Citation: Abdul Rahim M. Salih. The Use of the Insertion Sequence (IS6110) to characterize *Mycobacterium tuberculosis* detected among HIV Patients in Sudan. 2017, 2 (3). ajmsc.info

The Use of the Insertion Sequence (IS6110) to characterize *Mycobacterium tuberculosis* detected among HIV Patients in Sudan

Abdul Rahim M. Salih

Sudan University of Science and Technology, Khartoum, Sudan

Abstract

Background: National AIDS Control Program Report of 2002 showed that the prevalence of HIV infection in Sudan may be 1.6% of the population, which translates to 540.000 people living with HIV/AIDS in Sudan. Surveys indicate that HIV/AIDS in Sudan is low/concentrated rather than generalized epidemic. Tuberculosis is particularly insidious problem to those who have AIDS. In these patients, the immune system cells that normally mount a response to *M. tuberculosis*, are being destroyed, and the patient cannot respond to the bacterial infection.

Objective: To use the insertion sequence (IS6110) to characterize *Mycobacterium tuberculosis* detected among HIV patients in Sudan.

Materials and methods: The study was conducted in West Darfur State (Sudan) from 2012 through to 2015. The sputum specimens were collected from two groups of patients: 158 patients living with HIV/AIDS (PLWHA), 1160 tuberculosis (TB) suspected patients who were negative for HIV. All specimens had been stained by Zeihl Neelsen (ZN) stain and screened microscopically for the presence of acid fast bacilli (AFB). ZN stain positive samples and other negative samples collected from PLWHA patients were subjected to DNA extraction by the modified Guanidine Chloride Method. The samples were then processed by polymerase chain reaction (PCR) technique. Selected PCR amplified DNA samples were sent to Macrogen Company in Korea for DNA sequencing.

Results: Six of the (PLWHA) patients were found to be positive for AFB, of whom (66.7%) were males and (33.3%) were females. The most affected age group was 31-40 years (50%) , followed by 41-50 years (33.3%) Of the total sputum specimens collected from TB suspected patients. 200 samples were found positive for AFB (17.2%). The results achieved revealed high similarity to the reference laboratory strain of *Mycobacterium Tuberculosis* (*H37Rv*).

Conclusion: The prevalence of TB among PLWHA participants was low. Molecular techniques are rapid and sensitive diagnostic tools for diagnosis of tuberculous infection and determination of the major phylogenetic families.

Key words: Insertion sequence (IS6110), *Mycobacterium tuberculosis*, HIV patients.

Introduction

The etiologic agent of AIDS has infected an estimated 30 to 40 million people worldwide. Of the two major forms of HIV, infection with type 1 (HIV-1) is prevalent throughout the world and is characterized by a slow, progressive deterioration of the immune system that is almost uniformly fatal. By contrast, infection with type 2 (HIV-2), generally has a more benign clinical course. HIV-1 is a retrovirus in the *Lentivirus* family that establishes a persistent infection in humans, which is ultimately associated with the development of acquired immunodeficiency syndrome (AIDS). Importantly, infection continues to spread at an alarming rate; in sub- Saharan Africa it is estimated that 3 million people become infected in 2003 alone. Although antiretroviral therapy has been a key in reducing the morbidity and mortality associated with HIV infection, this is not a long term-solution for curbing the pandemic.

Human immunodeficiency virus (HIV) and acquired immunodeficiency syndrome (AIDS) epidemic is now a global crisis, and constitutes one of the most formidable challenges to development and social progress. In the most affected countries, the epidemic is eroding decades of development gains, undermining economies, threatening security and destabilizing societies. In addition, HIV/AIDS is affecting fundamental rights, particularly with respect to discrimination and stigmatization aimed at people living with and affected by HIV/AIDS. The epidemic and its impact strikes had at vulnerable groups including women and children, thereby increasing existing gender inequalities and exacerbating the child labor problem.

Despite the fact that TB is a disease of antiquity and it is probably one of the illnesses most dealt with in the literature, there has been surprisingly little sound knowledge of the disease through the course of history, which has not helped contemporary efforts to combat the illness. From the time of Hippocrates (460-377 BC) up until the nineteenth century, the infectious nature of the disease was not even acknowledged; rather, TB was considered a hereditary disorder. However, air-a common vehicle for the transmission of live germs-was included among the possible origin of the disease.

