Thesis Title:

The Role of Natural Resistance-Associated Macrophage Protein I (NRAMP1) Gene Polymorphism in Development of Pulmonary Tuberculosis in River Nile State Patients, Sudan.

A thesis submitted in fulfillment for the requirement of PhD degree in medical laboratory science (Medical microbiology)

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2016
قال تعالى:

بسم الله الرحمن الرحيم

[قالوا سبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الحَكِيمُ]

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Authorship Declaration

I am certify that this thesis submitted for the degree of phD in medical microbiology is the result of my own research, except where otherwise acknowledged, and that this thesis or any of its parts has not been submitted for higher degree in this University.

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Date:  16/2/2017
DEDICATION

To my beloved parents for their care and support

To my lovely daughter Fatima

To all my sisters and brothers who prayed for my success

To all whom I love and respect

Ahmed
ACKNOWLEDGEMENT

• First of all I would like to thank AL MIGHTY ALLAH who gave me the strength and patience to finish this work.

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• Again I would like to thank all staff of the Research Laboratory at the College of Medical Laboratory Sciences(Sudan University of Science and Technology) for their technical assistance.

• Also I am thankful to the staff of the Tuberculosis Centers at Shendi, Al Damer and Atbara for their help in specimens and data collection.
# LIST OF CONTENTS

<table>
<thead>
<tr>
<th>items</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quran Verse</td>
<td>I</td>
</tr>
<tr>
<td>Examination committee member (jury)</td>
<td>II</td>
</tr>
<tr>
<td>Bibliographic Entry</td>
<td>III</td>
</tr>
<tr>
<td>Authorship Declaration</td>
<td>IV</td>
</tr>
<tr>
<td>Dedication</td>
<td>V</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>VI</td>
</tr>
<tr>
<td>List of contents</td>
<td>VII</td>
</tr>
<tr>
<td>List of Tables</td>
<td>X</td>
</tr>
<tr>
<td>List of figures</td>
<td>XI</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>XII</td>
</tr>
<tr>
<td>Glossary</td>
<td>XV</td>
</tr>
<tr>
<td>Abstract (English)</td>
<td>XX</td>
</tr>
<tr>
<td>Abstract (Arabic)</td>
<td>XXII</td>
</tr>
</tbody>
</table>

**CHAPTER ONE: INTRODUCTION:**

1.1. Introduction: ................................................................. 1
1.2. Rationale: ................................................................. 3
1.3. Objectives: ................................................................. 4
  1.3.1. General objective: .................................................. 4
  1.3.2. Specific objectives: ............................................... 4
CHAPTER TWO: LITERATURE REVIEW:

2.1. History of Tuberculosis: .................................................. 5
2.2. Transmission of TB: ......................................................... 5
2.3. Immune response against \textit{M. tuberculosis}: ...................... 5
2.4. Tuberculosis disease: ...................................................... 9
2.5. Symptoms and signs of tuberculosis: .................................. 20
2.6. Diagnosis of Tuberculosis: .............................................. 22
2.7. Treatment and prevention of tuberculosis: ......................... 29
2.8. Host Genetics and Susceptibility: ..................................... 33
2.9. NRAMP-1 gene: ............................................................. 37

CHAPTER THREE: MATERIALS AND METHODS:

3.1 Study design: ................................................................. 43
3.2 Study area: ................................................................. 43
3.3 Study population: .......................................................... 43
3.4 Sample size: ................................................................. 43
3.5 Inclusion Criteria of Index cases: ...................................... 44
3.6 Inclusion Criteria of control individuals: ............................ 44
3.7 Ethical considerations: .................................................... 44
3.8 Validity and pre-testing: .................................................. 44
3.9 Collection of the specimens: ............................................. 44
3.9.1 Sputum specimens: ..................................................... 44
3.9.2 Blood specimens: ....................................................... 45
3.10 Preparation of smears: .................................................. 45
3.11 Ziehl-Neelsen (ZN) staining method: ................................ 45
3.12 DNA extraction: .......................................................... 46
3.13 Primers preparation: ...................................................... 47
3.14 DNA amplification: ....................................................... 48
3.15 Preparation of agarose gel: ........................................... 50
3.16 Restriction fragment length polymorphism: ...................... 50
3.17 Method of data collection: ............................................. 51
3.18 Statistical analysis: ...................................................... 51

CHAPTER FOUR: RESULTS:

4.1 The socio-demographic characteristics of the Study population . .52
4.2 Clinical characteristics of tuberculosis cases: ....................... 52
4.3 PCR amplification of the NRAMP1 locus: ..........................57
4.4 Allelic frequencies of the NRAMP1 loci: .............................. 59

CHAPTER FIVE:

5.1. Discussion: ............................................................. 63
5.2. Conclusion: ............................................................. 67
5.3. Recommendations: ..................................................... 68

CHAPTER SIX:

6.1. References: .............................................................. 69
6.2. Appendices: ............................................................... 81
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Descriptions</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1:</td>
<td>Quantitation scale recommended by the WHO and the IU against Tuberculosis and Lung Disease</td>
<td>25</td>
</tr>
<tr>
<td>Table 3.1:</td>
<td><em>PCR</em> primers and restriction enzymes employed in PCR-RFLP study of NRAMP-1</td>
<td>48</td>
</tr>
<tr>
<td>Table 3.2:</td>
<td><em>DNA</em> amplification program of <em>D543N</em> polymorphism</td>
<td>49</td>
</tr>
<tr>
<td>Table 3.3:</td>
<td><em>DNA</em> amplification program of <em>3’UTR</em> polymorphism</td>
<td>49</td>
</tr>
<tr>
<td>Table 3.4:</td>
<td><em>DNA</em> amplification program of <em>INT4</em> polymorphism</td>
<td>49</td>
</tr>
<tr>
<td>Table 4.1:</td>
<td>Distribution of TB cases and controls according to gender</td>
<td>53</td>
</tr>
<tr>
<td>Table 4.2:</td>
<td>Age group of TB cases and control distribution</td>
<td>54</td>
</tr>
<tr>
<td>Table 4.3:</td>
<td>Distribution of TB cases according to district of residence and age groups</td>
<td>55</td>
</tr>
<tr>
<td>Table 4.4:</td>
<td>Educational level of the TB cases</td>
<td>55</td>
</tr>
<tr>
<td>Table 4.5:</td>
<td>Demonstration the Duration of TB symptoms</td>
<td>57</td>
</tr>
<tr>
<td>Table 4.6:</td>
<td>Allelic frequencies of the three loci INT4, D543N and 3’UTR in sputum smear positive tuberculosis patients and health controls</td>
<td>62</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1:</td>
<td>World Map showing the estimated incidence rates of TB</td>
</tr>
<tr>
<td>Figure 4.1:</td>
<td>Distribution of TB cases and controls according to gender</td>
</tr>
<tr>
<td>Figure 4.2:</td>
<td>Residency percentage of TB cases</td>
</tr>
<tr>
<td>Figure 4.3:</td>
<td>Illustrate the family history of TB disease.</td>
</tr>
<tr>
<td>Figure 4.4:</td>
<td>Display the symptoms which were appeared in TB cases</td>
</tr>
<tr>
<td>Figure 4.5:</td>
<td>Agarose gel electrophoresis detection of the PCR amplification of the NRAMP1 loci</td>
</tr>
<tr>
<td>Figure 4.6:</td>
<td>Agarose gel electrophoresis analysis of the RFLP patterns observed from ApaI digestion of the locus Intron4</td>
</tr>
<tr>
<td>Figure 4.7:</td>
<td>Agarose gel electrophoresis analysis of the RFLP patterns observed from AvaII digestion of the locus D543N</td>
</tr>
<tr>
<td>Figure 4.8:</td>
<td>Agarose gel electrophoresis analysis of the RFLP patterns observed from FokI digestion of the locus 3′UTR</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’ UTR</td>
<td>3’ untranslated region</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>AFB</td>
<td>Acid Fast Bacilli</td>
</tr>
<tr>
<td>Ava II</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>Apa I</td>
<td>Restriction enzyme</td>
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<tr>
<td>BCG</td>
<td>Bacille Chalmette-Guerin</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>C3b</td>
<td>Complement factor 3</td>
</tr>
<tr>
<td>CD4+</td>
<td>T-helper cells expressing cluster of differentiation 4</td>
</tr>
<tr>
<td>CD8+</td>
<td>T-helper cells expressing cluster of differentiation 8</td>
</tr>
<tr>
<td>CPC</td>
<td>Acetylpyridium chloride</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocytes</td>
</tr>
<tr>
<td>CXR</td>
<td>Chest X-Ray</td>
</tr>
<tr>
<td>dNTP</td>
<td>2’,-deoxyribonucleotide 5’-triphosphate</td>
</tr>
<tr>
<td>del</td>
<td>Deletion</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell specific intercellular adhesion molecule</td>
</tr>
<tr>
<td>DTH</td>
<td>Delay type hypersensitivity</td>
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<tr>
<td></td>
<td>3 grabbing non-integrin</td>
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<td></td>
<td></td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>DR</td>
<td>Direct repeat</td>
</tr>
<tr>
<td>EB</td>
<td>Elution buffer</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetra-acetic Acid</td>
</tr>
<tr>
<td>EMB</td>
<td>Ethambutol</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>Fok I</td>
<td>Restriction enzyme</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>INT 4</td>
<td>Intron 4</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthases</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MOTT</td>
<td>Mycobacteria other than <em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>NRAMP1</td>
<td>Natural Resistance-Associated Macrophage Protein I</td>
</tr>
<tr>
<td>PAS</td>
<td>Paraaminosalicylic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Pmol</td>
<td>Picomole</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified Protein Derivative</td>
</tr>
<tr>
<td>PZA</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>rpm</td>
<td>Round per minutes</td>
</tr>
<tr>
<td>Sec</td>
<td>Second</td>
</tr>
<tr>
<td>T-cell</td>
<td>Thymus derived lymphocytes</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>Th-1</td>
<td>T- helper cells subset 1</td>
</tr>
<tr>
<td>Th-2</td>
<td>T- helper cells subset 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>Treg</td>
<td>T- regulatory cells</td>
</tr>
<tr>
<td>TST</td>
<td>Tuberculin skin test</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>ZN</td>
<td>Ziehl-Neelsen’s</td>
</tr>
</tbody>
</table>
GLOSSARY

**Acid fast:** Describes an organism that resists acid decolorization after staining.

**Allele:** Alternate forms of a gene at the same chromosomal locus.

**Alveoli (lung):** Microscopic air sacs in lung.

**Amplicon:** A defined stretch of (chromosomal) DNA that undergoes amplification.

**Amplification:** Genetic mechanism by which the copy number of a gene is increased above its normal level in the diploid genome.

**Apoptosis:** Complex program of cellular self-destruction, triggered by a variety of stimuli and involving the activation of caspase enzymes, that results in rapid fragmentation of a cell and phagocytosis of resulting cell fragments by neighboring cells.

**Chi-square test:** A statistical test for measuring the significance of the discrepancy between observed and expected results; also written $\chi^2$.

**Cytokines:** (1) Growth factors that stimulate one or several of the cell types constituting the hematopoietic system. (2) Regulatory factors of the immune system, including interferons and interleukins that, like mitogenic growth factors, convey signals between cells.

**Deletion:** A loss of a part of a chromosome.

**Denaturation:** Process that causes a molecule, such as a macromolecule (DNA, RNA, or protein), to lose its natural three-dimensional structure.

**Deoxyribonucleic acid (DNA):** Organic molecule that is the basis of heredity. A double-stranded structure composed of nucleotides (phosphate group, 5- carbon Deoxyribose sugar and purine or pyrimidine base).
**Dideoxyribonucleotides:** Analogues of the normal DNA precursor molecules that lack 3-hydroxyl residues. Incorporation of dideoxyribonucleotides into DNA terminates chain elongation and is the basis for the DNA sequencing technology developed by Frederick Sanger.

**DNA sequencing:** A technique that permits the base-by-base determination of the linear array of nucleotides within a DNA chain.

**Duplication:** A part of a chromosome in which the genetic material is repeated; also the process of forming a duplication.

**Electrophoresis:** A technique used to detect variation in proteins, involving the use of an electric field to cause the proteins to migrate along a gel and then observing their relative positions on the gel by protein-specific staining reactions.

**Facultative:** When describing bacteria without a qualification means ability to grow aerobically or anaerobically.

**Family history:** A record of the relationships among family members along with their medical histories. This includes current and past illnesses. A family history may show a pattern of certain diseases in a family.

**Gene:** a sequence of DNA that usually encodes a polypeptide.

**Gene amplification:** Increase in the number of copies of a gene normally present in the diploid genome.

**Genetic polymorphism:** A variant sequence element in an organism’s genome that has no effect on phenotype yet is transmitted genetically as a Mendelian determinant.

**Genotype:** The genetic constitution of an organism.
Growth factor: Protein that is able to stimulate the growth and/or proliferation of a cell by binding to a specific cell surface receptor displayed by that cell.

Heterozygosity: Genotypic situation in which two homologous loci in a given chromosome pair each carry a different allele.

Heterozygote: An organism having different alleles at a given locus on homologous Chromosomes.

Heterozygous: Referring to the configuration of a genetic locus in which the two copies of the associated gene carry different versions (alleles) of the gene.

Homologous: (1) Referring to the relationship between a pair of chromosomes that carry the same set of genes within a diploid cell or organism. (2) Referring to genes or characteristics that are similar in related organisms because of shared descent from a common precursor. (3) Referring to two nucleic acids having similar nucleotide sequences.

Homozygosity: Presence of identical alleles on both chromosomes in a given pair; this term may apply to the genotype of individuals who have inherited a double dose of an abnormal allele, whether the mutated version is the same or different on each chromosome.

Homozygote: An organism having the same allele at a given locus on homologous chromosomes.

Homozygous: Referring to the configuration of a genetic locus in which the two copies of the gene carry identical versions (alleles) of the gene.

Immunocompromise: Deficiency in some components of the body’s immune mechanisms.

Inflammation: A process in which certain cellular components of the immune system are involved in the remodeling of a tissue in response to wounding, irritation, or infection.
**Intron:** Portion of a primary *RNA* transcript that is deleted during the process of splicing. An intervening sequence.

**Kinase:** Enzyme that covalently attaches phosphate groups to substrate molecules, often proteins.

**Mutation:** any detectable and heritable change in the genetic material that is not caused by segregation or recombination.

**Nucleotide:** The monomeric unit that makes up *DNA* or *RNA*, formed by the addition of a phosphate group to a nucleoside.

**Pathogen:** an organism with the potential to cause disease.

**Phagolysosome:** The digestive vacuole formed by fusion of the cell lysosomes with the phagocytic vacuole.

**Phagocytosis:** The process of internalization of particulate matter by cells, e.g. microbes and dead cells.

**Polymorphism:** Genetic polymorphism is where a gene has several allelic forms present at a single gene locus (e.g. blood groups, MHC).

**Polymerase chain reaction:** Continuous enzyme-mediated amplification of a nucleotide sequence that allows its detection and analysis.

