بسم الله الرحمن الرحيم

Republic of Sudan

Ministry of Higher Education and Scientific Research

Shendi University

Faculty of Graduate Studies and Scientific Research

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

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[قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ العَلِيمُ الحَكِيمُ]

صدق الله العظيم

{سورة البقرة الاية: 32}

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Thesis author:Ahmed Ali Alamin Abd Alsalam

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.

BSc degree field of study: Medical Microbiology and chemical pathology

Master degree field of study: Medical Microbiology

Degree required:PhD

PhD field of Study: Medical Microbiology

Supervisor:Prof. ELFADHIL ALOBEID OMER

Thesis duration: 3 years and 3 months

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.
- I would like to express my deepest gratitude to my supervisor Professor Al Fadhil Al Obeid Omer, for his invaluable advice, continuous guidance, encouragement and patience throughout the period of my research.
- Also grateful thanks go to Dr. Mogahid El Hassan, Dr. Ibrahim Bahkit,Dr. Mutaz Hassan and Mr. Khalid Saeed for their cooperation and guidance.
- 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.

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LIST OF ABBREVIATIONS

3′ UTR	3’ untranslated region
µL	Microliter
AFB	Acid Fast Bacilli
Ava II	Restriction enzyme
Apa I	Restriction enzyme
BCG	Bacille Chalmette-Guerin
bp	Base pair
C3b	Complement factor 3
CD4+	T-helper cells expressing cluster of differentiation 4
CD8+	T-helper cells expressing cluster of differentiation 8
CPC	Acetylpyridium chloride
CTL	Cytotoxic T lymphocytes
CXR	Chest X-Ray
dNTP	2,-deoxyribonucleotide 5'-triphosphate
del	Deletion
DNA	Deoxyribonucleic Acid
DC-SIGN	Dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin
DTH	Delay type hypersensitivity

DR	Direct repeat
EB	Elution buffer
EDTA	Ethylenediamine Tetra-acetic Acid
EMB	Ethambutol
GM-CSF	Granulocyte macrophage colony stimulating factor
HLA	Human leukocyte antigen
Fok I	Restriction enzyme
JNK	Jun N-terminal kinase
IFN-γ	Interferon gamma
IL	Interleukin
INT 4	Intron 4
INH	Isoniazid
iNOS	Inducible nitric oxide synthases
mm	Millimeter
min	Minutes
mL	Milliliter
MOTT	Mycobacteria other than *Mycobacterium tuberculosis*
MTB	*Mycobacterium tuberculosis*
NRAMP1	Natural Resistance-Associated Macrophage Protein I
PAS	Paraaminosalicylic acid

PCR	Polymerase Chain Reaction
Pmol	Picomole
PPD	Purified Protein Derivative
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNS	Reactive nitrogen species
rpm	Round per minutes
Sec	Second
T-cell	Thymus derived lymphocytes
TB	Tuberculosis
TBE	Tris-Borate-EDTA
Th-1	T- helper cells subset 1
Th-2	T- helper cells subset 2
TLR	Toll-like receptors
Treg	T- regulatory cells
TST	Tuberculin skin test
UV	Ultra violet
V	Volts
WHO	World Health Organization
ZN	Ziehl-Neelsen′s

GLOSSARY

Acid fast: Describes an organism that resists acid decolorizationafter staining.

Allele: Alternate forms of a gene at the same chromosomallocus.

Alveoli (lung): Microscopic air sacs in lung.

Amplicon: A defined stretch of (chromosomal) *DNA* thatundergoes 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, andcytotoxic 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 NRAMPl 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 I* 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%) tuberculosed patients were reported as positive family history of TB. In the group of tuberculosis patients, the

results of the statistical analysis for the 3 NRAMPl loci were found to be: INT4 (p-value = 0.604), D543N (p-value = 0.001) and 3'UTR (p-value = 0.003). Therefore the NRAMPl 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، تسببه جرثومه تسمي بالمتفطرة السليه والتي لها المقدرة للنمو داخل الخلايا البلعميه ، السل هو مرض مزمن يتسبب في قتل عدد كبير من الناس اكثر من أي مرض معدي اخر. ثلث سكان العالم مصاب بهذا المرض. هنالك جين يسمي بالبروتين الاول للمقاومة الطبيعية للخلايا البلعميه وهو مرتبط بالقابلية للإصابة بالسل. وقد تم العثور على أدلة عن ارتباط الجين بالقابلية للإصابة بمرض السل في افريقيا الغربية، السكان الأصليين الكنديين والكوريين واليابانيين.

الاهداف: الهدف من هذه الدراسة هو تحديد العوامل الاجتماعية والاقتصادية لمرضى السل والتحقيق من الأشكال الجينية الثلاثة الموجودة في جين البروتين الاول للمقاومة الطبيعية للخلايا البلعميه وهي INT4 ، D543N وUTR'3، لمعرفة قابلية المرضي لمرض السل بين سكان ولاية نهر النيل في السودان خلال سبتمبر 2013 حتى يناير عام 2017.

المواد والطرق: تم استخدام تقنية PCR-RFLP لتحديد الانماط الجينية الثلاثة من الجين في عينات كل من المرضي و الحالات الضابطة.

النتائج: استخلصت النتائج على أساس دراسة الحالات والشواهد عن طريق تحليل 143 من مرضى السل و 126 من الاصحاء. 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 عالي بين مجموعة دارسي الخلوة و الأساس (40٪)، ومجموعة الأميين (33.6٪). كما تم الإبلاغ عن 24٪ من مرضى السل كان لهم تاريخ عائلي إيجابي من السل.

في مجموعة مرضى السل، نتائج التحليل الإحصائي للجين فيالمواضع الثلاثة كانت علي النحو التالي: INT4 (القيمة الاحتمالية = 0.604)، D543N (القيمة الاحتمالية = 0.001) وUTR'3 (القيمة الاحتمالية = 0.003).لذلك كانت هنالك علاقه ذات دلالة إحصائية قوية بين النمطين D543N وUTR'3 وقابلية المضيف لمرض السل.

الاستنتاجات: خلصت الدراسة إلى أن النمطين D543N و3' UTR من جين البروتين الاول للمقاومة الطبيعية للخلايا البلعميه قد يكون له دور في تطوير مرض السل الرئوي ، كما لوحظ عدم وجود اي 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, *M. tuberculosis* can cause active pulmonary or extra *pulmonary TB*(Smith 2003). Therefore, it is expected that the genetic variants of molecules involved in innate host-defense mechanisms are associated with host *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 *tuberculosis infection*(Murray 1990). Differential rates of tuberculosis infection and clinical outcomes among races, ethnicities, and families suggest a plausible genetic contribution toward *tuberculosis susceptibility*(Azad 2012). Complex interactions of *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 *TB susceptibility*(Moller 2010).

NRAMP-I 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). *NRAMP-I* acts as a divalent cation transporter or antiporter across phagosomalmembranes that are expressed only in reticuloendothelial cells (Azad 2012). These facts suggest that *NRAMP-I* may inhibit the replication of intracellular pathogens by altering the phagolysosomal environment. *NRAMP-I* is a critical mediator in the innate immune response to tuberculosis infection which leads to decreased *DNAreplication* and respiratory chain function in *M. tuberculosis*, but the precise function of this protein remains unclear (Courville*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 polymorphismwithin these genes to the common *phenotype of TB* remainsunclear.

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 *developingTB* 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, paracoccidioidomycosis, 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 *primaryTB* 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 *secondaryTB*, 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 bacilli*microscopically 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, andlipoarabinomannan. 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 *MHC1* molecules. This causes stimulation of the *T cells* and the macrophagewhich 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 Cabec*et al*., 2000; Astarie-Dequeker*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 (Vergne*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. *DC*is able to activate *T cells* and can polarize the *T cell response* towards *Th1 or Th2phenotypes* (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 defensing production and recruit neutrophils and monocytes to the site of inflammation, and are involved in the early phase of host defense.