For this reason, the dietary regimen proposed by Hippocrates and Galen (130-200 AD) remained the basis of treatment up until the Renaissance. This practice changed very little in the seventeenth century, the sole difference being the recommendation of physical exercise and the use (as with other diseases) of new medicinal substances introduced in Europe at the time, such as quinine, coffee, tea, cocoa, and even tobacco. Such lack of understanding partly explains why humankind has been unable to defend itself against this terrible illness for the most of history-the only option being to fall ill and ultimately die. Only towards the latter half of the nineteenth century did the infectious nature of TB become apparent, as a result of the studies by Villemin (1865) and, particularly, Robert Koch (1843-1910).

Koch was the first to suggest the possibility of controlling this endemic disease, with the presentation of the results of his research (in 1882) that showed that TB was a contagious disease. He not only isolated the bacterium, which was latter named after him (Koch's bacillus),

from the sputum of infected patients, but also proposed that the principal measure for controlling the disease in the community would be to isolate affected patients. This suggestion paved the way for the "sanatorium" era of TB, during which prolonged confinement of patients in sanatoriums was believed to be the only effective way to cure TB and control its transmission. HIV/AIDS and tuberculosis became a major global health problem .The geographic location of West Darfur State which has international open borders with Republic of Chad and the Republic of Central Africa, where people from most West African countries with high prevalence of HIV/AIDS infections, travel freely across these borders. In addition, the current conflicts in Darfur create suitable environment for HIV/AIDS transmission.

Molecular diagnosis will lead to good results compares to the previous conventional procedures. The proper diagnosis of disease will help the health professions to be sure that they are treating the patients properly. This study will follow the feasible and the most adequate diagnostic procedures for HIV and TB.

Furthermore, PCR helps in determining the real prevalence of tuberculosis among people living with HIV/AIDS (PLWHA) and the determination of *Mycobacterium tuberculosis* to the genus level. These findings will add to our understanding of the co-pathogenesis of these two infections and may have therapeutic implications.

Materials and methods

This was a qualitative, prospective, hospital-based, analytical, descriptive, and cross-sectional study. Specimens were collected from participants with tuberculosis and HIV infections. The study was conducted during the period from August 2012 to June 2015. It was carried out in West Darfur State which is located in the Sudan's far western border. Population studied was divided into two groups: patients with HIV and AIDS and tuberculous patients negative for HIV and AIDS; covering males and females at different age groups. Inclusion criteria included all HIV positive patients, TB positive, and HIV negative patients in West Darfur State. Exclusion criteria included HIV-negative individuals, and TB-negative individuals. The software used for the analysis of data was the Statistical Package for Social Sciences (SPSS) program (version 14). For categorical variables, proportions were compared by the Chi-square test as appropriate. The means and medians of the continuous variables were compared by Student's *t* test program depending on the sample distribution. Frequencies, percentages, tables and graphs were used for presentation of the data. Confidentiality of information obtained from participants investigated was maintained. Consent of the participants was taken before being enrolled in the study. Laboratory results of specimens collected were handed to all participants included in the study or dispatched to physicians treating those participants for prescription. Permission to collect the specimens was obtained from the Federal Ministry of Health (Khartoum) and Algeneina TB Center at Algeneina Hospital, West Darfur State (Sudan). Complete information regarding risk factors, if any, was handed to all participants under the study and no concealment what so ever. Sampling was a non- probability purposive sampling type, and sample strategy was convenience where participants were chosen on the basis of accessibility. The sample frame was participants

