999). Efforts to improve the sensitivity of the test using these two antigens led to mixing the two antigens to increase diagnostic performance (Anderesen *et al.*, 2000; van Pinxteren *et al*., 2000). A commonly used commercial test kit

for TB diagnosis (Quantiferon –GOLD in tube) resulted from this innovation. However, its low sensitivity (Yoshihiro *et al.,* 2009) prompted further investigations on *M. tuberculosis* genome to improve on its diagnostic performance. ELLI, a new antigen (LIONEX Inc.Germany) produced from genome of *M. tuberculosis* is being assessed in this study for its diagnostic performance.

In severe cases of TB especially in co-infections with HIV, subjects have severe immune suppression and malnutrition. The cells of the immune system especially the T-cells (T-Lymphocytes) become suppressed, lethargic or depleted (Geldmacher *et al.,* 2008). This makes immunological technique involving the use of T-cells unsuitable for TB diagnosis in immune-compromised patients. This demands the employment of devices that can improve the response of immune cells when challenged. This study is based on the concept that using Rosewell Park Memorial Institute (RPMI) 1640 as an enriched medium to culture the immune cells and Fetal Calf Serum (FCS) as an inducing agent will improve the response of the immune cells and thus TB case detection in suspected TB and HIV+TB subjects when stimulated with the genomic antigens. The demonstration of these concepts would improve TB case detection rate and thus management of the disease.

1.2 STATEMENT OF THE PROBLEM

There are myriads of problems and limitations associated with conventional tests for diagnosis of TB especially with non pulmonary or latent TB. The report that 40% of TB victims with HIV who were not able to present with pulmonary TB were missed until autopsy at death (WHO, 2005b) showed the peculiarity of Immune-compromised subjects (HIV, malnutrition, aged) and limitation of sampling for extra-pulmonary TB which should be overcome. This situation is a major challenge to achieving the WHO standard set for diagnosis of TB which is 70% active TB case detection. Hence there is need to improve TB diagnosis by exploring use of new TB specific antigens and improving diagnostic procedure especially in immune compromised subjects.

1.3 AIM OF THE STUDY

The study aims to evaluate ELLI (the new antigen product from LIONEX in Germany) as TB antigen and then investigate if inducement of compromised T-cells using fetal calf serum can improve TB diagnosis by immunodetection method.

1.4 OBJECTIVES OF THE STUDY ARE TO:

1. Evaluate the diagnostic performance of ELLI (the new TB antigen product from LIONEX Company in Germany) for TB detection against ESAT-6, CFP-10, Culture and Sputum smear microscopy tests.
2. Determine the minimum level (percentage) of Fetal Calf Serum (as an inducing agent) that gives optimal inducement of T-cells.
3. Determine the performance of induced T-cells with the test antigens ELLI, ESAT-6+CFP-10 in detecting active and non pulmonary TB in subjects with or without HIV and TB contacts.
4. Compare the diagnostic performance of the different tests in detecting TB in HIV+TB, TB subjects compared to Culture (as Gold Standard).

1.5 SIGNIFICANCE OF THE STUDY:

The contribution of this study to the improvement of TB diagnosis is the revitalization of compromised T-cells from immune compromised subjects such as in HIV/AIDS made by incubation of T-cells with RPMI1640 and inducing them with FCS.

This is to cause a better response of the weak T-cells to low antigen stimulation that will lead to increased production of IFN-γ. By inducement, more T-cells will be available to produce IFN-γ the biomarker for TB in IGRA test. This will make IGRA for TB case detection more sensitive in the immune-compromised, in latent and non pulmonary TB cases.

1.6 OPERATIONAL DEFINITION OF TERMS

SENSITIVITY = The probability (percentage) that patients with the infection (determined by the result of the reference or gold standard test) will have a positive result using the test under evaluation.

SPECIFICITY = The probability (percentage) that patients without the infection (determined by the result of the reference or gold standard test) will have a negative result using the test under evaluation.

POSITIVE PREDICTIVE VALUE (PPV) = Probability that a positive result accurately indicates the presence of infection.

NEGATIVE PREDICTIVE VALUE (NPV) = Probability that a negative result accurately indicates the absence of infection.

PREVALENCE = Proportion of a given population with an infection at given time.

1.7 LIST OF ABBREVIATIONS

AFB = Acid Fast Bacilli.

APC = Antigen Presenting Cells

BCG = Bacillus Calmette Guerin

CFP-10 = Culture Filtrate Protein

CTL = Cytotoxic T- Lymphocytes

DC = Dendritic Cells.

DOTS = Directly Observed Treatment Short course

ELLI = Acronym given as trade name by LIONEX the producer of the new antigen.

ESAT-6 = Early Secreted Antigens Target

FCS = Fetal Calf Serum

IFN-Y = Interferon gamma

IGRA = Interferon Gamma Release Assay.

IL = Interleukin

LJ = Lowenstein- Jensen

MDR-TB = Multi Drug Resistance- Tuberculosis

NK = Natural Killer

NTBLCP =National Tuberculosis Leprosy Control Programme.

PBMC = Peripheral Blood Monocytic Cells.

PCR = Polymerase Chain Reaction

PLWHA = People Living with HIV and AIDS

PPD = Purified Protein Derivative

QFT- GIT = Quantiferon Test-Gold in Tube

RPMI = Rosewell Park Memorial Institute.

T-CELLS = Also called T-lymphocytes in Immune System

TMB = Tetramethylbenzidine

TST = Tuberculosis Skin Test

ZN = Ziehl-Neelsen

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. AETIOLOGY OF TUBERCULOSIS

Tuberculosis is caused by *M. tuberculosis* in man. It is a small non motile bacillus (Fig 2.1) (Dolin *et al.*, 2010). The bacterial cell outer membrane has a lipid bilayer. The bacterium is weakly gram positive because it does not retain stain due to the high lipid and mycolic content of its cell wall (Madison, 2001). However it retains certain stains after being treated with acidic solution, which made it to be classified as an acid fast bacillus (AFB) (Kumar *et al.,* 2007). The most common acid fast staining techniques are the *Ziehl Neelsen* stain that the acid fast bacillus a bright red standing out in a blue background, the *Auramine rhodamine* stain followed by fluorescent microscopy (Piot *et al.,* 2008). Other tuberculosis causing mycobacteria include *M. bovis, M. africanum, M. canetii* and *M. microti.* Some of these are not wide spread such as *M. africanus* while some like *M. bovis* are food bound which after pasteurization of milk has caused its elimination as public health problem (Acton Ashton, 2011). Other pathogenic mycobacteria include *M. avium, M. kasansii*, which are classified as non-tuberculous mycobacteria (NTM) but they cause pulmonary disease resembling tuberculosis (American Thoracic Society, 2000).

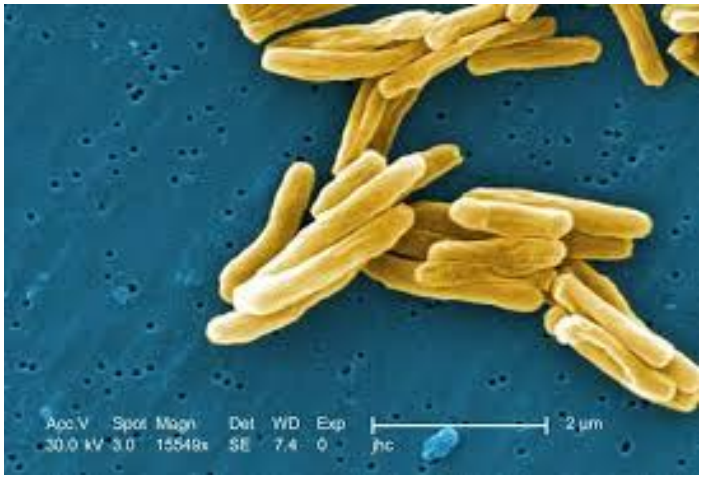

Fig. 2.1 Tubercule bacilli (Electron Microscope)

Source: Dolin *et al*. 2010

2.2 PATHOGENESIS OF MYCOBACTERIUM TUBERCULOSIS

In majority of cases, including all sputum smear-positive and a significant proportion of smear-negative cases, *M. tuberculosis* resides in the lung, which therefore serves as the origin of transmission via aerosols. Enclosed in minute droplets, bacteria are expelled by coughing of an active pulmonary TB patient and in this way enter lung alveoli of other individuals. There, alveolar macrophages, as well as interstitial dendritic cells engulf *M. tuberculosis***.** In the alveolar macrophages, the TB bacteria replicate within the endosomes. The primary site of infection in the lungs is called the **Ghon focus** and is generally located in either upper part of lower lobe or the lower part of the upper lobe. Some bacteria are transported to draining lymph nodes, while others are trapped in the lung parenchyma. Monocytes and granulocytes are attracted to the site of bacterial deposition in the lung parenchyma where accumulating cells form loosely packed lesions. Tuberculosis infection of the lungs can also come through the blood stream. This is **Simon focus** and is found on top of the lung (Khan, 2011). The transmission through the blood can spread TB infection to other tissues and organs where secondary TB lesions can develop in other parts of the lung, peripheral lymph nodes, kidney, brain and the bones (Herrmann & Lagrange, 2005; Kumar *et al.,* 2007).

About 90% of people with *M. tuberculosis* are asymptomatic and therefore classified as latent TB infection (Skolnik and Richard, 2011), only 10% of those infected will have a life time chance of progressing to active TB (Mainous and Pomeroy, 2009). However in those with the HIV, 10% of them stand the risk of developing active disease within a year of infection. The TB infection starts when the *M. tuberculosis* reaches the pulmonary alveoli where they invade and replicate within endosome of alveolar macrophages (Houben *et al.,* 2006). The alveolar macrophages identify the *M. tuberculosis* as foreign and attempt to eliminate it by process of phagocytosis. During this process, the bacterium is enveloped by the macrophage and stored temporarily in a membrane bound vesicle called phagosome. The phagosome then combines with lysosome to form phagolysosome. In the phagolysosome, the macrophage cell attempt to

use reactive oxygen (H_2O_2) species and reactive nitrogen (nitric acid) to kill the bacterium. However *M. tuberculosis* has a thick, waxy mycolic acid capsule that protects it from those toxic substances. It then reproduces inside the macrophage cell and kill the cell.

2.2.1 Tuberculosis Granuloma

Tuberculosis is classified as one of the granulomatous inflammatory diseases. Tuberculosis granuloma is defined as organized immune cell aggregates that are formed in response to persistent TB infection (Ramakrishnan, 2012). The cellular composition of TB granuloma include macrophages, neutrophills, monocytes, dendritic cells, B and T–cells, fibroblasts and epithelial cells (Russel, 2007, Ramakrishnan, 2012). The granuloma is the site of infection, persistence, pathology and protection. Tuberculosis granuloma morphology is characterized by a central necrotic core surrounded by concentric layers of macrophages, epitheloid cells, multinucleated Langahan's giant cells and lymphocytes Fig 2.2 (Kaufman 2001; Helming & Gordon, 2007; Ramakrishnan, 2012). The macrophages can also differentiate into foamy cells with a high lipid content (Russel *et al.,* 2009) The containment of *M .tuberculosis* at the site of primary infection by a cellular wall and a fibrotic outer layer prevents the pathogen from dissemination throughout the host and focuses the immune response to the site of mycobacterial persistence. Effector T-cells including conventional CD4+ and CD8+ T-cells and unconventional T-cells that recognize antigen by the dendritic cells and macrophages participate in the control of tuberculosis. Interferon gamma and TNF-α produced by the T-cells are important macrophage activators. Macrophage activation permits phagosomal maturation and the production of antimicrobial molecules such as reactive nitrogen intermediates (RNI) that produces nitric acid and reactive oxygen (ROI) that produces H_2O_2 (Fig 2.3) (Ulrich & Kaufman 2006; Bikle, 2008). This co-ordinated activation of cells is crucial for a productive granuloma to develop and thus for a long containment of mycobacteria at distinct sites of infection. Successful containment of the pathogen to the site of primary lesion results in latent infection often seen

as Calcified Granulomatous lesion. However more recent evidence suggests that the bacteria use the granulomas to avoid destruction by the host's immune system. Macrophages and Dendritic cells in the granulomas are unable to present antigen to lymphocytes; thus the immune response is suppressed (Bozzano , 2014).

Granuloma formation may fail in individuals with a compromised immune system and there are several hypothesis about HIV exacerbating TB pathology through the manipulation of granuloma (Diedrich & Flynn, 2011). Specifically, TB patients with AIDS present a dominant granulocytic infiltrate and necrosis without the typical caseous necrosis seen in non-HIV infected TB granulomas. This has been associated with the killing of CD4+ T-cells in the granuloma, probably resulting in direct disruption of granuloma structure and abolition of the containment of infection. Cavity lesions are seldom seen in patients with CD4+ T-lymphocytes count <200/mm^3 (Sharma *et al.,* 2005). As a result of this, while in majority of adult patients TB is confined preferentially to the lungs, in HIV infected patients, TB can be a systemic disease involving multiple organs that lack well defined granulomas and instead develop more diffuse lesions (de Noronha *et al.,* 2008). All forms of extra pulmonary TB have been described in patients with HIV.

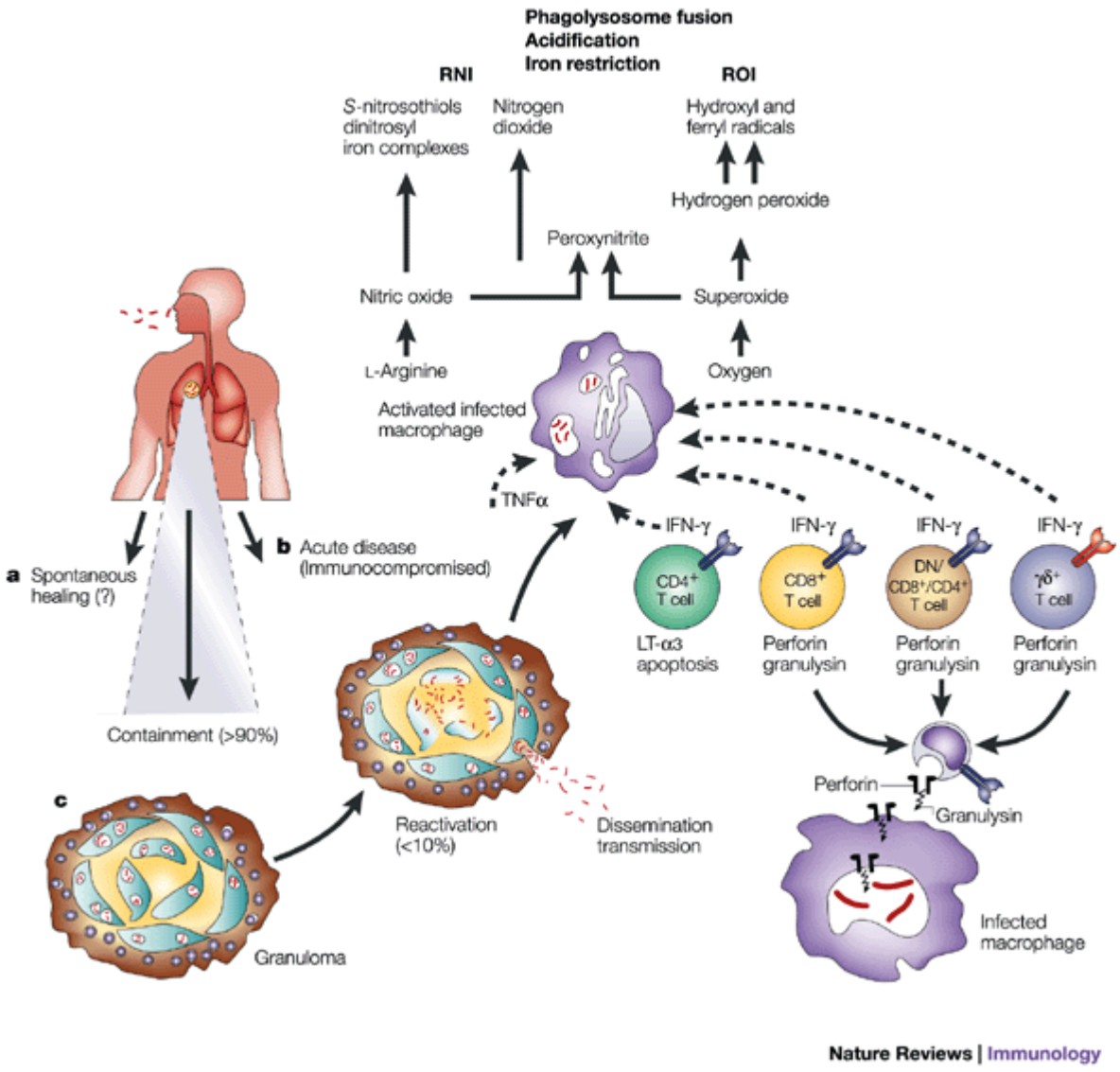

Fig. 2.2 Three potential outcomes of infection of the human host in Mycobacterium tuberculosis.

Source: Kaufman 2001.

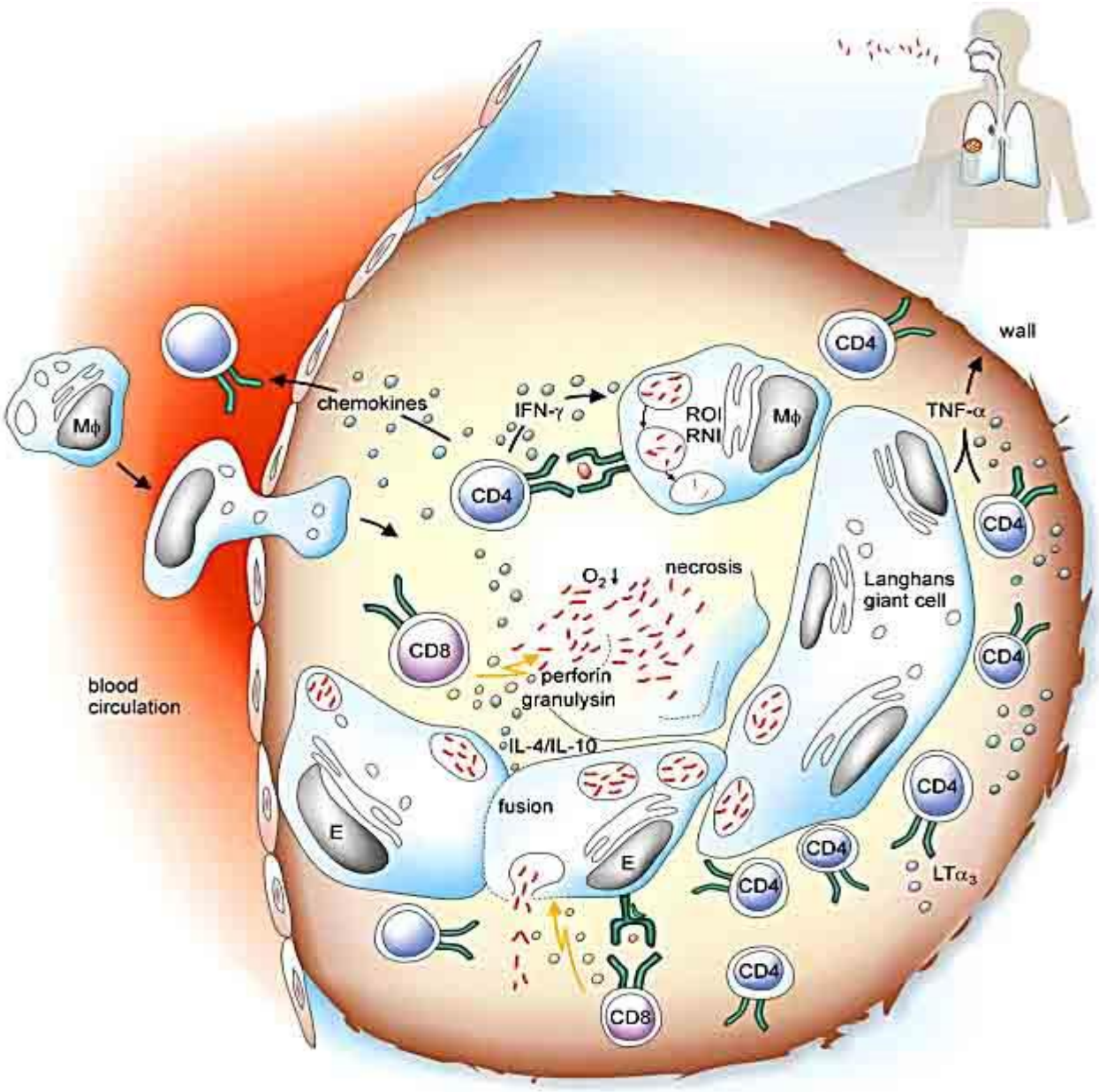

Fig. 2.3 Host response and Granuloma formation

Source: Ulrichs and Kaufmann 2006**.**

2.3 EPIDEMIOLOGY OF TUBERCULOSIS

One third of the world's populations is infected with *M. tuberculosis* (WHO, 2011) with new infections at a rate of one per second (WHO, 2006a) and with most infections developing as latent form, 10% of which progresses to active disease that results in death. *M. tuberculosis* is an obligatory pathogen which has a predilection site for lung tissue that is rich in oxygen supply. An estimated 8.6 million TB cases were reported in 2012 with 940,000 deaths of which 27% were in Africa and 58% in Asia (WHO, 2013). Of these new cases, 12.8% were HIV positive. Tuberculosis is therefore reported as commonest cause of death among people living with HIV/AIDS with 17 deaths per 100,000 (WHO, 2013). Global incidence of TB has decreased marginally from 142 per 100,000 in 2004 to 125 per 100,000 in 2011. Also in Nigeria, decrease in the incidence of TB from 449,000 new cases in 2006 to 180,000 cases in 2012 was reported (WHO, 2013). Out of 300,000 cases of multidrug resistant TB (MDR-TB) reported globally in 2012, 38,000 cases were from Africa and 3,600 cases were reported from Nigeria (WHO, 2013). In the breakdown, for global report, an estimate percentage of new cases with MDR-TB was 3.6% and the percentage for retreatment cases with MDR-TB was 20%. For Africa, this was 2.3% for new TB cases with MDR-TB and 11% for retreatment cases with MDR-TB and for Nigeria, 2.9% of new TB cases with MDR-TB and 14% of retreatment TB cases with MDR-TB (WHO, 2013).