M. bovisBacilleChalmette -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).

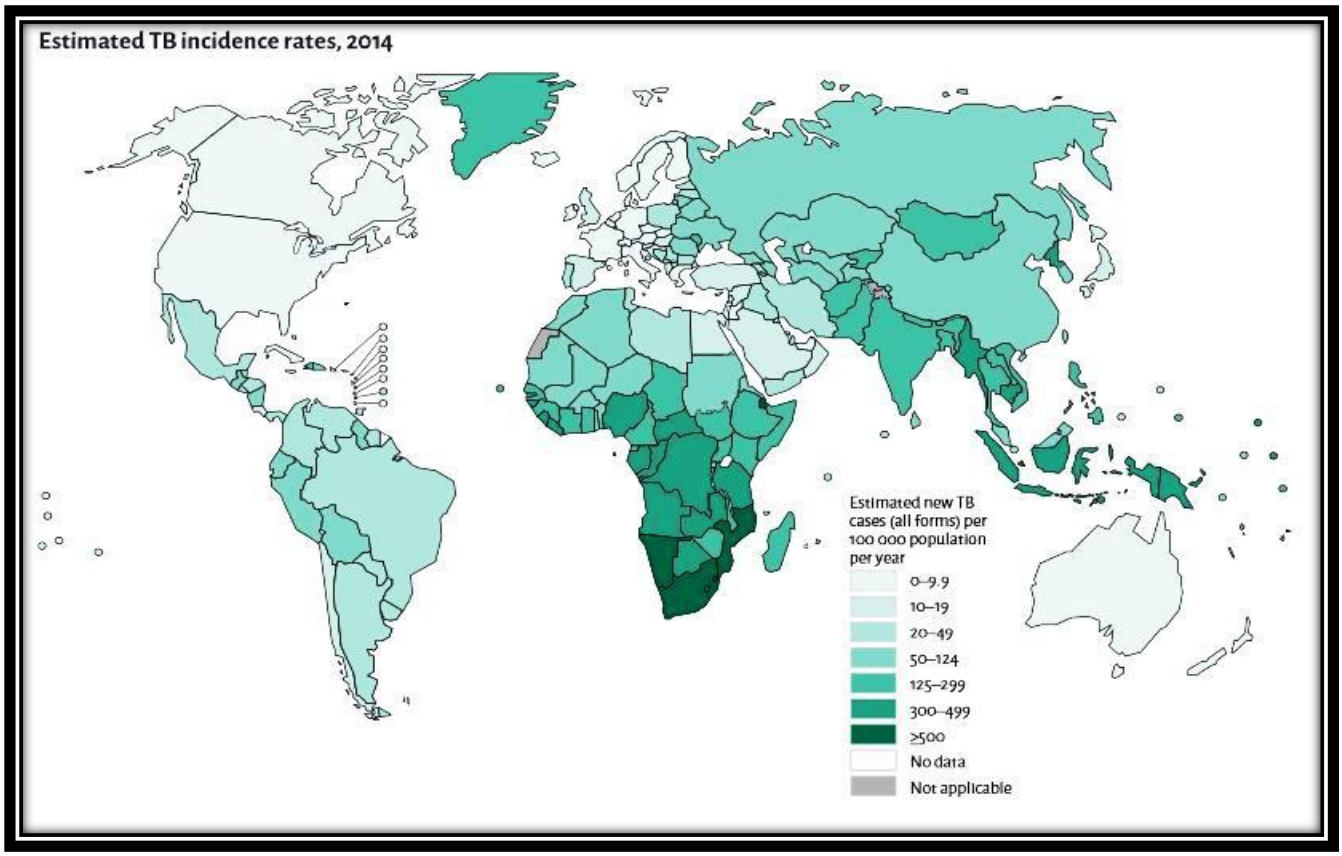


Fig (2.1) theestimated incidence rates of tuberculosis(WHO report 2014).

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 mucocilliary 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 (Verver 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-primaryTB* 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 *activeTB* 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 developmentof the specific immune response. After this, *tubercle bacilli* can multiply atany time when there is a decrease in the host's immune capacity to contain thebacilli in their implantation sites. The specific signs and symptoms will depend onthe affected organ or system, and are characterized by inflammatory or obstructivephenomena. 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 ≥ 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. Miliary tuberculosis:

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 *extrapulmonaryTB*, 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 *pulmonaryTB* case in the two years preceding the current episode. The simultaneous presence of active *pulmonaryTB* 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 *HIV*sero-negativepatients 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 developfistulas. Patients mainly complain of fever and the increasing volumeof lymph nodes, but other symptoms may be absent. In general, the *TST* isstrongly positive, except in immunosuppressed patients. The etiological diagnosis canbe made by aspiration puncture biopsy, which is*AFBpositive* in only (10 % to25 %) of cases, but*M. tuberculosis* may be isolated by culture in (50 % to 85 %) ofcases(Ikezoe 1992).

2.4.5.4. Renal tuberculosis:

Renal TB is rare in children and predominantly affects individuals in the fourthdecade of life. Renal disease occurs after a long latency period and is frequentlysecondary to hematogenous dissemination. The localization is almost always bilateral,but can be asymmetric. The lesions often start in the renal cortex and progressslowly toward the central region. Dissemination can occur to the bladder and evento the genital system.Symptoms and signs may vary in duration and severity. The patient generally complainsof dysuria, polacyuria, and lumbar pain, whereas systemic symptoms occurless frequently. Frequently, the disease presents as a urinary infection that does notrespond to routine broad spectrum antimicrobial treatment. Purulent urine is frequentlyfound, 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: meningoencephalitisand intracranial tuberculoma. Since the introduction of modernchemotherapy and especially massive *BCG vaccination*, a lower proportion of themeningoencephalitis has been observed, but the frequency of this form of *TB* ishigher among young adults with *HIV/AIDS*.The clinical manifestations are due to the inflammatoryprocess induced by themycobacterial infection, and the symptoms depend on the site and intensity ofinflammation. Granulomas can be located in the

cerebral cortex or in the meninges.Meningoencephalitis generally has an insidious onset and a slowly progressivecourse, with symptoms including lethargy, fever, and mental disturbancessuch as irritability, understanding difficulties, personality alterations, disorientation,and progressive mental confusion.The cerebrospinal fluid is generally clear, with a predominance of lymphocytes, anincrease 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, Smith1994).

2.4.5.6. Osteoarticular tuberculosis:

Involvement of the osteoarticular system is most commonly found in children andthe elderly, and is generally secondary to hematogenous seeding, but can also occuras a consequence of lymphatic dissemination or direct spread from a contiguouslesion. Bone involvement consists of osteomyelitis, and arthritis can occur either byextension of the osseous lesion to the joint or by directshematogenic 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 multiarticular disease.The diagnosis of *osteoarticularTB* is usually delayed because this etiology is oftenoverlooked in the differential diagnosis of joint disease. In most cases, the *TST* ispositive, and approximately (50 %) of cases also have abnormal *chest X-rays*, suggestingprevious pulmonary disease. Cold abscesses occurring in the advancedphase of *osteoarticularTB* can develop into cutaneous fistulae, which are frequentin this form of the disease. The diagnosis is established by puncture, biopsy, histopathologicalexamination, and culture (Zylbersztejn 1993, Davidson 1994, Ridley1998, 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.*IntestinalTB* can be acquired by the oral route and in countries with a high prevalenceof *bovine TB.* Before the generalization of milk pasteurization, this was arather common form of *zoonotic TB* (produced by *Mycobacterium bovis*), particularlyin infants. *Eye and skinTB* may be the consequence of accidental inoculation, particularlyamong medical and veterinary professionals, and *genital TB* may be produced byspread from *renalTB* (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'slymphoma), malnutrition (more than 15 % loss of usual weight), old age,Diabetes mellitus and silicosis. The occurrence of *TB* in these patients is likely tobe the result of both increased susceptibility and longer patient survival, due to theincreased 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 toconsume those affected, and anorexia and weight loss are still frequent in *TBpatients*(about 70 % of the cases). Weight loss is proportional to the duration andextent of the disease and is frequently accompanied by anemia(Palomino 2007).