with HIV and AIDS and tuberculous patients negative for HIV and AIDS. Sample size was a 1318 sputum specimens collected from the two population groups. Demographic and clinical data were collected from all participants using a structured questionnaire with a written informed consent. Specimens collected from patients with HIV and AIDS were 158. Patients of this group were attending Algeneina Hospital. Specimens were microscopically examined for AFB. TB positive sputum samples were stored at -20°C until transported to Khartoum (Sudan) for further molecular techniques. Samples collected from TB patients were 1160. Patients of this group were pulmonary TB patients attending Algeneina TB Center. Specimens were microscopically examined for AFB. TB positive sputum samples were stored at -20°C until transported to Khartoum for further molecular techniques. Two consecutive sputum specimens (spot-morning) were collected and examined for the presence of acid fast bacilli (AFB) using the standard Ziehl-Neelsen (ZN) method. The specimens were transported to the laboratory for immediate processing while any sample that was poorly collected (sputum containing saliva) was discarded. Diagnosis of TB among TB suspects was performed as per WHO guidelines. All ZN stain positive TB patients were subjected to DNA extraction by the Guanidine chloride method. Sputum samples were taken in a tube and equal volume of sodium hydroxide (4%) was added for decontamination, then transferred to a Falcon tube and mixed well and centrifuged for 15min-at 4000rpm. The supernatant was discarded and the quantities of the following reagents were added: WBCs lysis buffer which contains (Tris-HCl + EDTA + NaCl + SDS), 10 µl of proteinase K, ammonium acetate, and guanidine chloride. After incubation at 65^{o} C for 2 hours, chloroform was added, mixed and centrifuged. The upper layer was carefully transferred to another clean Falcon tube, and cold ethanol was added and incubated overnight at -20^{o}C. Then the tubes were centrifuged and the supernatant was discarded off, and the tubes were then dried in a vertical position. Ethanol was added to the tubes, mixed well and centrifuged and the supernatant was discarded. The pellet was allowed to dry and distilled water was added. The concentration of the DNA and its purity were measured using the Nanodrop machine (Germany). The samples with DNA concentration above 50 and purity above 1.7 were used for next steps. The extracted DNA was stored at -20^{o}C till used. The extracted DNA was processed by PCR technique using the primers design of the oligonucleotides derived from the IS6110 sequence (Eurofins Genomics). DNA amplification was performed using the primers:
IS6110-Forward 5′-CCTGCGAGCGTAGGCGTCGG-3′
IS6110-Revers- 5′CTCGTCCAGCGCCGCTTCGG-3′
To amplify 123 bp fragment of insertion element IS6110 of *M. tuberculosis* complex a template DNA and primers were added to Maxime PCR Pre-Mixtubes (i-Taq) which contains i-Taq TMDNA Polymerase, dNTP mixture and reaction buffer. Then a volume of 16 µl distilled water was added into the tubes to a total volume of 20µl. Then the PCR procedure was performed in line with 30 cycles including denaturation, annealing, and extension cycling parameters. The samples were then loaded on 2.0% agarose gel without adding a loading-dye buffer and electrophoresis was performed. The electrophoresis product was visualized under ultra-violet (UV) light. Electrophoresis technique for DNA detection was performed on gels and stained with ethidium bromide, which has an intense fluorescence excited by ultra-violet radiation when it

complexes with nucleic acids. A molecular weight DNA marker (Ladder) was run on every gel. The gel was run in 1X TBE running buffer and electrophoresis was carried out at 100 volts for 20 min. then the gel is viewed under UV light and photographed. The amplified DNA extracted was sequenced by Macrogen Company (Korea).

Results:

Of the total 158 PLWHA patients tested for TB in this study 6/158 (3.8%) were found smear positive by microscopic ZN examination. The frequency rate of TB among HIV-infected patients in this study was significantly higher in males (66.7%) compared with females (33.3%). The TB frequency rate among PLWHA patients was observed to be higher in the age group 31-40 years (50%) and 41-50 years (33.3%) compared to younger age groups of 21-30 years (16.7%).
Of the total 1160 sputum samples collected from TB patients, 200 samples were smear positive by ZN microscopy. From these males were more infected by TB than females. The TB frequency rate among TB patients was higher in the age group 31-45 years followed by that in the age group 46-60 years. It was also noticed that most of the patients studied were residents of rural villages around Algeneina City. Of the 200 control smear-positive sputum samples collected from the TB patients who were negative for HIV infection, 85 % (170/200) were found PCR positive. PCR-IS6110 primer used for detecting *M. tuberculosis* in this study showed clear and obvious bands on gel electrophoresis. 83 % (5/6) of the TB positive PLWHA patients were from the VCT Center and only one patient (17%) was from the PLWHA Association Center.
BLAST technique was used to identify the sequence polymorphisms of selected genes related to *Mycobacterium tuberculosis* strain H37Rv. The DNA sequencing results showed that the DNA sequence of these selected samples had a pattern matching those genes in the database and close to or identical to the *M. tuberculosis* H37Rv genome by 100% for sample 1, by 99% for samples 2, 3, 4 respectively, and by 96% for sample 5. A high similarity of sequences with deletion of C nucleotide in position 5 (in samples 2, 3 and 4) and with deletion of 4 nucleotides (in sample 5) was observed. The alignment sequence of the four nitrogenous bases was drawn by base spacing computerized programs showing the steady distribution of these bases along a piece of *M. tuberculosis* H37Rv genome colored with distinct green, blue, black and red colors (Fig. 1).