Globally, TB case detection rate has improved from 40% in 1995 to 56% in 2005 and 66% in 2012. In Africa, TB case detection rate has also improved from 32% in 1995 to 52% in 2005 and to 59% in 2012. In Nigeria, Tb case detection rate improved from 8.9% in 1995 to 26% in 2005 and to 51% in 2012 (WHO, 2013).

2.3.1 Highlights of Tuberculosis in Nigeria

Nigeria ranks 4th among the 22 high burden of TB countries in the World (WHO, 2013). World Health Organization estimated 108,000 new cases of all forms of TB occurring in the country in 2012. The

prevalence of TB cases was estimated to be 270,000 in 2012 by WHO. This was an equivalent of 161/100,000 of TB cases in the country (WHO, 2013). There were 97,853 TB cases notified in 2012 with 52,901cases as new smear positive and a case detection rate of all forms of TB at 51%. The death rate as at 2012 was 16/100,000 cases.

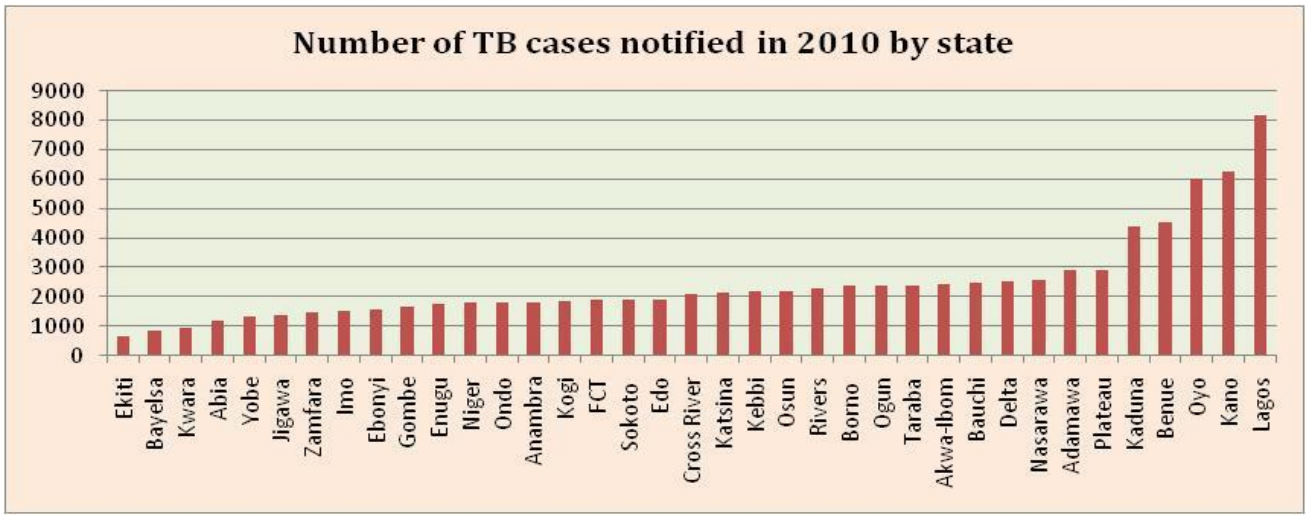

Fig 2.4. TB prevalence rate in the States

Source: Nigeria TB Factsheet; USAID 2012

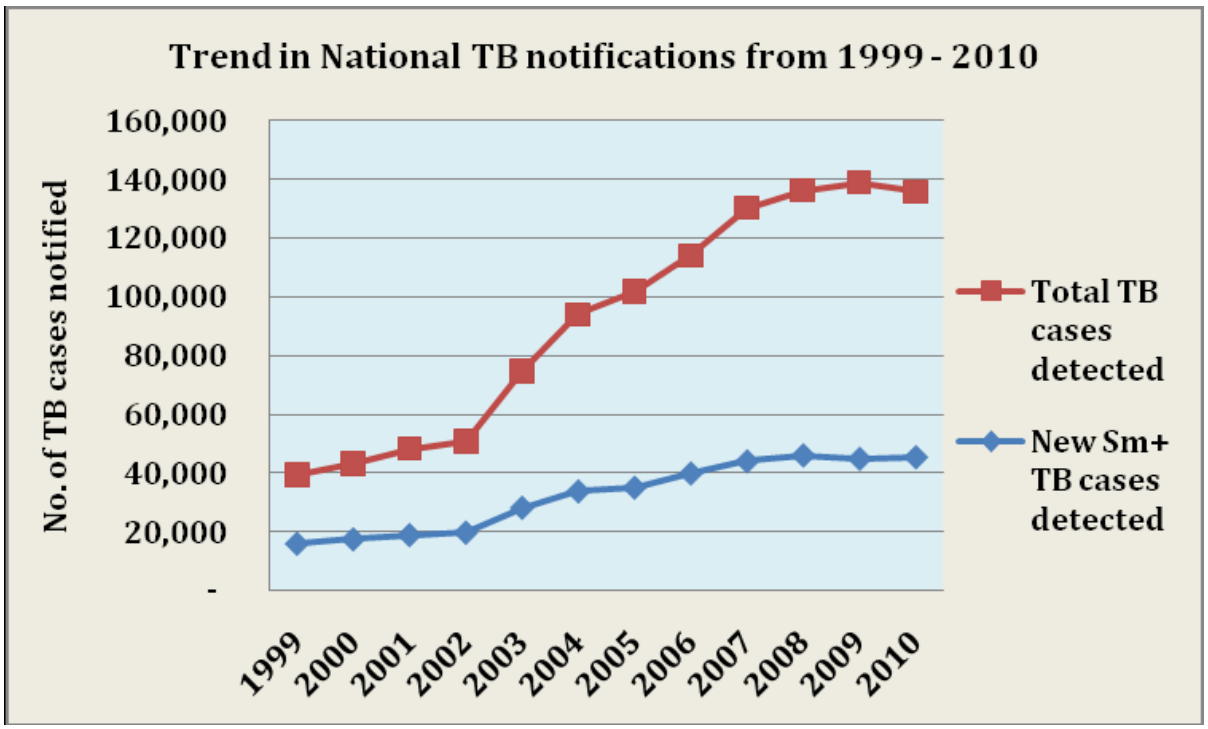

Fig. 2.5 Trend in National TB notification from 1999 to 2010

Source: Nigeria TB Factsheet; USAID 2012

2.3.2 TB-HIV cases in Nigeria

An individual that is HIV positive has 10 times risk of developing TB compared to an HIV negative individual (WHO 2011). The life time risk is 50% for an HIV positive individual compared with 5-10% risk for an HIV negative person (Dosumu 2006). In Nigeria the rate of TB increase is at 7% annually (USAID 2012). This was in contrast to global annual rate of TB increase at 0.4% (Dauda 2010). HIV has been the driving factor of the increase in incidence of TB of which the overwhelming effect of immune suppression of HIV infection on active TB has increased the recurrence rate of TB (Dauda 2010).

In a study conducted in Benin City to determine the pattern of presentation and prevalence of TB in HIV positive patients, a prevalence of 33.9% was found. Pulmonary TB was the common type found (78.9%) while extra-pulmonary was 21.4% (Affusin *et al*., 2012). In another study of HIV prevalence among TB patients, in some States in Nigeria, a median prevalence of 17% (4.2%- 35.1%) among the TB patients was found. The highest prevalence was recorded in North Central State of Benue State and the least was in the Southwest State of Oyo State. Kano State recorded 12.4% in the survey (Ekanem *et al*., 2004).

The estimated HIV positive incidence in TB cases for 2012 was 46,000. The proportion of TB cases tested for HIV was 84% and 80% of these cases were started on Cotrimazole prophylaxis, while 2,300 were provided with Isoniazide (IPT) prophylaxis. The proportion of TB-HIV co-infected patients on anti-retroviral (ARV) therapy was 56%. The number of HIV registered cases screened for TB was 140,460 in 2012 (WHO, 2013).

2.3.3 MDR-TB cases in Nigeria

The emergence of MDR-TB is posing a threat to the control programme of TB in Nigeria and if it is not addressed with utmost attention, could wipe out the achievement made so far on the control of TB. MDR-TB is defined as a disease condition caused by strains of Mycobacterium tuberculosis that are resistant to both rifampicin and isoniazid with or without resistance to other TB drugs. It is observed that treatment of

MDR-TB is usually prolonged and has the potential of developing resistance to second line of drugs. Nigeria was reported to have an estimated MDR-TB rate of 2.2% and 9.4% among new and re-treatment TB cases, respectively and is therefore ranked 15th among the 27 high burden countries for MDR-TB (WHO, 2011). Nigeria is among the 4 African countries with the highest burden of drug resistant TB. According to the national TB drug resistance survey, MDR-TB prevalence rates was 2.9% among new patients and 14.3% among patients who had previous exposure to anti TB drugs in 2012 (FMOH, 2012). This indicated that MDR-TB in Nigeria is poorly documented. However, WHO report showed that MDR-TB cases among notified pulmonary TB cases were 2,500 new cases and 1,100 from retreatment cases (WHO, 2013). In a study conducted in Abeokuta Ogun State, the prevalence of MDR-TB found was 5.8%. This was found to be significantly associated with HIV sero-positive patients having 32% rate when compared with HIV negative rate of 2.2% ($P<0.05$) (Ejilude *et al*., 2013).

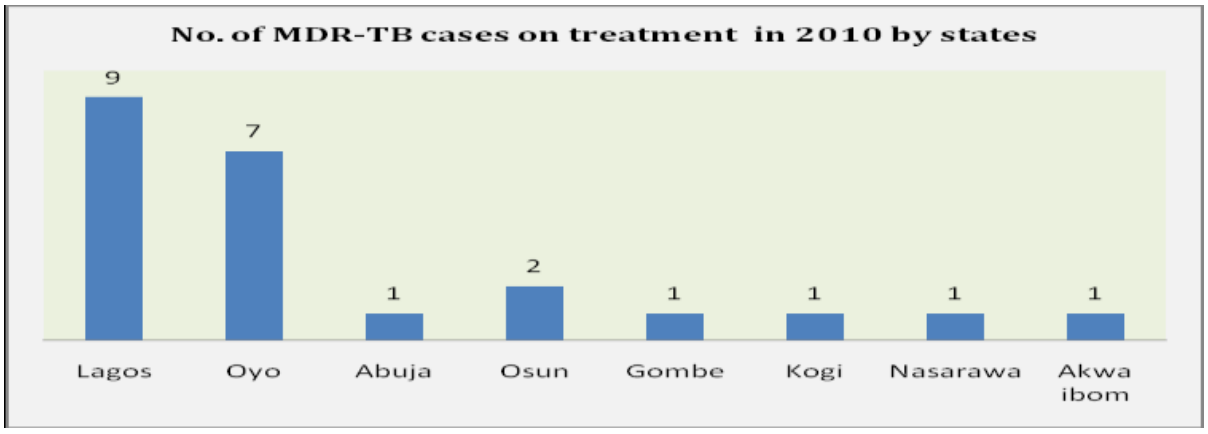

Fig 2.6. Number of MDR-TB cases on treatment in 2010 by States

Source: Nigeria TB Factsheet; USAID 2012

2.3.4 Tuberculosis transmission:

Tuberculosis spread through the air when people with active TB disease cough, sneeze or spit. They expel infectious aerosol droplets of 0.5 to 5mm in diameter. A single sneeze can release up to 40,000 droplets that may transmit the disease, since the infectious dose is very low an inhalation of just a single bacterium can cause a new infection (Nicas *et al* ., 2005; Konstantinos , 2010). People with prolonged, frequent or intense contact are particularly at high risk of becoming infected, with an estimated 22% infection rate (WHO, 2006a). Others at risk include health care givers, household contacts, patients with immune compromised conditions such as HIV / AIDS and those on immuno suppressive drugs.

Transmission can only occur from people with active TB and it depends on number of infectious droplets expelled by the carrier, effectiveness of ventilation, duration of exposure and strain of mycobacterium.

2.3.5 Incidence of TB Infection with age:

The incidence of TB varies with age. In Africa, TB primarily affects adolescents and young adults (WHO, 2006b). However in countries where TB has gone from high to low incidence, such as United States, TB is mainly a disease of older people or of the immunocompromised (CDC, 2006).

Clinical features of TB in the elderly can be atypical, non specific and confused with concomitant age related diseases. Underlying acute or chronic diseases, malnutrition and biological changes with aging can disrupt integument barrier, impair microbial clearance mechanisms and contribution to the expected age associated decline in cellular immune response to infecting agents such as *M. tuberculosis.* Therapy of tuberculosis in elderly is challenging due to incidence of drug reactions (Rajagopalan and Yoshikawa, 2000).

Tuberculosis in children reflects the prevalence of the disease in adult as well as current transmission rates. Africa and the South East Asia have the largest number of tuberculosis cases and the situation in these areas had been worsened by the HIV epidemic.

Children born to HIV infected parents whether infected or not, are at high risk of developing tuberculosis because of the increased risk of exposure to the disease.

Tuberculosis is more common among the disadvantaged and vulnerable groups in each society and the impact of overcrowding, under nutrition and poverty is particularly severe on children. Infected children and those with large tuberculosis reaction (>18mm) are at increased risk for progression and should be followed closely. Mortality from tuberculosis is also highest in early childhood, mainly due to disseminated forms like meningeal and miliary tuberculosis (Datta and Swaminathan, 2001).

In Nigeria, an eighteen month retrospective study was carried out to determine the prevalence rate of TB in HIV children in Abuja Nigeria, 19.5% of 210 HIV infected children were found to have TB (Okechukwu and Okechukwu, 2011). TB co-infection with HIV in children was found most common in the environment. Severe weight loss was an indicator to identify HIV infected children at risk with TB. Before then, Ugochukwu (2010) did a retrospective review of mortality rate among HIV positive children diagnosed with TB and found a range of 20-35%. However both studies concluded that diagnosing TB in HIV positive children was found challenging especially in poor resource setting because of non availability of new methods of diagnosing TB of which most diagnosis were based on combination of old methods.

Early detection of TB at the latent level is therefore very important to prevent the stage of active TB that could lead to the experience of infection in children as expressed above. For the children living with parents having active TB, contact tracing will be very useful to detect patients with latent TB infection and should be followed by preventive therapy. When properly applied, it can have an impact on morbidity and mortality especially among children. In most industrialized countries, the decline in the risk of infection due to better overall health and effective TB control measures is likely to result in the disappearance of TB as a public health problem, therefore the cost benefits of contact tracing is likely to be less than in the developing world.

In the developing countries, effective application of DOTS strategy for the prevention and control of TB in both adults and children can be more beneficial. However, diagnosis and treatment of known active infectious cases must precede contact tracing especially in children (Nair, 2001). In Nigeria, DOTS coverage has expanded from 2,780 in 2008 to 3,931 in 2009. This subsequently increased notified cases of all forms of TB from 86,294 in 2007 to 90,447 TB cases in 2010 (USAID TB fact sheet, 2012).

2.3. 6 TB in health workers:

With the incidence of TB rising, more frightening is the emergence of drug resistance including multi-drug resistance TB. In this changing situation, the potential threat of nosocomial spread of tuberculosis to other patients and health care workers has been poorly addressed.

A retrospective study was carried out to investigate the risk of tuberculosis in health workers in Estonia. Sixty-seven health care workers (23 doctors, 23 Nurses, 7 Laboratory technicians, 12 Assistant Nurses and 2 Cleaners) all of whom tested negative for HIV were diagnosed as having active TB. The incidence among health care workers (HCW) was 1.5 to 3 times higher than in general population (Tekkel *et al.,* 2002).

A study carried out to ascertain the magnitude of occupationally acquired pulmonary TB (PTB) among health workers was conducted at 2 designated DOTS centers in Ibadan Nigeria. Nine (3.3%) of the 271 subjects were positive for AFB microscopy, while 262 (96.7%) were negative. Six (2.2%) samples were positive for culture and 260 (95.9%) were negative. Six (2.2%) were both AFB and culture positive. These TB positives were found in the age range of 21-50 years, while only one was in adolescent age group (Kehinde *et al.,* 2011). The TB status of health care workers deserves more attention in the developing countries like Nigeria, where drug supply to TB patients with active TB had been epileptic.

2.3.7 Risk Factors for Tuberculosis:

Many factors make people vulnerable to tuberculosis infections. Most important of this is the global pandemic HIV infections of which 13% of the people with TB are infected with the virus (WHO, 2011). Of the people without HIV who are infected with TB, about 5-10% develop active disease during their life time, which is a contrast to 30% of those co-infected with HIV developing active disease in a short time (WHO, 2005b).

Overcrowding and malnutrition have made TB a disease of poverty (Lawn and Zumla, 2011). People in this category include those in prisons and homeless shelters (Souza *et al.,* 2000). Also vulnerable are the medically under privileged in resource poor communities. Children in close contact with TB subjects, house care givers and health care providers for the TB subjects are susceptible to TB especially when the contact is of long term (Griffith and Kerr, 1996). Chronic lung disease is another risk factor subjecting the victim to TB infection as a result of weak capacity of the protective mechanism of the respiratory pathway. Smokers are in this category as they stand the risk of contracting TB as compared to non-smokers (van Zyl Smit *et al.,* 2010). Other disease conditions that can subject people to the risk of TB infection include alcoholism and use of immunosuppressive drugs such as corticosteroids (Lawn and Zumla, 2011).

2.3.8 TB-HIV Co-infection

HIV is considered to be the most potent risk factor for progression to active TB among those infected with both TB and HIV. As a result, TB is the most common life threatening opportunistic infection associated with HIV and biggest cause of death among patients with AIDS (Corbett *et al.,* 2003; Getahun *et al.,* 2010). In areas hard hit by HIV, TB is increasing - leading to a greater case load, thereby overstretching the already fragile health infrastructure (Narain and Lo, 2004). The co-infection poses both diagnostic and therapeutic challenges in the health systems of most developing countries. The co-infection potentiates

one another by accelerating the deterioration of immunological functions and resulting in premature deaths if untreated. Tuberculosis causes 26% of AIDS related deaths (Corbett *et al.,* 2003) and this mostly occur in developing countries (WHO, 2013). Pulmonary TB is the most common manifestation in adults with HIV. This occurs at various stages of HIV infection. The clinical pattern of this co-infection correlates with the subjects' immune status. Not only does HIV increase the risk of reactivating a latent TB, it also increases the risk of rapid TB progression (WHO, 2009; Getahun *et al.,* 2010). Tuberculosis has also been reported to exacerbate HIV infection (Modjarad and Vermund, 2010).

Both TB and HIV have profound effect on the immune system as they are capable of disarming the host's immune responses through the mechanism that is not yet clear. At the early stage of HIV infection when immune status is only partially compromised, the clinical features are more typical of tuberculosis with upper lobe cavitations resembling the pre HIV era. As immune deficiency advances, HIV patients present as a typical primary pulmonary TB disease, as extra pulmonary and disseminated disease is commonly associated with hilar adenopathy and lower lobe infection (Collins *et al.,* 2002, Pawlowski *et al.,* 2012).