2.5.3. **Cough**:

Cough is present in virtually all patients with *pulmonaryTB*. Cough results fromthe stimulus caused by the alveolar inflammatory process or from the granulomatousimpingement into the respiratory airways. At the onset of the disease, thecough is dry; but with progression, it becomes productive with mucous or mucopurulentexpectoration, generally in small amounts, and sometimes with blood. Coughis less frequent in the pleural form of the disease. It is worth mentioning that coughtends to be ignored or minimized by smokers, who may have a chronic cough, soquestions about changes in the usual pattern can be of great value in increasingsuspicion of *pulmonaryTB*(Palomino 2007).

2.5.4. **Hemoptysis**:

When hemoptysis occurs, the blood volume is variable, from bloody streaks mixedin 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 *TBpathologicalprocess* begins in the alveoli, very close to the pleural surface, this is an early andrelatively frequent symptom. Generally of low intensity, it disappears within two orthree 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 *Mycobacteriumavium*infection 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, *AFBsmear* microscopyis 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 (NaClO*or 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.

Count on Ziehl-Neelsen /Kinyoun stain (1000x)	Report
0	Non AFB observed
1-9/100 fields	Exact count
10-99/100 fields	+1
1-10/field	+2
> 10/field	+3

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 micro-organisms 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 since1898, when crude cell preparations containing carbohydrates, lipids, and

proteinsfrom *M. tuberculosis* or *M. bovisBCG* were used as antigen preparations showinghigh sensitivity but low specificity (Arloing 1898). Modern developments in thepurification of antigens, generation of monoclonal antibodies and chromatographictechniques, have led to a considerable improvement in specificity. During the lastthree decades, a large number of purified (native and recombinant) antigens havebeen assessed, showing substantial progress in the serodiagnosis of *TB*(Jackett1988).

In *TBpatients,* 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. AlsoImmunochromatographic 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(Jackett1988).

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, *TBpatients* 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 (*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 (*PPD*) (Palomino 2007).

TST has been used to identify patients actively infected with *TB*, to measure the prevalence of infection in a community, and to select susceptible or high-risk patients for *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).

TST works by intradermally injecting (0.1 mL) of 5 *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 *BCG vaccine* and other mycobacteria common in the environment, the *TST*is often falsely positive. It is currently estimated that almost one third of people positive to *TST* do not actually have *TB infection*. The sensitivity of the skin test is estimated to be around just 7(0 %) in known active *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).

2.7. Treatment and prevention of tuberculosis:

2.7.1. Treatment of tuberculosis:

The history of*TB* changed dramatically after the introduction of anti-mycobacterial agents. Drug treatment is fundamental for controlling *TB*, promoting the cure of the patients and breaking the chain of transmission when the anti-tuberculosis drug

regimen is completely and correctly followed. Anti-tuberculosis drug treatment started in 1944, when streptomycin (*SM*) and 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 months). In 1960, ethambutol (*EMB*) substituted *PAS*, and the treatment course was reduced to (18 months). In the ’70s, with the introduction of rifampicin (*RIF*) into the combination, treatment was shortened to just (9) 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 *TBrelapses*. 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 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 *TBsymptoms* 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 i*nfectiousTB*, their close contacts are screened for the illness, this is known as contact tracing.

iv. Managing your environment:

As *TB* is an airborne infection, *TBbacteria* 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 *TBbacteria*.
- Good hygiene: covering the mouth and nose when coughing or sneezing reduces the spread of *TBbacteria*.

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 problemin Europe and America in the 18th , 19th, and early 20th centuries, and considerableeffort was spent trying to understand it. Many of the questions investigated inthe past are now being re-addressed at the molecular level. One of the principal questions that occupied earlier researchers were the interplay ofbacterial 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 inheriteddisorder; a contagious disease; and a disease caused by poor living conditions.Hippocrates thought it was inherited, while Aristotle and Galen believed itwas contagious (Smith 2003). As the disease was most common in the urban poor,crowded into the rapidly growing cities of the recently industrialized Europe, socialreformers of the time believed that *TB* was caused by the deplorable living conditionsof the working class and rejected a contagious explanation.

While the discovery of the *TB* bacillus by Koch in 1882 disproved the notion thatthe disease had a purely hereditary etiology, or was caused solely by the unhealthyliving conditions of the lower classes in the early industrial age (Hass 1996), severalaspects of *TB* epidemiology are not explained by the germ theory and suggestthat there are individual differences in susceptibility: not everyone exposed to *M.tuberculosis* becomes infected; even when infection can be demonstrated with *apositiveTST,* only about one in ten infected individuals becomesill; the course of the disease varies in different individuals - before

antibiotics some tuberculars died rapidly of “galloping consumption” while others recoveredor lived a relatively long life with chronic disease; and some infected individualsdevelop 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 hasdefied solid experimental proof, perhaps due to the difficulty in eliminating theconfounding biases of environment and exposure. In 1912, the statistician Karl Pearson, attempting to demonstrate racial differences in *TB* susceptibility, statedthe basic question, “*We have to inquire whether persons living habitually in thesame environment and with practically the same risk of infection have the samechance of developing phthisis whatever be their stock*” (Puffer 1946).

Since the mid (80s), there have been many studies that have tried to identify genesthat might be associated with *TBsusceptibility*, as well as those testing the validityof 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 predispositionpositive (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 IgElevels, 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 bemild or variable, as many *hyper IgE patients* have been vaccinated with *BCG* andsurvived 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 *TBsusceptibility* 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-I*gene:

NRAMP-I (natural resistance-associated macrophage protein l), 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-1* 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 *NRAMPI*-transfected macrophage. It has been found that *NRAMPI*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-l* 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. Immunoflorescence studies in primary macrophages indicated that *NRAMP-I*is not located at the plasma membrane (Gruenheid*et 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 cationtransporter. 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 (Kontoghioghes*et al*, 1995) by acting as important cofactors for the production of toxic hydroxyl radicals or for bacterially encoded detoxifying metalloenzymes such as superoxide dismutase (Jabado*et 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-I*was 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-I*and 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-I*gene 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-I*genotypes ($p<0.001$). The authors concluded that genetic variations at *NRAMP-I*affect 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 *TB*in West Africans. Four families from Guinea-Conakry were analyzed by Cervino et al, in the year 2000, to test for the association between *NRAMPIpolymorphisms* 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-I*gene. 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'UTRloci*. 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 andShendi 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 hundredand 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: +.

- ✓ 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 (60^0) C for (10 min).
iv. (350 µl) of binding solution were added to the mixture andmixed thoroughly.
v. This mixture was centrifuged at (12,000) rpm for (60 sec), and flow-through was discarded.
vi. (400 µl) washing solutionwas added to reaction tubes andcentrifuged 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 werecentrifuged 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.