**Primer:** a short sequence of single-stranded DNA or RNA required by DNA polymerase as a starting point for chain extension.

**Prognosis:** A prediction about the future clinical course of a disease often influenced by detailed analyses of its existing attributes, such as histopathology and biochemical markers.

**Prophylaxis:** Measures or treatments designed to prevent disease.

**Receptor:** Component of the cell surface to which another substance or organism attaches specifically.
**Replication:** the process by which the two strands of DNA separate and each serves as a template for the synthesis of a new complementary strand.

**Restriction endonuclease:** an enzyme that internally cleaves double-stranded DNA molecule after binding to a specific nucleotide recognition sequence.

**Restriction fragment length polymorphism:** Variation in DNA sequence that can be detected through its effect of allowing or preventing cleavage of a chromosomal DNA segment by a restriction enzyme.

**Serology:** The use of antibodies to detect and measure antigens, e.g. in the typing of infectious agents.

**T cells:** Thymus derived immunocytes: helper, suppressor, and cytotoxic T cells.

**Vaccination:** inoculation with a vaccine to provide protective immunity.
ABSTRACT

Background: Tuberculosis, caused by the human pathogen *Mycobacterium tuberculosis* (MTB) which is harbored by macrophages, is a chronic disease which kills more people than any other infectious agents. It has been estimated that one third of the whole world's population has been infected with MTB. The candidate gene natural-resistance-associated macrophage protein one, NRAMP1, which is associated with host susceptibility to the pathogen MTB. Evidence for the gene associated with host susceptibility to tuberculosis has been found in Western Africans, Aboriginal Canadians, Koreans and Japanese.

Objective: The aim of this study was to determine the socio-economic factors of diagnosed TB patients and to investigate polymorphisms of three regions of the NRAMP1 gene: INT4, D543N and 3'UTR, in association with the host susceptibility to tuberculosis among River Nile State population in Sudan during September 2013 through January 2017.

Materials and methods: PCR based restriction fragment length polymorphism (*RFLP*) analysis was used to type the polymorphisms and to determine the allelic frequencies of the 3 loci of the *NRAMP 1* gene amongst patients and controls.

Results: An association based case-control study has been done by analyzing (143) tuberculosis patients and (126) healthy controls. The age groups most commonly affected by tuberculosis were adults in their productive years (16 – 30 years, 28.7 %). Male to female ratio (1.5:1). Prevalence was higher among the Khalwa & Primary group (40%) and illiterate group (33.6%). (24%) tuberculed patients were reported as positive family history of TB. In the group of tuberculosis patients, the
results of the statistical analysis for the 3 NRAMP1 loci were found to be: INT4 (p-value = 0.604), D543N (p-value = 0.001) and 3'UTR (p-value = 0.003). Therefore the NRAMP1 gene exhibits strong statistically significant associations between D543N and 3'UTR polymorphisms and the host susceptibility to tuberculosis.

**Conclusion:** The study concluded that D543N and 3'UTR NRAMP1 polymorphisms may have a role in the development of pulmonary tuberculosis and a lack of association was observed between INT4 polymorphism and susceptibility to the development of pulmonary tuberculosis.
الخلاصة

الخلفية: السل، تسبب جرثومه تسمى بالمتفرزة السلية والتي لها المقدرة للنمو داخل الخلايا البلعمية، السل هو مرض مزمن يتسبب في قتل عدد كبير من الناس أكثر من أي مرض مغذي آخر. تثلث سكان العالم مصاب بهذا المرض. هنالك جين يسمى البروتين الأول للمقاومة الطبيعية للخلايا البلعمية وهو مرتبط بالقابلية للإصابة بالسل. وقد تم العثور على أداة عن ارتباط الجين بالقابلية للإصابة بمرض السل في إفريقيا الغربية، السكان الأصليين الكنديين والكوريين واليابانيين.

الأهداف: هدف من هذه الدراسة هو تحديد العوامل الاجتماعية والاقتصادية لمرضى السل والتحقيق من الأشكال الجينية الثلاثة الموجودة في جين البروتين الأول للمقاومة الطبيعية للخلايا البلعمية وهي D543N و UTR'3 و INT4، لمعرفة قابلية المرضى لمرض السل بين سكان ولاية نهر النيل في السودان خلال سبتمبر 2013 حتى يناير عام 2017.

المواد والطرق: تم استخدام تقنية PCR-RFLP لتحديد الأنماط الجينية الثلاثة من الجين في عينات كل من المرضى والحالات الضابطة.

النتائج: استخلصت النتائج على أساس دراسة الحالات والشواهد عن طريق تحليل 143 من مرضى السل و 126 من الإصحاء. وكانت الفئات العمرية الأكثر إصابة بالسل البالغين في سنواتهم الإنتاجية (16-30 سنة 28.7%). كما كانت نسبة الذكور إلى الإناث 1:5.1. وكان انتشار السل العالمي بين مجموعة دارسي الخلوة والأساس 40٪، ومجموعة الأميين 33.6٪.

كما تم الإبلاغ عن 24٪ من مرضى السل كان لهم تاريخ إيجابي من السل.

في مجموعة مرضى السل، نتائج التحليل الإحصائي للجين في مواقع الثلاثة كانت على النحو التالي: D543N (القيمة الاحتمالية = 0.604)، UTR'3 (القيمة الاحتمالية = 0.001) و INT4 (القيمة الاحتمالية = 0.003). لذلك كانت هناك علاقة ذات دلالة إحصائية قوية بين النمط المض뒤 للمرض والتسبب في النجاة.

الاستنتاجات: خلصت الدراسة إلى أن النمط D543N UTR'3 من جين البروتين الأول للمقاومة الطبيعية للخلايا البلعمية قد يكون له دور في تطوير مرض السل الرئوي، كما لوحظ عدم وجود أي ارتباط بين النمط INT4 والقابلية لتطوير السل الرئوي.
Chapter One

Introduction

Rationale

Objectives
1.1. INTRODUCTION

*Mycobacterium* is a genus of Gram-positive bacilli that demonstrate the staining characteristic of acid-fastness. It’s most important species, *Mycobacterium tuberculosis*, is the etiologic agent of tuberculosis. Tuberculosis (*TB*), one of the oldest known human diseases, still is one of the major causes of mortality. *TB* remains the single largest infectious disease causing high mortality in humans, leading to (3) million deaths annually, about five deaths every minute. Approximately (8-10) million people are infected with this pathogen every year (*WHO* 1999). Out of the total number of cases, (40) per cent of cases are accommodated in South East Asia alone. In India, there are about (500,000) deaths occurring annually due to *TB* (*RNTCP* 1999), with the incidence and prevalence being 1.5 and 3.5 million per year. *TB* has many manifestations, affecting bone, the central nervous system, and many other organ systems, but it is primarily a pulmonary disease that is initiated by the deposition of *M. tuberculosis*, contained in aerosol droplets, onto lung alveolar surfaces. From this point, the progression of the disease can have several outcomes, determined largely by the response of the host immune system. The efficacy of this response is affected by intrinsic factors such as the genetics of the immune system as well as extrinsic factors, *e.g.*, insults to the immune system and the nutritional and physiological state of the host. In addition, the pathogen may play a role in disease progression since some *M. tuberculosis* strains are reportedly more virulent than others, as defined by increased transmissibility as well as being associated with higher morbidity and mortality in infected individuals (*WHO* 1999).

In tuberculosis pathogenesis, the host cellular immune response determines whether an infection is arrested as latent or persistent infection or progresses to the next stages, active tuberculosis infection. Efficient cell-mediated immunity hinders tuberculosis infection by permanently arresting the infection at latent or persistent
stage, but if the initial infection in the lung is not controlled or if the immune system becomes weakened, \textit{M. tuberculosis} can cause active pulmonary or extra\textit{pulmonary TB}(Smith 2003). Therefore, it is expected that the genetic variants of molecules involved in innate host-defense mechanisms are associated with host\textit{susceptibility to TB}(Murray 1990).

Approximately (90%) of tuberculosis-infected individuals may remain asymptomatic with latent infection and only (10%) will develop active disease, again, suggesting that host genetic factors play an important role to regulate the progression of\textit{tuberculosis infection}(Murray 1990). Differential rates of tuberculosis infection and clinical outcomes among races, ethnicities, and families suggest a plausible genetic contribution toward\textit{tuberculosis susceptibility}(Azad 2012). Complex interactions of \textit{M. tuberculosis} with environmental and host genetic factors play a critical role in tuberculosis infection (Azad 2012). Several genomic studies demonstrate that host genetics strongly influence \textit{TB susceptibility}(Moller 2010).

\textit{NRAMP-1} is located on the endocytic compartment of resting macrophages and is recruited to the membrane of the phagosome depending on the pH gradient (Hatta 2010). \textit{NRAMP-1} acts as a divalent cation transporter or antiporter across phagosomal membranes that are expressed only in reticuloendothelial cells (Azad 2012). These facts suggest that \textit{NRAMP-1} may inhibit the replication of intracellular pathogens by altering the phagolysosomal environment. \textit{NRAMP-1} is a critical mediator in the innate immune response to tuberculosis infection which leads to decreased\textit{DNAreplication} and respiratory chain function in \textit{M. tuberculosis}, but the precise function of this protein remains unclear (Courvilleet \textit{et al} 2000).
1.2. Rationale:

Tuberculosis (TB) remains a major global health problem by causing ill-health among millions of people each year and ranking as the second leading cause of death from an infectious disease worldwide. The latest estimates were almost (9) million new tuberculosis cases and (1.4) million tuberculosis-related deaths in 2011. The complex interactions of M. tuberculosis with environmental and host genetic factors play a critical role in tuberculosis infection, also host genetics strongly influence tuberculosis susceptibility. Unraveling the mechanisms underlying the genetic variations that influence the susceptibility or resistance to tuberculosis may lead to better understanding TB pathogenesis and the development of novel strategies for prevention and treatment of tuberculosis. Assessing the contributions and functional consequences of human genetic polymorphisms to TB susceptibility or disease progression remains a major challenge. In spite of that fact only a few researches had been done in Sudan. This research will be used to facilitate the prevention of TB (inform physician to decide whether antituberculous drugs should be prescribed) by detection of polymorphisms in NRAMP-I gene, which may play a role in the development of tuberculosis, but the relevance of polymorphism within these genes to the common phenotype of TB remains unclear.
local perfusion: this is an ideal association that hinders the access of defense cells (Russell 2007).

2.4.2. The inflammatory response:
With the development of specific cellular immune response and production of interferon-gamma (IFN-γ), a mature stable granuloma is formed, which is responsible for the immune containment of the pathogen. Mature granulomas present neovascularization, epithelioid and giant multinucleated cells. An extensive fibrotic capsule develops and infected macrophages trapped inside granulomas eventually die. *Tubercle bacilli* tend to locate in the center of the granuloma, but bacteria and antigens are also associated with macrophages in the peripheral infiltrate (Russell2007). The necrotic material present in the center of *TB lesions* contains high amounts of fat representing the lipids liberated from bacillary catabolism. This material, which has a soft, dry and cottage cheese texture, is known as caseous necrosis. On microscopy, large amounts of epithelioid and giant multinucleated cells can be observed in the granulomas, located mainly around the caseous material. The nature of the host immune response will determine whether the infection will progress or be contained(Russell 2007).

Individuals who are able to mount an immune response adequate enough to contain *M. tuberculosis bacilli* at this stage will develop a clinical form of infection, characterized as latent infection, in which bacilli will stay for an undetermined period of time. The risk of progressing to clinical *TB* is highest during the first(3-5 years) after the infection, especially among immunosuppressed individuals(Russell 2007).

In most individuals, *TB infection* is clinically irrelevant and seldom recognized. It commonly occurs during childhood, and may occasionally cause malaise, low-grade fever and cough. In most individuals, however, primary *TB infection* causes no apparent symptoms and the infection stays latent for life or until reactivation. *Chest X-rays* can present several manifestations. The classical presentation is
known as the primary Ranke’s complex, including a calcified peripheral lung nodule (Gohn’s primary focus), lymphangitis, and enlarged local lymph nodes (Bates 1980, Melo 1993, Lima 1993).

The development of clinical TB will occur in (5 -10 %) of infected persons at some point in their lives, for reasons that are not completely clear. Some factors involved in increased risk of developing TB have been established, of which the most important are those interfering directly with host immunity. Diseases and conditions that weaken immunity, such as malnutrition, alcoholism, advanced age, HIV/AIDS, diabetes, gastrectomy, chronic renal insufficiency, silicosis, paracoccidiomycosis, leukemia, solid tumors, immunosuppressive drug treatments, and hereditary features, are factors that facilitate the development of TB disease. Additional factors include the infective bacterial load, pathogenicity/virulence of bacilli, and host genetic susceptibility.

2.4.3. The primary disease:

Adult primary TB is paucibacillary, practically non-contagious, difficult to diagnose and have variable severity. In seriously immunodepressed patients, but also in individuals with IFN-γ or IL-12 receptor deficiency, it can develop into a disseminated form, which is sometimes fatal. High morbidity in the primary form was also observed in patients whose ancestors were not previously exposed to the tubercle bacillus, as reported in the Yanomami Indians in the Amazon Region (Souza 1997).

2.4.4. The post-primary disease:

The existence of post-primary TB, also known as secondary TB, means that the infection can progress after the development of an adequate specific immune response. This TB episode can develop in two ways: by inhalation of new bacilli or by reactivation of the primary focus. Recently, in African countries, using molecular typing methods, it has been shown that the transmission is community driven, and not solely through households, and that reinfection with novel M.
1.3. Objectives:

1.3.1. General objective:

To determine the role of NRAMP-Igene polymorphism in development of pulmonary TB among patients in River Nile State.

1.3.2. Specific objectives:

- To detect the *M. tuberculosis* bacillimicroscopically in sputum using ziehl - Neelsen (ZN) smears.
- To assess the association of *M. tuberculosis* infection with age and gender incidences.
- To check the association of TB with environmental factors (work, education, and family history)
- To detect polymorphism of NRAMP-1 gene by using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP).
- To study the association of polymorphismof NRAMP-1 gene with pulmonary TB.
Chapter two

Literature Review
2. Literature Review

2.1. History of tuberculosis:
Tuberculosis (TB) can present in various forms, including one that attacks bone and causes skeletal deformities. Hard tissues like bone can be preserved for thousands of years, allowing the almost certain identification of individuals with bone TB who died more than (4,000 years) ago. The frequency of unearthed skeletons with apparent tubercular deformities in ancient Egypt suggests that the disease was common among that population. The discovery of similarly deformed bones in various Neolithic sites in Italy, Denmark, and countries in the Middle East also indicates that TB was found throughout the world up to (4,000 years) ago (Stead 1997).

2.2. Transmission of TB:
TB can be contracted when M. tuberculosis is ingested (Rua-Domenech, 2006) or when it is introduced through the skin (Kramer et al., 1993). However, M. tuberculosis is predominately an airborne pathogen that spreads between hosts by inhalation. Droplets of moisture from the respiratory tract are expectorated when people cough, sneeze or talk. It was found that when a person is talking, (60%) of the droplets that are expelled from the mouth are smaller than (100 µm) in diameter, which means that they evaporate rapidly (Xie et al., 2009; Xie et al., 2007). Droplets produced by people with active pulmonary TB contain bacilli and so an infectious core is left behind, following evaporation, which is known as a droplet nucleus. Droplet nuclei can travel on air currents and remain airborne for several hours. It is estimated that eight hours of exposure to an infectious person are required for TB transmission to occur (Musher, 2003; Riley et al., 1962).