2.4 IMMUNOLOGY OF TUBERCULOSIS AND HIV-TB CO-INFECTION

2.4.1 Immune response to *M. tuberculosis*

There are three potential outcomes of *M. tuberculosis* infection in the human host; a) The frequency of abortive infection resulting in spontaneous healing is unknown, but is assumed to be minute. b) In the immunocompromised host, disease can develop directly after infection. c) In most cases, mycobacteria are initially contained and disease develops later as a result of reactivation. The granuloma is the site of infection, persistence, pathology and protection. Effector T cells (including conventional $CD4^+$ and $CD8^+$ T cells, and unconventional T cells, such as γδT cells, and double-negative or CD4/CD8 single-positive T cells that recognize antigen in the context of CD1) and macrophages participate in the control of tuberculosis. Interferon-γ (IFN-γ) and tumour-necrosis factor-α (TNF-α), produced by T cells and

dendritic cells respectively, are important macrophage activators. Macrophage activation permits phagosomal maturation and the production of antimicrobial molecules such as reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). LT-3, lymphotoxin-3 (Kaufman, 2001).

2.4.2 Innate immune response

The innate immune response plays an important role in the protection against TB as it provides the first line of defense that the invading pathogen meets. When the bacilli are inhaled, the alveolar macrophages are the primary target cells for this intracellular pathogen. *M. tuberculosis* is detected by these innate cells through recognizing the pathogen –associated molecular patterns via toll-like receptors (TLRs) and interacting with these cells by binding to receptors on their cell surfaces. The innate immune system is expected to clear the infection in many cases if activated correctly. However, because *M. tuberculosis* has evolved strategies to manipulate the macrophage, allowing intracellular survival and replication thus making the innate immune system to become a prerequisite for mycobacterial pathogenesis. The innate immune system comprised anatomical barriers such as the skin as well as the complement system and several types of innate immune cells. *M. tuberculosis* interact with these cells and binds to receptors on their cell surfaces. These receptors include TLR, complement receptor (CR) 3, mannose receptor, scavengers receptors and DC-specific intercellular-adhesion-molecule-3-grabbing non-integrin (DC-SIGN), and engagement of these leads to clearance of the bacilli or drive granuloma formation (Davis & Ramankrishnan, 2009; Dheda *et al.,* 2010). The alveolar macrophages that first ingest the bacilli and arriving monocytes from the blood stream provide the bacteria with its niche, but may also be able to disarm the pathogen if stimulated correctly. The DC that ingest the *M. tuberculosis* can also provide a replication niche, simultaneously being essential for antigen presentation to T-cells in the draining lymph node (Dheda *et al.,* 2010; Korbel *et al.,* 2008). On the other hand, *M. tuberculosis* has developed mechanisms to prevent both DC migration and antigen presentation (Wolf *et al.,* 2007). This highlights the complexity of the interaction between *M. tuberculosis* and the innate immune system. Apart from

macrophages and DC, neutrophils and NK cells have been implicated in immune response against *M. tuberculosis*. Neutrophils are among the first cells to respond to inflammatory stimuli by migrating to the site of infection and *M. tuberculosis* infection is followed by massive influx of neutrophils (Korbel *et al.,* 2008). It has been shown that neutrophils can be activated in response to *M .tuberculosis* and kill the pathogen using its range of antimicrobial molecules contained in their granules including defensins, lactoferrin, cathelicidin and lysozyme, which is transferred into *M.tuberculosis*-containing phagosome upon fusion with granules (Korbel *et al.,* 2008; Martineau *et al.,* 2007). Neutrophils also exert efficient killing of microbes through the assembly of the NAPDH oxidase in the phagosomal membrane, which leads to the generation of superoxide followed by reactive oxygen species (ROS) in the phagosome (Faurschou & Borregaard, 2003) and have been shown to be able to kill *M .tuberculosis* in a Ca^{2+}-dependent manner (Majeed *et al.,* 1998). Furthermore, neutrophils are able to activate macrophages through release of granule proteins (Tan *et al.,* 2006) and heat shock proteins 72(hsp72). Hsp72 is released from apoptotic neutrophils which has been found to be able to induce macrophage activation in addition to having an inflammation resolving role (Persson *et al.,* 2008). However, in-vivo studies are giving conflicting evidence as to whether neutrophils have protective or tissue –damaging effect during *M. tuberculosis* infection (Dheda *et al.,* 2010; Korbel *et al.,* 2008; Martineau *et al.,* 2007).

In animal studies, NK cells that are granular lymphocytes of the innate immune system that have cytotoxic functions, exerted through perforin and granzyme or granulysin (Korbel *et al*., 2008) and provide the macrophage with a signal through IFN-Y during *M. tuberculosis* infection(Ducati *et al.,* 2006). They are activated through complex interactions between IL-12, IL-18, IFN-α and a range of activating and inhibitory receptors. *M. tuberculosis* infected macrophages can be lysed directly by NK cells and the NK cells mount a pro-inflammatory response to *M. tuberculosis* in-vitro, restricting *M. tuberculosis* growth in an apoptosis – dependent manner (Korbel *et al.,* 2008). Furthermore NK cells can kill regulatory T-cells that are at risk of dampening the immune response to *M. tuberculosis* (Roy *et al.,*

2008). However, the role of NK cells in *M. tuberculosis* infection is not very clear an observed defect in the functionality of NK cells in TB patients was found to be due to effect of *M. tuberculosis* infection rather than cause of the disease (Raja, 2004).

2.4.3 Adaptive immune response

Cell mediated immunity is essential for control of *M. tuberculosis* infection; activation of both the CD4+ and CD8+ T–cells is seen in active TB in human and experimental mice infection (Cooper, 2009). CD4+T-helper cells of type Th1 are the most critical (North and Jung, 2004).

In experimental mice infection, adaptive immune response was mounted two weeks after *M. tuberculosis* infection and this is accompanied by a drop in bacterial replication (Ducati *et al.*, 2006; Dheda *et al.*, 2010). Infected DC and macrophages present *M. tuberculosis* antigen to T cells through MHC class 1 to CD8+ cytotoxic cells and through MHC class 2 to CD4+ T helper cells, which lead to the activation and proliferation of lymphocytes. In addition, CD restricted T-cells can be activated through presentation of glycolipid antigens by DC and γδ T-cells through presentation of phospholigands and these contribute to protective immunity against TB by producing IFN-Y or exerting cytotoxic activity (Raja 2004; Dheda *et al.*, 2010). Memory T-cells also form upon *M. tuberculosis* infection. The infected macrophages and DC secrete cytokines including IL-12, IL-23, IL-7, IL-15, and TNF-α leading to attraction of more leucocytes to the infection site (Dheda *et al.*, 2010). Depending on the cytokine environment, the CD4+ T cells can mount Th1 response (IL-12, IL-18, IFN-Y) or a Th2 response (IL-4,IL-5, IL-13). A Th1 response leads to the release of pro-inflammatory cytokines including IFN-Y, which is thought to enhance killing of intra-macrophage mycobacteria through NO and ROS production (Russel, 2007; Rohde *et al.*, 2007; Dheda *et al.*, 2010). A Th2 response on the other hand, leads to release of IL-4, IL-5, IL-10 and IL-13 promoting B lymphocyte activation leading to an antibody response, and promoting an anti-inflammatory macrophage response. Th17 cells, stimulated by IL-23, IL-6, IL-21 and low TGF-β levels, are involved in recruitment

of cells of the innate immune system and Th1 cells and also secrete IL-17 (Dheda *et al.,* 2010). Regulatory T-cells (Treg), stimulated by IL-2 and high TGF-β can be stimulated. Treg produce anti-inflammatory cytokines such as IL-10 and can suppress microbicidal mechanism in the macrophage, and the activity of these cells is elevated in TB patients (Dheda *et al.,* 2010).

Specific activation of CD8+ cytotoxic T cells have been reported to lead to killing of *M. tuberculosis* through a perforin and granulysin-mediated pathway by which the infected macrophage undergoes cell death, or by induction of apoptosis through the extrinsic pathway via Fas ligand (Weerdenburg *et al.,* 2010; Dheda *et al.,* 2010). It is known that TB patients display a defect in the killing capacity of their cytotoxic T cells (Brighenti & Andersson, 2010).

The role of B-cells and humoral response in protection against TB is unclear and their importance in *M. tuberculosis* infection has been played down due to intracellular nature of the infection. However experimental evidence suggests that an antibody response can have an immune modulating effect on cellular immunity through cytokine signaling, as well as a protective role against infection inhibiting bacterial replication, neutralizing bacterial products, triggering of the complement system and promoting antibody-dependent cellular cytotoxicity. Antibody response results in opsonization of extracellular bacilli with IgG leading to phagocytosis by macrophages and DC through FcyR (Abebe & Bjune, 2009).

2.4.4 Role of macrophages.

Macrophages are large mononuclear cells of the innate immune system that function as professional phagocytes, they are capable of engulfing particles larger than 0.5um, including microbes. The role of macrophage is to internalize debris and apoptotic cells in a non-inflammatory manner (Rohde *et al.,* 2007). During infection, their role is to ingest and destroy pathogens, recruit other cells of the immune system and present antigens from the microbe to cells of the adaptive immune system. Resident macrophages are terminally differentiated and have a fixed location in the body, at strategic points where

infection can occur e.g alveolar macrophages are stationed in the lungs, Kupfer cells in the liver, microglia cells in the nervous system and mesangial cells in the kidney. The precursor of macrophages, the monocyte, circulates in the blood stream and is recruited into sites of infection or tissue damage when stimulated. It then differentiates into a macrophage, with increased phagocytic capacity and different morphology and adhesive properties. Macrophages can become activated upon inflammatory or microbial stimulation. When a macrophage is activated by microbial products , such as Lipopolysaccharide (LPS), the cell acquires the antimicrobial properties necessary for elimination of the invader, although some pathogens including *M. tuberculosis,* have found a way of circumventing this response (Benoit *et al.,* 2008; Flannagan *et al.,* 2009). The mechanism of containing tuberculosis by macrophages is discussed as follows: surface binding of *M. tuberculosis* to macrophages; phagosome-lysosome fusion; mycobacterial growth inhibition/killing (Raja, 2004).

2.4.4.1 Binding of *M. tuberculosis* to monocytes /macrophages:

Complement receptors (CR1, CR2, CR 3, and CR 4), mannose receptors (MR) and other cell surface receptor molecules play important roles in binding of the organisms to phagocytes bearing the receptors (Dheda *et al.,* 2010). The interaction between mannose receptors on phagocytic cells and mycobacterial cell wall is mediated through the mycobacterial surface glycoprotein, lipoarabinomannan (LAM) (Nigou *et al.,* 2001). Prostaglandin E2 (PGE2) and interleukin-4 (IL-4), a Th_2-type cytokine, up regulate complement and mannose receptor expression and function in the binding process. But Interferon gamma (IFN-Y) produced by lymphocytes decreases this receptor expression thereby resulting in diminished ability of the mycobacterial organism to adhere to macrophages (Dheda *et al.,* 2010).

2.4.4.2 Phagosome-lysosome fusion:

Phagocytosed microorganisms are subjected to degradation by intralysosomal acidic hydrolases upon phagolysosome fusion. This highly regulated event constitutes a significant antimicrobial mechanism of phagocytes. However it has been reported that mycobacterial sulphatides which are derivatives of multiacylated trehalose 2-sulphate have the ability to inhibit phagolysomal fusion (Roberts and Deretic, 2008). *M. tuberculosis* also blocks phagosome maturation after entry into the host macrophage thereby promoting environment that supports bacillary replication (Lee *et al.,* 2009). In vitro studies also showed that *M. tuberculosis* generates copius amount of ammonia in cultures which is thought to be responsible for the inhibitory effect on macrophages (Gordon *et al.,* 1980).

2.4.4.3 How do the Macrophages handle the engulfed *M. tuberculosis*:

Macrophages produce antimicrobial reactive oxygen and nitrogen species (ROS and RNS) via NADPH oxidase and inducible nitric oxide synthase (iNOS) (Ehrt and Schnappinger, 2009). The antimycobacterial effector functions of macrophages is in the generation of Reactive Oxygen Intermediates (ROI) and Reactive Nitrogen Intermediates (RNI), mechanisms mediated by cellular enzymes and cytokines described below.

***Reactive Oxygen Intermediates (ROI)*:**

Macrophages use the NADPH oxidase NOX2 to generate ROS from O_2 for killing microorganisms, upon activation, the enzyme complex subunits of the NADPH oxidase assemble in the phagosomal membrane in a Rac1- or Rac2-dependent manner. The active NADPH oxidase transfers cytosolic NADPH electrons to O_2 in the phagosome, producing superoxide (O_2-) which forms hydrogen peroxide (H_2O_2) through dismutation in the phagosome. H_2O_2 in turn further reacts with O_2 -, generating different ROS, which can kill the intraphagosomal pathogen (Flannagan *et al.,* 2009; Robinson, 2009). This hydrogen peroxide

(H_2O_2), generated by macrophages via oxidative burst is the first effector molecule that mediated mycobactericidal effect of mononuclear phagocytes (Rohde *et al.,* 2007; Dheda *et al.,* 2010). Studies have shown that *M. tuberculosis* infection induces accumulation of macrophages in the lungs and also H_2O_2 production *(*Dheda *et al.,* 2010).

Reactive Nitrogen Intermediates (RNI):

Phagocytes, upon activation by T-cell derived cytokine IFN-Y and Tumor Necrosis Factor alpha(TNF-α) generate nitric oxide (NO) and related RNI via inducible Nitric Oxide Synthase (iNOS2) using L-arginine as the substrate. This iNOS, also termed NOS2 in phagocytes, synthesised upon microbial stimulation of macrophages, functions to produce nitrogen radicals on the cytoplasmic side of the phagosome, which can then diffuse into the phagosome. iNOS has two subunits, which act in concert to produce NO• and citrulline from L-arginine and O2. Upon reaction with oxygen radicals produced by the NADPH oxidase, NO is converted to reactive nitrogen intermediates (RNI), which are very toxic to microbes in the phagosome, and can damage DNA, lipids and proteins (Flannagan *et al.,* 2009). The importance of RNI in protection against microbial infection in mouse macrophages of generating the peroxynitrite and killing peptides, the defensin and cathelicidin when induced for antimicrobial activity by combination of T-cell derived cytokine and TNF-α. (Yang *et al.,* 2009; Jozefowski & Marcinkiewicz 2010) have been documented experimentally and in human macrophage infection as well (Schon *et al.,* 2004).

Superoxide anion produced in the phagocytes are to sterilize the bacteria, but these anions are not strong enough to kill tuberculosis bacilli because of strong catalase activity and superoxide dismutase (SOD) action of the bacilli, through which the bacilli directly scavenge oxygen radicals (Casbon *et al.,* 2012). The significance of these toxic nitrogen oxides in host defense against *M. tuberculosis* has been well documented both in vitro and in vivo (Benoit *et al.,* 2008; Pawlowski *et al.,* 2012). In genetically altered

INOS gene knockout mice, *M. tuberculosis* replicates much faster than in wild type animals, thus implying the significant role of NO in mycobacterial host defense (MacMicking *et al.*, 1997).

The role of RNI in human infection has been widely discussed and it differs from that of mice. 1, 25-dihydroxy vitamin D3(1,25-$(OH)_2D_3$) was reported to induce the expression of the NOS2 and *M. tuberculosis* anti inhibitory activity in the human HL-60 macrophage –like cell line (Liu and Modlin, 2008*).* This observation thus identifies NO and related RNI as the putative antimycobacterial effectors produced by human macrophages. This fact was further supported by another study in which IFN-Y stimulated human macrophages co-cultured with lymphocytes (*M. tuberculosis* lysate/IFN-Y primed) exhibited mycobactericidal activity concomitant with the expression of NOS2 *(*Bikle, 2008*).*

2.4.4.4 Macrophage Apoptosis:

Another potential mechanism involved in macrophage defense against *M. tuberculosis* is apoptosis or programmed cell death. Apoptosis was found in TB infection to be induced in a dose dependent fashion in BAL cells recovered from patients with TB, especially in macrophages from HIV infected patients (Placido *et al.*, 1997*).* Within the granuloma, apoptosis is prominent in the epitheloid cells. This is demonstrated by condensed chromatin as viewed by light microscope (Keane *et al*., 1997). The macrophage apoptosis results in reduced viability of mycobacteria and it leads to the formation of vesicles containing mycobacterial antigens. Dendritic cells in the vicinity of such apoptotic macrophages engulf the vesicles and present the antigens in the context of MHC-11, MHC-1 and CD1 in a highly efficacious way *(*Schaible *et al.*,2003; Winau *et al.*, 2006*)* This process termed cross priming probably plays a major role in T cell stimulation during *M. tuberculosis* infection..

2.4.5 TNF-α Activity:

IFN-Y and TNF-α are mainly produced from dendritic cells and activated macrophages (Fig 2.7) (Roach *et al.,* 2002; Kozo Yasui 2014). One of the major functions of TNF-α is the recruitment of monocytes and circulating antigen specific T-lymphocytes to the site of TB infection. TNF-α directs leukocyte movement, including its action on the vascular endothelium (intracellular adhesion molecule) and on the establishment of chemochine gradients (Roach *et al.,* 2002; Stenger, 2005). TNF-α additionally activates CD8+ cytotoxic T-cells (CTLs) that may be important because these release granulysin and directly kill intracellular bacteria (Stenger, 2005). TNF-α also promotes the maturation of monocytes to dendritic cells and or macrophages inducing the antigen presentation of intracellular mycobacteria. TNF-α produced in a local infection site allows macrophages, natural killer (NK) cells and γδ T-cells gather at the infection site and bring their activation. The activated CTL cells have the ability to produce perforin protein and TNF-α by itself, which guide TB-infected monocytes to apoptosis, which involves intracellular living TB bacilli and to induce the autophagy of infected cells via activated macrophages (Gautam *et al.,* 2014).

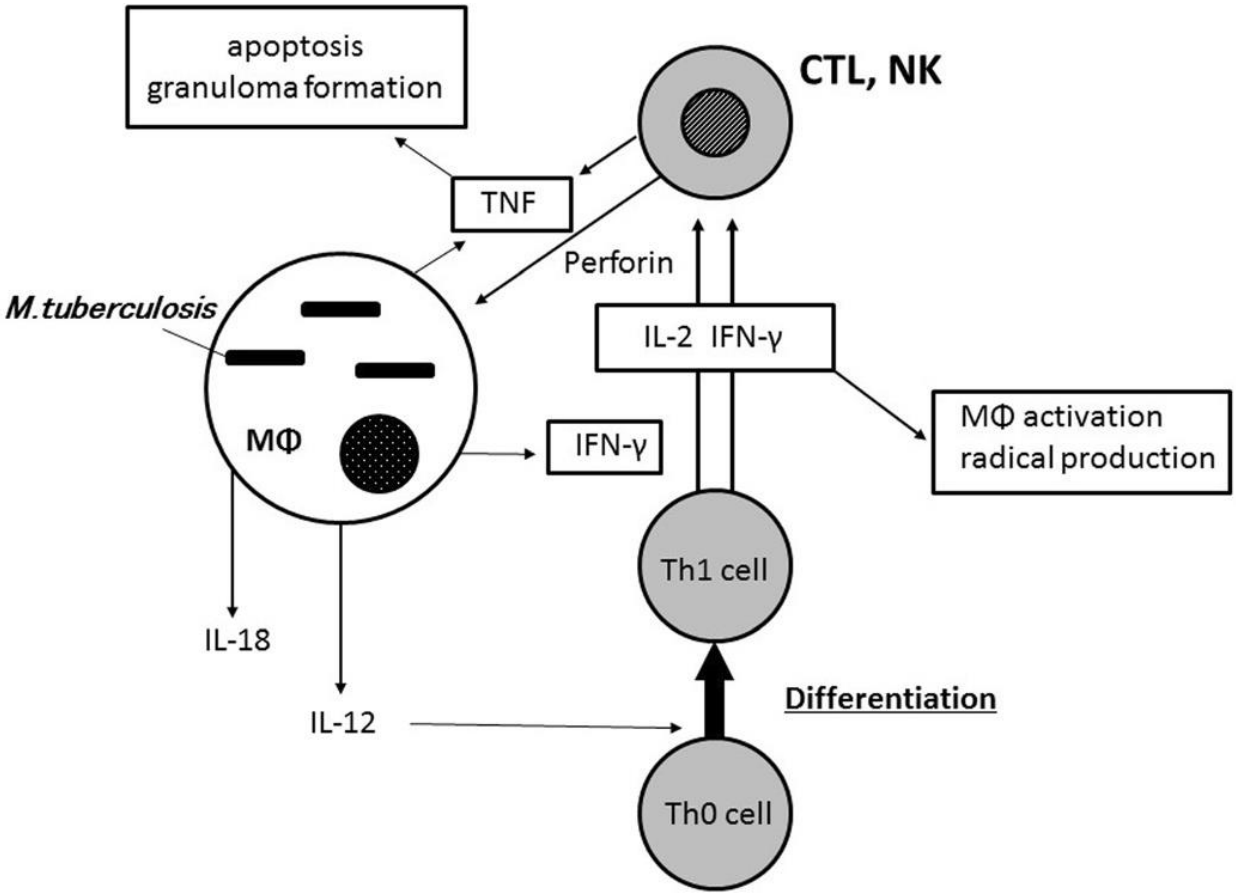

Fig 2.7. The network of Innate and Adaptive Immunity.