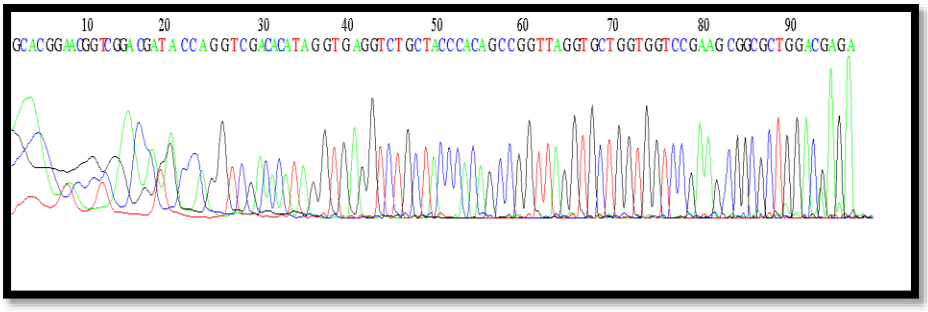

Fig. (1): A chromatogram showing the four nitrogenous bases distribution in different colors

The phylogenetic tree of the selected study strains revealed the evolutionary relation between the sequences. The phylogenetic analysis of revealed two distinct phylogroups which include some MTB mutant strains with the deletion of one or more nucleotides (Fig. 2).

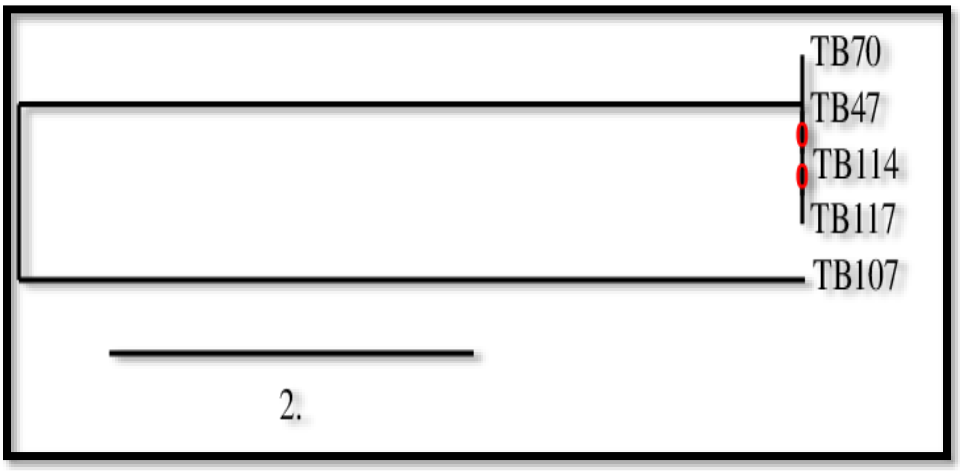

Fig. (2): The phylogenic tree of the sequenced *Mycobacterium tuberculosis* strains with code numbers

Discussion

A total 158 PLWHA patients were investigated for TB infection in this study. The prevalence rate found was 6/158 (3.8%). This result reflects a very high TB prevalence rate among PLWHA in Gujarat region in India. Our Study results also disagreed with other findings reported Cain and his colleagues from Rural Cambodia where the TB prevalence rate was 38.0% among HIV infected patients[1]; and with the result (33.0%) reported by Mihir and his co-workers among HIV-sero-positives attending a counseling center in Kolkata, India[2].