2.3. Immune response against Mycobacterium tuberculosis:
The immune response against TB plays a fundamental role in the outcome of M. tuberculosis infection. It is clear that the immune system reacts efficiently in the
vast majority of infections. This is particularly evident in the case of *TB*, where most people infected by the *tubercle bacillus* (~ 90 %) do not develop the disease throughout their lifetimes. Nevertheless, the risk of developing the disease increases considerably when *TB infection* co-exists with an alteration in the immune system, such as co-infection with human immunodeficiency virus (*HIV*) (Palomino *et al* 2007).

Elimination of *M. tuberculosis* infection mainly depends on the success of the interaction between infected macrophages and *T lymphocytes*. Primary as well as acquired immunodeficiency, especially human immunodeficiency virus infection, has dramatically shown the importance of cellular immunity in tuberculosis. (*CD4*⁺ *T cells*) exert their protective effect by the production of cytokines, primarily gamma interferon (*IFN-γ*), after stimulation with mycobacterial antigens. Other *T-cell* subsets, like (*CD8*⁺ *T cells*), are likely to contribute as well, by secreting cytokines and lysing infected cells (Geluk 2000, Stenger 1999).

**2.3.1. Innate immune response:**

Upon entry into the host lungs by aerosol inhalation, *M. tuberculosis* interacts with various receptors such as pattern recognition receptors such as toll-like receptors (*TLR*), complement receptor 3, mannose receptor, scavenger receptor, on the surface of macrophages and dendritic cells (*DC*). These receptors recognize components of *M. tuberculosis* such as lipoprotein, and lipoarabinomannan. Lung surfactant protein D binds *M. tuberculosis* surface lipoarabinomannan and limits the intracellular growth of *M. tuberculosis* by increasing phagosome lysosome fusion (Ferguson 2006).

**2.3.2. Initial infection:**

Free macrophages are present in the alveoli. Their function is to ingest particles and destroy bacteria. Ingestion occurs when receptor molecules on the surface of the macrophage bind to the surface of a bacterium. *M. tuberculosis* is a facultative intracellular pathogen, which primarily inhabits macrophages. The outcome of the
deposition of *M. tuberculosis* in the alveoli depends on the virulence of the bacteria and the microbicidal capacity of the alveolar macrophage. However, if the response is excessive the inflammation caused can lead to permanent tissue damage (Tomlinson *et al.*, 2012).

The macrophages of people who are exposed to *TB* but do not become infected are able to produce toxic reactive nitrogen and oxygen intermediates in response to ingestion of *M. tuberculosis*. The phagosome, containing the ingested bacteria, fuses with lysosomes, which introduces digestive enzymes that destroy the bacteria (Hope *et al.*, 2004). Proteins from dead bacteria are displayed on the surface of the macrophage, in association with major histocompatibility complex (*MHC*) class II. This allows the antigen to be presented to *T cell* receptors on (*CD4*+ *T cells*). Lipids can be presented to different sets of *T cells*, including (*CD8*+ *T cells*) by *MHC* I molecules. This causes stimulation of the *T cells* and the macrophage which presented the antigen and results in further activation of the immune response (Pieters, 2001).

In (30%) of individuals, the actions of the alveolar macrophage are not sufficient to kill the *M. tuberculosis*. It has been suggested that the type of macrophage receptor that a bacterium binds can determine the response that is generated by the macrophage (Le Cabecet *et al.*, 2000; Astarie-Dequekeret *et al.*, 1999). Another study suggests that when macrophages are unable to destroy *M. tuberculosis* the bacteria prevent the normal maturation of the phagolysosome. Some studies have shown that mycobacteria prevent the fusion of the phagosome with the lysosome (Vergneet *et al.*, 2005). Ingested *M. tuberculosis* remains viable and bacterial antigens are not presented on the surface of the infected macrophage. Macrophages burst when the number of intracellular bacteria becomes too large. The content of the burst macrophage is discharged into the alveoli, releasing the bacteria, which are ingested by other alveolar macrophages, or monocytes that migrate from the
blood stream. These cells are not activated and so the bacteria continue to divide and more monocytes are recruited to the area. A primary complex forms, which consists of a small lesion at the site of infection and enlarged regional lymph nodes. DCs are a small population of cells that line the trachea and can also phagocytose mycobacteria. Once mycobacteria have been ingested, the dendritic cell travels to the draining lymph node and displays mycobacterial antigens on the cell surface. DCis able to activate T cells and can polarize the T cell response towards Th1 or Th2 phenotypes (Hope et al., 2004).

2.3.3. Adaptive Immune response:
Mycobacteria-infected macrophages and DC of the innate immunity present antigens to T cells and B cells that belong to adaptive immunity. Cytokine IL-12 plays a fundamental role in the pathogen induced activation of pulmonary DC. The (CD4+ T helper cells) can be differentiated into Th1, Th2, Th17 and Treg cells. The Th1 cells produce cytokines, notably IFN-γ, TNF-α, IL-2, lymphotoxin and granulocyte-macrophage colony-stimulating factor (GM-CSF), which prompts stimulation of Th1 cells, CTL, and maturation and activation of macrophages as well as granulocytes. The Th2 cells produce B cell-stimulation factors such as IL-4, IL-5, IL-10 and IL-13, which promote antibody production but suppress theTh1 typeimmuneresponse. The Th17 cells, a distinct subset of helper T cells, produce unique cytokines of IL-17, IL-17F, IL-21 and IL-22, which stimulate defending production and recruit neutrophils and monocytes to the site of inflammation, and are involved in the early phase of host defense.

M. bovisBacilleCalmette-Guerin (BCG) directly activates the classical, lectins and alternative pathways, resulting in fixation of C3b onto macromolecules of the mycobacterial surface, which will contribute to mycobacterial killing (Carroll 2009).
MemoryT (TM) cells form after *M. tuberculosis* exposure or infection. *TM cells* proliferate promptly after encounter with antigens, and produce multiple cytokines such as IFN-γ, IL-2, and TNF-α, lymphotoxin and/or GM-CSF (Kaufmann 2008).

2.4. **Tuberculosis disease:**

*TB* is a disease with deep social and economic roots. Low-income people with large families, living in dense urban communities with deficient housing conditions, have a high probability of becoming infected, developing active disease, and dying from *TB*. Also, the risk of becoming infected and ill with *TB* is higher among people that live in congregated institutions, such as prisons, youth correctional facilities, nursing homes for elderly people, social shelters, day nurseries and schools; the same is valid for elderly people, diabetics and people living with Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (*HIV/AIDS*) (WHO 2006).

The lung is the main entrance gate of the tuberculous bacillus, which causes a focal infection in the site where it is deposited after inhalation. If the infection cannot be contained at the local level, bacilli dissemination is produced initially by hematogenic route, probably inside phagocytic cells, towards different organs and, eventually, to the contiguous pleura. It reaches hilar lymph nodes via the lymphatic route, and from there, a second systemic dissemination can occur, through the thoracic duct and superior vena cava, with the development of local foci in the lungs. Extrapulmonary foci can also be produced by hematogenic and lymphatic dissemination. The clinical manifestations of *TB* depend on the local organic defenses on the sites of bacilli multiplication. It has been emphasized that the use of *BCG* vaccination may play a role in this phase, avoiding dissemination and the occurrence of extrapulmonary forms of *TB* (Rich 1994, Bates 1980, Stead 1989).

In (2015), there were an estimated (10.4) million new (incident) *TB* cases worldwide, of which (5.9) million (56%) were among men, (3.5) million (34%)
among women and (1.0) million (10%) among children. The world map illustrates the global epidemic situation of *MTB* (WHO, Global Tuberculosis Report 2016) Figure (2.1).

Fig (2.1) the estimated incidence rates of tuberculosis (WHO report 2014).

### 2.4.1. The initial lesion:

Once inhaled, most tubercle bacilli are trapped in the mucosa of the upper respiratory tract, trachea and bronchi, and are eliminated by the mucociliary defense mechanisms. Tiny particles or droplet nuclei smaller than (5)μm behave as a gas and overcome this barrier and reach the inferior respiratory tract, especially inside the alveoli, where they are readily phagocytosed by alveolar macrophages.
The survival of the infectious agent in the lung will depend on its pathogenicity/virulence and on the ability of the host cells to eliminate it. The alveolar macrophages are the first line of defense against *M. tuberculosis*. This initial response, if completely effective, will cause the elimination of the pathogen through the phagocytic action inherent to such macrophages. If the alveolar macrophage is not capable of arresting bacterial growth, a localized proinflammatory response is formed through the activity of *TLR agonists*, abundant on the surface of bacteria. Tumor necrosis factor alpha (*TNF-α*) and inflammatory chemokines produced by the infected macrophages recruit white blood cells, which phagocytose bacilli and eventually return to the bloodstream causing the primary hematogenic dissemination. The recruited cells produce their own complement of chemokines and cytokines that amplify cellular recruitment and remodel the infection site into a cellular mass, the tubercle or granuloma. The granuloma initially formed consists of a core of infected macrophages surrounded by foamy macrophages, with an external layer of lymphocytes encircled by collagen and other extracellular matrix components (Russell 2007).

*Tubercle bacilli* can disseminate by the lymphatic route to regional lymph nodes, constituting the tuberculous primary complex of Ranke, composed by the original granuloma at the inoculation site (Gohn’s nodule), the lymphangitis and the hilar lymph node enlargement. Although in some cases these lesions may become evident on *chest X-ray*, most cases of primary tuberculous infection are clinically and radiologically unapparent, with a positive *TST* being the only indication of the occurrence of the infection. From the hilar lymph nodes, tubercle bacilli disseminate to tracheal and vertebral lymph nodes. Through the thoracic duct, they reach the blood stream, spreading to the upper areas of the lungs or to different organs, such as kidney, brain, and bones. At these sites, they find a favorable atmosphere for implantation that combines a satisfactory oxygen tension and a low
tuberculosis strains may occur in (40%) of relapsing cases. The recurrence/relapse caused by new strains highlights the possibility that the progression to disease can be enhanced by multiple infections, especially among high-risk persons, such as HIV infected individuals (Verwer 2005).

Pulmonary TB is the most common form of post-primary disease. Lymphatic dissemination can occur, but in this case the hilar lymph nodes are usually not affected. The response to bacillary multiplication provokes caseous necrosis that eventually blends and progresses to liquefaction. Tubercle bacilli multiplication had been until then inhibited by granuloma formation, find favorable conditions for population growth after liquefaction of the caseum and subsequent cavitation, and may produce more than (10^8) bacilli per cavity with a diameter of less than (2 cm). The development of tuberculous cavities in the lung characterizes the post-primary TB and, from this lesion, infectious material can spread through bronchi, resulting in the continuous production and elimination of sputum. The natural evolution of post-primary lesions in immunocompetent persons can lead to dissemination and death in about (50%) of cases, and to chronicity in about (25% to 30%). Natural cure can also occur in (20% to 25%) of cases, when the host immune response is able to re-establish control of the disease (Bates 1980, Melo 1993).

In most non-immunosuppressed persons infected by the tubercle bacillus, disease will occur in the first three to five years after the initial exposure. In HIV positive persons infected with the tubercle bacillus, however, (7% to 10%) will develop active TB annually (ATS 2000). The remaining cases occur at any time during a lifetime, especially when there are other diseases or weakening conditions, for example malnutrition, diabetes, prolonged treatment with corticosteroids, immunosuppressive therapy, chronic renal disease, gastrectomy, and others. The post-primary disease presents a great spectrum of manifestations, which are related to the affected organ. The lungs are most commonly affected, usually in the upper
lobes or apical segments of inferior lobes. The disease can also affect other organs, including lymph nodes, pleura, kidneys, the central nervous system, and bones. In *pulmonary TB*, the patients often present with an insidious clinical onset, sometimes with minimal or non-specific complaints in the initial phase. With the development of the disease, two types of signs and symptoms can be recognized. The most frequent are: lack of appetite, low-grade evening fevers, and night sweats. With respect to respiratory signs and symptoms, the patient may complain of cough, at any hour of the day, which as initially dry and later on productive with purulent or mucous expectoration. Hemoptysis and bloody sputum occur in less than a quarter of patients, with the worst cases originating from lesions invading blood vessels. Chest pain can be localized and dependent on breathing movements. In most cases, the patient may be symptomatic for (1 to 3) months before diagnosis. Such delays in diagnosis may be due to low diagnostic suspicion by the medical personnel, lack of access to health services, because the patient may not acknowledge being sick or may not seek medical help due to economic or cultural reasons. An early diagnosis is critical for controlling transmission of the disease in the community, especially in congregated institutions, such as hospitals, prisons, and shelters(Hopewell 2006).

### 2.4.5. Extrapulmonary Tuberculosis:

After penetration into the organism through the respiratory route, *M. tuberculosis* can settle and multiply in any organ during the primary infection, before development of the specific immune response. After this, *tubercle bacilli* can multiply at any time when there is a decrease in the host’s immune capacity to contain the bacilli in their implantation sites. The specific signs and symptoms will depend on the affected organ or system, and are characterized by inflammatory or obstructive phenomena. Systemic symptoms are much less frequent than in *pulmonary TB*, except in the disseminated form of the disease.
The majority of the extrapulmonary forms of \( TB \) affect organs with sub-optimal conditions for bacillary growth. For this reason, the extrapulmonary disease generally has an insidious presentation, a slow evolution and paucibacillary lesions and/or fluids. Access to the lesions through secretions and body fluids is not always possible, and for this reason, invasive techniques may be necessary in many cases, to obtain material for diagnostic investigation. Tissues and/or body fluids should be submitted to laboratory examination, in particular bacteriological culture for mycobacteria and histopathological analysis. In the immunocompetent patient, the \( TST \) response is usually positive (induration \( \geq 10 \) mm).

Imaging studies provide valuable information for the diagnosis of \( extrapulmonary \) \( TB \), although specific radiological patterns are not observed. In immunocompetent patients, the extrapulmonary forms only occasionally coexist with active pulmonary \( TB \). Nevertheless, the \( chest \) \( X\)-ray is mandatory for the evaluation of evidence of primary infection lesions, which provide a good verification to support the diagnosis (Rottenberg 1996).