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 thestudy were in the form of lyophilized powderpurchased 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 hoodusingautomatic pipette withsterile filter tips, labeled with date of preparation.

iii. Then the primers solutionwas 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 Eppendorfof tubeto get a final concentration of (10 pmol/µl). After dilution, the primer was labeled andstored at (-20°C) until later usein *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*

Name	Primer sequence	Annealing temperature	Product length	Restriction Enzyme
D543N	5'-GCATCTCCCCAATTCATGGT-3' 5'-AACTGTCCCCACCTATCCTG-3'	56ºC	244 bp	*Ava*II
3'UTR	5'-GCATCTCCCCAATTCATGGT-3' 5'-AACTGTCCCCACCTATCCTG-3'	56ºC	240 or 244 bp	*Fok*I
INT4	5'-TCTCTGGCTGAAGGCTCTCC-3' 5'-TGTGCTATCAGTTTGAGCCTC-3'	59ºC	624 bp	*Apa*I

3.14. *DNA* amplification:

The (4ul) 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 programmedto run to each polymorphism (Tables 3.2, 3.3 and 3.4).

Table (3.2):*DNA* amplification program of D543N polymorphism

Step	Time	Temp.	Cycle
Initial denaturation	3 min.	95°C	1
Denaturation Annealing Extension	30 seconds 30 seconds 30 seconds	95°C 56°C 72°C	30
Final extension	10 min.	72°C	1

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

Step	Time	Temp.	Cycle
Initial denaturation	5 min.	95°C	1
Denaturation Annealing Extension	30 seconds 30 seconds 30 seconds	95°C 56°C 72°C	30
Final extension	10 min.	72°C	1

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

Step	Time	Temp.	Cycle
Initial denaturation	3 min.	95°C	1
Denaturation Annealing Extension	30 seconds 45 seconds 60 seconds	95°C 59°C 72°C	37
Final extension	10 min.	72°C	1

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-Ipolymorphisms* 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 *Ava*II, *Fok*I and *Apa*I 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

Gender	TB cases	Control
Male	87 (60.8%)	64 (50.8%)
Female	56 (39.2%)	66 (49.2%)
Total	143 (100%)	126 (100%)

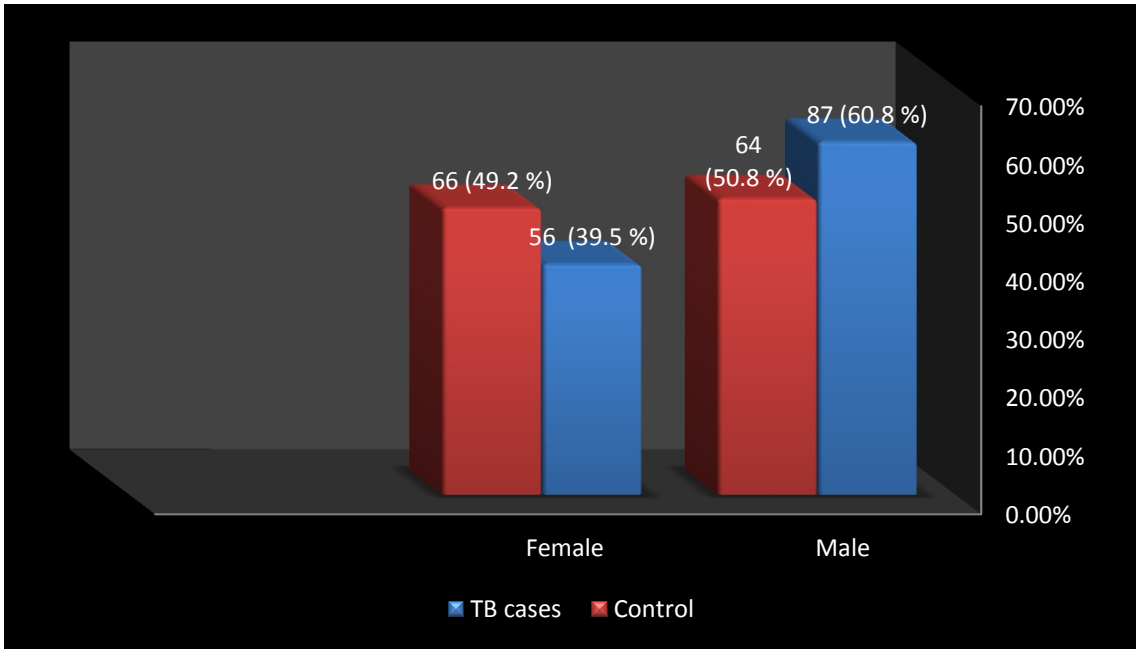
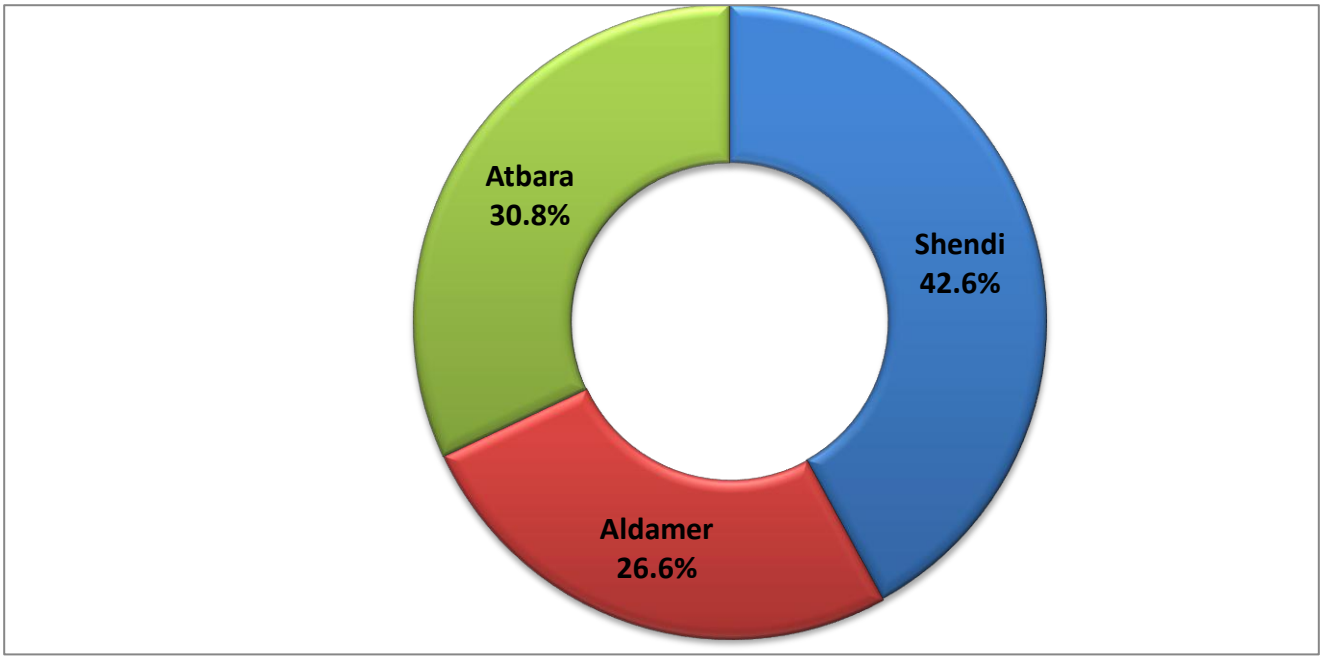