The variations in results of this study results and the above-mentioned findings is multi-factorial. They may be due to the different methodologies carried out in these studies; or due to the diagnostic techniques adopted; or due to the different populations studied. Most of these studies covered all forms of tuberculosis, while our present study focused on pulmonary tuberculosis only. Also the variation could be due to the difference in the general HIV prevalence rates reported in these countries. Furthermore, the differences between the prevalence rate of this study and the other studies mentioned above may be due to differences in the selection of the inclusion and exclusion criteria. In turn, the diagnosis of TB in PLWHA patients can be problematic, depending on the degree of the immuno- suppression state at the time of diagnosis. In HIV-infected patients with good immune defenses (CD4+lymphocyte counts >300 cells/mm3), the microbiological tests afford similar performance and maximum care is necessary

to ensure collection of good multiple samples. This makes it necessary to apply the threefold WHO strategy (2004). This strategy aimed to enhance collaborative efforts between TB and HIV/AIDS programs, to decrease the burden of TB in PLWHA patients, and to decrease the burden of HIV in TB patients (USIDS, 2014). This may be probably attributed to the type, time, and area of studies. Our findings are closer to the 3.4% result reported by Sudre and his colleagues in Switzerland[3]. The decrease in the prevalence rate of TB in HIV-infected patients may be due to the introduction of HAART which was found to have remarkable changes towards improving the management of HIV patients. We believe that, the rate of pulmonary tuberculosis could be higher if new diagnostics, particularly Xpert MTB/RIF was performed routinely for detection of *Mycobacterium tuberculosis*.

This result was obviously higher compared to our 3.8% prevalence rate.

WHO guidelines call for culture of sputum-smear-negative HIV-infected patients especially those with a clinical suspicion of TB. In our study area it was difficult to apply due to the limited resources. The clinical care and close follow-up provided for HIV infected patients in the VCT centers and clinics in our study area might have further reduced transmission and might explain the lower prevalence of TB among HIV-infected patients compared to HIV-negative patients.

Concerning the high TB infectivity among the rural residences compared to the urban may be due to the poverty, malnutrition, and the lack the health care facilities more prevalent in the rural areas.

The IS6110 gene was used as a target by the majority of the investigators performing PCR-based diagnosis of TB. The principal reason for using IS6110 gene is because it is considered to be a good target for application as an insertion sequence in most strains of *Mycobacterium tuberculosis* liable to confer high sensitivity[4].

Concerning the 85% positivity of our study control samples collected from TB-negative HIV patients was closer to that reported by El Dawi and his colleagues in their study that aimed to assess and evaluate a PCR-amplified IS6110 insertion element in the rapid diagnosis of pulmonary tuberculosis in comparison to microscopic methods in Sudan. They reported a 88.5% sensitivity and 98.6% specificity rates for IS6110-based PCR for identification of *Mycobacterium. tuberculosis* isolates in Khartoum[5]. Also ZN microscopy detects most acid fast bacilli in the sputum samples examined. These points indicate that the techniques used in this study are reliable and can be used for diagnosis and characterization of *Mycobacterium* strains.

In this study the TB infectivity rate was found higher (66.7%) among males as compared with females (33.3%).

The globally low frequency of women TB infection may be due to the gender factor as a biological determinant; or as a socio-cultural determinant factor influencing access to TB examination. Some workers attribute that to social barriers and others ascribe it to the natural epidemiology of the disease[6]. In this study area it is socially unwise for women to cough or to spit up sputum deeply on collection of sputum samples. Hence the probability of finding positive AFB in the sputum given by women will be lower than by men.

In this study the TB prevalence among PLWHA patients was found to be higher (50%) in the age group 31-40 years and (33.3%) in the age range 41-50 years as compared to younger