2.4.5.1. \textbf{Miliary tuberculosis:}

\textit{MiliaryTB} results from the massive hematogenic dissemination of the Koch bacillus during the primary infection. Its onset may be either insidious or abrupt, depending on the bacillary load and/or the host immune situation, with unvaccinated infants, elderly and immunodeficient patients being the most susceptible. The variable and often nonspecific symptoms include fever, anorexia, weight loss, and asthenia. Other specific symptoms depend on the organs affected, and involvement of the central nervous system occurs in (30 \%) of cases. The physical examination is unspecific, and the patient can present with variable degrees of wasting, fever, tachycardia and toxemia. The observation of bacilli on smear microscopy examination is rare, and culturing mycobacteria provides a higher probability of bacteriological confirmation of the diagnosis of \( TB \). In the advanced stages of \( HIV/AIDS \) (\( CD4+ \) \( T \) cell) count lower than (200 cells/mm3) or
peripheral blood lymphocyte count lower than (1000/mm3), the bacilli circulate in
the bloodstream, and *tubercle bacilli* are often isolated from blood when
appropriate culture media are used (Lester 1980, Thornton 1995).

2.4.5.2. Pleural tuberculosis
This is the most common form of *extrapulmonary TB*, and can either result from
the rupture of a primary sub-pleural lung focus (evident or not on conventional
chest X-ray) or be secondary to lymphohematogenic dissemination. The presence
of a *pleural TB effusion* has also been related to hypersensitivity (Light 1990).
Most cases occur several months after the primary infection, and frequently the
patient relates having contact with an active *pulmonary TB* case in the two years
preceding the current episode. The simultaneous presence of active *pulmonary TB*
may be related to recent infection followed by disease. The onset of the disease
may be insidious or abrupt, with fever, systemic complaints, dyspnea, dry coughs,
and pleuritic thoracic pain. The physical examination shows signs characteristic of
pleural effusion. With regard to diagnosis, the result of the *TST* may be negative at
the diagnosis of the disease and become positive during anti-tuberculosis
treatment. The pleural effusion is generally unilateral and moderate, and can easily
be detected by conventional *chest X-ray* examination. The pleural liquid has a
typically citrine yellow aspect and sometimes may be sero-hemorrhagic. It is
generally an exudate with a predominance of lymphomononuclear cells, often
negative for *acid-fast bacilli (AFB)* on microscopic examination. The etiological
diagnosis is confirmed by the isolation of *M. tuberculosis* by culture of the fluid.
The histopathological finding of granulomatous lesions in the pleural biopsy also
confirms diagnosis, especially in countries with a high *TB prevalence* (Light 1990,
Uehara 1993).

2.4.5.3. Lymph node tuberculosis:
This is the second most common form of *extrapulmonary TB* in HIVsero-
negative patients and the most frequent in patients living with *HIV/AIDS*. Initially,
lymph nodes grow slowly, and are painless and mobile. Later on, as their volume increases, they tend to coalesce and some develop fistulas. Patients mainly complain of fever and the increasing volume of lymph nodes, but other symptoms may be absent. In general, the TST is strongly positive, except in immunosuppressed patients. The etiological diagnosis can be made by aspiration puncture biopsy, which is AFB positive in only (10% to 25%) of cases, but *M. tuberculosis* may be isolated by culture in (50% to 85%) of cases (Ikezoe 1992).

2.4.5.4. Renal tuberculosis:

*Renal TB* is rare in children and predominantly affects individuals in the fourth decade of life. Renal disease occurs after a long latency period and is frequently secondary to hematogenous dissemination. The localization is almost always bilateral, but can be asymmetric. The lesions often start in the renal cortex and progress slowly toward the central region. Dissemination can occur to the bladder and eventually to the genital system. Symptoms and signs may vary in duration and severity. The patient generally complains of dysuria, polacyuria, and lumbar pain, whereas systemic symptoms occur less frequently. Frequently, the disease presents as a urinary infection that does not respond to routine broad spectrum antimicrobial treatment. Purulent urine is frequently found, with urine culture negative for common germs (aseptic pyuria). Hematuria occurs in (10% to 15%) of the cases (Erkoc 2004).

2.4.5.5. Tuberculosis of the central nervous system:

The compromise of the central nervous system occurs in two basic forms: meningoencephalitis and intracranial tuberculoma. Since the introduction of modern chemotherapy and especially massive *BCG vaccination*, a lower proportion of the meningoencephalitis has been observed, but the frequency of this form of *TB* is higher among young adults with *HIV/AIDS*. The clinical manifestations are due to the inflammatory process induced by the mycobacterial infection, and the symptoms depend on the site and intensity of inflammation. Granulomas can be located in the
cerebral cortex or in the meninges. Meningoencephalitis generally has an insidious onset and a slowly progressive course, with symptoms including lethargy, fever, and mental disturbances such as irritability, understanding difficulties, personality alterations, disorientation, and progressive mental confusion. The cerebrospinal fluid is generally clear, with a predominance of lymphocytes, an increase in proteins and a decrease in glucose levels. Microscopic examination for AFB is generally negative and cultures are positive in only (15 % to 30 %) of cases (Simon 1977, Smith 1994).

2.4.5.6. Osteoarticular tuberculosis:
Involvement of the osteoarticular system is most commonly found in children and the elderly, and is generally secondary to hematogenous seeding, but can also occur as a consequence of lymphatic dissemination or direct spread from a contiguous lesion. Bone involvement consists of osteomyelitis, and arthritis can occur either by extension of the osseous lesion to the joint or by direct hematogenous inoculation. The peripheral joints most frequently affected by TB are the hip and the knee. Pain, with or without movement limitation, fever and systemic symptoms are frequent. Monoarticular involvement is much more frequent than multiaxial disease. The diagnosis of osteoarticular TB is usually delayed because this etiology is often overlooked in the differential diagnosis of joint disease. In most cases, the TST is positive, and approximately (50 %) of cases also have abnormal chest X-rays, suggesting previous pulmonary disease. Cold abscesses occurring in the advanced phase of osteoarticular TB can develop into cutaneous fistulae, which are frequent in this form of the disease. The diagnosis is established by puncture, biopsy, histopathological examination, and culture (Zylbersztejn 1993, Davidson 1994, Ridley 1998, Schlesinger 2005).

2.4.5.7. Other extrapulmonary localizations:
Tuberculous involvement of other tissues, such the eye, skin (Lupus vulgaris), genital, and digestive tract, may also be the result of hematogenous
dissemination, but there are other possible routes of infection. Intestinal TB can be acquired by the oral route and in countries with a high prevalence of bovine TB. Before the generalization of milk pasteurization, this was a rather common form of zoonotic TB (produced by Mycobacterium bovis), particularly in infants. Eye and skin TB may be the consequence of accidental inoculation, particularly among medical and veterinary professionals, and genital TB may be produced by spread from renal TB (Singla 2006, Moore 2002, Erkoc 2004).

2.4.5.8. Special conditions:

During the past few decades, TB has been observed in association with immunosuppression, malignant neoplasms (i.e. lung cancer, head and neck cancer, Hodgkin’s lymphoma), malnutrition (more than 15% loss of usual weight), old age, diabetes mellitus and silicosis. The occurrence of TB in these patients is likely to be the result of both increased susceptibility and longer patient survival, due to the increased frequency of organ transplantation and the accompanying immune suppression, more effective cytotoxic treatments for neoplastic disease, and immunosuppressive treatment of autoimmune diseases, such as systemic Lupus erythematosus (SLE) or the anti-TNF antibodies used against chronic inflammatory arthritis (Al-Wabel 1997, John 2002).

2.5. Symptoms and signs of tuberculosis:

2.5.1. Fever and sweating:

It is believed that bacillary multiplication increases in the afternoon, with the daily circadian rhythm cortisol peak, which is followed by the evening fever characteristic of the disease. M. tuberculosis multiplies at a slow pace in comparison with other bacteria and therefore the inflammatory process is moderate and is accompanied by a low-grade fever. The body responds to the evening fever with night sweats to maintain the body temperature. However, when there is massive hematogenous or endobronchial dissemination, peaks of high fever can occur at any time of the day and are accompanied by chills (Palomino 2007).
2.5.2. Weight loss:
Consumption was the name given to TB many years ago because it appeared to consume those affected, and anorexia and weight loss are still frequent in TB patients (about 70% of the cases). Weight loss is proportional to the duration and extent of the disease and is frequently accompanied by anemia (Palomino 2007).

2.5.3. Cough:
Cough is present in virtually all patients with pulmonary TB. Cough results from the stimulus caused by the alveolar inflammatory process or from the granulomatous impingement into the respiratory airways. At the onset of the disease, the cough is dry; but with progression, it becomes productive with mucous or mucopurulent expectoration, generally in small amounts, and sometimes with blood. Cough is less frequent in the pleural form of the disease. It is worth mentioning that cough tends to be ignored or minimized by smokers, who may have a chronic cough, so questions about changes in the usual pattern can be of great value in increasing suspicion of pulmonary TB (Palomino 2007).

2.5.4. Hemoptysis:
When hemoptysis occurs, the blood volume is variable, from bloody streaks mixed in the sputum (hemoptoic sputum) to massive hemoptysis (more than 400 mL/day), which is rare. Bleeding can also occur in small lesions during the formation of the cavities. Hemoptysis can be the first manifestation of the disease (Palomino 2007).

2.5.5. Thoracic pain:
Thoracic pain occurs when there is pleural involvement, but as the TB pathological process begins in the alveoli, very close to the pleural surface, this is an early and relatively frequent symptom. Generally of low intensity, it disappears within two or three weeks after effective treatment has begun (Palomino 2007).
2.6. Diagnosis of Tuberculosis:

2.6.1. Specimens:

The successful isolation of the pathogen requires that the best specimen be properly collected, promptly transported and carefully processed. If pulmonaryTB is suspected, specimens originating from the respiratory tract should be collected, i.e. sputum, induced sputum, bronchoalveolar lavage or a lung biopsy. For the diagnosis of pulmonary TB, three first-morning sputum specimens (not saliva) obtained after a deep, productive cough on non-consecutive days are usually recommended. Several studies have shown, however, that the value of the third sputum is negligible for the diagnosis of TB, as virtually all cases are identified from the first and/or the second specimen (Yassin 2003, Nelson 1998, Dorronsoro 2000, Finch 1997). Before processing, sputum specimens must be classified at the laboratory with regard to their quality, i.e. bloody, purulent, mucopurulent, saliva. In patients who cannot produce it spontaneously, the sputum can be induced by inhalation of hypertonic saline solution. Otherwise, the specimen can be collected from bronchoscopy. This intervention usually provokes cough and postbronchoscopy expectorated sputum specimens should be collected because they often provide satisfactory microorganism yields (Sarkar 1980, de Gracia 1988). Some studies suggest that a single induced sputum specimen is equally effective as bronchoscopy for diagnosing pulmonaryTB (Conde 2000). A recent study demonstrated that the most cost-effective strategy is to perform three induced sputum tests without bronchoscopy (MacWilliams 2002).

Specimens to be collected for the diagnosis of extrapulmonary disease depend on the site of the disease. The most common specimens received in the laboratory are biopsies, aspirates, pus, urine, and normally sterile body fluids, including cerebrospinal fluid, synovial, pleural, pericardial, and peritoneal liquid. Stool can be collected when intestinal TB is suspected and also in the case of suspected Mycobacteriumaviuminfection in AIDS patients. Whole blood and/or bone marrow
specimens are collected only if disseminated TB is suspected, mainly in patients with an underlying severe immunosuppressive condition such as AIDS. Bone biopsies are the specimen of choice when skeletalTB is suspected (Valdez 1998).

2.6.2. Specimen transport:
Specimens should be transported rapidly to the laboratory to avoid overgrowth by other microorganisms. This is particularly true for specimens from non-sterile sites, such as sputum. When the transport or the processing is delayed, specimens should be stored for not more than five days at (4°C) until transported or presented for bacteriological processing. The acetylpyridium chloride (CPC) method is widely used for the transport of sputum specimens (Smithwick 1975). CPC eliminates the associated flora in sputum specimens and treated specimens should not be submitted to further decontamination prior to cultivation. The detection of AFB with ZN staining can be significantly reduced in specimens preserved by this method (Selvakumar 2004, Selvakumar 2006).

2.6.3. Smear staining:

2.6.3.1. AFB smears staining
AFB smear microscopy plays an important role in the early diagnosis of mycobacterial infections because most mycobacteria grow slowly and culture results become available only after weeks of incubation. In addition, AFB smear microscopy is often the only available diagnostic method in developing countries. Smear staining is based on the high lipid content of the cell wall of mycobacteria which makes them resistant to decolorization by acid-alcohol after the primary staining. To determine that a clinical specimen contains AFB, the specimen is spread onto a microscope slide, heat-fixed, stained with a primary staining, decolorized with acid-alcohol solution and counterstained with a contrasting dye in order to obtain better differentiation between the microorganism and the background. The slide is observed under the microscope for the detection of AFB. Several methods can be used for determining the acid-fast nature of an organism.
Two methods, ZN and Kinyoun, utilize basic fuchsin in ethanol for primary staining. In both cases, AFB appears red after decolorization with acidalcohol. ZN is a hot acid-fast stain because the slide has to be heated during incubation with fuchsin. In contrast, Kinyoun staining is a cold acid-fast staining procedure and therefore does not require heating. Kinyoun's cold carbolfuchsin method is inferior to the ZNstaining (Somoskovi 2001, Van Deun 2005).

The results of the smear microscopy should be reported according to an internationally agreed quantitation scale (Table 2.1)

2.6.3.2. Concentrated sputum smears:

Sputum is the most common specimen received for TBdiagnosis. The minimum number of bacilli needed to detect their presence in stained smears has been estimated to be (5,000-10,000 per mL) of sputum. For diagnosis, the sensitivity of AFB smear staining relative to culture has been estimated to vary from (50 %) to over (80 %). Several studies have been published on improving smear microscopy performance using methods that concentrate the bacilli present in the sputum specimen. The methods consist of submitting the specimen to a liquefaction step prior to concentrating it by sedimentation or centrifugation. The smears are then performed from the sediment and stained for microscopic examination. The chemical method used for the liquefaction depends on the next step following concentration; smear staining only or smear staining followed by culturing.

The best known concentration procedure is the 'bleach microscopy method', in which the sputum is liquefied with sodium hypochlorite (NaClOor household bleach), and concentrated by centrifugation before AFBstaining. This technique is inexpensive and easy to perform. In addition, NaClO is a potent disinfectant that also kills mycobacteria, thus reducing the risk of laboratory-acquired infection but, at the same time, rendering the method unsuitable for culturing (Angeby 2004).

Other concentration methods should be used if the specimen is to be cultured. The sediment of a sputum specimen liquefied and decontaminated with sodium
hydroxide- N-acetyl-L-cysteine method and concentrated by centrifugation can also be examined by AFBsmear staining. An advantage of this method is that the same sediment can be cultured, in contrast to those liquefied with the NaClO method described above (Steingart 2006).