Fig. (4.1): Distribution of *TB* cases and control according to gender

Table (4.2): age group of *TB* cases and control distribution

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



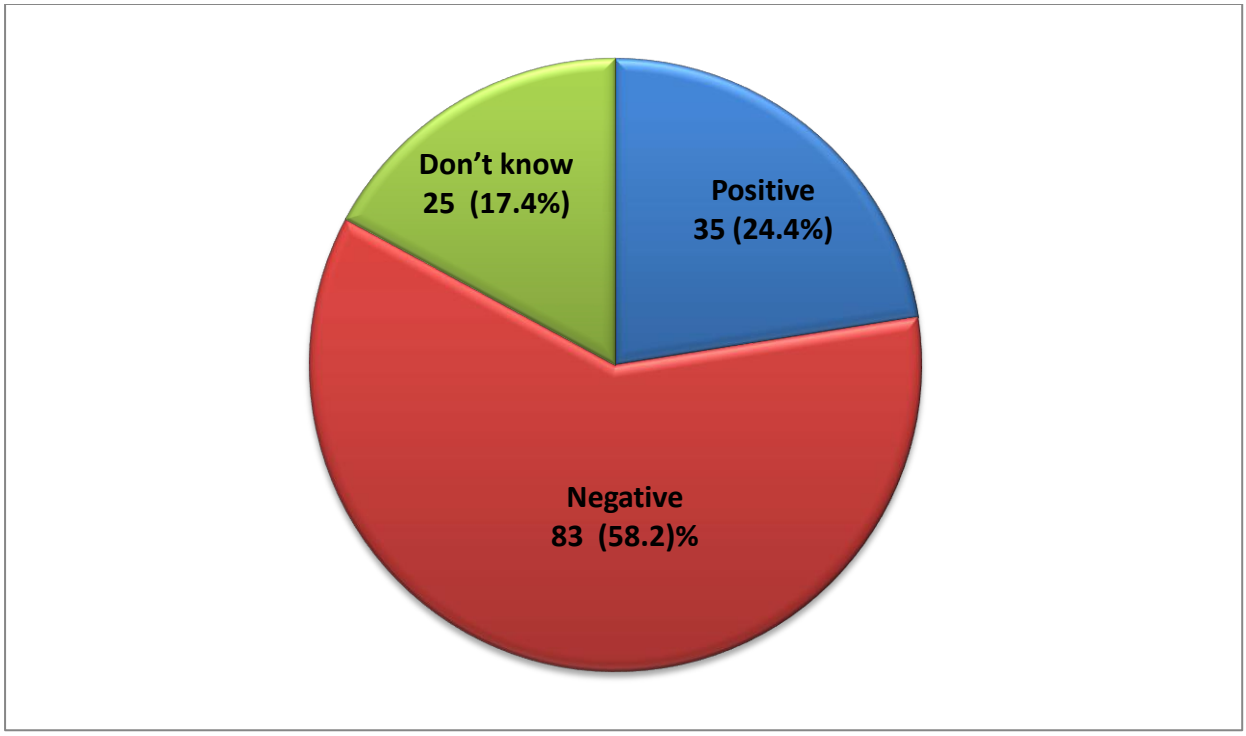
Fig(4.2): Residency percentage of *TB* cases

Table (4.3): Distribution of *TB* cases according to residence and age incidence

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

Table(4.4): Educational level of the *TB* cases

Educational level	Percentage of TB cases
Illiterate	48 (33.5 %)
Khalwa& Primary	58 (40.6 %)
Secondary	31 (21.7%)
Higher (university)	6 (4.2 %)
Total	143 (100 %)



Fig(4.3): Illustrate the family history of *TB* disease.

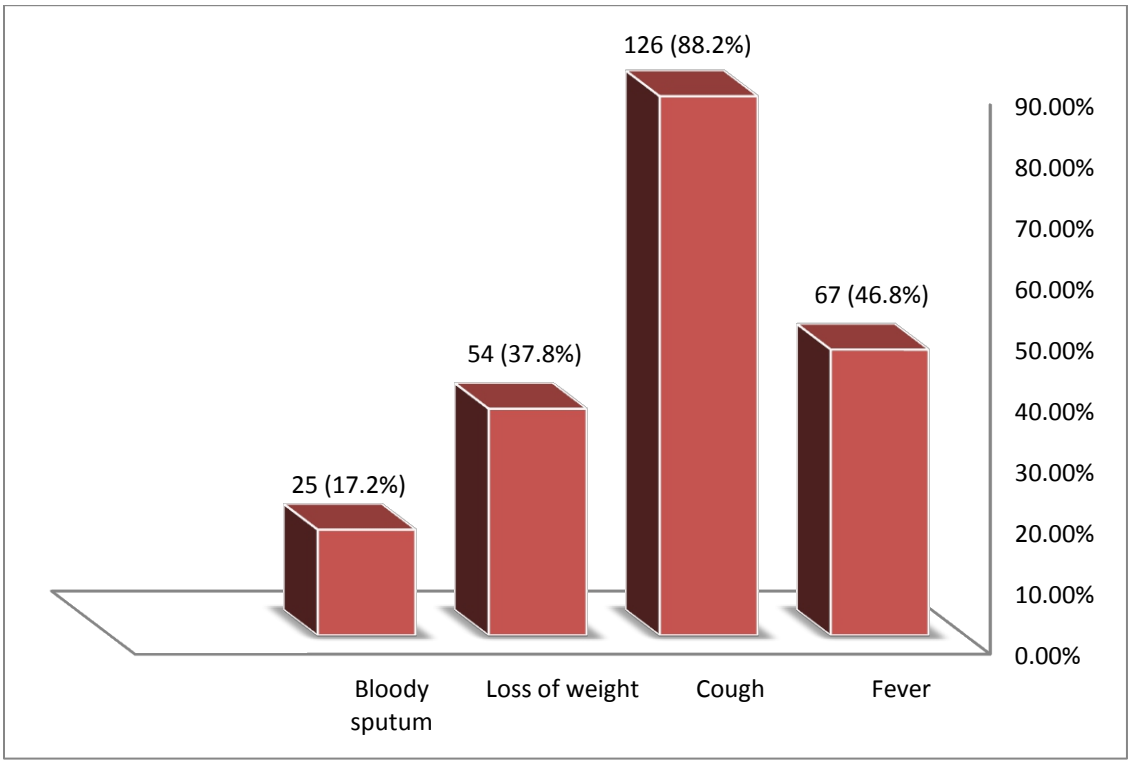


Fig (4.4): Display the symptoms which were appeared in *TB* cases

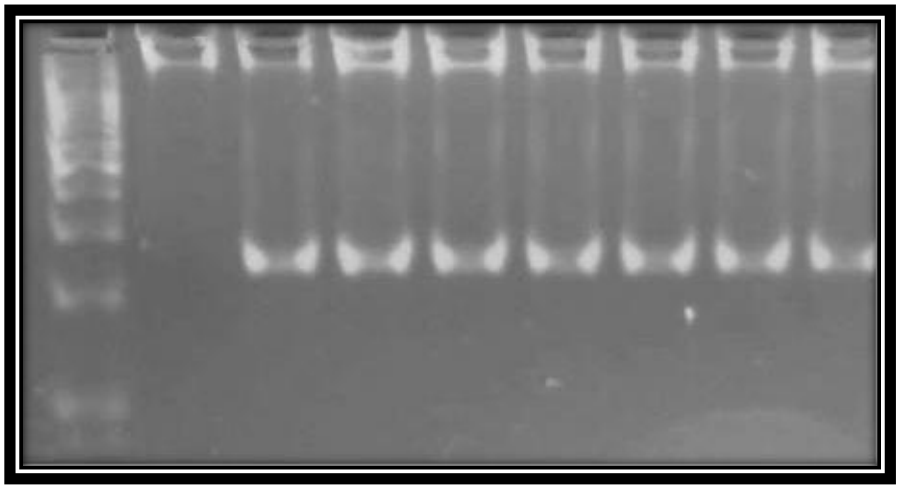
Table (4.5): Demonstrate the Duration of *TB* symptoms

Duration of *TB* symptoms	Percentage of *TB* cases
2 weeks or less	18 (12.6%)
3-5 weeks	29 (20.2%)
6-8 weeks	34 (23.8%)
More than 8 weeks	62 (43.4%)