Source: Yasui, 2014

2.4.6 Dendritic Cells:

Priming of naïve T lymphocytes against mycobacterial antigens is thought to occur in the proximal draining lymph nodes and to rely on a particular subset of phagocytic cells, the **dendritic cells (DCs).** Dendritic cells have the unique ability to activate naïve lymphocytes after their migration from infectious sites. They capture antigens and take them to the lymph nodes, where they will express high amount of presentation molecules such as MHC-1 or 2 as well as co-stimulatory molecules, such as CD80 and CD86 (Mellman and Steinman, 2001*)* and soluble factors such as IL-12, IL-18, or IL-23. Dendritic cells are present in the epithelial layers including the alveolar spaces in the lung, where they create a tight surveillance network around the airways, the vessels, and loose connective tissues (Uehira *et al.,* 2002). It has been shown that *M. tuberculosis* enters dendritic cells after binding to lectin DC specific inter-cellular adhesion molecular -3 grabbing non-integrin (DC-SIGN). By contrast complement receptor 3 (CR 3) and the mannose receptors, which are the main *M. tuberculosis* receptors on macrophages play a minor role if any in mycobacterial binding to DCs (Tailleux *et al.,* 2003*)*. The mycobacterial specific lipoglycan lipoarabinomannan (LAM) is identified as a key ligand of DC-SIGN. Also it appears that huma n immunodeficiency virus (HIV) is captured by the same receptor DC-SIGN (Geijtenbeek *et al*., 2000*)*, allowing the entry of both HIV and *M. tuberculosis* in DCs in vivo. This is likely to influence bacterial persistence and compromise host immunity against *M. tuberculosis* (*T*ailleux *et al.,* 2003*)*. Therefore, DC-SIGN might account for several pathological and immunological aspects of *M. tuberculosis* infection in subjects co-infected with HIV and unable to provide an adequate immune response, which leads to enhanced incidence of mediastinal adenitis and disseminated tuberculosis (Nicod, 2007; Gringhuis *et al.,* 2009). Toll-like Receptor-2 (TLR-2) has been shown to play an important role, as it is able to mature DC myeloid precursors into competent antigen –presenting cells expressing CD 1 (Cluster of Difference) proteins. Mycobacteria were shown to provide two signals for the activation of lipid reactive T cell

receptors, and the lipoid adjuvant that activates antigen presenting cells (APCs) through TLR-2 (Roura-Mir *et al*., 2005).

After their priming in lymph nodes, memory CD4+ and CD8+T cells become central components of the acquired immune system and are therefore the basis for successful immunity/vaccination. The need for DCs to prime CD4+ and CD8+ T cell response following *M. tuberculosis* infection was confirmed using a selective depletion of DCs in an animal experiment (Tian *et al.*, 2005)

2.4.7 Evasion of host Immune response by *M. tuberculosis*:

M. tuberculosis is equipped with numerous immune evasion strategies, including modulation of antigen presentation to avoid elimination by T-cells. These are from a gene family occupying 2% of the mycobacterial genome that is required for synthesis of complex lipids that play both structural and immunomodulatory roles in *M. tuberculosis* (Nicod, 2007). These mycobacterial components such as sulphatides, Lipoarabinomannan(LAM) and phenolic –glycolipid 1 (PGL-I) are potent oxygen radical scavengers (Briken *et al.,* 2004). The glycolipids are well known stimulators of immune-suppressive mechanisms with mannose–capped LAM being of particular importance. The LAM molecule is known to stimulate suppressive mechanisms by signaling through DC-SIGN (Geijtenbeek *et al.,* 2003; van Kooyk and Rabinovich, 2008) By this mechanism *M. tuberculosis* infected macrophages appear to be diminished in their ability to present antigens to CD 4+ T-cells, which leads to persistent infection (Geijtenbeek *et al.,* 2003*).* Also proteins secreted by *M. tuberculosis* such as superoxide dismutase and catalase are antagonistic to Reactive Oxygen Intermediates (Casbon *et al.,* 2012). These affect the phagocytic capability of macrophages in containing *M. tuberculosis.* When macrophages become foamy under this condition, the bacteria become persistent and thus contribute to tissue pathology that leads to cavitation and release of infectious bacilli (Russelli, 2009).

Another mechanism by which antigen presenting cells (APCs) contribute to defective T cell proliferation and function is by the production of cytokines, including TGF-β, IL-10 (Rojas *et al.,* 1999) or IL-6 (van Heyningen *et al.,* 1997). These are immune suppressive in their activities.

2.4.8 Immune response to HIV infection.

HIV-1 persists as chronic infection even though the virus elicits strong innate and adaptive cellular and humoral immunity. The explanation to this may be linked to virus genomic integration and subsequent cellular latency as well as extreme genetic variability which translate into constant immune escape. HIV specific CD8+ T-cells play key role in the initial reduction of vireamia during acute infection, but become increasingly dysfunctional and exhausted under conditions of chronic antigen persistence (Hess *et al.,* 2004; Streeck *et al*, 2008). Virus neutralizing antibodies are also produced but are frequently accompanied by immune escape (Euler *et al.,* 2010).

The hallmark of HIV infection is the depletion of CD4+ T-cells. During the primary HIV infection, the cells that are preferentially depleted are the effector memory CD4+ T-cells in the gut mucosa (Brenchley & Douek, 2008). These immunopathogenic features together with the systemic and chronic state of immune activation including accelerated T-cell turnover are thought to contribute to progression of HIV disease (Moir *et al.,* 2011). This constant antigenic stimulation is characterized by a dysfunctional T-cell population displaying loss of functional potential i.e cytokine production and cytotoxic activity and proliferative ability in response to antigen stimulation. In addition, the loss of immune balance between Th17 and regulatory T-cells (T-reg) during HIV disease progression has recently been implicated in permeability of gut integrity and the pathogenesis of HIV (Kanwar *et al.,* 2010). Microbial translocation caused by gut permeability has also been suggested to contribute to systemic immune activation observed during chronic HIV infection (Brenchley *et al.,* 2006).

2.4.8.1 Reactivation of Tuberculosis by HIV

The rate of progression from infection to disease varies greatly with immune status of the infected host. Approximately 10% of *M. tuberculosis* infected individuals are thought to develop overt clinical disease (Aaron *et al.,* 2004; Pawlowski *et al.,* 2012). The depletion of CD4+ T-cells in HIV/AIDS is an important contributor to the increased risk of reactivation of latent TB and susceptibility to the new *M. tuberculosis* infection. There is also some evidence that CD8+T-cells play a role in the control of latent TB (Lewinsohn *et al.,* 2007; Chen *et al.,* 2009). Other mechanism reported to enhance *M. tuberculosis* infection and disease in individuals with HIV are up regulation of *M. tuberculosis* entry receptors on macrophages (Rosas-Taraco *et al.,* 2006), HIV manipulation of macrophage bactericidal pathways, deregulated chemotaxis and a tipped Th1/Th2 balance (Pawlowski *et al.,* 2012).

It has been shown that HIV impairs tumor necrosis factor (TNF) which mediated macrophage apoptotic response to *M. tuberculosis* and this enhances bacterial survival (Patel *et al.,* 2007). In the latent phase of tuberculosis, the bacteria are not completely eradicated despite the Th1 immune response. A failure or alteration of the quality of levels of the protective adaptive immune response or of the involvement with innate immune responses leads to reactivation of *M. tuberculosis* infection.

2.4.8.2 Exacerbation of HIV infection by *M. tuberculosis.*

The incidence and mortality rates of new AIDS opportunistic infections have been shown to be higher in individuals with HIV that are co-infected with TB (Narain and Lo, 2004).The function of many immune cells including macrophages and dendritic cells is modulated by both HIV and *M. tuberculosis*. *M. tuberculosis* has been reported to up regulate HIV-1replication in chronically or acutely infected T-cells or macrophages as well as ex-vivo in alveolar macrophages and lymphocytes from patients with HIV (Pawlowski *et al.,* 2012).

The primary target for *M. tuberculosis*, which is alveolar macrophages, can also be infected with HIV.

Mycobacterium exacerbates HIV replication in macrophages and lung cells obtained from co-infected individuals (Orenstein *et al.,* 1997, Abbas *et al.,* 2012). Monocytes from HIV positive patients display an impaired response to TLR ligands (Jiang *et al.,* 2005) and viral proteins can also interfere with both monocytes derived macrophages and dendritic cell maturation and function in-vitro, including their ability to phagocytose mycobacteria and kill intracellular bacteria(Sacchi *et al.,* 2007).

TNF production in response to *M. tuberculosis* infection is required for control of bacterial growth, but TNF is also known to activate HIV replication in macrophages (Kedzieska *et al.,* 2003), this is indicating that the host immune response initiated against one pathogen may promote the replication of another. Thus both HIV and *M. tuberculosis* stimulate TNF release from infected cells and TNF inhibit bacterial growth while enhancing HIV replication. *M. tuberculosis* survives in dendritic cells (DC) and actively down regulates their pro-inflammatory activity and antigen presenting function with concurrent induction of anti-inflammatory cytokines (Jiao *et al.,* 2002). Similarly, HIV can infect and also manipulate DCs and compromise T-cell functions (Donaghy *et al.,* 2004). In HIV infection, not only is DC mediated activation of T-cells impaired, but the migration of infected DCs can also contribute to pathogen distribution especially during antigen presentation when they do infect naïve T-cells by virtue of their close contact with the T-cells (Abbas *et al.,* 2012).

The DC-expressed C-type lectin receptor DC-SIGN (DC specific intercellular –adhesion molecule 3 grabbing non-integrin) has been suggested to facilitate transmission and immune escape of both *M. tuberculosis* and HIV (van Kooyk *et al.,* 2003). HIV attaches to DC-SIGN through interaction with the viral envelope glycoprotein gp120 and this interaction is thought to contribute to efficient spread and transmission of the virus to CD4+ T-cells (Kwon *et al.,* 2002). *M. tuberculosis* has been reported to target DC-SIGN by a mechanism that is distinct from that of HIV leading to inhibition of pro-inflammatory IL-12 and TNF production and induction of IL-10 by DCs (Geijtenbeek *et al.,* 2003) and hence down regulation of protective immune responses.

2.5 TB CASE DETECTION

Fast and accurate diagnosis of tuberculosis is a key factor of global health measure to control the disease. The gold standard remains clinical examination combined with direct microscopic examination of sputum and culture of mycobacteria. However the mandate of WHO on diagnosis is to detect 70% of active infectious TB cases and treat 85% of this 70% case detection. This may be difficult for Nigeria to attain with the present method of smear microscopy and culture employed in the country. Presently, Nigeria TB case detection rate is 51% with effective treatment rate of 83% (WHO, 2013).

For Nigeria to improve on its record, there is need to revisit the present methods and adopt new techniques to attain the WHO mandate. Details of present techniques used for TB case detection are described below.

2.5.1 TB Case detection by Sputum Smear Examination and Microscopy

The Ziehl -Neelsen (ZN) smear microscopy:

This is in common use to diagnose TB and was used by Robert Kosch 125 years ago. It is widely used in Nigeria. The test requires the submission of 3 samples collected over two days by a patient suspected of having pulmonary TB. The test is simple, economical and reproducible in any laboratory, but it fails to identify about 50% of TB cases (since it requires about 10,000 bacilli/ml of sample), who are likely to have smear negative TB.

The current guidelines of the World Health Organization (WHO, 2012) and the International Union Against Tuberculosis and Lung Disease (The Union) (Reider *et al.*, 2007) specify that the essential step in the investigation of patients who are suspected of having pulmonary tuberculosis should be the microscopic examination of their sputum samples. Standard 2 of the International Standards for Tuberculosis Care categorically states that all patients (adults, adolescents, and children who are capable of producing sputum) suspected of having pulmonary tuberculosis should have at least two, and

preferably three, sputum specimens obtained for microscopic examination (International Standard for TB care 2013; WHO, 2013). However, in the current era of molecular diagnostics, the role of sputum smear microscopy should be redefined. It is important to consider the role of smear microscopy, particularly in view of the recent WHO endorsement of the new rapid, automated nucleic acid amplification test, Xpert MTB/RIF (Reider *et al.*, 2007).

Sputum smear microscopy has been the primary method for diagnosis of pulmonary tuberculosis in low and middle income countries (International Standard for TB care, 2013) which is where nearly 95 per cent of TB cases and 98 per cent of deaths due to TB occur. It is a simple, rapid and inexpensive technique which is highly specific in areas with a very high prevalence of tuberculosis (Fig 2.8) (Reider *et al.,* 2007; Escalante 2009). It also identifies the most infectious patients and is widely applicable in various populations with different socio-economic levels (Reider *et al.*, 2007; WHO, 2012). Hence, it has been an integral part of the global strategy for TB control. However, sputum smear microscopy has significant limitations in its performance. The sensitivity is grossly compromised when the bacterial load is less than 10,000 organisms/ml sputum sample. It also has a poor track record in extra-pulmonary tuberculosis, paediatric tuberculosis and in patients co-infected with HIV and tuberculosis (Luemo, 2004). Due to the requirement of serial sputum examinations, some patients who do not come back for repeated sputum examinations become "diagnostic defaulters'' (Harries, 1998). Some do not come back for results, and are lost to treatment and follow up. A personal observation showed that limited resources, large numbers of samples, all combined together often reduce the observation time per slide to less than 60 seconds and this also contributes to reduction in the sensitivity of the test. Therefore, techniques for optimization of smear microscopy are under active investigation. There has been an attempt to reduce diagnostic defaulting by assessing the feasibility of diagnosing pulmonary tuberculosis by collecting two sputum samples on a single day (1-day protocol), and comparing this protocol with the national policy of collecting samples on consecutive days (2-day protocol). It was felt that since the 2-day protocol did not show a statistically

significant difference in diagnostic performance compared with the 1-day protocol, the latter may be adopted as an alternative protocol, particularly for patients who are more likely to default (Shingadia and Novelli, 2003).

Fluorescence microscopy was introduced in the 1930s, in an attempt to improve outcomes of smear microscopy. Fluorochrome dyes are used to stain the smear. A halogen or high-pressure mercury vapour lamp is traditionally used to excite the dye, and make it fluoresce. A meta analysis of studies comparing fluorescent and conventional microscopy found that the sensitivity of fluorescent microscopy was 10 per cent higher than that of conventional microscopy, and that it remains high even after concentration of the samples (Reider *et al.,* 2007). Sensitivity was found to be higher particularly in low grade smear positive sputum. Specificity estimates, however, were similar to conventional microscopy, though turnaround times were shorter. This meta- analysis concluded that the successful and widespread implementation of fluorescence microscopy might be expected to improve case finding through an expected increase in sensitivity and decrease in time spent on microscopic examination. Although fluorescence microscopy increases the sensitivity of sputum smear microscopy, additional data on specificity and on the clinical consequences associated with false-positive results are needed to guide implementation of this technology in high HIV prevalence settings (Rawat *et al.,* 2012). Cost constraints are major issues with fluorescent microscopy. This may be circumvented by the use of light-emitting diodes (LEDs) which cost less than 10 per cent of a mercury vapour lamp (Rawat *et al.,* 2010). Despite these qualities, all positive readings must still be confirmed by ZN stain, since the ZN stain allows improved visualization of certain details of bacterial morphology that can provide clues to the identity of mycobacterium responsible for the disease. This test, as laudable as it is, has produced false positives and false negatives.

Front loaded smear Microscopy:

In 2007, WHO recommended that countries with well functioning facilities with good quality assurance could reduce the number of samples collected from patient from three to two bacillary load sputum

samples which would be sufficient to diagnose a case of TB. The front-loaded smear microscopy technique takes the second sputum sample one hour after the first, this is eliminating drop out from the need for the second visit by the patient.

WHO through preliminary studies provided evidences to validate the technique in a study of 923 symptomatic patients with chronic cough. The result of TB detection using this technique compared with the conventional method was similar. Significant advantage of same day result for persons with TB symptoms is preventing drop outs from diagnosis schedule. (Lei Chou *et al.,* 2009).

Factors predisposing to false positive include:

1. Staining acid fast particles other than tubercle bacilli which may include food particles, organic materials, micro-organism and artifacts.

2. Smear contamination through transfer of bacilli from one smear to another.

Factors predisposing to false negative include:

1. Poor quality of sputum sample.

2. Improper examination of the smear-fault of examiner. However several physical & chemical sputum processing methods including centrifugation, sedimentation and bleaching have been developed to improve sensitivity of smear microscopy and have been found to be working (Steingart *et al.,* 2006). Further studies have shown the possibility of reducing the number of days for samples collected (Hiraol *et al.,* 2007, Mase *et al.,* 2007*).*

Fig 2.8. *M. tuberculosis* (stained red) in sputum

Source: Escalante 2009.

2.5.2 TB Case detection by culture

2.5.2.1 *Solid Media Culture (Lowenstein-Jensen Media)*

Culturing of *M. tuberculosis* from clinical samples in solid media is the gold standard for detecting active TB. This method can detect limit of 100 bacilli organisms per ml as compared to that of 5000 – 10,000 bacilli organisms per ml needed for smear microscopy. It also makes it possible to identify the mycobacterial species on the basis of biochemical and other properties. The method involves growing the bacteria on media that provided nutrients, usually the solid Lowenstein – Jensen (L J) media. The culture is 100% sensitive. Due to its higher sensitivity, *Mycobacterium tuberculosis* culture is more susceptible to reduced specificity as a result of contamination and failure of the bacilli to grow (Kanwar, 2012). *M. tuberculosis* grows very slowly and this makes it to take between 4 to 6 weeks for the organism to grow sufficiently to be identified (Adler *et al.*, 2005).

In Nigeria, tertiary centers operate the culture method as well as the drug susceptibility testing (DST). But the length of time it takes to culture mycobacteria on solid media led to the development of the liquid media with its various modifications.

Liquid culture system offers the advantage of increased sensitivity over solid media especially faster detection of bacterial growth, reducing the time of result to 2-3 weeks. The yield of culture may increase by 10% and also reduce delays of result to about 10 days compared to 28-42 days with conventional solid media, but the liquid systems have been associated with increased contamination rate, difficulty in identifying mixed cultures and the inability to observe colony morphology.

There are 2 types of liquid culture media systems developed. These are 1. Radiometric (Bactec 460TB), with its modifications 2. Non-radiometric (MGIT, ESP, MB /Bast.)

2.5.2.2 Radiometric methods Liquid culture media:

Bactec TB – 460 (Becton Dickinson, Sparks, MD. USA) provides the most useful diagnostic advancement in clinical microbiology for reference laboratories.

The technique automatically detects mycobacterial growth, measuring the amount of Co_2 generated by the metabolisation of 14c – radiolabelled substrate (fatty acids). The vials used contain 4ml of Middlebrook 7HI2 medium and the radio labeled fatty acids, admitting inocula of about 0.4ml. The Bactec method compared with the solid culture system by saving time (15-20 days) of detecting bacterial growth, it increases sensitivity for detecting *M. tuberculosis* as well as for identifying *M. tuberculosis* in 4 to 5 days. It is possible to perform drug susceptibility testing for first-line drugs (isoniazid, rifampicin, ethambutol, streptomycin and pyrazinamide) in 3 to 6 days instead of 21 to 42 days for solid media.

The limitations of this method include the need to use radio isotopes, the high cost of equipment, reagents and maintenance, the need to use syringes that can cause possible cross-contamination between samples, as well as the potential formation of aerosols. It is also laborious, because a semi-auto mated system is used .Caminero- Luna, 2004).

2.5.2.3Non-radiometric Liquid Culture media.

This uses BACTEC 460 system that showed a sensitivity and specificity of 85.8% and 99.9% respectively in detecting mycobacteria. It involves automatic reading designed to resolve the disadvantages of the BACTEC system. Manipulation of the vials is simple because it is automatic and thereby saves more time. This has all the advantages of Bactec system and added to this is the benefit of continuous readings of mycobacterial growth. The limitation is the high cost which makes it unaffordable to the developing world (Caminero-Luna, 2004)

2.5.2.4 MGIT liquid culture.

This is Mycobacteria Growth Indicator Tube system (MGIT). It is based on a glass tube containing a modified Middlebrook 7H9 broth with a fluorescence quenching – based oxygen sensor embedded at the bottom of the tube. When inoculated with *M. tuberculosis,* consumption of the dissolved oxygen produces fluorescence when illuminated by a UV lamp.

More recently, the MGIT system has been fully automated and turned into the BACTEC MGIT 960 system, which is non radiometric, non invasive system with the tubes incubated in compact system that reads them automatically. A meta analysis of 10 studies, comparing the BACTEC 960/MGIT and BACTEC 460 systems showed a sensitivity and specificity in detecting mycobacteria (1,381 strains from 14,745 clinical specimens) of 81.5 and 99.6% and 85.8 and 99.9% respectively. When combined with solid media, the sensitivity of the two systems increased to 87.7% and 89.7% respectively (Cruciani *et al.,* 2004).