**Table 2.1: Quantitation scale recommended by the WHO and the International Union against Tuberculosis and Lung Disease.**

<table>
<thead>
<tr>
<th>Count on Ziehl-Neelsen /Kinyoun stain (1000x)</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Non AFB observed</td>
</tr>
<tr>
<td>1-9/100 fields</td>
<td>Exact count</td>
</tr>
<tr>
<td>10-99/100 fields</td>
<td>+1</td>
</tr>
<tr>
<td>1-10/field</td>
<td>+2</td>
</tr>
<tr>
<td>&gt; 10/field</td>
<td>+3</td>
</tr>
</tbody>
</table>

**2.6.4. Culture:**

**2.6.4.1. Sterile or contaminated specimens:**

Acid-fast microscopy is easy and quick, but it does not confirm TBdiagnosis because mycobacteria other than *M. tuberculosis* are also AFB in the smear microscopic examination. In addition, a high bacterial load is needed in the specimen to render an AFBmicroscopy result positive. A positive culture for *M. tuberculosis* confirms the diagnosis of active disease. For culturing of mycobacteria, two types of clinical specimens are considered: contaminated specimens and specimens collected aseptically from normally sterile sites. Sterile
Specimens can be inoculated directly onto the culture medium. Specimens from non-sterile bodily sites are considered contaminated and therefore require processing before culturing in order to eliminate the associated flora. If not properly eliminated, this flora will overgrow the culture medium long before mycobacteria have the chance to develop visible colonies.

Several methods have been used to minimize culture contamination when specimens from non-sterile body sites are processed. Most of these methods include the digestion of mucus or organic debris and treatment to eliminate microorganisms from the normal flora. Both steps are done to maximize the probability of isolating mycobacteria in culture. No single decontamination method is applicable to all circumstances, laboratories and clinical specimens; therefore, a laboratory should use the best suited method that keeps the contamination rate between (3 %) and (5 %). A contamination rate lower than (3 %) may indicate that the procedure used is too harsh and may be killing the mycobacteria (Della Latta 2004). The decontamination methods most commonly used is Sodium hydroxide (NaOH): This method uses NaOH at concentrations ranging between (2 %) and (4 %) to digest and, at the same time, decontaminate the specimen. Each laboratory should determine the lowest concentration for optimal digestion and decontamination (Della Latta 2004).

2.6.4.2. Culture media:
Different Culture Medias are in use for the isolation of mycobacteria. The most common are based on egg and also contain high concentrations of malachite green to overcome contamination with other bacteria. In general, after the centrifugation step, sediments are inoculated onto two Löwenstein- Jensen slants (Drancourt 2003).
2.6.4.2.1. Reading of culture results:
Conventional culture media such as those based on egg and agar should be examined for growth twice a week for the first four weeks starting on day (3 to 5) postinoculation, and thereafter, once a week until the eighth week. All specimens showing growth in culture should be confirmed as AFB by smear microscopy of the colonies and reported immediately as “culture positive for mycobacteria pending identification”. All cultures reported positive for mycobacteria should be identified to the level of species using either biochemical or molecular methods. *M. tuberculosis* bacilli are slow-growing mycobacteria. This means that, in primary isolation, they hardly show any visible growth during the first week of culture. On egg-based media they produce characteristic non-pigmented colonies, with a general rough and dry appearance simulating breadcrumbs. On agar based media, the colonies appear flat, dry and rough with irregular edges (Palomino 2007).

2.6.4.3. Identification:

2.6.4.3.1. Biochemical procedures:
The final species identification of *M. tuberculosis* is based on characteristics such as slow growth, colony morphology, and biochemical tests. From a practical point of view, most isolates from human disease belong to the species *M. tuberculosis*. However, depending on geographical and epidemiological circumstances, it may be necessary to differentiate species within the *M. tuberculosis* complex. An initial identification as *M. tuberculosis* is defined on AFBbacilli from slow-growing, non-pigmented colonies that are niacin positive, are inhibited by p-nitro-benzoic acid and display nitrates activity. Additional tests that confirm an isolate as *M. tuberculosis* are susceptibility to pyrazinamide, absence of catalase production at (68°C) and absence of iron uptake (Vincent 2003).

2.6.4.3.2. Serological diagnosis:
Historically speaking, serology for the diagnosis of TB has been explored since 1898, when crude cell preparations containing carbohydrates, lipids, and
proteins from *M. tuberculosis* or *M. bovis* BCG were used as antigen preparations showing high sensitivity but low specificity (Arloing 1898). Modern developments in the purification of antigens, generation of monoclonal antibodies and chromatographic techniques, have led to a considerable improvement in specificity. During the last three decades, a large number of purified (native and recombinant) antigens have been assessed, showing substantial progress in the serodiagnosis of TB (Jackett 1988).

In *TB patients*, the serological response to mycobacterial antigens has been primarily evaluated using standard ELISA within house methodologies and protocols which certainly differ from laboratory to laboratory. Also, immunochromatographic assays are used also called simply strip tests. The benefits of immunochromatographic tests include: very short time to test result and relatively inexpensive to make (Jackett 1988).

**2.6.4.3.2.1. Tuberculin skin test (TST):**

In 1882, about eight years after the discovery of the tubercle bacillus, Robert Koch announced a cure for TB. He obtained a heat-inactivated filtrate from cultures of *M. tuberculosis*, and found that this material would protect guinea pigs from experimental TB. This product, known as “Koch’s Old Tuberculin”, was then administered to patients with TB, and Koch claimed that this treatment resulted in the cure of the disease (Kaufmann 2000, Gradmann 2001, and Gradmann 2006). However, *TB patients* who received tuberculin had generalized systemic reactions, including fever, muscle aches, and abdominal discomfort with nausea and vomiting, in contrast to people without TB, who did not develop this violent reaction. These observations were the basis for the proposal of the use of tuberculin as a diagnostic test, despite its failure as a therapeutic substance. The intradermal injection of tuberculin was described by Mantoux, and his method became widespread because of the reproducibility of the results. After local application of the product injected intradermally, a hallmark response is elicited
within (24 to 72) hours, which includes induration, swelling and monocytic infiltration into the site of the injection. The skin reaction, classified as delayed type hypersensitivity (\textit{DTH}), has been used since then to test if prior exposure to an antigen has occurred. Koch's tuberculin was an impure extract of boiled cultured tubercle bacilli. In 1934, Siebert made a simple protein precipitate of the old tuberculin and named it purified protein derivative (\textit{PPD}) (Palomino 2007).

\textit{TST} has been used to identify patients actively infected with \textit{TB}, to measure the prevalence of infection in a community, and to select susceptible or high-risk patients for \textit{BCG} vaccination. The test has been in existence for more than (100 years) and has remained more or less unchanged for the last (60 years) (Huebner 1993, Curley 2003).

\textit{TST} works by intradermally injecting (0.1 mL) of 5 \textit{TU PPD} on the forearm. On examination, after (48-72 hours), a positive reaction is indicated by erythema and induration of (> 10 mm) in size. Erythema (redness) alone is not taken as a positive reaction. All persons with prior infection with tubercle bacilli will mount an immune response to bacilli proteins (Curley 2003).

As the active ingredient used in the skin test contains a whole series of proteins that are shared with the \textit{BCG vaccine} and other mycobacteria common in the environment, the \textit{TST}s is often falsely positive. It is currently estimated that almost one third of people positive to \textit{TST} do not actually have \textit{TB infection}. The sensitivity of the skin test is estimated to be around just 7(0 \%) in known active \textit{TB cases}; so the test misses up to (30 \%) of people who are infected. This sensitivity decreases to as low as (30 \%) in immunocompromised people (Palomino 2007).

\textbf{2.7. Treatment and prevention of tuberculosis:}

\textbf{2.7.1. Treatment of tuberculosis:}

The history of\textit{TB} changed dramatically after the introduction of anti-mycobacterial agents. Drug treatment is fundamental for controlling \textit{TB}, promoting the cure of the patients and breaking the chain of transmission when the anti-tuberculosis drug
regimen is completely and correctly followed. Anti-tuberculosis drug treatment started in 1944, when streptomycin \((SM)\) and paraaminosalicylic acid \((PAS)\) were discovered. In 1950, the first trial was performed comparing the efficacy of \(SM\) and \(PAS\) both as monotherapy or combined. The study demonstrated that combined therapy was more effective and resulted in the first multidrug antituberculosis treatment that consisted of a long course of both drugs. In 1952, a third drug, isoniazid \((INH)\), was added to the previous combination, greatly improving the efficacy of treatment, but which still had to be administered for \((18-24\, \text{months})\). In 1960, ethambutol \((EMB)\) substituted \(PAS\), and the treatment course was reduced to \((18\, \text{months})\). In the ’70s, with the introduction of rifampicin \((RIF)\) into the combination, treatment was shortened to just \((9\, \text{months})\). Finally, in 1980, pyrazinamide \((PZA)\) was introduced into the anti-tuberculosis treatment, which could be reduced further to only six months. Anti-tuberculosis treatment has two main objectives (Onyebujoh 2005). First, there is a need to rapidly kill those bacilli living extracellularly in lung cavities, which are metabolically active and are dividing continuously; this is required in order to attain the negativization of sputum and therefore to prevent further transmission of the disease. Second, it is necessary to achieve complete sterilization and elimination of those bacilli replicating less actively in acidic and anoxic closed lesions, and to kill semi-dormant bacilli living intracellularly in other host tissues, otherwise these bacilli may persist and will be responsible for subsequent \(T\!B\text{relapses}\). The current short-course treatment for the complete elimination of active and dormant bacilli involves two phases:

- **Initial phase**: three or more drugs (usually isoniazid, rifampicin, pyrazinamide and ethambutol or streptomycin) are used for \((2\, \text{months})\), and allow a rapid killing of actively dividing bacteria, resulting in the negativization of sputum.
• **Continuation phase:** fewer drugs (usually isoniazid and rifampicin) are used for (4 to 7 months), aimed at killing any remaining or dormant bacilli and preventing recurrence(Onyebujoh 2005).

### 2.7.2. Prevention of tuberculosis:

As with all health conditions, *prevention of TB* is always better than a cure. Although there is no sure-fire way to completely prevent the spread of *TB* at this point in time, there are a number of measures that can be put in place to reduce the spread of the illness

i. The *BCG* vaccination:

The *BCG* is a live vaccine against tuberculosis. The vaccine is prepared from a strain of the weakened bovine tuberculosis bacillus, *Mycobacterium bovis*.

The *BCG* is currently the only licensed vaccine against *TB*, and has been in use since 1921. It is one of the most widely used vaccines worldwide, yet we still see around (9 million) new cases of *TB* annually – a testament to the *BCG*’s limited effectiveness. The *BCG* is:

- (80%) effective in preventing *TB* for (15 years).
- More effective against complex forms of *TB* in children.
- Of limited effectiveness in people over the (age of 35).
- Less effective when given in equatorial regions (due to high levels of naturally occurring environmental mycobacteria).

ii. Early diagnosis:

Early diagnosis and treatment is the most effective way to prevent the spread of tuberculosis.
A person with infectious tuberculosis can infect up to (10–15) other people per year. But once diagnosed with TB, and started on treatment, the majority of patients are no longer infectious after just two weeks of taking the medication.

iii. Case finding:

Limiting the spread of TB depends on successfully finding and treating people with the illness, to prevent them from passing it on to others.

This can be done through raising awareness of TB, so people with TB symptoms know to seek help. Outreach workers and volunteers also work within communities with high rates of TB to find people with symptoms and refer them for testing.

When someone is diagnosed with infectious TB, their close contacts are screened for the illness, this is known as contact tracing.

iv. Managing your environment:

As TB is an airborne infection, TB bacteria are released into the air when someone with infectious TB coughs or sneezes. The risk of infection can be reduced by using a few simple precautions:

- Good ventilation: as TB can remain suspended in the air for several hours with no ventilation.
- Natural light: UV light kills off TB bacteria.
- Good hygiene: covering the mouth and nose when coughing or sneezing reduces the spread of TB bacteria.

In healthcare settings, the spread of TB is reduced through the use of protective masks, ventilation systems, keeping potentially infectious patients separate from other patients, and the regular screening of healthcare workers for TB.
v. A healthy immune system:

Having a healthy immune system is the best form of defense against TB: (60%) of adults with a healthy immune system can completely kill TB bacteria.

2.8. Host Genetics and Susceptibility:

Tuberculosis (TB), “The White Plague” was a predominant public health problem in Europe and America in the 18th, 19th, and early 20th centuries, and considerable effort was spent trying to understand it. Many of the questions investigated in the past are now being re-addressed at the molecular level. One of the principal questions that occupied earlier researchers were the interplay of bacterial and host factors that determine which one becomes infected and which one develops TB. The discussion over the causes of TB goes back at least as far as the ancient Greeks and Romans, and basically consists of three different explanations: an inherited disorder; a contagious disease; and a disease caused by poor living conditions. Hippocrates thought it was inherited, while Aristotle and Galen believed it was contagious (Smith 2003). As the disease was most common in the urban poor, crowded into the rapidly growing cities of the recently industrialized Europe, social reformers of the time believed that TB was caused by the deplorable living conditions of the working class and rejected a contagious explanation.

While the discovery of the TB bacillus by Koch in 1882 disproved the notion that the disease had a purely hereditary etiology, or was caused solely by the unhealthy living conditions of the lower classes in the early industrial age (Hass 1996), several aspects of TB epidemiology are not explained by the germ theory and suggest that there are individual differences in susceptibility: not everyone exposed to M. tuberculosis becomes infected; even when infection can be demonstrated with a positive TST, only about one in ten infected individuals becomes ill; the course of the disease varies in different individuals - before
antibiotics some tuberculars died rapidly of “galloping consumption” while others recovered or lived a relatively long life with chronic disease; and some infected individuals develop the disease only many years after the initial infection. Without treatment, *TB* is fatal in about half of the patients who develop the disease (Rich 1951).

As the disease was more common in particular families and racial or ethnic groups, a heritable component to susceptibility was a plausible assumption, but one that has defied solid experimental proof, perhaps due to the difficulty in eliminating the confounding biases of environment and exposure. In 1912, the statistician Karl Pearson, attempting to demonstrate racial differences in *TB* susceptibility, stated the basic question, “*We have to inquire whether persons living habitually in the same environment and with practically the same risk of infection have the same chance of developing phthisis whatever be their stock*” (Puffer 1946).

Since the mid (80s), there have been many studies that have tried to identify genes that might be associated with *TB susceptibility*, as well as those testing the validity of published associations.

**2.8.1. Early studies of *TB* in families:**

Many early studies of *TB* in families compared the cumulative incidence of disease in the offspring of couples where one, both, or neither had *TB*, also noting other family history of *TB*, and whether cases were sputum. While these studies clearly demonstrated that living in a house with a tubercular person increased the chances of developing *TB*, most investigators accepted that their results represented a combination of the effects of exposure and hereditary predisposition positive (Puffer 1946, Stocks 1928, Frost 1933).

**2.8.2. Racial differences:**

Much of the controversy about genetic susceptibility to *TB* in the early part of the 20th century was concerned with allegations of racial differences, or more specifically, that Asians and especially Africans and African Americans had less
innate resistance than Whites. The higher rates of TB in Africans and African-Americans predominantly to the effects of genetic composition. Liu (Liu 2006) was cited examples of higher TB rates in Africans; he really concentrated on the more severe nature of the pathology of the disease. He proposed that because of Africa’s short history of exposure to TB, Africans have not developed genetic resistance to the bacillus, and therefore many Africans, even as adults develop a systemic TB(Palomino 2007).