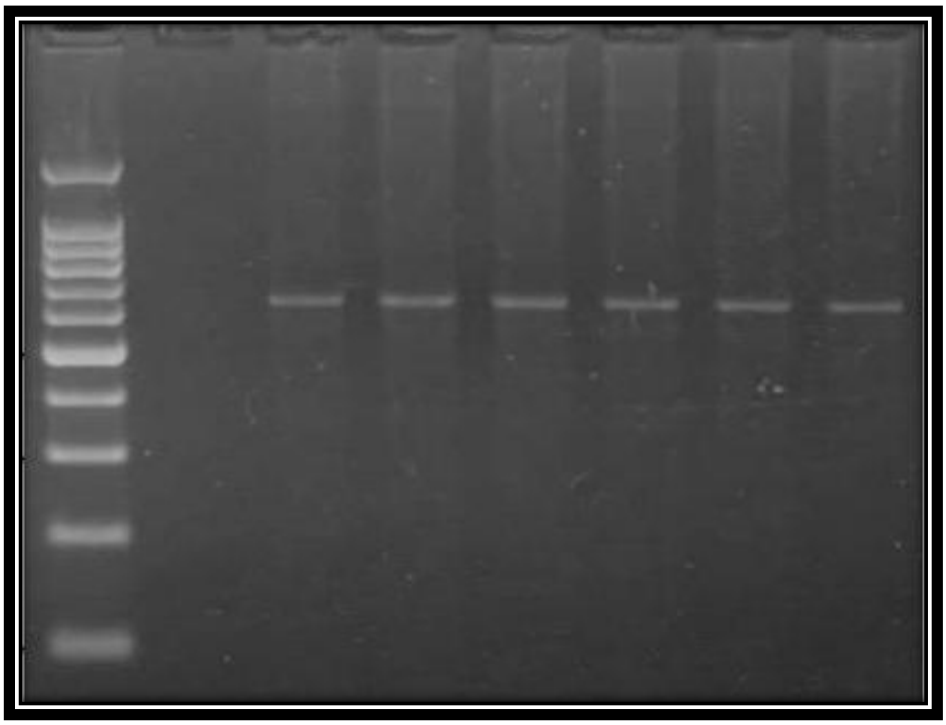
4.3. *PCR* Amplification of the *NRAMP-I* locus:

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).

A



B



C

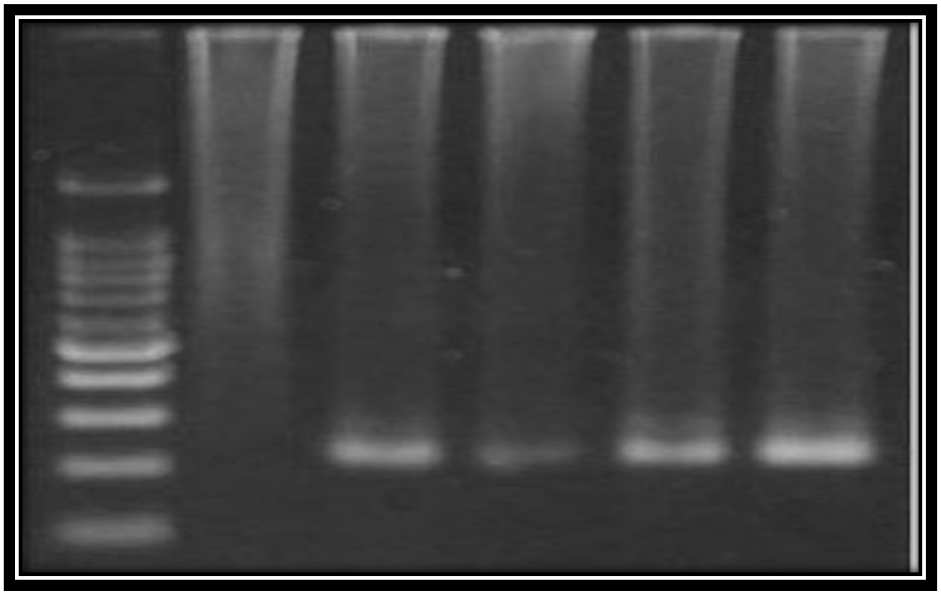


Fig (4.5):Agarose gel electrophoresis detection of the *PCR* amplification of the*NRAMP-I*loci. A: *PCR* amplification of D543N , B: *PCR* amplification of INT4 and C: *PCR* amplification of 3'UTR.

4.4. Allelic frequencies of the *NRAMPI*loci:

Allelic frequencies of the three *NRAMPIloci 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-I*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.***

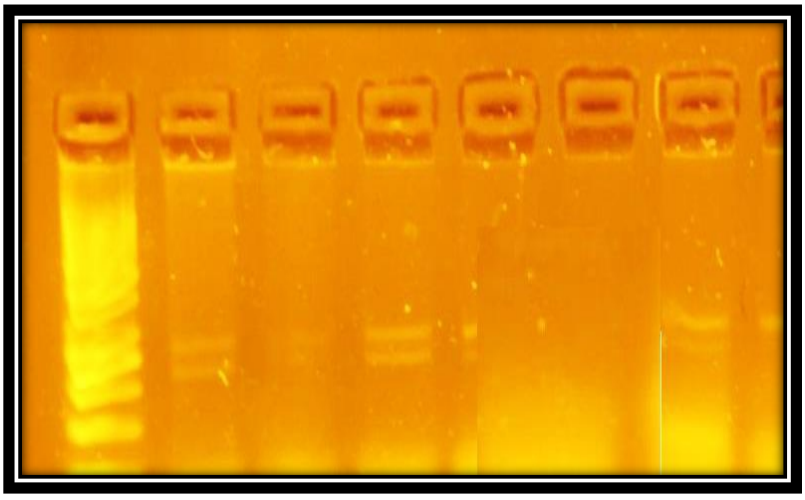
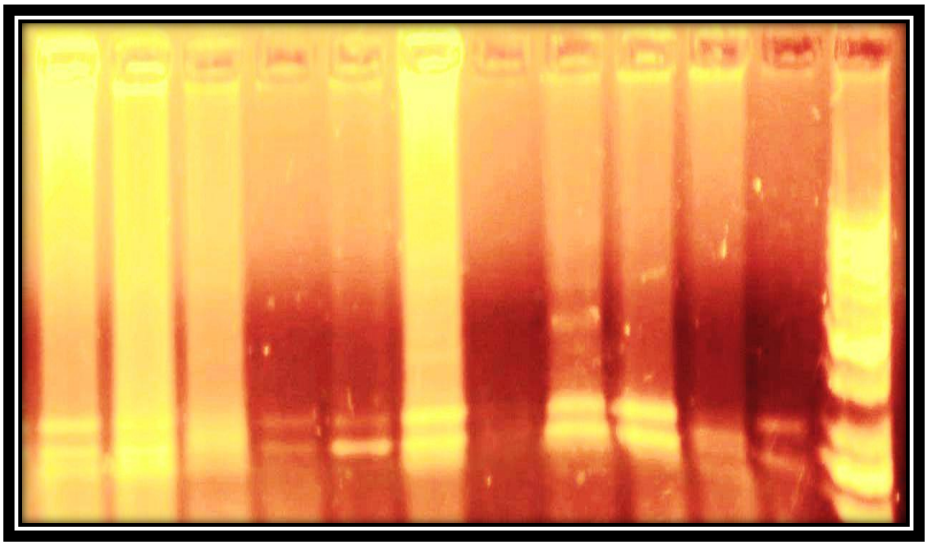


Fig (4.6):Agarose gel electrophoresis analysis of the *RFLP* patterns observed from *ApaI* digestion of the locus *Intron4*

4.4.2. D543N:

Out of the (143) patients, (40) were homozygous for the common allele with the ***genotype G / G***. ***(99) AFBpositive*** 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 ***homozgyous*** 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.***