2.5.3 Molecular Method of TB Case Detection

2.5.3.1 Nucleic Acid Amplification Test. (NAAT).

Genetic probes are tests that allow the identification of DNA or RNA sequence specific to each mycobacterial species. An example of this method is the Accuprobe (Gene-probe inc. Sandrego CA. USA), a commercial method based on species-specific DNA probes that hybridize to rRNA for the identification of important mycobacteria, including the *M. tuberculosis,* complex, *M. kansasii*, and *M. gordonae*. Result is given in about 2hours from culture positive specimens. Found to be sensitive and specific (Caminero-Luna, 2004).

New techniques are now being made available to optimize the culture system as highlighted in the remodification of culture (Singh *et al.,* 2013). Various steps of culture were optimized in the requirement

and monitored to effect the change observed. First step is the optimization of culture medium. Blood medium was used and among the compounds added to the blood at each time and at varying concentration, egg lecithin was found to promote mycobacterial growth (Drancourt & Raoult, 2007). Second step is the strict and effective control of oxygen tension which has been found to have critical influence on intracellular bacterial growth and optimize the cultures. An improved growth was found in the solid medium under a microaerophilic atmosphere (2.5-5% O_2, 2.5-5% O_2 plus 5% CO_2) compared to normal oxygen tension. The primary cultures were detected one day earlier under the microaerophilic condition than under normal atmosphere (Singh *et al.,* 2013). The third step is the early detection of growth. The detection of microcolonies has been found to save time for diagnosis (Leung *et al.,* 2012). Use of naked eye, binocular microscope and autofluoresence to evaluate the number of mycobacteria per colony by cytometer. The analysis showed the rough hemispheric form and structure of a 90 um microscopy. Fourth step is the use of matrix-assisted laser desorption/ionization time of flight mass spectrometry for identification (Elkhechine *et al.,* 2009). These steps were experimentally investigated to obtain the optimal culture conditions for antibiotic susceptibility testing which yielded results in less than 7 days. Combining an optimal medium, optimized incubation conditions and powerful detecting tools, a primo-isolation from clinical sputum was obtained in 4.75±1.3 days compared to 12.55±4.6 days with Bactec MGIT ($P<0.05$) (Ghodbane *et al.,* 2014). Findings from these investigations showed that *M. tuberculosis* culture could progress quickly from isolating clinical specimen to antibiotic testing using the modification of current methods.

2.5.3.2Polymerase Chain Reaction (PCR):

This is used for nucleic acid amplification. It is used for species identification. The rational for using PCR is to (a) diagnose TB rapidly by identifying DNA from *M. tuberculosis* in clinical sample that are negative by microscopic examination.(b) identify the species of mycobacteria (c) identify the genetic modification

that is associated with resistance to antimycobacterial agents. Sensitivity is low (56.8%) and the specificity is high (80 to 100%) (Gomez Pastrana *et al*., 1999, Montenegro *et al*., 2003*)*. The PCR uses the sputum material and this is obtainable from adults but for the pediatric age group, this might be difficult. Also studies have shown variable sensitivity (Daphne *et al*., 2008). Limitations are variability of results from different laboratories, high cost and in ability to identify species within the *M. tuberculosis* complex.

2.5.3.3 HAIN Assay:

The GenoType Mycobacterium assay (Hain Diagnostikia, Nehren Germany) is for *M. tuberculosis* identification from clinical samples and liquid cultures . It allows a specific MTB diagnosis together with detection of rifampicin and isoniazid resistance strains achieved by PCR amplification of the 16S-23S ribosomal DNA spacer region. This is followed by hybridisation of the amplified DNA product with specific oligonucleotide probes. It has the advantage of detecting mixed mycobacterial infections. In a study of sputum samples in South Africa, the Genotype MTB-DR plus assay demonstrated excellent sensitivity and specificity for the detection of MDR-TB (Barnard. *et al*., 2008). A meta-analysis showed that the HAIN Assay demonstrates excellent sensitivity for rifampicin though variable sensitivity for isoniazid. The test showed very high sensitivity (Ling *et al.*, 2008).

2.5.4 TB Case detection by radiography.

Subject radiography and mass miniature radiography (MMR) are used for detecting TB in an individual or an entire community. But radiological shadows are not specific, neither any radiographic pattern to the diagnosis of TB. Many of the diseases of the lump show similar radiographic appearance and can easily mimic tuberculosis. (American Thoracic Society, 2000).

Limitations of this method include observer error which is mostly subjective. The ability to detect abnormal opacities and interpret them correctly vary with readers (inter observer variation and also between viewings of the same film by a single observer)

Level of Disagreement on the Interpretation of Chest Radiographs and Conclusions*:*

Areas of disagreement on the interpretation of chest radiographs have been identified by International Union Against Tuberculosis. These include:

- Abnormality in lymph nodes
- Abnormality in the lung, probably tuberculosis
- Checking if the film used is normal
- Need for medical action
- Presence of any cavity

These areas were selected with a view of using them for classification of radiographic findings (*Kioppaka and Bock*, 2004). Co-Infection of TB with HIV may further diminish the reliability of chest radiography for the diagnosis of pulmonary tuberculosis.

Since the emergence of HIV/AIDS, clinical studies have consistently documented the typical radiographic pattern seen in patients with both pulmonary middle or lower lung field infiltrates. The absence of pulmonary infiltrates and cavities are common in such patients, as are in normal and minimally abnormal chest radiographs (Greenberg *et al.,* 1994). In conclusion – the experience of many decades or detailed data collection and analysis indicate that chest radiography for diagnosis or follow-up of pulmonary tuberculosis cases with or without HIV co-infection is unreliable (Tomans, 2004b).

2.5.5 TB Case detection by Immunological techniques.

2.5.5.1 Tuberculosis Skin Test (TST) in TB case detection:

Tuberculin is a purified protein Derivative (PPD) of *M. tuberculosis* in a glycerin-broth culture evaporated to $1/10^{th}$ volume at $100^{o}C$ and filtered. This was introduced by Robert Koch over 90 years ago. This is still being used for skin test in the screening of tuberculosis. It is measured in tuberculin units and to perform the skin test, 5 tuberculin units is used. This standard dose contains 0.1µg/0.1ml dose of PPD. It is injected intradermally into either the volar or dorsal surfaces of the forearm.

Following correct administration of PPD, the injection site is examined 48-72hrs. Typical reaction may begin 5-6hrs as a lump and may maximize at 48-72hrs and may gradually wane over a period of days. Reading is performed in good light by palpating the area of induration. The diameter of induration is measured transversely to the long axis of the forearm. Erythema or bruising is disregarded. If induration is 10mm or more, the test is positive in a person at risk of TB infection.(Tomans, 2004c)

A positive TST may indicate any of the following (1) Exposure and infection with *M. tuberculosis* (True positive) (2) Exposure and sensitization of the immune system to non tuberculosis mycobacteria. Vast majority of antigenic protein present in PPD prepared from *M. tuberculosis* are present in extracts from other atypical mycobacteria common in the environment (false positive). (3) BCG (Bacillus Calmette – Guerin) vaccination in the past (false positive) (4) false positive response that can be due to incorrect measurement of induration or over reaction in some disease conditions such as psoariasis (Tsiouri *et al*, 2009). (5) TST can be negative in the immunocompromised due to lack of immunocompetence (Richeldi *et al.*, 2009)

The TST is measuring the induction of induration as a result of delayed hypersensitivity reactions after intradermal injection of purified protein derivative (PPD). However, this test has significant limitations because the PPD used for the TST is a crude precipitate of filtered *M. tuberculosis* that contains more than 200 antigens widely shared among environmental mycobacteria (Wang *et al.*, 2002). In patients with

active TB, TST is 75% to 90% sensitive, but among those with disseminated disease, the sensitivity falls to 50% (Heubner *et al.,* 1993). The TST sensitivity is low in immunocompromised patients such as in HIV positive patients following varying degree of immune suppression (Stavri *et al.,* 2009). A major drawback to TST is that a positive tuberculin skin test does not discriminate between latent and active TB, hence limited application in HIV / TB global control. It has been suggested that TST, when combined with direct smear examination (AFB microscopy) can allow an immediate diagnosis of TB infections. The major draw backs in this regard are the specificity of the test and the fact that 10 to 25% of TB patients do not react to PPD thereby decreasing test specificity to less than 50% in patients with advance disease (Cobelens *et al.,* 2006).

2.5.5.2 T-cell Interferon Gamma Release Assay for TB detection:

T-cell Interferon Gamma Release Assays (IGRAs) have been developed as an alternative immunodiagnostic approach to the TST for detecting *M. tuberculosis* infection. The history of this assay stemmed from the use of PPD in-vitro in whole blood stimulation using ELISA method to detect specific IFN-γ secreting lymphocytes sensitized by mycobacterial antigens. This results in IFN-γ production from healthy individuals not vaccinated with BCG correlating with TST (Pottumarthy *et al.,* 1999; Mori *et al*., 2004) and detecting more positives among intravenous drug users than TST (Converse *et al.,* 1997). It is however not specific for TB diagnosis (Andersen, *et al.,* 2000).

The development of IGRA also stemmed from the advances made in mycobacterial genomics which identified a genomic segment (Region of Difference 1) that is deleted from all strains of BCG (Mahairas *et al.,* 1996) and the majority of environmental mycobacteria (except *M. kansasi*; *M. szulgai*, *M. marinum*, *M. flavescens*, and *M. gastrii*) (Harboe *et al.,* 1996). Two antigens encoded by this segment Early Secretory Antigen Target 6(ESAT-6) and Culture Filtrate Protein 10 (CFP-10) are strong targets of Th1 T-cells in *M. tuberculosis* infection. Their ability to elicit strong specific T-cell responses reduces the

frequency of false positive TST results in individuals who have previously received BCG vaccination (Lavani., 2007;Lavani & Millington, 2008). These two antigens ESAT-6 and CFP-10 are considered in this study to be useful as tools for immunodetection of *M .tuberculosis*.

2.5.5.3 Studies with Antigen Mixtures of ESAT-6 and CFP-10:

Combining tuberculosis specific antigens into antigen mixtures to increase diagnostic performance is a practice that has become realistic option. The goal is to attain higher sensitivity than achieved with single antigens without jeopardizing specificity (Andersen *et al.,* 2000). In two studies conducted, combinations of CFP-10 and ESAT-6 antigens increased the sensitivity of the assay over that provided by the individual antigens by about 10% in both cases (up to 73% and 84% respectively) without jeopardizing specificity (van Pinxteren *et al.,* 2000). The modified antigen mixture is being produced commercially as QuantiFERON GOLD TEST (QFT-Gold) Immmunotest kit. It is produced by Cellestis incorporation in Australia. It is approved for use in some parts of the world such as Centre for Disease Control (CDC) in United States of America. A modification of this test kit is QFT Gold in a test tube (QFT GIT). This contains coating of *M. tuberculosis* antigen mixture in a test tube. The blood sample is collected in the tube and is processed for antigen stimulation by adding the antigen mixture of ESAT-6 and CFP-10 in equal ratio and then incubate for 16 to 24 hours at 37^{o}C in a humidified Co_2 incubator. The tubes need to be incubated within 16 hours of blood collection. The presence of IFN-γ produced resulting from antigen stimulation of T-lymphocytes in the blood is measured by an ELISA method. The sensitivity and specificity claims of this test are 89% and 99% respectively. It is being proffered as alternative to TST, but the rate of agreement between QFT and TST in studies published are varying (Menzies *et al*., 2007, Connell *et al*., 2006, Rangaka *et al.,* 2007, Madhukar *et al.,* 2008). However, false negative result is now being reported using this Immuno test kit in patients with active tuberculosis especially in immune suppressed patients. Two reasons were likely attributed to this (1) decrease in IFN-γ produced due to

advanced patient age or lymphocytopenia and (2) lymphocytes inability to produce IFN-γ for *M. tuberculosis* specific antigen in young patients without underlying disease (Yoshihiro *et al*., 2009). The prospect of addressing the causes of these false negatives is being considered in this study. ESAT-6, CFP-10 and ELLI (new antigen product from LIONEX Inc. Germany) are being employed for the immune- detection exploration. The exploration is carried out in patients with active TB, HIV/TB, latent TB in TB contacts and TB asymptomatic HIV positives.

2.5.5.4 Clinical performance of interferon–gamma release assay in immunocompromised individuals.

Correctly identifying and treating Latent TB infection in high risk individuals with impaired cell mediated immunity is a concern for clinicians due to poor sensitivity of TST diagnostic tool that is commonly used. The groups that are at risk of progressing from latent TB infection to active TB disease are HIV-positive individuals and those on immunosuppressive drugs. Therefore to have alternative to TST there is need to evaluate the diagnostic performance of the IGRAs in these populations.

A number of studies have evaluated IGRA whole blood ELISA in HIV positive individuals of which data obtained seem variable. A study of individuals with active smear positive TB in Zambia compared IGRA whole blood ELISA and TST positivity in HIV positive and negative individuals. The result obtained revealed that the sensitivity of the IGRA whole blood ELISA in HIV positive individuals (63%) was significantly lower than in HIV negative individuals (84%) Raby *et al.*2008). In another cross sectional study of HIV infected individuals in a high prevalence setting, the ELISA result was found to have a lower rate of positivity than TST (Mandalakas *et al.,* 2008). Conversely, in a Chilean study of HIV positive adults, the ELISA had a higher positivity rate than the TST and significantly correlated to levels of TB exposure while TST did not (Balcells *et al.*, 2008). However these authors did not find the impact of CD4+ cell count on ELISA result (Balcells *et al.,* 2008), but other authors have found that in HIV

positive individuals with very low CD4+ cell count, the ELISA's performance is adversely affected as exemplified by a high proportion of negative and indeterminate results (Raby *et al.,* 2008; Matulis *et al.,* 2008). A significant association was found between low CD4+ cell count and indeterminate results (Brock *et al.,* 2006).

2.6 CULTURE MEDIA

2.6.1 Fetal Calf Serum

Background

Fetal Calf Serum comes from blood drawn from the unborn bovine fetus via a close system of venopuncture at the abattoir. Fetal Calf Serum is the most widely used serum due to being low in antibodies and containing more growth factors, allowing for versatility in many different applications such as in the culturing of eukaryotic cells. Fetal Calf Serum (FCS) is known to contain growth factors and cytokines necessary for cell division (Sasse *et al.,* 2000). It is used in many Culture media for successful culturing of cells (Cuchet *et al.,* 2005). Addition of FCS to certain fibroblastic cells results in activation of DNA synthesis by increasing the concentration of intracellular Ca^{++}. This observation clarifies the mechanism of growth activation (Bertsch & Marks 1974). It is also used in cell proliferation (Cuchet *et al.,* 2005, Naseer *et al.,* 2009). The rich variety of proteins in Fetal Calf Serum maintains Cultured Cells in a medium in which they can survive, grow and divide.

2.6.1.1 Application of Fetal Calf Serum

Fetal Calf Serum (FCS) is used in many Culture media for successful culturing (Cuchet *et al.,* 2005, Sagirkaya *et al.,* 2004). It is known to contain growth factors and cytokines necessary for cell division (Sasse *et al.,* 2000). Supplementation of FCS in developing embryos results in DNA Synthesis and cell proliferation (Cuchet *et al.,* 2005).

Addition of FCS to certain fibroblastic cells results in activation of DNA synthesis by increasing the concentration of intracellular Ca++. This observation clarifies the mechanisms of growth activation (Bertsch and Marks, 1974).

FCS contained growth factors necessary for cell division and is a strong inducer of cell proliferation (Naseer *et al.,* 2009).

Incubation of normal human peripheral blood lymphoid cells in Fetal Calf Serum supplemented media initiates blastogenic and non specific cytotoxic responses. FCS sensitized human lymphoid cells were active in cytotoxicity assay against a wide variety of a cultured human turnover and normal target cells. Cytotoxicity continued to increase from 3 to 9 days of incubation in fetal calf serum supplemented media, while blastogenic response peaked at 6 days of incubation (Zielske and Golub, 1976).

Dendritic Cells (DC) can be successfully generated by cultivating peripheral blood monocytes in FCS containing medium in the presence of granulocyte macrophage colony stimulating factor (GMCSF) and interleukin-4(Pietschmann *et al.,* 2000). This can be used for in vitro diagnosis, but clinical application of FCS is being avoided due to xenogenic protein complications. As a result of this, alternative protocols are human plasma instead of FCS.

2.6.2 RPMI-1640 MEDIUM

RPMI-1640 medium was developed by Moore et al at Rosewell Park Memorial Institute, hence the acronym RPMI. The formulation is based on the RPMI-1630 series of media utilizing a bicarbonate buffering system and alterations of the amount of amino acids and vitamins. RPMI-1640 medium has been used for the culture of human normal and neoplastic leucocytes (Moore *et al*., 1967). RPMI -1640, when properly supplemented has demonstrated wide applicability for supporting growth of many types of cell cultures, including fresh human lymphocytes in the 72-hour Phytohemagglutinin (PHA) stimulation assay (Moore and Woods, 1976).

These culture media are intended to be used as trial in improving the response of the lymphocytes to in-vitro stimulation during immune-detection of TB and they were selected relative to their properties and function

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

The study is a descriptive, case control prospective study involving subjects suspected of having active tuberculosis and those at risk of latent TB or asymptomatic tuberculosis with or without HIV infection. The control group comprised volunteers that were Industrial Training students and healthy workers of similar demographic characteristics.

3.1.1 Study Population

Subjects were recruited from Mainland General Hospital (MGH) and Nigerian Institute of Medical Research (NIMR) Yaba, Lagos. A non-probability method known as convenience sampling method and willingness to participate in the study were used to select the subjects.

3.1.2 Inclusion /Exclusion Criteria for Subject Selection*:*

Inclusion criteria were subjects suspected to have active TB with and without HIV, those with HIV but asymptomatic of TB and individuals at risk. This includes: 1) Subjects presenting with chronic cough, weight loss, fatigue and anaemia at MGH. 2) Individuals at risk comprising health professionals such as Doctors, nurses and laboratory workers at MGH Yaba Lagos and laboratory workers processing TB sputum samples at National reference laboratory at NIMR Yaba, Lagos and house hold contacts caring for TB patients at home. 3) Control group which comprised healthy volunteers: students on industrial training and staff of NIMR. . Excluded from the study were pregnant women and patients on immunosuppressive drugs.

3.2 Ethical Consideration

Ethical approval was obtained from NIMR-Research Ethics Committee. Subjects were taken through a study review and the informed consent process which included the type of investigation, the need for blood samples and, benefits of the study. Confidentiality was assured while enrollment was purely on consent. (**Appendix 1**-Ethical Approval)

3.3 Sample Size Estimation and Sampling Technique

The sample size was determined using the formula n = $n = \frac{(Z_\alpha + Z_\beta)^2 \times \{(p_1q_1)+(p_2q_2)\}}{(p_1 - p_2)^2}$

with a disease prevalence of 16.7% of HIV-TB (Jemikalajah & Okogun, 2009). (**Appendix -3**) Convenience sampling method was employed to select the subjects. The calculated sample size was 66 subjects and a total of 88 subjects were studied. The eighty eight subjects selected were grouped as follows: **Group 1** consists of 24 subjects suspected to have active TB without HIV, **Group 2** consists of 12 subjects suspected to have TB with HIV, **Group 3** consists of 19 HIV subjects with no symptoms of TB, **Group 4** consists of 17 individuals at risk and **Group 5** consists of 16 controls.

3.4 Socio-Demographic Information:

Profile of each subject was obtained through interview conducted using structured questionnaires that captured data on age, sex, socio-economic and household status.

Questionnaire Administration: This was used to collect information on demographic and social characteristic profile of tested subjects including patients that presented at the hospital. Relevant data were collected from 88 subjects with the aid of questionnaire. This data included demography (age, sex), socio-

economic status, household status, BCG vaccination, history of chest pain, coughing especially coughing up blood that will indicate tuberculosis infection. (**Appendix 4**)

3.5 Collection of sputum sample:

Sputum samples collected from subjects presenting with cough, weight loss, fatigue and anaemia, and individuals at risk were processed for smear microscopy and culture.

Sputum samples were collected from a) subjects presenting with cough, weight loss, fatigue and anaemia, b) individuals at risk.

Three samples were collected from each subject as the policy of the laboratory. The samples were of three types:

Spot Sample*:* This was collected on the spot from the subject when given the universal bottle. Each subject was trained to have deep gentle inbreathing three times before coughing the sputum into a sterile universal bottle. The mechanism is to activate and put pressure on inter-coastal muscles on the lungs to enable the subject to cough the quality sputum.

Early Morning Sample*:* The subject was given the universal bottle to take home and produce the sputum very early in the morning the same way he was trained to produce the spot sputum in the laboratory.

Spot Sample*:* When the same subject brought the sputum from home, he was given another universal bottle to produce the sputum the same way he produced the other two samples. The samples were collected and submitted to the bench to process for smear microscopy and culture.

The samples were processed for smear microscopy and culture.