2.8.3. Mutations and polymorphisms that increase susceptibility:

2.8.3.1. Tuberculosis susceptibility in generalized immune deficiencies: Considerable insight has been obtained by studying humans with immunological deficiencies, and determining which genetic defects lead to increased risk of mycobacterial infections (Picard 2006). Undoubtedly, the largest group of highly susceptible persons is individuals infected with the HIV virus, who are prone to develop TB early in the course of the disease. After the onset of AIDS, they are also susceptible to atypical or environmental mycobacteria as well as many other pathogenic and opportunistic agents. TB takes the lives of a large percentage of AIDS patients in Africa (Cantwell 1996), and the early susceptibility underlines the overwhelming importance of (CD4+ T cells) in immunity to TB.

Disseminated BCG infection, pneumonia with M. intracellulare, and a M. tuberculosis brain abscess (Metin 2004) have been described in individuals with hyper-IgE syndrome, a rare autosomal dominant disorder characterized by high serum IgE levels, eczema, and susceptibility to bacterial and fungal infections (Casanova2002). Reports have described low levels of IL-12 and IFN-γ in several of these patients, but this defect must be mild or variable, as many hyper IgE patients have been vaccinated with BCG and survived into adulthood without mycobacterial infections. Overall, mycobacterial infections occur in perhaps a third of patients with severe combined immunodeficiency (Netea 2005).
2.8.3.2. Candidate genes in common tuberculosis:

The identification of the genes where mutations lead to extreme susceptibility has helped to identify the essential components of the human immune defense to mycobacteria. These genes and several others thought to play a role in the human defense against TB have also been studied to see if there might exist different alleles or polymorphisms that cause subtle changes in function that could account for individual variation in susceptibility to commonTB.

2.8.3.2.1. Human leukocyte antigens (HLA):

HLA alleles have been associated with susceptibility to several infectious diseases, including severe malaria, HIV progression, and hepatitis B and C persistence (Hill 2006, Yee 2004). HLA studies have also shown an association of HLA-DR2 with either leprosy per se or the type of leprosy - tuberculoid or lepromatous - in both case-control and family linkage studies, and in Asian, African, and American populations (Geluk 2006). Many studies have looked for associations of TB susceptibility with particular HLA alleles of the major (MHC). The MHC loci are divided into class I and class II alleles. The class I, HLA-A, B, and C, are thought to be principally involved in the presentation of peptides generated in the cytosol by virus-infected cells to (CD8+ T cells), while the class II molecules, DR, DQ and DP, present antigens of phagocytosed pathogens, such as mycobacteria, to (CD4+ T cells). In the human immune response to TB, (CD4+ T cells) seem to be of primary importance (Flynn 2001).

2.8.3.2.2. Cytokines and cytokine receptors:

Many studies have looked for an association of TB susceptibility with polymorphisms in genes encoding other elements of the immune system thought to be important in controlling mycobacterial infections. These different polymorphisms, or alleles, which coexist in the population, are generally changes in a single nucleotide (Single Nucleotide Polymorphism, or SNP). They have mainly been evaluated in case control studies, but some have also been tested in
family studies correlating the inheritance of particular parental alleles with the development of TB in both the parents and offspring.

2.8.3.2.3. IFN-γ:
From studies in mice and investigations in humans, it is clear that IFN-γ is critical for the defense against mycobacteria, and therefore, the gene encoding it was an obvious candidate for polymorphisms that might slightly affect its function and alter susceptibility to common TB (Flynn 1993).

2.8.3.2.4. Pattern recognition receptors:
One of the first lines of defense of the immune system is the recognition and uptake of microorganisms by professional phagocytes: macrophages and dendritic cells. On the surface of phagocytic cells are several different pattern recognition receptors, which, in the absence of adaptive immunity, bind to different patterns on microbes to promote phagocytosis and activate signaling that leads to cytokine production, antigen presentation, and the development of adaptive immunity. These pattern recognition receptors include TLR, scavenger receptors, the complement receptors, the macrophage mannose-binding lectin (MBL), the dendritic-cell-specific intercellular adhesion molecule-3, called DC-SIGN, and others. Several of these have been shown to mediate the phagocytosis of M. tuberculosis (Ernst 1998).

2.9. NRAMP-Igene:
NRAMP-I (natural resistance-associated macrophage protein 1), was originally termed the BCG gene (Skamene 1994). It was found to be responsible for the abnormal sensitivity of a strain of mice to infections with BCG, Salmonella, and Leishmania. The encoded protein is a divalent cation transporter that appears to play a role in macrophage activation (Nevo 2006). It may also alter the phagosome environment to affect anti-microbial capacity, and regulate the levels of cations,
especially iron. A number of studies have looked at genetic markers to see whether in humans, as in mice, there are variants that confer different levels of resistance to TB. Initial studies suggested a minor effect, with about a two-fold increase in susceptibility to TB for each of two polymorphisms, and a four-fold difference when both were present (Bellamy 1998). Results of subsequent studies have varied, and several have shown no effect (Li 2006).

2.7.1. Structure of NRAMP-I:

The NRAMP-I gene encodes a highly hydrophobic protein of predicted molecular mass 60 KD. The protein has characteristics of an integral membrane protein, with twelve putative transmembrane domains and a glycosylated extracellular loop (Barton et al, 1994). This suggests that NRAMP-I may play a role in the membrane transport processes of the macrophage (Govoni, 1998).

Blackwell in 1989 showed that the NRAMP-I gene plays an important role early in the macrophage activation pathway through the functional studies which utilized congenic mouse strains and NRAMP-I transfected macrophage. It has been found that NRAMP-I has many effects on macrophage function including regulation of the chemokines, (IL-1) inducible nitric oxide synthase (iNOS), (MHC), (TNF-α), nitric oxide (NO) release, oxidative burst, and tumouricidal as well as antimicrobial activity (Blackwell et al, 2000).

Macrophages carrying the mutated infection susceptible NRAMP-I allele were found to have a defect in the ability to process antigen, which was compounded by the influence of the gene on molecules regulating TNF-α, IL1, or directly involved in MHC class II antigen presentation. All of these effects suggest that NRAMP-I is important in processing the pathogens engulfed in macrophages, which may therefore affect the host susceptibility to the infectious pathogens that harbor macrophages as the host cell (Lang et al, 1997).
2.7.2. Localization of NRAMP-I molecule: In fact, no NRAMP-I protein has been identified on the outer macrophage membrane in any studies. Immunofluorescence studies in primary macrophages indicated that NRAMP-I is not located at the plasma membrane (Gruenheidet al, 1989). It was also found that upon phagocytosis, NRAMP-I is recruited to the membrane of the phagosome and remains associated with this structure during its maturation to phagolysosome. After phagocytosis, NRAMP-I is acquired by the phagosomal membrane. The targeting of NRAMP-I from endocytic vesicles to the phagosomal membrane supports the hypothesis that NRAMP-I controls the replication of intracellular parasites by directly or indirectly altering the intravascular environment of the microbe-containing phagosome (Blackwell et al, 2000). Besides, evidence have found on the role of NRAMP-I as a cation transporter. Indeed, it is possible that NRAMP-I is mechanistically involved in delivering intracellular signals for endosomal / lysosomal migration and for phagosome / endosome / lysosome fusions to occur. (Brown et al, 1995).

2.7.3. NRAMP-I function as metal ion transporter:

Divalent metals such as Fe$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ are known to play an important role in host defense against infections (Kontogiogheset al, 1995) by acting as important cofactors for the production of toxic hydroxyl radicals or for bacterially encoded detoxifying metalloenzymes such as superoxide dismutase (Jabadoet al, 2000). These functions in innate immunity make divalent metals attractive substrates for NRAMP-I. In addition, macrophages are also essential for the recycling of iron from the breakdown of red cells (Kondo et al, 1988). This suggested that NRAMP-I may function as a macrophage-specific iron transporter at the phagosomal membrane (Andrews and Levy, 1998).
2.7.4. The association between NRAMP-I polymorphism and tuberculosis:

Associations between susceptibility to mycobacterial infections and NRAMP-Igene have been found in different populations. Linkage between leprosy susceptibility and NRAMP-Iwas detected in Vietnamese families (Abel et al, 1998; Alcais, 2000), however, other studies failed to detect the association in leprosy families from Brazil (Blackwell, 1998) and French Polynesia (Roger et al, 1997). In contrast, associations between NRAMP-Iand susceptibility to tuberculosis have been detected in many different populations including Aboriginal Canadian (Greenwood et al, 2000), Western Africans (Bellamy et al, 1998; Bellamy et al, 2000; Cervino et al, 2000), Koreans (Ryu et al, 2000) and Japanese (Gao et al, 2000).

2.7.4.1. Study in Western Africans: In a case-control study of tuberculosis in Gambia, West Africa, the association of NRAMP-Ipolymorphic variants with susceptibility to the infection of MTB was found in (410) adults with smear-positive pulmonary tuberculosis and (417) ethnically matched healthy controls. Patients with human immunodeficiency virus infection were excluded. The allelic frequencies of polymorphic variants of the NRAMP-Igene was studied: a polymorphism in INT 4, D543N and a polymorphic variant in the 3' end. Patients with polymorphisms in INT 4and in the 3'UTRregion of the gene were particularly overrepresented in the TB population, as compared with those with most common NRAMP-Igenotypes (p<0.001). The authors concluded that genetic variations at NRAMP-Iaffect susceptibility to TB in West Africans.

A family based study was then performed in the same population to further confirm the association between the NRAMP-Ipolymorphisms and TBin West Africans. Four families from Guinea-Conakry were analyzed by Cervino et al, in the year 2000, to test for the association between NRAMP-Ipolymorphisms and TB
and the single nucleotide substitution in INT 4 was found to be significantly associated with tuberculosis (value = 0.036).

**2.7.4.2. Study of aboriginal Canadians:** In the year 2000, Greenwood et al. collected genetic and epidemiologic data to assessed evidence for linkage between NRAMP-I and MTB infection. The major gene identified in the Canadian studies by Greenwood et al in the year 2000 appears to control the progression from infected status (individuals with positive TST) to affected status (individuals with tuberculosis) in a dominant manner. This mode of inheritance is consistent with the results observed in the Gambian casecontrol study (Bellamy et al, 1998).

**2.7.4.3. Study of Koreans:** Studies similar to the study done in the population in Gambia, West Africa, on the association between NRAMP-Igene polymorphisms and host susceptibility to MTB were also done in Koreans. In the Korean study, a significant association was found between the smear-positive tuberculosis patients and polymorphisms in the UTR and D543N loci of the NRAMP-Igene. It was also found that the 3′, UTR TGTG allele was always associated with the D543N G allele (p<0.001), indicating a strong linkage disequilibrium between them. Another additional polymorphism in the NRAMP-Igene was also typed in Korean population. However, the polymorphism in the (INT4 locus) was not found to be associated with tuberculosis, this suggests that the (INT4 locus) and the (3′UTR locus) are independent of each other (Ryu et al, 2000).

**2.7.4.4. Study of Japanese:** Different results were found from the other similar study in the Japanese population. To test whether variants of NRAMP-I relate to active tuberculosis in Japanese populations, a study using two independent populations from Tokyo and Osaka were conducted (GAO et al 2000). Perfect linkage disequilibrium was found between the 5′ promoter (GT) and the INT4 loci, as well as the D543N and 3′UTR loci. Genotyping of the 5′ promoter (GT) locus and D543N locus found that there was a weak association between D543N and
tuberculosis in the Tokyo population. In contrast, there was a significant association with the 5' promoter (GT) in both populations. However, it was found that there was no interaction between D543N and the 5' promoter (GT) loci in the Japanese populations.
Chapter Three

Materials and Methods
3. Materials and Methods:

3.1. Study design:

This is a case-control study was carried out during the period from September 2013 to January 2017, including the pilot study, literature review, specimen collection, laboratory investigations, data analysis, and thesis writing.

3.2. Study area:

This study was conducted in River Nile State, north of Sudan. River Nile State lies between latitudes (16°-22°) and longitudes (32°-36°), covering an area of about (122,000) km². The population is about (1,250,000). More than two thirds of the population is resident in the rural areas. Al Damer is the capital city of the state. Slightly north of Al Damer is the important rail junction town of Atbara the other towns (localities) are Shendi, Berber and Abu Hamed.

3.3. Study population: The study was conducted in collaboration with the Tuberculosis Centers of Ministry of Health in River Nile State, Sudan. All smear-positive patients who reported in the TB Centers in Al Damer, Atbarah and Shendi during the period of April 2014 to August 2015 and agreed to participate were chosen as index cases (ICs). Appropriate control individuals were also enrolled following the normal consenting procedure.

3.4. Sample size:

Two hundred and sixty nine (269) specimens were collected (143 tuberculosis patients and 126 healthy controls), based on non-probability convenience sampling technique during admission to the TB centers.
3.5. Inclusion Criteria of Index cases:

The clinical characteristics of the tuberculosis patients included in this study were well defined. All of them had clinical symptoms of tuberculosis and were all diagnosed to have tuberculosis by radiology, AFBstaining of sputum smear.

3.6. Inclusion Criteria of control individuals:

Sex un-matched controls were enrolled aged (≥15 years). No symptoms of tuberculosis, no history of tuberculosis in his/her household. Giving informed consent.

3.7. Ethical considerations:

The study protocol was scientifically reviewed and approved by the Medical Research Committees of the Shendi University and the Ethical Committees of the Ministry of Health, River Nile State, Sudan. Before questionnaires were administered to any eligible patient, the latter was provided with a consent form to sign or thumbprint after the study was explained to them in details.

3.8. Validity and pre-testing:

All reagents and primers were pre-tested before using and equipment was calibrated.

3.9. Collection of the specimens:

3.9.1. Sputum specimens:

Three consecutive sputum specimens were collected from each patient in plastic, clean, dry, wide-mouth, screw capped containers to avoid leaks and aerosol formation. Patients were advised to collect the sputum samples on early morning before any mouth washing, following deep cough to produce sputum and not
saliva. The containers were labeled by patient's name, serial number and date of collection.

3.9.2. Blood specimens:

Whole *EDTA-blood samples* were taken from (143) lung *TB* patients and (126) health individuals as control for gene extraction.

3.10. Preparation of smears:

The work was carried out in preparation room. The purulent part of each sputum sample was selected and picked by applicator wood sticks and applied in to the slide. The smear was prepared neither thin nor thick. Smears were left to dry and then fixed by (70%) alcohol.

3.11. Ziehl-Neelsen (ZN) staining method:

Smear slides were placed on the staining rack and covered with carbolfuchsin as a primary stain. The slides were heated until vapor rises and left for (5 min). The slides were washed by distilled water until all deposit disappears. Then the decolorizer(3%) acid-alcohol was added for (2 min). The slides were washed and counter stain malachite green was added and the slides were washed by distilled water until no stain was visualized, and no deposit remained. The slides were then air-dried. Positive and negative control smears were prepared as above. The slides were examined by a light microscope, oil immersion objective lens (X100) Acid fast bacilli were viewed as bright red against a green background. The result was recorded as follows:

- No acid fast bacilli per 100 fields: negative.
- 1-9 acid fast bacilli per 100 fields: scantly.
- 10-100 acid fast bacilli per 100 fields: +.