age groups of 21-30 years (16.7%). This age distribution is similar to the global and national frequency where the majority of TB cases are in their productive ages (SNTCP, 2011).
The official prevalence rate of TB infection in Sudan is about 209 cases per 100,000 of the population in 2009 (SNTCP, 2011). However, the prevalence rates of TB/HIV co-infection in West Darfur State in the years 2013 and 2014 was 3.0 % and 4.5% respectively (W.D.TB Records, 2015). This frequency rate is very closer to the results of the present study. Thus this study would probably give a representative sample of tuberculosis prevalence among HIV co-infection in West Darfur State.
The 100% and 99% similarity of the genetic sequence detected in this study as regard *M. tuberculosis* H37Rv strain strongly indicate that all sample processing procedures (i.e. ZN microscopy and molecular techniques) were performed properly. Pair-wise alignment of the sequences showed that the studied TB strains were closely related to each other. The 99% and 96% similarity *M. tuberculosis* genetic sequence reported in this study among PLWHA patients were closely similar to that found among negative HIV-TB patients. The deletion of one and four nucleotides observed in the present study as compared to the reference strain nucleotide sequence is a common phenomenon. However, it has been observed that many of the differences between strains within the *M. tuberculosis* complex involve just single nucleotide, the so-called single nucleotide polymorphisms (SNP). These differences between the *M. tuberculosis* strains of this study were small on considering that there are over 4 million base pair (bp) in the genome of members of *M. tuberculosis* complex[7].
The genotyping results of the present study agreed with recently published work reported by Brudey and his colleagues. These authors commented on the genetic diversity of *Mycobacterium. tuberculosis* genomes and hence their population structure are strongly linked to geography at a fine geographical scale.
The mechanisms of the virulence of *M. tuberculosis* may vary according to the genotyping lineages of the strains which may have diversified as adaptations to human population of differing genetic constitution, as this study was aimed to characterize the predominant genotypes responsible for TB in the study area and to generate a preliminary baseline data for further epidemiological and infection control studies.
This reported pattern of phylogenic diversity may be due to the results of both a deep ecological differentiation and a more recent demographic and epidemic history. This observation may suggest the introduction of new genotypes due to casual contact and/or increased international travel. Also, these findings suggest that the evolutionary characteristics of tuberculosis bacteria could synergize with the effects of increasing globalization and human travel to enhance the global spread of drug-resistant tuberculosis.
It is of great importance to say that, should the TB/HIV co-infection rate increases, it may be anticipated that providing comprehensive HIV/AIDS care and support to HIV-positive TB patients such as antiretroviral therapy (ART) and monitoring and management co-infection cases, will be made more difficult. On top of this, health care services in remote areas of the study area suffer from a lack of infrastructure, aging equipment and a shortage of adequately trained healthcare workers, resulting in additional overburdening of already strained health care institutions. Because the data of *Mycobacterium tuberculosis* sequencing in Sudan is very

limited, a comprehensive national and state level epidemiological surveys are needed applying the combination of genomics and bioinformatics which has the potential to generate the information and knowledge that will enable the conception and development of new therapies; and interventions needed to treat this airborne disease and to elucidate the unusual biology of its etiological agent.
Conclusion: Conventional and molecular techniques performed in this study showed that the people living with HIV/AIDS in West Darfur State are also co-infected with tuberculosis. Molecular techniques are rapid and sensitive diagnostic tools for diagnosis of tuberculous infection; and the distribution of the major phylogenetic families.

References

1. Cain KP, Kanara N, Laserson KF, Vannarith C, Sameourn K, Samnang K, Qualls ML, Wells CD, Varma JK, The Epidemiology of HIV-Associated Tuberculosis in Rural Cambodia., *Int J Tuberc Lung Dis.*, 2007; 11(9):1008-13.
2. Mihir K. Bhattacharya, Trailokya N. Naik, Mrinmoy Ghosh, Sarojit Jana, Phalguni Dutta. Pulmonary ,Tuberculosis among HIV Seropositives Attending a Counseling Center in Kolkata, *Indian Journal of Public Health*, 2011; Volume 55, Issue 4.
3. Sudre P., Hirschel B., Toscani L., Ledergerber B., Rieder H.L. Risk Factors for Tuberculosis among HIV-Infected Patients in Switzerland, , *European Respiratory Journal*, 2002; 1(2), 27-33.
4. Gasmelseed .N ,Aljak. M.A, Elmadani .A.E., Elgaili . M.E., and Saeed, O.K, Assessment of different samples for molecular diagnosis of extra-pulmonary tuberculosis, *International Journal for Biotechnology and Molecular Biology Research*, .2014; 5 (5): 41-47
5. El-Dawi T.G., Saeed el N.S., Hamid M.E., Evaluation of a PCR-Amplified IS6110 Insertion Element in The Rapid Diagnosis of Pulmonary Tuberculosis in Comparison to Microscopic Methods in Sudan. *Saudi Med. J.* 2004; 25(11):1644-7.
6. Ottmani S. E., M. W. Uplekar, Gender and TB: Pointers from Routine Records and Reports, *The International Journal of Tuberculosis and Lung Disease.* 2008; 12(7) : 825 -826
7. Schaaf H. Simon, Zumla I. Alimuddin. *Tuberculosis: A Comprehensive Clinical Reference*, Elsevier, 2009.