3.5.1 Smear Preparation

Direct Smear Preparation using *Ziehl-Neelsen* staining technique was carried out on the samples as follows:

Clean greese free unscratched slides were selected for the smears of the samples. The wire loop was used to pick up the yellow purulent particles of the sputum and was spread on the glass slide evenly by smearing repeatedly in coil like patterns to a length of 2-3cm and 1-2cm width. The wire loop was heated in a flame until red hot after each smearing was completed.

Each smear was allowed to dry completely at room temperature and the slide was then passed over a flame 2 or 3 times for about 3 seconds.

3.5.2 Staining

The slide placed on the staining bridge was stained by covering the surface with filtered Ziehl-Carbol fuchsin solution. The slide was heated from underneath with a flame from an alcohol lamp, until steam rose from the stain. The slide was left for 5 minutes without allowing it to dry, the excess stain was drained off and the staining solution was washed off with gentle running water. Excess water was also drained off.

The slide was covered with 25% Sulphuric acid for 3 minutes to decolorize the stain. The slide was washed again with running water and then drained off the water.

3.5.2.1 Counter Staining and drying

Counter Staining was effected by covering the slides surface with 0.3% methylene blue and left for 30 seconds. Methylene blue was drained off and the slide was washed with gentle running water after which it was dried.

3.5.3 Microscopic Examination

Immersion oil was dropped on the stained smear and then examined under high power of 100 x objective with the 10x eye piece. A hundred visual fields were read to determine if the slide was positive or negative.

3.5.3.1 Acid Fast Bacilli (AFB) appearance in the smear

AFB appeared stained pink against a blue background when stained with Ziehl-Neelsen stain. The bacilli appeared rod shaped.

3.6 Culture

Sputum samples were treated using concentration method (Modified Petroff Technique). Equal volumes of sterile 4% NaOH to sputum sample were mixed and then vortex mixed for 15 minutes. The content was then centrifuged at 3000 rpm for 10 minutes. The supernatant from the centrifuged content was decanted into a jar containing 5% phenol.

Sterile distilled water was dispensed into the cell deposit left in the container. This was again vortex mixed and the content was again centrifuged and the supernatant was decanted into the 5% phenol solution. The resultant cell deposit was used for culture.

With the aid of Pasteur pipette, the cell deposit was stroke into 2 slopes of Lowensten Jensen medium.

The culture medium was incubated at 37˚C in a normal incubator. The incubation was for 8 weeks. The culture isolates were confirmed morphologically.

3.6.1 Quality Control

Two Lowestein –Jensen (LJ) slopes were prepared. One taken as positive control was inoculated with control strain of *M. tuberculosis (RV37),* while the other taken as negative control was left blank to check for sterility of the LJ slope. Both were incubated at 37^0 C for 24 hours.

In the positive control, growth of the control strain RV37 was observed. If there was no growth, two reasons could be given for that; 1.) Temperature of the incubator could be higher or lower than normal. 2.) It could be that the LJ slope lacked the nutrient that will support the growth of the organism.

In the negative control, no growth was found. If there was any, contamination would have been queried.

3.7 Blood Collection and Processing for HIV-1 testing, Whole Blood cell Stimulation and Immunoassay:

3.7.1 Collection of Blood Sample:

Five milliter of venous blood were collected from all categories of study population comprising subjects reporting at MGH, individuals at risk (doctors, nurses, laboratory workers and household TB contacts) and the controls. The blood collected was divided into heparinised and plain bottles in ratio of 2:1. The blood collected in heparinised bottle was used for whole blood stimulation, while the blood collected in plain bottle was centrifuged at 3000 revolution per minute (rpm) for 30 minutes. Serum obtained was used for HIV test and determining the gamma interferon value before antigen stimulation.

3.7.2 HIV Status determination:

The sera from the subjects were tested for HIV-1 using 2 rapid test kits-Determine and Capillus test kits. The two kits were used to confirm HIV result, as the two have different antigenic determinants with different procedures. This is in line with Nigerian HIV testing algorithm. (**Appendices 5&**6 described the procedure for the tests) (**Appendix 7 –**Nigerian HIV testing algorithm)

3.8 Antigen Preparation:

3.8.1 Antigens Used for Stimulation:

ESAT-6: Early Secretory Antigen Target (ESAT) is a recombinant protein of *M .tuberculosis*. with N-terminal His Tag. Alternative name is Rv3875. (See **Appendix 8**)

CFP-10: Culture Filtrate Protein (CFP) is a recombinant protein of *M. tuberculosis* with C-terminal His-Tag. Alternative name is Rv3874.(**See Appendix 9**)

ELLI: It is a mixture of selected antigens in the Region of Difference (RD1) in *M. tuberculosis* genome. It is taken as positive control for assays measuring gamma interferon. The trade name given is presently used (See **Appendices 8, 9 & 10** Certificates of analysis obtained from LIONEX Inc. for TB antigens used)**.** All the antigens used were obtained from LIONEX Inc. Germany.

3.8.2 Preparation of Stock Antigen:

The lyophilized antigen was pelleted down by centrifugation at 5000 revolution per minute. One milliliter of 10mM NH_4HCO_3 was added to the pellet to dissolve the antigen. The mixture was vortex mixed until homogenous mixture was obtained.

Weight of pellet was 500µg = 0.5mg

When dissolved in 1ml = 500µg/ml or 0.5mg/ml

This was kept at room temperature of 25^0C for 3 hours before proceeding to use. This product was taken as stock antigen.

3.8.3 Preparation of Working Stock Antigen (2.5µg/ml):

This was prepared from stock antigen =500µg/ml

To prepare 2.5µg/ml from 500µg/ml, a 1:200 dilution was made from the stock antigen

by diluting 10µl of Stock Antigen with 1990µl of 10 mM of NH_4HCO_3.

Each antigen used was prepared by this procedure.

3.9 Whole Blood Stimulation

3.9.1 Whole Blood Stimulation Assay without Inducing T-cells*:*

One milliliter of the heparinised blood from each subject was taken and diluted 1:10 with RPMI 1640 with glutamine, supplemented with 40µg of streptomycin per ml and 40 units of penicillin per ml. Two hundred and fifty micro liter of diluted blood was dispensed into 24 wells of Tissue Culture plates. 2.5µg/ml of antigens ESAT-6, CFP-10, antigen mixture ESAT-6+CFP-10 and ELLI (Scholvinck, *et al.*, 2004) were added respectively to each processed diluted blood samples in a serial form. The plates were then incubated for 72 hours at 37^0C in humidified CO_2 incubator (**Appendix 11**-Standard Operation Procedure for whole blood stimulation). The supernatant from each well was drawn into 2ml Eppendorf tubes and stored at -20^0C until ready for gamma interferon assay using ELISA technique.

3.9.2 Whole Blood Stimulation Assay for Fetal Calf Serum Gradient Titration to Induce T-cells:

This assay was to estimate the percentage of Fetal Calf Serum (FCS) to be used to induce the T-cells. To do this estimation, a trial run of gradient percentages was made on a suspected TB subject blood sample. A range of 0 to 25% FCS concentration was prepared using RPMI 1640 with glutamine medium supplemented with 40µg/ml of streptomycin and 40 units of penicillin per ml. Each mixture medium was used to make 1:10 dilution of the subject's blood relative to FCS concentration and 250µl of the diluted blood was dispensed into wells of tissue culture plates according to the range of FCS concentration. Thereafter, 2.5µg/ml of each ESAT-6, CFP -10 and ELLI antigens was added to blood dilution in the wells designated for each antigen respectively. The plates were incubated at 37^0C in humidified CO_2 incubator for 72 hours. Supernatant from each well was drawn and stored at -20^0C until ready for IFN-γ assay.

3.9.3 Whole Blood Stimulation Assay with Inducement of T-cells using Fetal Calf Serum:

One percent of fetal calf serum resulting from gradient titration was used with RPMI1640 with glutamine supplemented as stated above, to prepare diluting medium. This diluting medium was used for dilution of one milliliter of heparinised blood from each subject. Two hundred and fifty micro liter of the diluted blood was dispensed into 24 wells of tissue culture plates. 2.5µg/ml of antigens ESAT-6+CFP-10 mixture and ELLI (new antigen) were added respectively to each processed diluted blood sample in a serial form. The plates were incubated at 37^0C in a humidified CO_2 incubator for 72 hours and the supernatant from each well was taken and stored at -20^0C until ready for gamma interferon assay by ELISA technique.

3.10 Gamma Interferon Immuno Assay (IFN-γ) (ELISA kit from BD Sciences Incorporation).

This is an ELISA technique utilizing monoclonal antibody specific for IFN-γ coated on 96 well plates carried out by strictly following manufacturer's instruction.

Standards and test samples were added to the ELISA well plates to bind IFN-γ present to the immobilized antibody. The wells were washed with washing buffer and Streptavidin horseradish peroxidase conjugate mixed with biotinylated anti-human IFN-γ antibody added to produce an antigen antibody sandwich. The wells were again washed with washing buffer, after which Tetramethylbenzidine (TMB) substrate solution was added. This addition produced a blue color in direct proportion to the amount of IFN-γ in the sample tested. The stop solution in the kit was added to each well to stop the reaction in the wells evidenced by color change from blue color to yellow. The micro well absorbance was read at 450 nm and the optical density (OD) values of the IFN-γ concentrations were recorded. The OD values of the standard were plotted against the concentration values of the standards processed along with the test samples using logarithm graph sheet. Using the OD values of the test samples, the concentration values of IFN-γ were obtained. A cut-off value above10 picogram per milliliter (pg/ml) was taken as positive for TB. (Scholvinck, *et al.*,2004).

3.11 Data Collection and Statistical Analysis: Results obtained from demography, laboratory processing of sputum and blood samples were collated and analyzed using SPSS version 16. Demographic data were presented as mean ± SD. Statistical significance within each group studied was analyzed using student's t-test, with P values < 0.05 considered as significant.

Performance characteristic of the tests in the study was determined using 2 by 2 contingency table.

3.12 Performance Characteristics using 2 by 2 contingency table:

Assessing performance characteristic of the tests used revealed the value of the tests carried out in the study. It is designed to distinguish infected from the uninfected individuals. The structure of Table 3.1described below was carried out.

Sensitivity = a/(a + c) Specificity =d/(b+ d)

Positive Predictive Value = a/(a+ b) Negative Predictive Value = c/(c+ d)

a) True Positives = Patients positive for both culture and gamma interferon

b) False Positives = Patients negative for culture but positive for gamma Interferon assay.

c) False Negatives = Patients positive for culture but negative for gamma interferon assay.

d) True Negatives = Patients negative for culture and negative for gamma Interferon assay.

Sensitivity: This is the ability of gamma interferon assay to detect correctly gamma interferon produced when sensitized T-cells in the patient are stimulated in-vitro. This sensitivity is the number of true positive patients identified by the gamma interferon assay (a), divided by the number of patients identified by culture and the assay (a + c). This is expressed as a percentage.

Specificity: This is the ability of the gamma interferon assay to detect correctly sera that do not contain gamma interferon (to recognized level of positivity). This specificity therefore is the number of true negative sera identified by the gamma interferon assay as negative (d) divided by the number of patients identified by culture and the assay. This is expressed as a percentage.

Positive Predictive Value (PPV): The probability that those testing positive by the test are truly infected. This is expressed as a percentage.

Negative Predictive Value (NPV): The probability that those testing negative by the test are truly uninfected. This is expressed as a percentage. (See Table 3.1)

Table 3.1 2 by 2 Contingency table.

		TRUE TB STATUS (CULTURE)	
GAMMA INTERFERON ASSAY	**a** True Positive	**b** False Positive	**a + b**
	c False Negative	**d** True Negative	
	a + c	**b + d**	

CHAPTER FOUR

4.0 RESULTS

4.1. Demographic and Social Characteristics Profile of the Subjects

The age of the subjects ranged between 18 and 47 years with a mean of 32 years. Many of the subjects with TB were in the low income group particularly the subjects with active TB and those with HIV+TB were in the low income group. Many of these subjects also lived in high density areas (Table 4.1).

Table 4.1. Demographic and Social profile of subjects tested.

Profile	GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5
Mean Age (X ± SD)	32± 1.4	29±1.7	30±1.08	35±1.5	35±1.8
Age Range (Yrs)	19-45	27-44	27-40	29-47	18-46
Sex:					
Male	11	6	10	8	7
Female	13	6	9	9	9
Total (n)	24	12	19	17	16
Socio-Economic Status:					
High >₦ 30,000	4.17%	16.7%	37%	53%	75%
Low ₦<30,000	95.8%	83.3%	63%	47%	25%
Household Status(%): High Density Cluster living	80	78	15	32	21
Low Density	20	22	85	68	79

KEYS: GROUP 1= (Subjects with active TB); GROUP 2 = (Subjects with active HIV+TB); GROUP 3 = (Subjects with HIV with no TB signs); GROUP 4 = (Subjects with TB contacts) ; GROUP 5 = (Control group).

4.2 Age Distribution of the subjects tested positive for TB and HIV-1:

Age profile of subjects infected with TB was analyzed with respect to age range that is mostly affected. Both age range of 19-25 years and 36-40 years had infection rate of 28% of TB infection and these were mostly affected. Age range of 41-45 years were least affected with an infection rate of 10% (Fig. 4.2).

The subjects confirmed to have HIV-1 were those tested positive with both the Determine test and Capillus test kits. This was according to the Nigerian HIV-1 Algorithm test.

Subjects with age range of 26-30years with HIV-1 infection rate of 26% and those with age range of 36-40 years having HIV -1infection rate of 29% were mostly affected. Those in age range of 41-45 years had the least infection rate of 13% as observed in figure (Fig. 4.2).

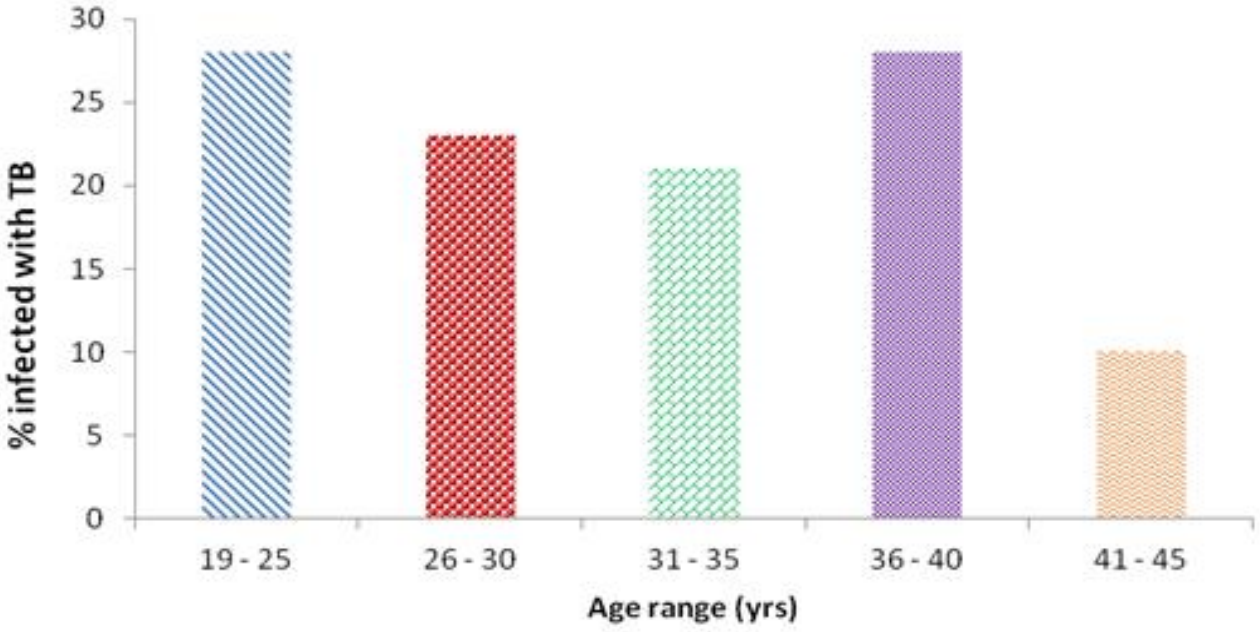

Fig 4.1. Age distribution of subjects found infected with tuberculosis.

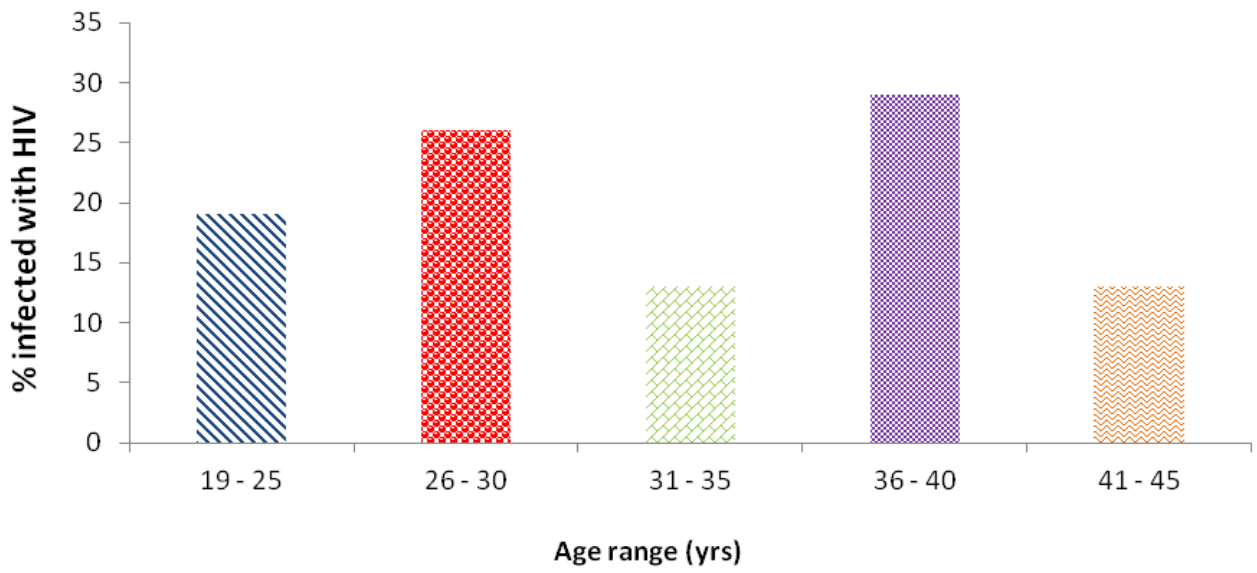

Fig. 4.2. Age distribution of subjects Infected with HIV-1.

4.3. Microscopy and Culture

4.3.1 Subjects with active tuberculosis:

The subjects positive for AFB microscopy were 32 out of 36 (88.9%). When sputum samples from the same subjects were cultured, 29 of 36 (80.6%) of them were positive for culture. One of thirty six subjects (2.78%) was negative for AFB but positive for culture, while 5 of 36 (13.9%) were positive for AFB but negative for culture (Table 4.2)

Table 4.2. Acid Fast Bacilli (AFB) Microscopy and Culture Tests of suspected Active TB subjects with and without HIV

	TEST	
	AFB	**CULTURE**
Positive	32	29
Negative	4	7
Total	36	36

4.4. Gamma Interferon (IFN-γ) Assay

The unit cut off value of the gamma interferon assay conducted was measured by ELISA (Kit from BD Sciences) in the three sets of experiments which are : Gamma Interferon produced from T-cell stimulation without inducing T-cells; Gradient titration of inducing agent- Fetal calf serum; and Gamma Interferon produced from T-cells when induced with Fetal Calf Serum, and the cut off value was 10pg/ml.

4.4.1 Gamma Interferon produced from T-cell stimulation without inducement of T-cells.

Gamma Interferon Assay on specimen from suspected active Tb subjects without HIV using ESAT-6, CFP-10 and ELLI antigens for T-cell stimulation:

Seventeen of the twenty four subjects suspected to have active TB tested using the whole blood stimulation assay were positive by the measure of IFN-γ produced when antigen mixture ESAT-6+CFP-10 was used, and 18 of the 24 tested positive when ELLI was used for T-cell stimulation. Only 14 of the subjects tested positive for TB when ESAT-6 and CFP-10 were initially used separately to stimulate the T-cells in the blood. Significant differences were observed in the IFN-γ produced when the results of individual antigens ESAT-6 and CFP-10 were compared with antigen mixture of ESAT-6+CFP-10 for antigen stimulation ($P<0.001$) and ELLI antigen for stimulation ($P< 0.0001$) in the IGRA test. Variation observed in IFN-γ level was found significant ($P<0.0001$) with ELLI effecting the strongest and CFP-10 the lowest stimulation.

The result of IFN-γ obtained from the use of antigen mixture of ESAT-6+CFP-10 led to the use of the mixture in subsequent testing of the subjects for TB (Fig 4.3).