45
1-10 acid fast bacilli per one field: ++.

More than 10 acid fast bacilli per one field: +++ (Wagne 1995).

3.12. DNA extraction:

The DNA was extracted from specimens according to manufacturer’s instructions (Analytik Jena AG, Germany). This was done as follows:

i. (200 µl) of whole blood were pipetted into (1.5 ml) reaction tube.

ii. (200 µl) of lysis solution and (20µl)ofproteaseKenzyme were added to reaction tube and the solution was mixed vigorously by pulsed vortexing for (10 sec).

iii. The reaction tubes were incubated at (60°) C for (10 min).

iv. (350 µl) of binding solution were added to the mixture and mixed thoroughly.

v. This mixture was centrifuged at (12,000) rpm for (60 sec), and flow-through was discarded.

vi. (400 µl) washing solution was added to reaction tubes and centrifuged at (12,000 rpm) for (60 sec). Then flow-through was discarded, again (600 µl) of washing solution were added, centrifuged at (12,000 rpm) for (60 sec), and flow-through was discarded.

vii. Then reaction tubes were centrifuged at (12,000 rpm) for (3 min) to remove all traces of ethanol.

viii. Then the spin filters(column) were placed into (1.5 ml) Elution tubes

ix. (200 µl) of pre-warmed (60°C)elution buffer (EB) were added and the reaction tubes were incubated at room temperature for (2 min) and centrifuged at (12,000 rpm) for (1 min). The spin filters were removed and elution tubes were closed.

46
x. Measurement of DNA concentration was performed by a NanoDrop spectrophotometer. Also the extracted DNA was electrophoresed on (2%) agarose gel to ensure the purity of the extraction.

xi. The DNA so extracted was stored at (-20°C) until PCR test was conducted.

3.13. Primers preparation:

i. The primers used in the study were in the form of lyophilized powder purchased from Macrogen (Korea).

ii. The primers sequences were first checked for quality assurance. Primer was spun for few seconds, the required volume of sterile water was added according to manufacturer’s instructions in a bio-safety hood using automatic pipette with sterile filter tips, labeled with date of preparation.

iii. Then the primers solution was mixed well and kept in a refrigerator at (4°C) overnight. The solution was vortex, and (2-3) primers aliquots were made.

iv. Primers dilution was made by taking (10 µl) of primers stock (100 pmol/µl) and added to (90 µl) sterile distilled water in a sterile Eppendorf tube to get a final concentration of (10 pmol/µl). After dilution, the primer was labeled and stored at (-20°C) until later use in PCR reactions.

v. Detection of NRAMP-1 gene polymorphisms was confirmed by the amplification of extracted DNA using the following primers and restriction enzymes (Table 3.1).
Table (3.1): *PCR* primers and restriction enzymes employed in *PCR- RFLP* study of *NRAmpi*

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
<th>Annealing temperature</th>
<th>Product length</th>
<th>Restriction Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>D543N</td>
<td>5'-GCATCTCCCCAATTCAATGGA-3' 5'-AACCTGATCCCAACCTATCCTG-3'</td>
<td>56°C</td>
<td>244 bp</td>
<td>AvaII</td>
</tr>
<tr>
<td>3'UTR</td>
<td>5'-GCATCTCCCCAATTCAATGGA-3' 5'-AACCTGATCCCAACCTATCCTG-3'</td>
<td>56°C</td>
<td>240 or 244 bp</td>
<td>FokI</td>
</tr>
<tr>
<td>INT4</td>
<td>5'-TCTCTGGCTGAGGCTCCTCC-3' 5'-TGTCCTATCATGTGACCGC-3'</td>
<td>59°C</td>
<td>624 bp</td>
<td>ApaI</td>
</tr>
</tbody>
</table>

3.14. DNA amplification:

The (4µl) specimen to be analyzed was adjusted to a total volume of (20 µl)*Maxime PCR PreMix* (iNtRON Technology, Korea). Ready composed i-Tag DNA polymerase (5U/ µl) equal to (2.5 U), dNTPs(2.5 mM) each, reaction buffer (10X) 1X and gel loading buffer 1X.

(One µl) of each primer was added to(14µl) nuclease- free water to get a final volume of (20µl) which was entered an automated DNA thermal cycler (Convergys ® TD Peltier thermal cycler-Germany). This cycler was programmed to run to each polymorphism (Tables 3.2, 3.3 and 3.4).
Table (3.2): *DNA amplification program of D543N polymorphism*

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp.</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>3 min.</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30 seconds</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>30 seconds</td>
<td>56°C</td>
<td>1</td>
</tr>
<tr>
<td>Extension</td>
<td>30 seconds</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td>Final extension</td>
<td>10 min.</td>
<td>72°C</td>
<td>1</td>
</tr>
</tbody>
</table>

Table (3.3): *DNA amplification program of 3’UTR polymorphism*

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp.</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>5 min.</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30 seconds</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>30 seconds</td>
<td>56°C</td>
<td>1</td>
</tr>
<tr>
<td>Extension</td>
<td>30 seconds</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td>Final extension</td>
<td>10 min.</td>
<td>72°C</td>
<td>1</td>
</tr>
</tbody>
</table>

Table (3.4): *DNA amplification program of INT4 polymorphism*

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temp.</th>
<th>Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>3 min.</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30 seconds</td>
<td>95°C</td>
<td>1</td>
</tr>
<tr>
<td>Annealing</td>
<td>45 seconds</td>
<td>59°C</td>
<td>1</td>
</tr>
<tr>
<td>Extension</td>
<td>60 seconds</td>
<td>72°C</td>
<td>1</td>
</tr>
<tr>
<td>Final extension</td>
<td>10 min.</td>
<td>72°C</td>
<td>1</td>
</tr>
</tbody>
</table>
PCR product was analyzed on a (2%) agarose gel and stained with ethidium bromide and photographed by UV rays.

3.15. Preparation of agarose gel:

i. Agarose gel (1.5 or 3%) was prepared by melting (1.5) or (3) grams of agarose powder in (100 ml) of Tris-borate EDTA (1X TBE) buffer and was dissolved by heating in a microwave. Agarose gel purchased from vivantis, Malaysia.

ii. The melted agarose was allowed to cool slightly, and then (3μl) ethidium bromide was added.

iii. The melted agarose was casted on (100x80 mm) gel casting trays into which a comb was inserted to form well in place. Any bubbles were removed and the gel was allowed to set at room temperature.

iv. The comb was removed and the gel was placed into the electrophoresis tank.

v. To visualize PCR product, the gel casting tray was flooded with 1xTBE buffer to cover the gel surface. (5 μl) of the PCR product from each specimen were loaded directly, and then the gel was electrophoresed at (100V) for (25min).

vi. The sizes of the PCR products were determined by comparison to the (100) base pairs ladder DNA marker.

vii. The band of amplified DNA was observed on UV trans-illuminator.

3.16. Restriction fragment length polymorphism:

i. NRAMP-I polymorphisms were investigated by PCR-RFLP. Amplicon of D543N, 3’UTR and INT4 was digested with restriction enzyme, AvaII, FokI,
and ApaI, respectively under conditions recommended by manufacturer (New England BioLabs, England).

ii. Ten microliters of PCR product was digested with (2 units) of restriction endonuclease AvaII, FokI and ApaI at (37°C) for (1½hr).

iii. The restriction digested fragments were electrophoresis on agarose gel and visualized under UV light.

3.17. Method of data collection:

A direct interviewing questionnaire was designed to collect demographical and clinical data and obtain all valuable information of each patient examined (Appendix).

3.18. Statistical analysis:

For each polymorphism, genotype frequency difference between patients and healthy controls was examined by Chi-square test. Difference was considered significant when $p$-value was (<0.05). Frequencies, percentages, and graphs were used for presentation of the data.
Chapter Four

Results
4. **RESULTS:**

4.1. **The socio-demographic characteristics of the study population:**

The total number of TB cases notified during the study period was (143), collected from the main centers in River Nile State Al Damer, Atbara and Shendi. In **(Table 4.1)**, from (143) smear-positive pulmonary TB patients in River Nile State, males were 87 (60.8%) and female were 56 (39.2 %). The male to female ratio was (1.5:1).

The age range was (9 to 80 years), mean (43.4 years). The results displayed in **(Table 4.2)** revealed that the most common age group exposed to TB was (16-30) years (28.7%).

The districts of residence of tuberculosis patients are shown in **(Fig. 4.2)** the highest proportion of tuberculosis cases (42.6%) came from Shendi district. According to districts of residence, age group (46-60) years (34.4%) was more exposure to TB in Shendi, while age group less than (15) years had no exposure in Shendi and AlDamer **(Table 4.3)**.

(40.6%) of the TB cases had Khalwa and Primary education; also (4.2 %) had University Education **(Table 4.4)**.

The results displayed in **(Fig. 4.3)** were shown (24.4 %) of patients had a positive family history of pulmonary TB.

4.2. **Clinical characteristics of tuberculosis cases:**

Cough and fever with sweating were the most common presenting clinical features **(Fig. 4.4)**.

The duration between onset of symptoms and presentation to the tuberculosis management unit in most cases (43.4%) was more than 8 weeks; however (12.1%) presented early, i.e. (2) week or less after the onset of symptoms **(Table 4.5)**.
Table (4.1): Distribution of TB cases and control according to gender

<table>
<thead>
<tr>
<th>Gender</th>
<th>TB cases</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>87 (60.8%)</td>
<td>64 (50.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>56 (39.2%)</td>
<td>66 (49.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>143 (100%)</td>
<td>126 (100%)</td>
</tr>
</tbody>
</table>

Fig. (4.1): Distribution of TB cases and control according to gender
Table (4.2): age group of TB cases and control distribution

<table>
<thead>
<tr>
<th>Age group</th>
<th>TB cases</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 15</td>
<td>1 (0.6 %)</td>
<td>8 (6.2%)</td>
</tr>
<tr>
<td>16 – 30</td>
<td>41 (28.7%)</td>
<td>61 (48.4%)</td>
</tr>
<tr>
<td>31 – 45</td>
<td>36 (25.2 %)</td>
<td>33 (26.2%)</td>
</tr>
<tr>
<td>46 – 60</td>
<td>38 (26.6 %)</td>
<td>16 (13%)</td>
</tr>
<tr>
<td>≥ 61</td>
<td>27 (18.9 %)</td>
<td>8 (6.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>143 (100%)</td>
<td>126 (100%)</td>
</tr>
<tr>
<td>Mean age</td>
<td>43.4 years</td>
<td>35.2 years</td>
</tr>
</tbody>
</table>

Fig(4.2): Residency percentage of TB cases
Table (4.3): Distribution of TB cases according to residence and age incidence

<table>
<thead>
<tr>
<th>Age (Yrs)</th>
<th>Shendi</th>
<th>AlDamer</th>
<th>Atbara</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 15</td>
<td>0 (0 %)</td>
<td>0(0 %)</td>
<td>1 (2.2 %)</td>
<td>1 (0.6 %)</td>
</tr>
<tr>
<td>16 – 30</td>
<td>13 (21.3 %)</td>
<td>17 (44.8 %)</td>
<td>11 (25 %)</td>
<td>41 (28.7 %)</td>
</tr>
<tr>
<td>31 – 45</td>
<td>17(27.8 %)</td>
<td>11 (28.9 %)</td>
<td>8 (18.3 %)</td>
<td>36 (25.2 %)</td>
</tr>
<tr>
<td>46 – 60</td>
<td>21 (34.4 %)</td>
<td>7 (18.5 %)</td>
<td>10 (22.7 %)</td>
<td>38 (26.6 %)</td>
</tr>
<tr>
<td>≥ 61</td>
<td>10 (16.5 %)</td>
<td>3 (7.8 %)</td>
<td>14 (31.8 %)</td>
<td>27 (18.9 %)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>61 (42.6%)</td>
<td>38 (26.6 %)</td>
<td>44 (30.8 %)</td>
<td>143 (100%)</td>
</tr>
</tbody>
</table>

Table(4.4): Educational level of the TB cases

<table>
<thead>
<tr>
<th>Educational level</th>
<th>Percentage of TB cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illiterate</td>
<td>48 (33.5 %)</td>
</tr>
<tr>
<td>Khalwa&amp; Primary</td>
<td>58 (40.6 %)</td>
</tr>
<tr>
<td>Secondary</td>
<td>31 (21.7%)</td>
</tr>
<tr>
<td>Higher ( university)</td>
<td>6 (4.2 %)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>143 (100 %)</td>
</tr>
</tbody>
</table>
Fig (4.3): Illustrate the family history of TB disease.

Fig (4.4): Display the symptoms which were appeared in TB cases.
Table (4.5): Demonstrate the Duration of TB symptoms

<table>
<thead>
<tr>
<th>Duration of TB symptoms</th>
<th>Percentage of TB cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks or less</td>
<td>18 (12.6%)</td>
</tr>
<tr>
<td>3-5 weeks</td>
<td>29 (20.2%)</td>
</tr>
<tr>
<td>6-8 weeks</td>
<td>34 (23.8%)</td>
</tr>
<tr>
<td>More than 8 weeks</td>
<td>62 (43.4%)</td>
</tr>
</tbody>
</table>

4.3. PCR Amplification of the NRAMP-I locus:

*PCR amplification* of the NRAMP-Iloci Intron 4, D543N and 3UTR using the pair of primers of each one produced a DNA fragment with size (624bp),(244bp) and (244 bp) respectively. The photograph of the ethidium bromide stained agarose gel is shown in (Figure 4.5).

![Image]
Fig (4.5): Agarose gel electrophoresis detection of the PCR amplification of the NRAMP-Iloci. A: PCR amplification of D543N, B: PCR amplification of INT4 and C: PCR amplification of 3’UTR.
4.4. Allelic frequencies of the NRAMP1 loci:

Allelic frequencies of the three NRAMP1 loci INT4, D543N and 3'UTR in the AFB positive patients and health controls were recorded and summarized (Table 3.2). The number of tuberculosis patients and health controls carrying different polymorphic alleles of the 3 NRAMP-1 loci was converted into percentage for easier comparison of the frequency of each allele.

4.4.1. INT 4:

For the INT 4 region, out of (143) tuberculosis patients, (135) were homozygous individuals having the genotype \( G/G \), which was the most common genotype. (8) of the tuberculosis patients were heterozygous individuals having the genotype \( G/C \), carrying both the common allele and the polymorphic allele. There is no tuberculosis patients have homozygous polymorphic allele or genotype \( C/C \). In the (126) health controls, (117) had the genotype \( G/G \), (9) had the genotype \( G/C \), and (0) had the genotype \( C/C \).

![Image](image-url)

Fig (4.6): Agarose gel electrophoresis analysis of the RFLP patterns observed from ApaI digestion of the locus Intr4
4.4.2. D543N:

Out of the (143) patients, (40) were homozygous for the common allele with the genotype $G/G$. (99) $AFB_{positive}$ samples were heterozygous individuals carrying both the common allele and the polymorphic allele, they had the genotype $G/A$. The remaining (4) patients were homozygous for the polymorphic allele having the genotype $A/A$. In health controls, only one out of the (126) had the genotype $G/G$, (124) had the genotype $G/A$ and only one had the genotype $A/A$.