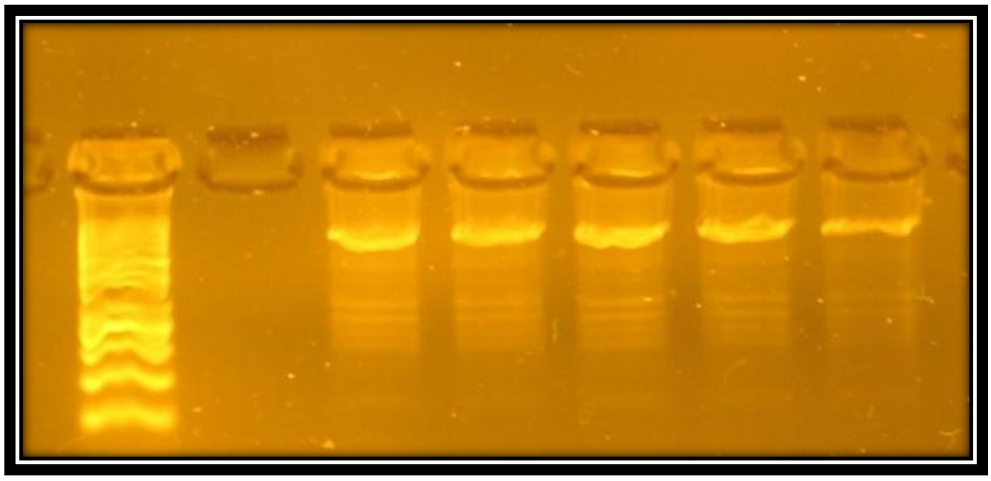


Fig (4.8): Agarosegel electrophoresis analysis of the *RFLP* patterns observed from *FokI* digestion of the locus *3'UTR*.

Table (4.6): Allelic frequencies of the three *loci INT4, D543N and 3'UTR* in sputum smear positive tuberculosis patients and health controls.

NRAMP 1 Locus	Polymorphisms	Tuberculosis Patient No %	Controls No %	P. value
D543N	G / G	40 (28 %)	1(0.8 %)	
	G / A	99 (69.2 %)	124(98.4%)	0.001
	A / A	4 (2.8 %)	1 (0.8 %)	
3'UTR	TGTG / TGTG	108 (75.5 %)	120 (95.2%)	
	TGTG / TGTGdel	35 (24.5 %)	6 (4.8%)	0.003
	TGTGdel /TGTGdel	0 (0 %)	0 (0 %)	
INT4	G / G	135 (94.4%)	117 (92.8%)	
	G / C	8 (5.6 %)	9 (7.2 %)	0.604
	C / C	0 (0 %)	0 (0 %)	
Total		143 (100 %)	126 (100 %)	

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, Elmadhoun W M *et al*2016 andGetahun 2011). *TB* affects the productive age group in River Nile State as seen also in some African countries (Uchimura2013). 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, AlDamer and Atbara respectively. Also age group less than (15 years) was found to be less exposure to tuberculosis infection (0%) in Shendi and Al Damerand (1.5 %) in Atbara.

This studyhad 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-I*gene 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-lpolymorphisms* 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-I*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-Iallele* 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-Igenotype* 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-Igenotype* 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 ofTB.*
- *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-I*variantsin *NRAMP-I*function.
- Identification of the *NRAMP-Igenotype*is 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:

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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:

Chronic Cough (more than two weeks) Yes ☐ NO ☐

Bloody sputum: Yes ☐ NO ☐

Fever: Yes ☐ NO ☐

Nocturnal Sweating: Yes ☐ NO ☐

Weight Loss: Yes ☐ NO ☐

Co-infection withHIV: Yes ☐ NO ☐

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): .....

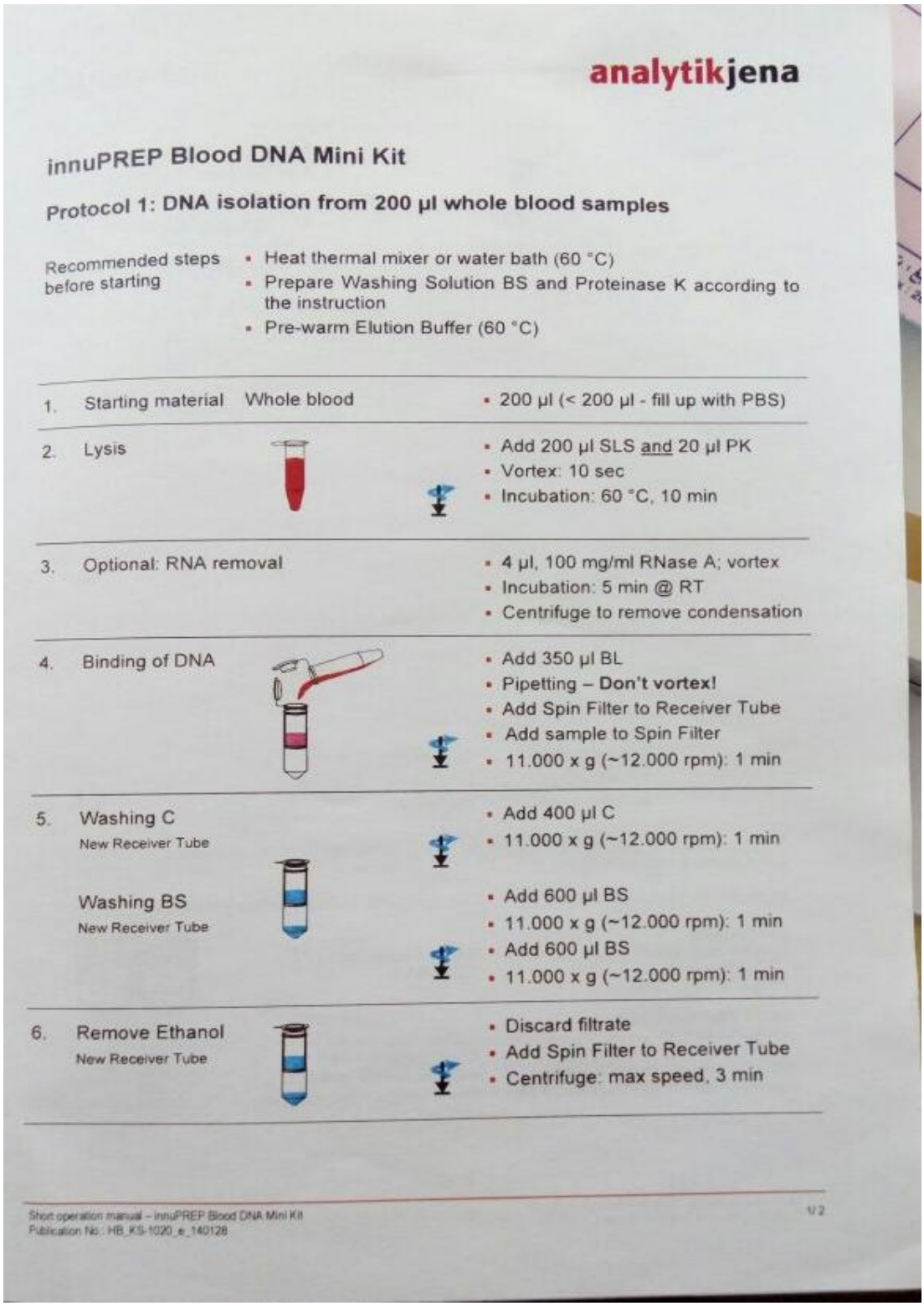
Drugs History:

a .Corticosteroids (Dose) ☐ b. Chemotherapy ☐

c. Alcohol ☐ d. I.V Drugs ☐

6.2.2. Appendix 2:

The bloodDNA extractionkits manufactured by Analytik Jena AG, Germany.