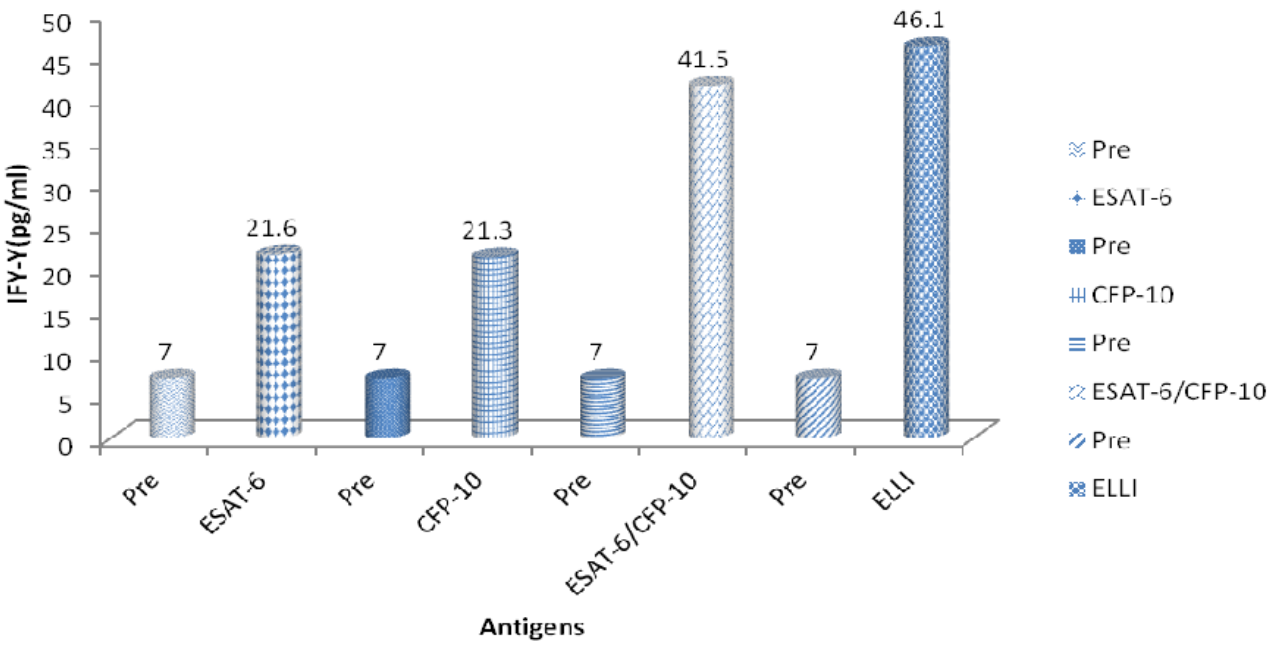

Fig 4.3: Mean levels of IFN-γ after antigen stimulation of T-cells from subjects with active TB using ESAT-6, CFP-10, ESAT-6+CFP-10 and ELLI antigens respectively.

4.4.2 Fetal Calf Serum Gradient Titration:

One percent fetal calf serum in the diluting mixture of RPMI 1640 with glutamine was observed to be the working percentage concentration obtained with each of the three antigens tested. Percentage concentration within the range of 2 % to 25% gave inconsistent readings (Table 4.3).

Table 4.3: Fetal Calf Serum Gradient Titration to Determine % of Working concentration.

ESAT-6 % conc^{n}	IFN-γ (pg/ml)	CFP – 10 % conc^{n}	IFN-γ (pg/ml)	ELLI %conc^{n}	IFN-γ (pg/ml)
1%	20	1%	20	1%	20
2%	20	2%	27	2%	13
3%	15	3%	20	3%	15
4%	20	4%	15	4%	13
5%	13	5%	13	5%	20
10%	10	10%	15	10%	10
15%	10	15%	18	15%	20
20%	18	20%	10	20%	10
25%	10	25%	10	25%	10

4.4.3 Gamma Interferon (IFN-γ) Assay produced with and without inducement of T-cells using Fetal Calf Serum:

4.4.3.1. IFN-γ assay of active TB subjects after inducement of T-cells and stimulating with antigen mixture ESAT-6+CFP-10 and ELLI antigen:

Eighteen of the twenty four subjects suspected of active TB tested were found positive by the value of gamma interferon produced. The concentration of gamma interferon produced with the induction of T-cells using fetal calf serum was significantly higher than when the T-cells were not induced and this determined the status of one of the subjects whose result was questionable before the inducement of T-cells. Significant increase was observed in the comparison of the use of antigen mixture of ESAT-6+CFP-10 and ELLI antigen to stimulate the T-cells with p values: $P<0.0001$,$P<0.0001$,respectively (Fig. 4.4). P values were calculated using Student's t-test.

Variations that were observed in IFN-γ levels in pre and post inducement were found significant ($P<0.0001$) with antigen mixture ESAT-6+CFP-10+FCS and ELLI +FCS effecting a higher stimulation than ESAT-6+CFP-10 and ELLI respectively.

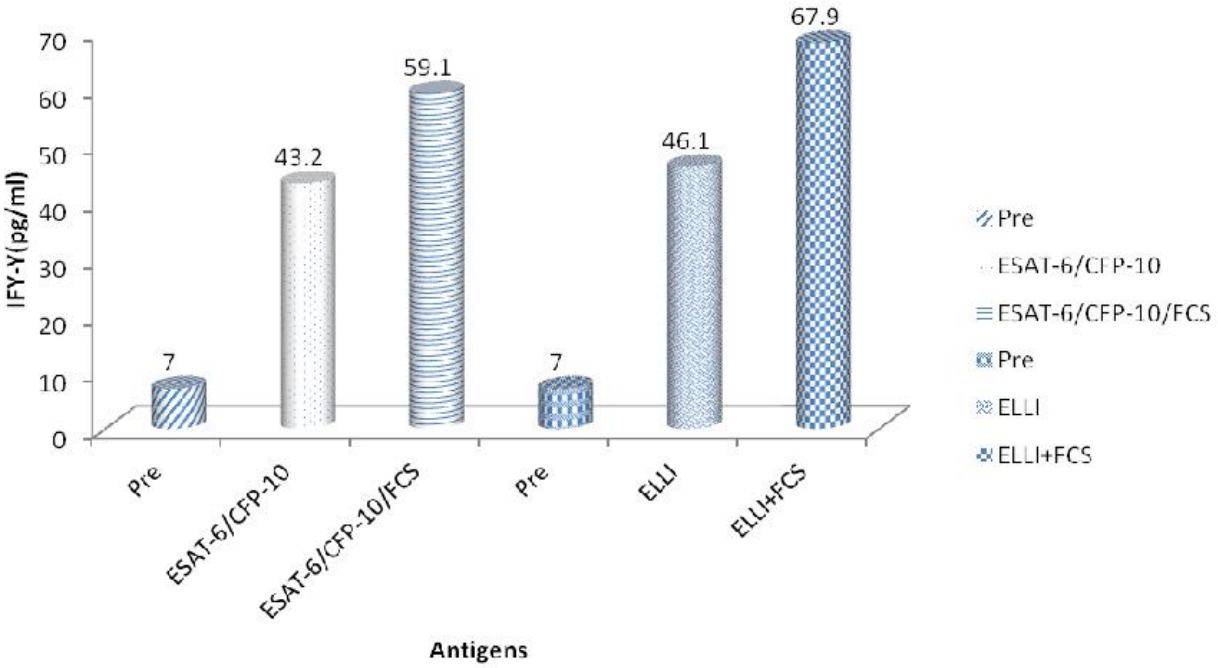

Fig 4.4. Comparison of Mean IFN-γ (pg/ml) produced by antigen mixture ESAT-6+CFP-10 and ELLI antigen stimulation pre and post inducement of T-cells in subjects with active TB.

4.4.3.2 IFN-γ assay of active HIV+TB subjects after the inducement of T-cells.

All the twelve subjects tested for TB using the gamma interferon assay were found positive for TB by the value of gamma interferon produced when T-cells were induced with FCS and stimulated using antigen mixture ESAT6+CFP-10 and ELLI antigen. The value of IFN-γ responses to antigen mixture and ELLI antigen at post inducement of T-cells were significantly higher than pre inducement states ($P<0.0001$). The P value was calculated using Student's t-test. (Fig 4.5).

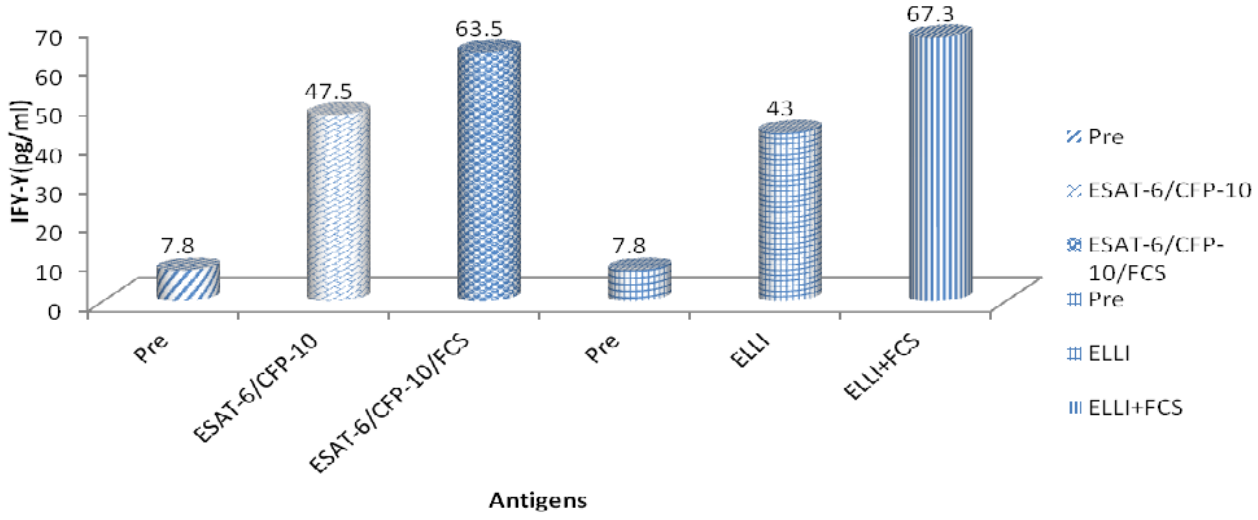

Fig.4.5 .Comparison of Mean IFN-γ(pg/ml) produced by combined antigen mixture ESAT-6+CFP-10 and ELLI antigen stimulation pre and post inducement of T-cells in subjects with active HIV+TB.

4.4.3.3 IFN-γ assay of HIV subjects with no pulmonary TB symptom after inducement of T-cells.

More TB positive subjects were identified after the inducement of T-cells. Eight (42.1%) of the nineteen subjects tested for latent TB were positive. Five subjects who previously tested negative became positive based on the value of IFN-γ produced when the T-cells were induced with fetal calf serum resulting in 13 (68.4%) of 19 subjects positive for TB in this group. A comparison of the two antigens used before and after the inducement of T-cells is shown in Fig.4.6.

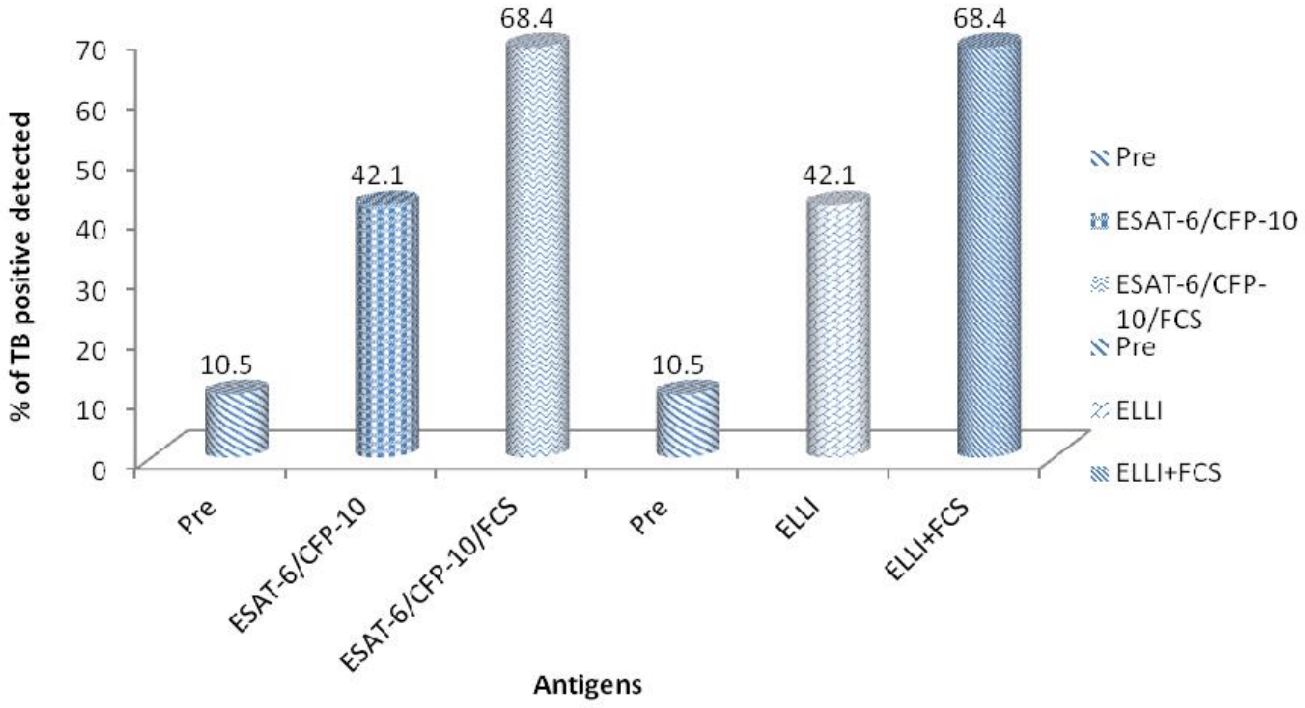

Fig 4.6. Percentage of TB cases detected by IFN-γ (pg/ml) assay pre and post-inducement of T-cells in HIV positive subjects with no TB symptoms.

4.4.3.4. IFN-γ assay of TB Contact subjects after inducement of T-cells:

Ten (58.8%) of the seventeen subjects tested positive for latent TB in this category, seven of whom were household members of TB patients and the remaining 3 were health care providers. The number of positives increased to 12 (70.6%) of 17 subjects after the inducement of T-cells, 8 of whom were household members of TB patients and the remaining 4, health care providers. There was significant difference ($P<0.0001$) in the use of antigens with and without inducement of T-cells using fetal calf serum in the gamma interferon produced (Fig.4.7).

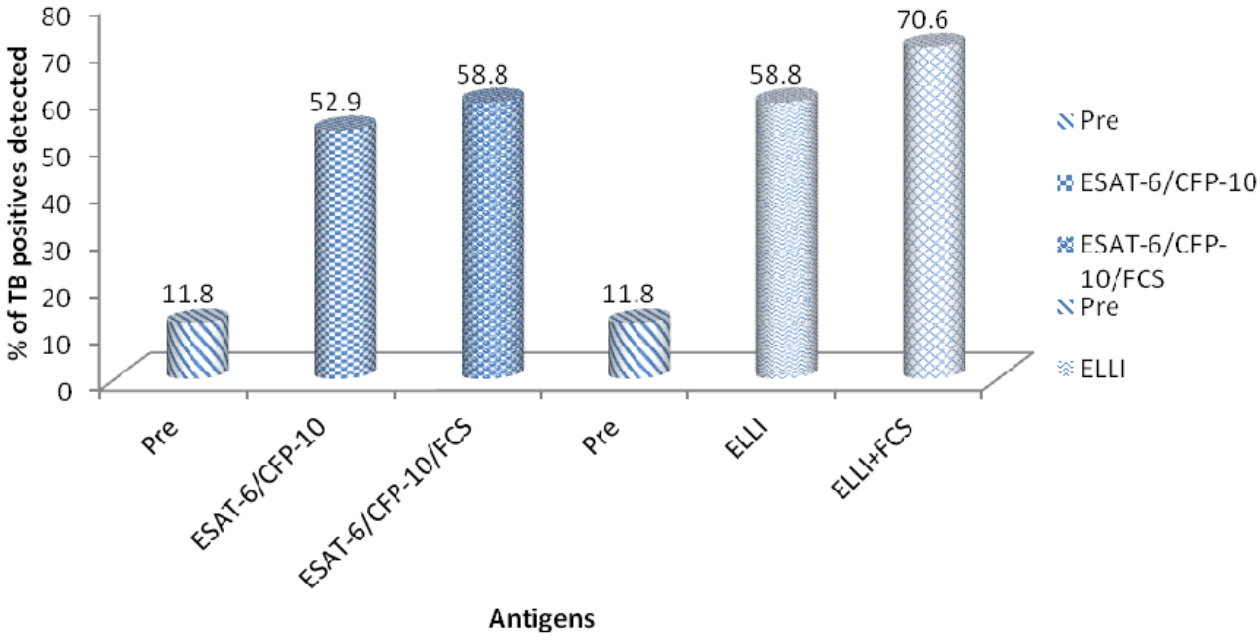

Fig.4.7 Percentage of TB positive cases detected by IFN-γ (pg/ml) assay pre and post-inducement of T-cells in TB contacts.

4.4.3.5 Control

All the sixteen healthy control subjects (without TB and with no TB contact) were screened for TB and they tested negative. The mean values of IFN-γ produced from stimulating their T-cells with genomic antigens ELLI and combined mixture of ESAT-6+CFP-10 were 3.6pg/ml and 3.54pg/ml respectively before inducement and these were observed to be non-significantly different from the mean values of 4.1pg/ml for ELLI and 3.79 pg/ml for combined mixture of ESAT-6+CFP-10 obtained after inducement.

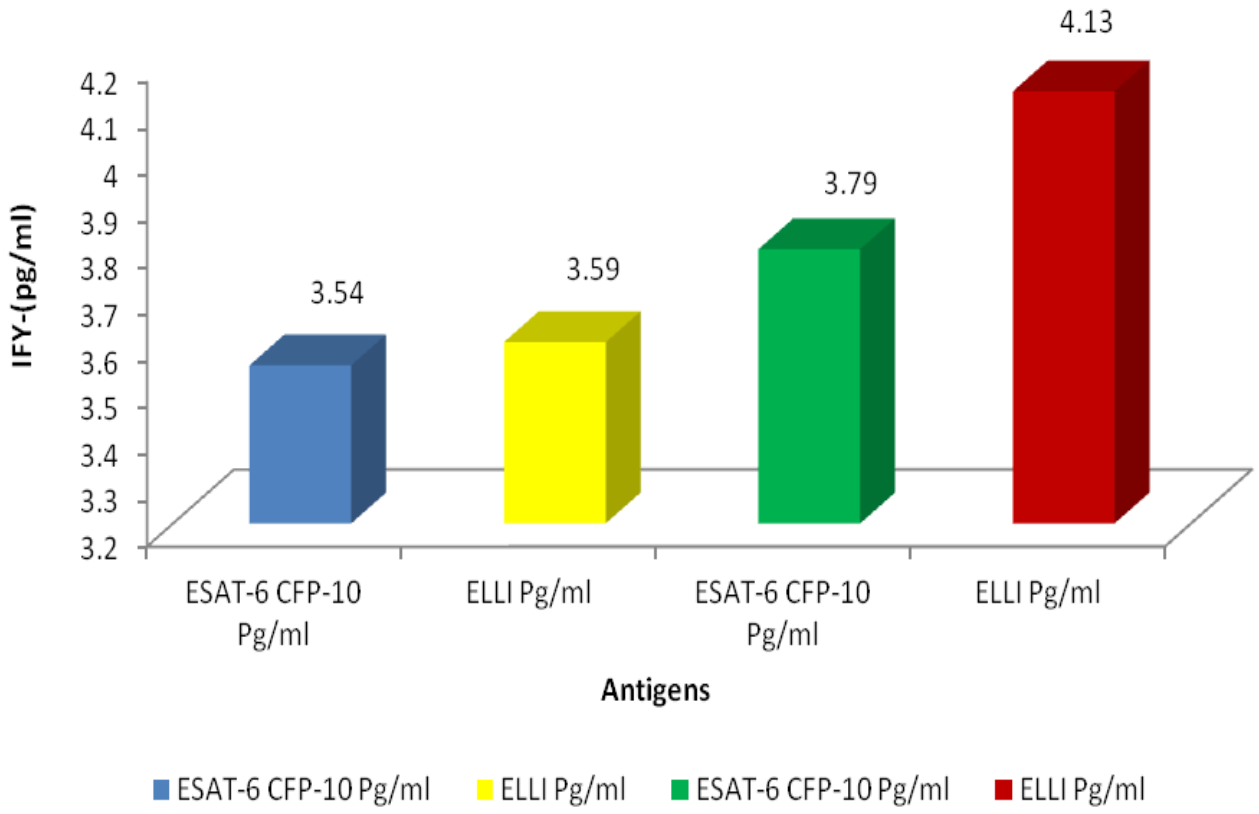

Fig.4.8. Comparison of Mean IFN-γ (pg/ml) produced by combined antigen mixture ESAT-6+CFP-10 and ELLI antigen stimulation pre and post inducement of T-cells in Control Subjects.