Fig(4.7): Agarose gel electrophoresis analysis of the $RFLP$ patterns observed from $AvaII$ digestion of the locus $D543N$
4.4.3. 3’UTR:

(108) of the (143) tuberculosis patients were homozygous for the common allele of the region 3' UTR. They had the genotype TGTG / TGTG which means that they did not have the polymorphism TGTG deletion in that region. There were (35) patients who were heterozygous individuals carrying both the common allele and the polymorphic allele, i.e. carrying the genotype TGTG / TGTG del. There was zero AFB positive patients were homozygous for the TGTG deletion allele. (120) of the (126) healthy blood donors had the common genotype TGTG /TGTG, (6) had the heterozygous genotype TGTG / TGTG del, and no AFB positive patients had the genotype TGTG del / TGTG del.

Fig (4.8): Agarosegel electrophoresis analysis of the RFLP patterns observed from FokI digestion of the locus 3’UTR.
Table (4.6): Allelic frequencies of the three loci *INT4*, *D543N* and 3'UTR in sputum smear positive tuberculosis patients and health controls.

<table>
<thead>
<tr>
<th>NRAMP 1 Locus</th>
<th>Polymorphisms</th>
<th>Tuberculosis Patient No</th>
<th>Controls No</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>D543N</td>
<td>G / G</td>
<td>40 (28 %)</td>
<td>1 (0.8 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G / A</td>
<td>99 (69.2 %)</td>
<td>124 (98.4 %)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>A / A</td>
<td>4 (2.8 %)</td>
<td>1 (0.8 %)</td>
<td></td>
</tr>
<tr>
<td>3'UTR</td>
<td>TGTG / TGTG</td>
<td>108 (75.5 %)</td>
<td>120 (95.2 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGTG / TGTGdel</td>
<td>35 (24.5 %)</td>
<td>6 (4.8 %)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>TGTGdel /TGTGdel</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td></td>
</tr>
<tr>
<td>INT4</td>
<td>G / G</td>
<td>135 (94.4 %)</td>
<td>117 (92.8 %)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G / C</td>
<td>8 (5.6 %)</td>
<td>9 (7.2 %)</td>
<td>0.604</td>
</tr>
<tr>
<td></td>
<td>C / C</td>
<td>0 (0 %)</td>
<td>0 (0 %)</td>
<td></td>
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<tr>
<td>Total</td>
<td></td>
<td>143 (100 %)</td>
<td>126 (100 %)</td>
<td></td>
</tr>
</tbody>
</table>
Chapter Five
Discussion
Conclusion
Recommendations
5. Discussion:

Tuberculosis (TB) remains the single largest infectious disease causing high mortality in humans, leading to (3) million deaths annually, about five deaths every minute. In 2015, there were an estimated (10.4) million new (incident) TB cases worldwide, of which (5.9) million (56%) were among men, (3.5) million (34%) among women and (1.0) million (10%) among children (WHO 2016). Sudan is a poor country whose healthcare delivery system has been placed under considerable strain in the past two decades through civil unrest, natural disasters and other economic factors. The susceptibility of an individual to M. tuberculosis is determined by the ability of the pulmonary macrophages to get rid of the pathogen or to inactivate the pathogen before it is able to create any further damage or lesion. Understanding the structural function relationship of NRAMP-Igene will help us understand its effects on the antimicrobial activity of macrophage and thus be able to explain its association with host susceptibility to tuberculosis. Thus, we present the first study to ascertain the role of NRAMP-Igene polymorphism in development of pulmonary tuberculosis in River Nile State patients.

In this study it was found that the Gender specific prevalence was higher in males than in females. In all studies, which contain data on prevalence of pulmonary tuberculosis in adults, males have a higher prevalence than females over all (Narang P 1992 and WHO 2016). Higher male prevalence than female prevalence rates were reported also in studies that were conducted on River Nile State by (Amel A S, et al 2013 and Elmadhoun W M et al 2016).

This study revealed that the female to male ratio was (1:1.5). Globally the ratio of female to male TB cases notified is 1: 1.5 to 2 (Borgdorff 1999). Literature written on TB has paid limited attention to gender related factors associated with the prevalence. This male predilection may be explained by the habit of smoking that
is more common in males, by different immunological factors, by greater outdoor exposure to infected individuals among men or by other unknown factors. The age groups most commonly affected by TB were adults in their productive years (16 – 30 years 28.7 %), a finding that is similar to other studies (Amel A S, Ermadoun W M et al 2016 and Getahun 2011). TB affects the productive age group in River Nile State as seen also in some African countries (Uchimura 2013). This fact may, partially, be explained by the fact that most populations of developing nations are among the young age groups.

The result displayed in (table 4.3) indicated that the age group (46-60 years) (34.4%), (16-30 years) (44.8%) and age group (more than 60 years) (31.8%) were found to be more exposed age groups to tuberculosis in Shendi, Al Damer and Atbara respectively. Also age group less than (15 years) was found to be less exposure to tuberculosis infection (0%) in Shendi and Al Damer and (1.5 %) in Atbara.

This study had shown a clear association between educational status of the TB cases and prevalence of tuberculosis (Table 4.4). Prevalence was higher among the Khalwa & Primary group (40.6%) and illiterate group (33.5%). Also low prevalence of tuberculosis was shown in higher educational level individuals (4.2 %). One explanation is that as they get education their knowledge about tuberculosis is increasing the chance of protection. Secondly the literate people do come closer to the schools where health facilities are also available. They are use the available health facilities thus detecting cases early and taking treatment. Instead the illiterate remain always in the deep interior village where they never reach to a health centre for diagnosis.

Nearly half of the patients (42.6%) were diagnosed in Shendi Teaching Hospital. These results were with disagreement with the study done in River Nile State by (Elmadhoun W M et al 2016) which found Atbara have higher prevalence of TB in River Nile State.
Findings from this study also suggest that (24.4%) of our study group reported a positive family history of TB. These results were in conformance with the known fact that the family history one of high risk of tuberculosis infection (Palomino J et al 2007).

Currently, there were(8–9) million new cases and (2–3) million deaths annually. In this regard, it is well known that approximately one-third of the world's population is infected with M. tuberculosis. However, most of the (90%) of infected individuals remain healthy, indicating the effectiveness of the different immune mechanisms in resistance against this mycobacterium. Although both acquired and innate mechanisms contribute to the killing of M. tuberculosis, the precise mechanisms that confer resistance against this infection have not been elucidated fully. Nevertheless, it seems evident that different genes are involved in susceptibility towards M. tuberculosis infection (Delgado, 2002 and Kramnik, 2000). In this work, we have explored the possible association between polymorphisms of NRAMP-I gene and pulmonary tuberculosis. Association between TB and the polymorphisms of the human homologueNRAMP-Igene was found in different populations including Aboriginal Canadians (Greenwood et al, 2000), Western Africans (Bellamy et al, 1998), Korean (Ryu et al, 2000) and Japanese (Gao et al, 2000). In this study, NRAMP-I polymorphisms were assessed (143) patients with clinical tuberculosis and (126) healthy controls, to determine whether the gene also governs host susceptibilities to pulmonary tuberculosis in River Nile State patients.

It has been shown from this study that the polymorphisms in (2) regions of the human NRAMP-Igene including D543N and 3'UTR, have statistically significant association with host susceptibility to pulmonary TB.

The results obtained results were in concordance with the previous study from Poland (Dubaniewics, 2005 and Jusak, 2011) showing that INT4 polymorphism is not associated with TB infection.
The study was done by Li et al 2006; it was found that the 3 gene polymorphisms that have been studied for NRAMP-1 [3'-UTR, D543N and INT4] do not show a significant association with TB in a European population. In contrast, a variable but significant association among most of these variants has been found in Asian and African subjects (Bellamy, 1998, Ryu, 2000 and Liaw, 2002). Our results show that in River Nile State population there is no apparent association between the INT4 variant of NRAMP-1 and pulmonary TB. Therefore, it is evident that the genetic background exerts a decisive role on the possible influence of this gene in susceptibility to infection by M. tuberculosis.

The 3'UTR variant allele associated with susceptibility to TB is very uncommon in Europeans (Liu, 1995) but was present in about a quarter of this West African population. These findings may in part explain why American blacks have greater susceptibility to tuberculosis than whites (Stead, 1990 and Stead, 1992) NRAMP-1 polymorphism will need to be assessed in large case–control studies of Africans, Europeans and Asians to determine whether the gene also governs susceptibility to tuberculosis in other racial groups.

The study of polymorphisms of NRAMP-Igene in association with host susceptibility to tuberculosis has potential contribution to the clinical management of tuberculosis. As individuals possessing uncommon polymorphic NRAMP-I allele or alleles are found more susceptible to pulmonary TB, the risk of contracting the disease would be relatively high. When a patient presents with symptoms of suspected tuberculosis but the infection is not yet confirmed microbiologically by the standard culture of sputum which normally takes (4 to 6) weeks, identification of the NRAMP-I genotype would be highly informative to the physicians and to decide whether anti-tuberculosis prophylaxis should be prescribed earlier. In addition, the identification of the NRAMP-I genotype would also help in estimating the risk of an individual to acquire infection when one of the family members of the individual is diagnosed of pulmonary TB.
5.2. Conclusion:

On the basis of the results of this study, it could be concluded that:

- Adult in their productive years (16 – 30 years; 28.7 %) were found to be more exposed to pulmonary TB.
- Strong association between educational statuses of the TB infection, the educational knowledge about TB is increasing the chance of protection.
- Approximately quarter of study group reported a positive family history of TB.
- D543N and 3'UTR polymorphisms had statistically significant association with host susceptibility to pulmonary TB.
- INT4 polymorphism was not associated with TB infection.
5.3. Recommendations:

On basis of the results of this study, it could be recommended that:

- Further studies should be done to assess the importance of the genetic variants in different populations and environments.
- Future studies on the NRAMP-Igene will be required to elucidate the direct effect of NRAMP-Ivariants in NRAMP-Ifunction.
- Identification of the NRAMP-Igenotypeis recommended to assess the risk of an individual to get infection when one of the family members of the individual is diagnosed of pulmonary TB.
- Further studies should be done with large samples size to determine the NRAMP-Igene in Sudanese people.
- Further large case-control studies should be done in African, European and Asians people to determine the association between NRAMP-Igene and racial groups.
- Public tuberculosis protection health education is recommended to increase the knowledge about pulmonary TB.
Chapter six

Reference

Appendices
6.1. References:


6.2.1. Appendix 1:

**Study questionnaire**

The Role of NRAMP 1 gene Polymorphism in Development of Pulmonary Tuberculosis in River Nile State Patients

**Part one: Details of the TB patient:**

Patient ID number: 

Patient Name: 

Patient age: 

Country of Birth: 

Sex: [1= male 2=female] 

**Part two: Social determinants:**

Nationality: Sudanese [ ] other (mention): 

City: Shendi [ ] Aldammer [ ] Atbara [ ]

If not Sudanese date of immigration: 

Education: Illiterate [ ] Khalwa & Primary [ ]

Secondary [ ] Higher (university): [ ]

Occupation:

Non-worker [ ] free worker [ ]

Farmer [ ] House wife [ ]

Employee [ ]
**Part three: Clinical information:**

History of Previous TB Disease & Treatment:  Yes [ ]  No [ ]

If Yes:

Type [1=pulmonary  2=extra-pulmonary] [ ]

Date of Diagnosis:......../......../.........

Symptoms of TB:

<table>
<thead>
<tr>
<th>Chronic Cough (more than two weeks)</th>
<th>Yes [ ]  NO [ ]</th>
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<tr>
<td>Bloody sputum:</td>
<td>Yes [ ]  NO [ ]</td>
</tr>
<tr>
<td>Fever:</td>
<td>Yes [ ]  NO [ ]</td>
</tr>
<tr>
<td>Nocturnal Sweating:</td>
<td>Yes [ ]  NO [ ]</td>
</tr>
<tr>
<td>Weight Loss:</td>
<td>Yes [ ]  NO [ ]</td>
</tr>
<tr>
<td>Co-infection with HIV: Yes</td>
<td>NO [ ]</td>
</tr>
</tbody>
</table>

Duration of TB symptoms:

| 2 weeks or less                      |          |
| 3-4 weeks                            |          |
| 5-6 weeks                            |          |
| More than 6 weeks                    |          |
History of Other Diseases:

a. Chronic Bronchitis □  b. Haemo-malignancy □  c. HIV □
d. Diabetes Mellitus □  e. Chronic Renal Failure □
f. Other Diseases (Mention): ........................................................................................................

Drugs History:

a. Corticosteroids (Dose) □  b. Chemotherapy □
c. Alcohol □  d. I.V Drugs □
6.2.2. Appendix 2:
The bloodDNA extraction kits manufactured by Analytik Jena AG, Germany.
7. Elution

- Add Spin Filter to an Elution Tube
- Add 200 µl Elution Buffer (60 °C)
- Incubation: 2 min @ RT
- 11,000 x g (~12,000 rpm): 1 min
6.2.3. Appendix 3:

Measurement of DNA concentration by a NanoDrop spectrophotometer.
### 6.2.4. Appendix 4:

The lyophilized powder primers purchased from Macrogen (Korea).

<table>
<thead>
<tr>
<th>Oligo</th>
<th>SEQ</th>
<th>GC%</th>
<th>MW</th>
<th>Yield</th>
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<th>Tm(c)</th>
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<td></td>
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<td></td>
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<td>nmol</td>
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<td></td>
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<tr>
<td>D543N</td>
<td>5'-GCA TCT CCC CAA TTC ATG GT-3' (20mer)</td>
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<td></td>
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<td>6028.0</td>
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<tr>
<td>1929a+ 55del4</td>
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<td></td>
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<td></td>
<td>55.0</td>
<td>5973.0</td>
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</table>

*Purification, Modification*
<table>
<thead>
<tr>
<th>Oligo</th>
<th>INT4</th>
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</thead>
<tbody>
<tr>
<td>SEQ</td>
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</tr>
<tr>
<td>GC%</td>
<td>calculated</td>
<td>measured</td>
<td>OD</td>
<td>nmol</td>
<td>scale (umoles)</td>
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<tr>
<td>60.0</td>
<td>6060.0</td>
<td>6052.9</td>
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<table>
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<th>Modification</th>
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<td>MOPC</td>
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<table>
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<th>Oligo</th>
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<th></th>
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</thead>
<tbody>
<tr>
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</tr>
<tr>
<td>GC%</td>
<td>calculated</td>
<td>measured</td>
<td>OD</td>
<td>nmol</td>
<td>scale (umoles)</td>
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<tr>
<td>47.62</td>
<td>6403.2</td>
<td>6389.7</td>
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</table>

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<th>vol. for 100pmol/ul</th>
<th>Purification</th>
<th>Modification</th>
</tr>
</thead>
<tbody>
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<td>Mf-PC</td>
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6.2.5. Appendix 5:
Maxime PCR PreMix (iNtRON Technology, Korea)
6.2.6. Appendix 6:

Map of River Nile State, Sudan.