4.5 Comparison of cumulative IFN-γ in five categories of subjects tested.

A comparison of the cumulative IFN-γ produced in the course of stimulation of the T-cells with ELLI antigen in the five categories of the subjects tested showed that those in the active TB group, both with and without HIV, had high concentration of IFN-γ, while those with no TB symptoms and TB contacts had lower concentration (Fig. 4.8). The control group has the lowest IFN-γ concentration as there was no TB infection.

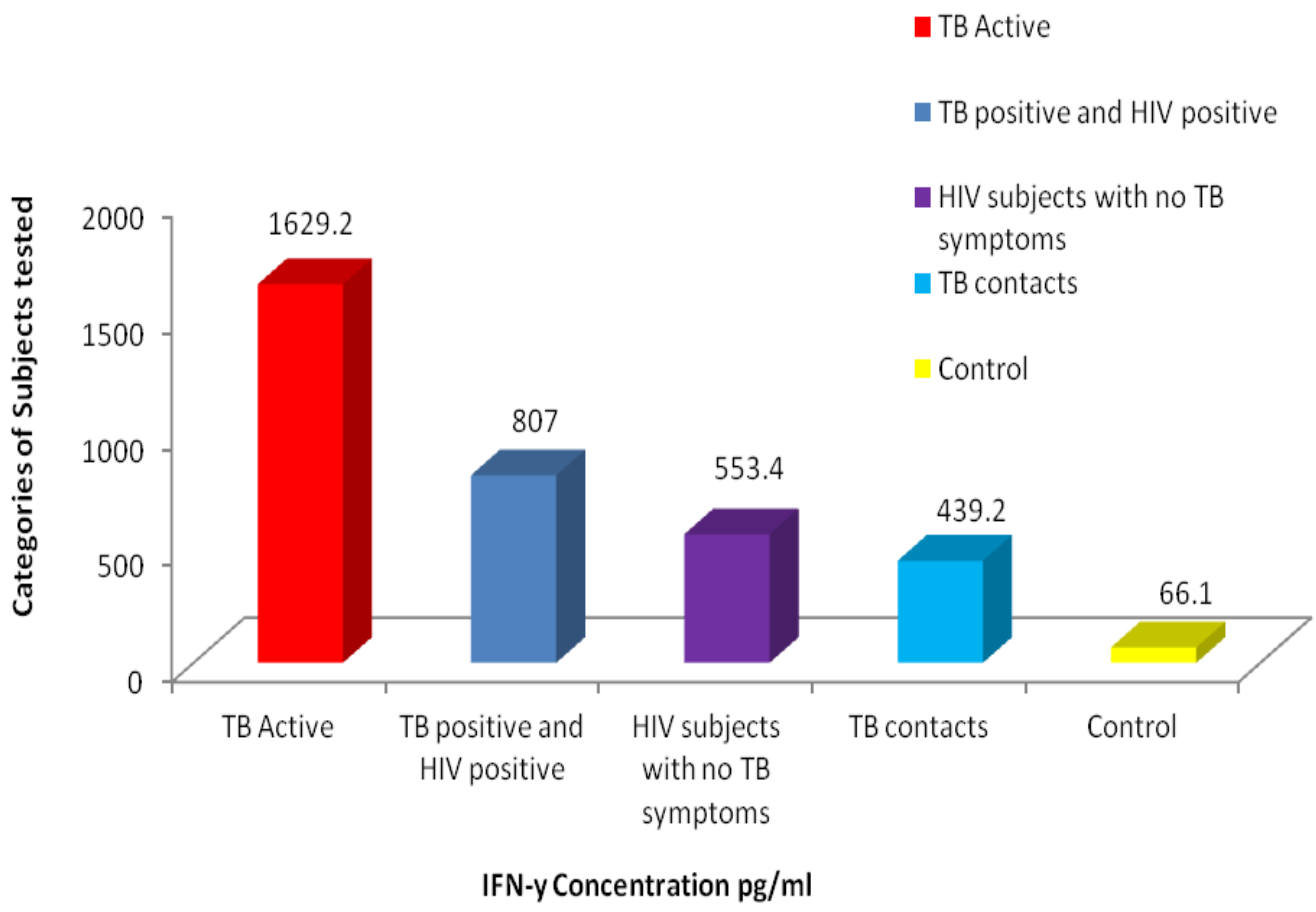

Fig 4.9. Comparison of cumulative IFN-γ (pg/ml) produced in five categories of subjects tested.

4.6 Performance characteristics of the diagnostic tests using TB culture as gold standard for TB diagnosis on subjects that produced sputum.

Evaluation of performance characteristics of the various diagnostic tests employed for TB diagnosis was carried out with respect to sensitivity, specificity, positive and negative predictive values of the tests using culture as the standard. The result showed that the conventional smear microscopy though had high sensitivity, it was with a low specificity. The use of genomic antigens showed improved specificity which ultimately improved after the inducement of T-cells.

Table 4.4: Performance characteristics of the diagnostic tests using TB culture as gold standard for TB diagnosis on suspected active TB subjects that produced sputum.

Diagnostic Test relative to culture	Sensitivity %	Specificity %	*PPV %	**NPV %	Confidence Interval.
CULTURE VS AFB Microscopy	90.6	50.0	76.2	57.1	74.36 - 103.4
CULTURE VS ESAT-6	77.7	83.3	93.3	44.4	58.56 – 96.98
CULTURE VS CFP-10	77.8	83.3	93.3	44.4	58.56 – 96.98
CULTURE VS ESAT-6+CFP-10	83.3	83.3	93.75	37.5	66.12 – 100.54
CULTURE VS ELLI	83.3	83.3	93.75	37.5	66.12 – 100.54
CULTURE VS ESAT-6+CFP-10-F	94.4	83.3	94.4	16.6	83.86 – 105.02
CULTURE VS ELLI-F	94.4	83.3	94.4	16.6	83.86 – 105.02

***PPV----Positive Predictive Value, **NPV----Negative Predictive Value**

CHAPTER FIVE

5.0 DISCUSSION

5.1 Demographic profile of the subjects studied.

Poverty, house hold crowding, poor nutrition and close contacts have been identified as risk factors of TB transmission (Frieden, 2009). In this study 95.8% of the subjects with active TB were in the low income group and 80% of this group live in high cluster households. Unfortunately this group of people have high tendency to disseminate the tubercle bacilli when they cough, sneeze or spit saliva. This is similar to the findings of Jumbo *et al*., (2013) in a study conducted at Ijebu-Ode General Hospital in Ogun State, Nigeria. Despite the fact that many of these active TB subjects have the potential to disseminate the disease, their low income status prevent them from appreciating the danger they constitute in the environment. This was also observed by Ford in Peru (Ford *et al.,* 2009).

In this study, 70.6% of the TB contacts studied were detected to have TB by the diagnosis made in the study. This group of people stayed on long term duration with active TB subjects. This included the health workers and the relations who were care givers at home with the TB subjects. Most of the subjects affected were in the middle age and low income group. The infectious aerosol droplets from the active TB subjects through coughing would have been the source of infection especially when the home care giver live in high clusters with the TB subjects or when the health facility is not highly ventilated. These conditions could predispose the care givers to TB infection. Ahmed and Hasnain (2011) gave an estimate of TB infection rate of 22% among such people with prolonged frequent or close contact with TB subjects being at high risk of becoming infected. Though many of the health workers live in low density areas of the community, their prolonged

frequent contact with the subjects with active TB in health facility could predispose them to TB infection. This infection can be latent and could make them a risk group (Desta *et al.*, 2009).

5.1.1 Age Distribution of subjects tested positive for TB and HIV-1

The age ranges that were mostly affected by the scourge of TB and HIV-1 in this study were 19-25 years (25.5%), 26-30years (21%) and 36-40years (25.5%). This could have happened by the individual's current and life time exposure to TB infection exacerbated by the endemicity of HIV-1(Abdool Karim *et al.,* 2009). This finding agreed with the study conducted in Enugu that found the model age group for TB cases associated with HIV prevalence for Enugu State to be 25-34 years (29.8%) and 35-44 years (20%) (Dim and Dim, 2013). This study and that of Enugu State are confirming the observation of a decade of trend of TB notification that was found to be affecting people in age group between 15 to 45 years (Linda-Gail Bekker and Robin Wood 2010). The adoption of DOTs strategy if adequately responded to by the TB subjects would be a good intervention process that could prevent TB spread.

5.2 Acid Fast Bacilli (AFB) and culture:

Direct Smear is known to detect AFB only at concentration of 10,000 bacilli/ml of specimen. Conversely as few as 100 bacilli/ml of specimen may be required for culture (Kant, 2001). The observation, in this study of two samples that tested negative for AFB by microscopy but found to be culture positive is in agreement with other reports (Tomans, 2004a; Desta *et al.*, 2009). This finding may interpret to be that the concentration of the bacilli could not have reached the level of detection by Smear Microscopy and this does not indicate that the TB infection has not taken place. The diagnostic value of sputum microscopy has been reportedly eroded in HIV related smear negative pulmonary cases (Kant, 2001; Valadas *et al.,* 2003). Unfortunately the danger of this is that TB transmission is known to be occurring before the level of bacilli reaches 10,000 bacilli/ml

of specimen (Kant, 2001). Desta *et al*., 2009 reported four cases which were AFB positive but culture negative. This may be attributed to failure of organism to grow in culture as reported by Pottumarthy *et al* (2000) or other resemblance of AFB not pointed by other methods. The sensitivity of AFB microscopy relative to culture was 90.6% with a specificity of 50%, which was observed and found similar to the finding of Desta *et al*. (Desta *et al*., 2009).

5.3 Using genomic Antigens for diagnosis of Tuberculosis.

The drawbacks of conventional methods of TB case detection have led to modifications of their processes and use of genomic TB antigens for immunodetection. In this study, the use of ESAT-6 and CFP-10 TB antigens in Interferon Gamma Release Assay (IGRA) showed these antigens to have a sensitivity of 77.7% and 77.8% respectively and specificity of 83.3% when compared with culture. The use of these TB antigens has an advantage in extra pulmonary TB case detection and when no sputum is produced. However the drawback of low sensitivity of these antigens led to evaluating the use of ELLI the new TB antigen along with the two other antigens. It was observed among the three genomic antigens used (ESAT-6, CFP-10 and ELLI) that ELLI the new antigen product, showed to be the most immunogenic in stimulating the T-cells of suspected TB subjects in the Interferon Gamma Release Assay (IGRA) test. It releases the highest concentration of IFN-γ which influences the degree of sensitivity of the test. The sensitivity value of 83.3% obtained was higher than the 77.7% and 77.8% obtained with ESAT-6 and CFP-10 respectively and specificity of 83.3% when compared to culture. The gamma interferon produced was significantly higher than those of ESAT-6 and CFP-10 ($P<0.0001$, $P<0.0001$ respectively). However when the combined antigen mixture of ESAT-6 and CFP-10 used to stimulate the T-cells of the subjects tested was compared with ELLI antigen, there was no significant difference in the amount of IFN-γ produced ($P>0.144$). This might be due to the source of ELLI antigen being a mixture of selected recombinant antigens obtained from genome of *M. tuberculosis.* The commercially produced antigen mixture of ESAT-6+CFP-10 Quantiferon Gold in Tube test kit used in diagnosing TB has been found to be of

low sensitivity (Yoshihiro *et al.,* 2009). This was reported in immune compromised subjects such as in situations of malnutrition, old age, immune suppression and emaciation, all of which could affect T-cells response to stimulation (Thomas *et al.*, 2010; Latore *et al.*, 2010; Hang *et al.*, 2011). This might be due to depleted T-cells or state of anergy (state of unresponsiveness to stimulation of the T-cells) (Geldmacher *et al*., 2008). This situation led to the use of fetal calf serum, which is known to contain growth factors and cytokines necessary for inducement of cells (Naseer *et al*., 2009), as an inducer in this study.

5.3.1 Fetal calf serum titration:

A gradient titration of fetal calf serum (FCS) was carried out using different line of antigens (ESAT-6, CFP-10 and ELLI the new antigen product) to determine working concentration applicable to all situations of TB including immune suppressed states. The finding in this study that one percent concentration (1%) of FCS induced the T-cells to cause effective stimulation by the antigens used to yield uniform maximum concentration of IFN-γ is in contrast to the finding of Nasser *et al* (2009) who found that proliferation of cells that could lead to IFN-γ production increased as FCS increased from 1% to 10%. However, findings in this study agreed with the findings of related studies on antibody response in immune compromised subjects (Semenova, 2008; Kunzi *et al*., 2009). When the immune competence is compromised, to induce or stimulate such cells, there is need to use low dose concentration to enable the cells to cope with antigen stimulation as they respond to it (Adeiga *et al.*, 1990). This could not have been taken into consideration in the commercially prepared kit for TB diagnosis such as in Quantiferon Gold IT test kit, since the test kit is for general use of detecting TB in the general population including the immune compromised. The implication of this is that most of the ELISA or antigen antibody based diagnostic kits for general population may require specific modification for optimum performance in immune suppressed subjects.

5.3.2 Inducing the T-cells for TB diagnosis.

Inducement of T-cells increased the production of IFN-γ when the T-cells were stimulated by the combined antigen mixture of ESAT-6+CFP-10 and ELLI antigen by improving the sensitivity of the IGRA assay for which the antigens were used. This led to an increase in the number of positives detected among the subjects with no TB symptoms from 8 to 13 with 5 of those who previously tested negative becoming positive after the inducement of T-cells which made the cells respond better to stimulation to produce higher concentration of IFN-γ. This is of great significance in TB detection in HIV subjects with disseminated TB (WHO, 2005) and it could enable diagnosis of TB before death. More TB positives were detected among the contacts after inducement thus improving detection of latent TB subjects. Hence improved method performance as demonstrated in this study have the potential to improve diagnosis and consequently reduce mortality and morbidity of TB disease.

5.4 Performance Characteristics:

The ability of a test to detect positive TB cases defines the sensitivity of the test. The sensitivity of the IGRA test using combined mixture of ESAT-6+CFP-10 antigen (83.3%) to stimulate T-cells was higher than that of the tests using individual ESAT-6(77.7%) and CFP-10 (77.8%) antigens. This is in agreement with the principle of van Pinxteren *et al.* (2000) of combining the two antigens. Also observed in this study was an increase in the sensitivity of the IGRA test (94.4%) with the use of combined mixture ESAT-6+CFP-10 antigen when T-cells were induced over the 92.3% reported by Hang *et al.* (2011). The similarity in sensitivity of IGRA test using ELLI the new antigen (94.4%) and that of combined mixture of ESAT-6+CFP-10 antigen, though higher than when individual ESAT-6 and CFP-10 antigens was used, is because ELLI the new antigen is also a product of selected recombinant antigen mixture of *M. tuberculosis*. The inducement of T-cells

increased the sensitivity of the test by 16.7% when ELLI and combined mixture of ESAT-6+CFP-10 were used as antigens over the use of individual antigens ESAT-6 and CFP-10 for the IGRA test. The use of IGRA test with either the combined mixture of ESAT-6+CFP-10 or ELLI as antigens to test for TB, results in higher sensitivity than with Tuberculin Skin Test (TST) with a lower sensitivity (<50%) in HIV positive subjects (Richeldi *et al.*, 2009; Stavri *et al.*, 2009). Thus, the IGRA using whole blood for test is of immense value in testing for non pulmonary TB among HIV subjects that are asymptomatic of TB and those at risk groups, especially the health workers. This is an advantage over sputum smear microscopy and culture because they use sputum for diagnosis. However, though the sensitivity of ESAT-6 and CFP-10 has been shown in this study to have improved the IGRA test, the presence of the genes encoding them are also in other mycobacterial organisms such as *M. kansasii, M. szulgai* and *M. marinum* (Mori *et al.*, 2004). This could have affected the specificity of the IGRA test with the antigens used in this study.

5.5 Summary of Findings

Objectives	**Findings**
Evaluate the immunodetection of performance of ELLI (new TB antigen) for TB detection in comparison with ESAT-6, CFP-10, culture and sputum smear microscopy.	Both conventional methods (microscopy and culture) showed limited sensitivity and specificity. Though improved diagnostic performance was achieved with ESAT-6 and CFP-10 but also demonstrated limitation in immune suppression. ELLI the new antigen product was the most immunogenic of the antigens used in IGRA test in this study, increasing the test's sensitivity to 94.4% relative to 77.7% and 77.8% obtained with ESAT-6 and CFP-10 respectively. Relative to culture as standard, the performance of ELLI was higher with 94.4% sensitivity and 83.3% specificity when compared with 90.6% sensitivity and 50% specificity of smear

	microscopy.
Determine the minimum level (%) of fetal calf serum (FCS) that gives optimal inducement of T-cells.	FCS induced T-cells optimally at 1% concentration in medium of RPMI 1640 with glutamine enriched with protein-vitamin that made the T-cells respond better to respective ESAT-6, CFP-10 and ELLI antigenic stimulation.
Determine the performance of induced T-cells with the test antigens ELLI, ESAT-6+CFP-10 in detecting active and non-pulmonary TB subjects with or without HIV and TB contacts.	The inducement of T-cells improved yield of IFN-γ when T-cells were stimulated with low dose antigen (2.5ug/ml) of ESAT-6+CFP-10 mixture and of ELLI antigen in a protein vitamin medium of RPMI 1640 with glutamine. This improved TB case detection. The sensitivity of IGRA test improved to 94.4% with specificity of 83.3%.
Assess the diagnostic performance of different tests in detecting TB in HIV+TB, asymptomatic TB and TB contact subjects	TB case detection could only be detected in HIV+TB subject that was able to produce sputum and the sensitivity of Smear microscopy was 90.6% with specificity of 50%. Interferon gamma release assay (IGRA) was used to test all the subjects suspected for TB. Inducement of T-cells improved diagnosis of TB in HIV subjects that were asymptomatic of TB. Five subjects that were negative at pre-inducing state became positive when the T-cells were induced.

6.0 CONCLUSION

To the best of my knowledge, this study investigated for the first time in our study population the immune detection performance of three genomic antigens compared with conventional diagnostic method of pulmonary TB (microscopy and culture) in immune suppressed subjects with or without TB. In addition, the study demonstrated inducement of T-cells with low concentration of FCS to improve diagnostic performance. ELLI the new antigen was found to be the most immunogenic in stimulating T-cells to produce high concentration of IFN-γ and increased the sensitivity of IGRA test to 94.4% to detect TB, in comparison with 77.7% and 77.8% for ESAT-6 and CFP-10 respectively.

Inducement of T-cells using FCS at 1% concentration improved sensitivity of IGRA test to detect TB among HIV positives that were asymptomatic of TB. These were suspected to be immune suppressed or have malnutrition which could confound IGRA's sensitivity (Latore *et al.*, 2010 Hang *et al.*, 2011).

Detecting TB in immune suppressed situation, low dose of antigens used (ESAT-6+CFP-10 and ELLI) was found useful to stimulate T- cells as this made weak cells to respond better than stimulating with large dose antigen that might cause immune paralysis. These procedure improved sensitivity of the IGRA assay by 16.7% and will address the issue of low sensitivity of commercial IGRA test kit that was reported by Yoshihiro *et al*, (2009). This could probably be significant and clinically important in diagnosis of TB cases in emaciated or immune suppressed subjects.

7.0 CONTRIBUTIONS TO KNOWLEDGE

This research work in its contribution to knowledge has:

1. Found that when T-cells are in a state of anergy (a state of unresponsiveness to stimulation), such as in HIV infection with no TB symptoms or chronic TB infections, inducement of T-cell can improve TB diagnosis.

2. Shown that using the fetal calf serum at low concentration (1% in this study) to induce the T-cells and stimulating them with low dose antigen (2.5ug/ml in this study) in a protein /vitamin enriched medium of RPMI 1640 with glutamine, the T-cells can respond better to antigen stimulation to improve sensitivity of Interferon Gamma Release Assay (IGRA) for TB case detection.

3. Affirmed that ELLI (the new antigen) increased the sensitivity of IGRA better than ESAT-6 and CFP-10 TB antigens that are currently in use and this could enhance early TB detection.

8.0 Recommendations:

1. In a failed diagnosis of TB and suspicion of disseminated cases, confounding factors causing the failed diagnosis should be identified.

2. Consideration of producing diagnostic kits that will make the proliferation and activation of the T-cells possible should be made. This will take care of the weak state of T- cells in the TB subjects.

3. In a situation of asymptomatic TB with possible dissemination of TB or extra-pulmonary TB, modified Interferon Gamma Release Assay (IGRA) test kit should be used for TB diagnosis.

4. Combination of TB antigens from broader epitopes of *M. tuberculosis* can improve IGRA to improve TB case detection as shown in the example of ELLI from LIONEX Inc. Germany.

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