analytikjena

innuPREP Blood DNA Mini Kit

Protocol 1: DNA isolation from 200 µl whole blood samples

Recommended steps before starting
- Heat thermal mixer or water bath (60 °C)
- Prepare Washing Solution BS and Proteinase K according to the instruction
- Pre-warm Elution Buffer (60 °C)

1.	Starting material	Whole blood	• 200 µl (< 200 µl - fill up with PBS)
2.	Lysis		• Add 200 µl SLS and 20 µl PK • Vortex: 10 sec • Incubation: 60 °C, 10 min
3.	Optional: RNA removal		• 4 µl, 100 mg/ml RNase A; vortex • Incubation: 5 min @ RT • Centrifuge to remove condensation
4.	Binding of DNA		• Add 350 µl BL • Pipetting – **Don't vortex!** • Add Spin Filter to Receiver Tube • Add sample to Spin Filter • 11.000 x g (~12.000 rpm): 1 min
5.	Washing C New Receiver Tube		• Add 400 µl C • 11.000 x g (~12.000 rpm): 1 min
	Washing BS New Receiver Tube		• Add 600 µl BS • 11.000 x g (~12.000 rpm): 1 min • Add 600 µl BS • 11.000 x g (~12.000 rpm): 1 min
6.	Remove Ethanol New Receiver Tube		• Discard filtrate • Add Spin Filter to Receiver Tube • Centrifuge: max speed, 3 min

Short operation manual – innuPREP Blood DNA Mini Kit
Publication No.: HB_KS-1020_e_140128

1/2

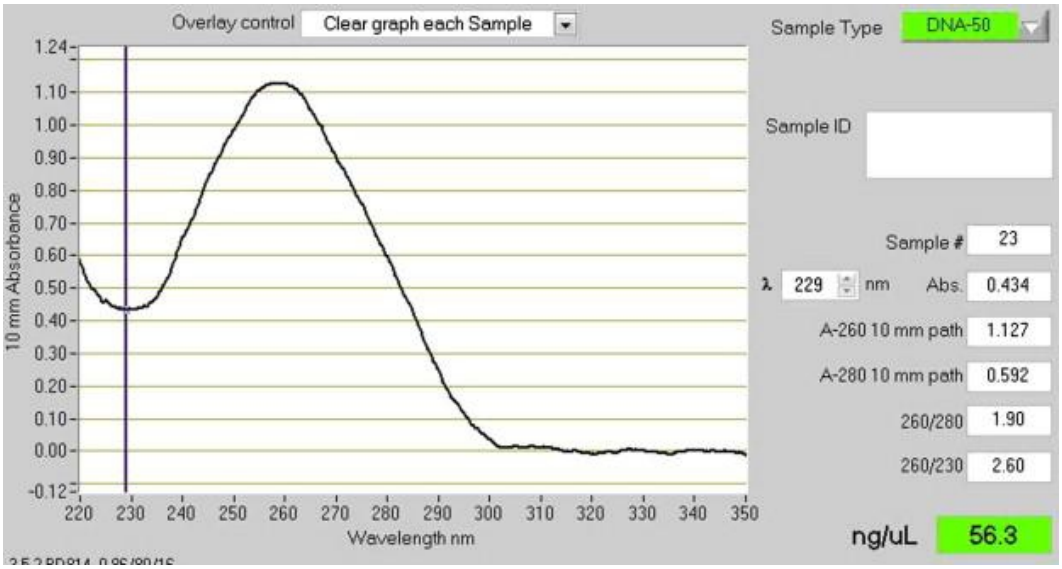
analytikjena

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

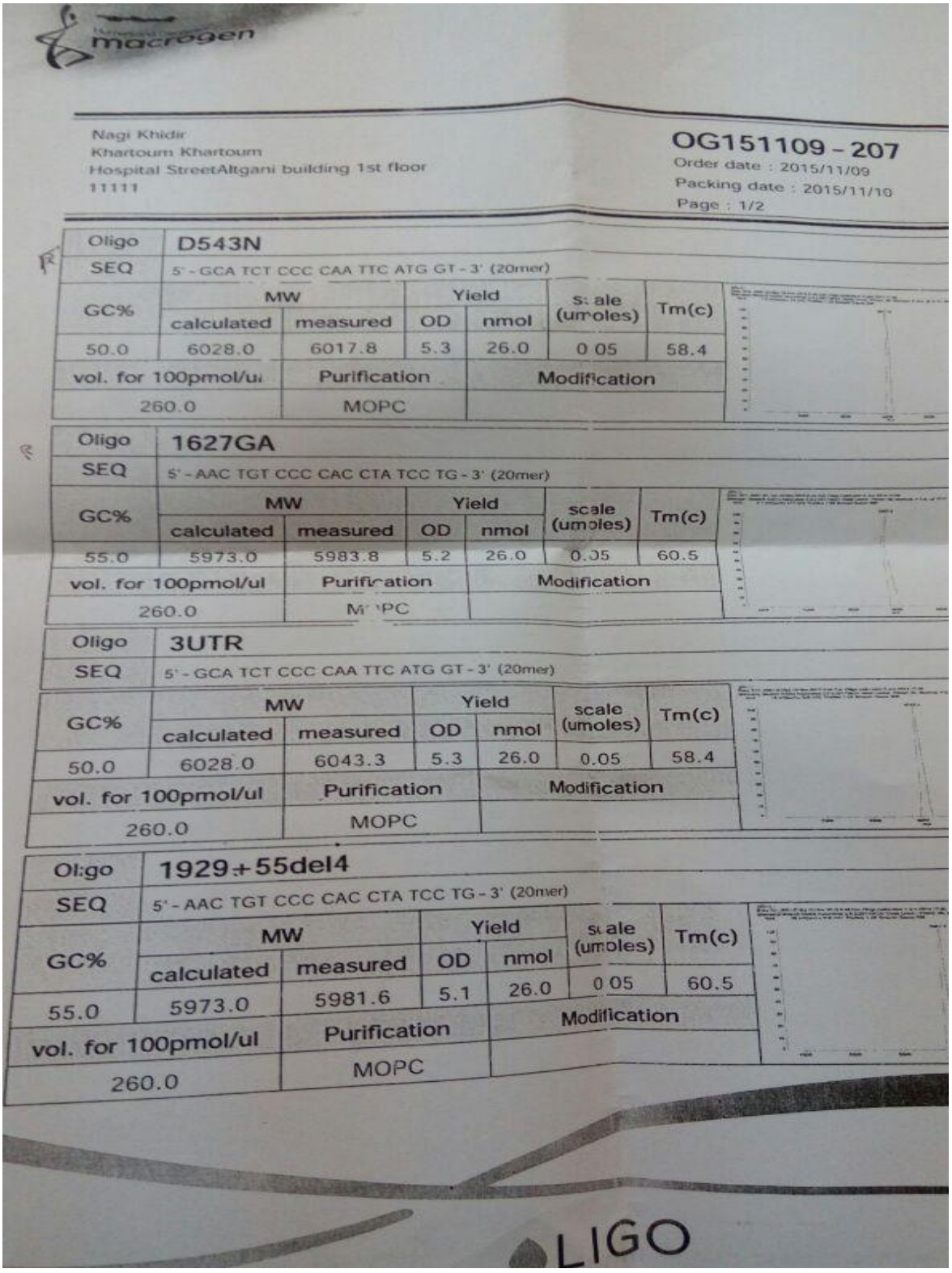
6.2.3. Appendix 3:

Measurement of DNA concentration by a NanoDrop spectrophotometer.



6.2.4. Appendix4:

The lyophilized powder primers purchased from Macrogen (Korea).



macrogen

Nagi Khidir
Khartoum Khartoum
Hospital StreetAltgani building 1st floor
11111

OG151109 - 207
Order date : 2015/11/09
Packing date : 2015/11/10
Page : 1/2

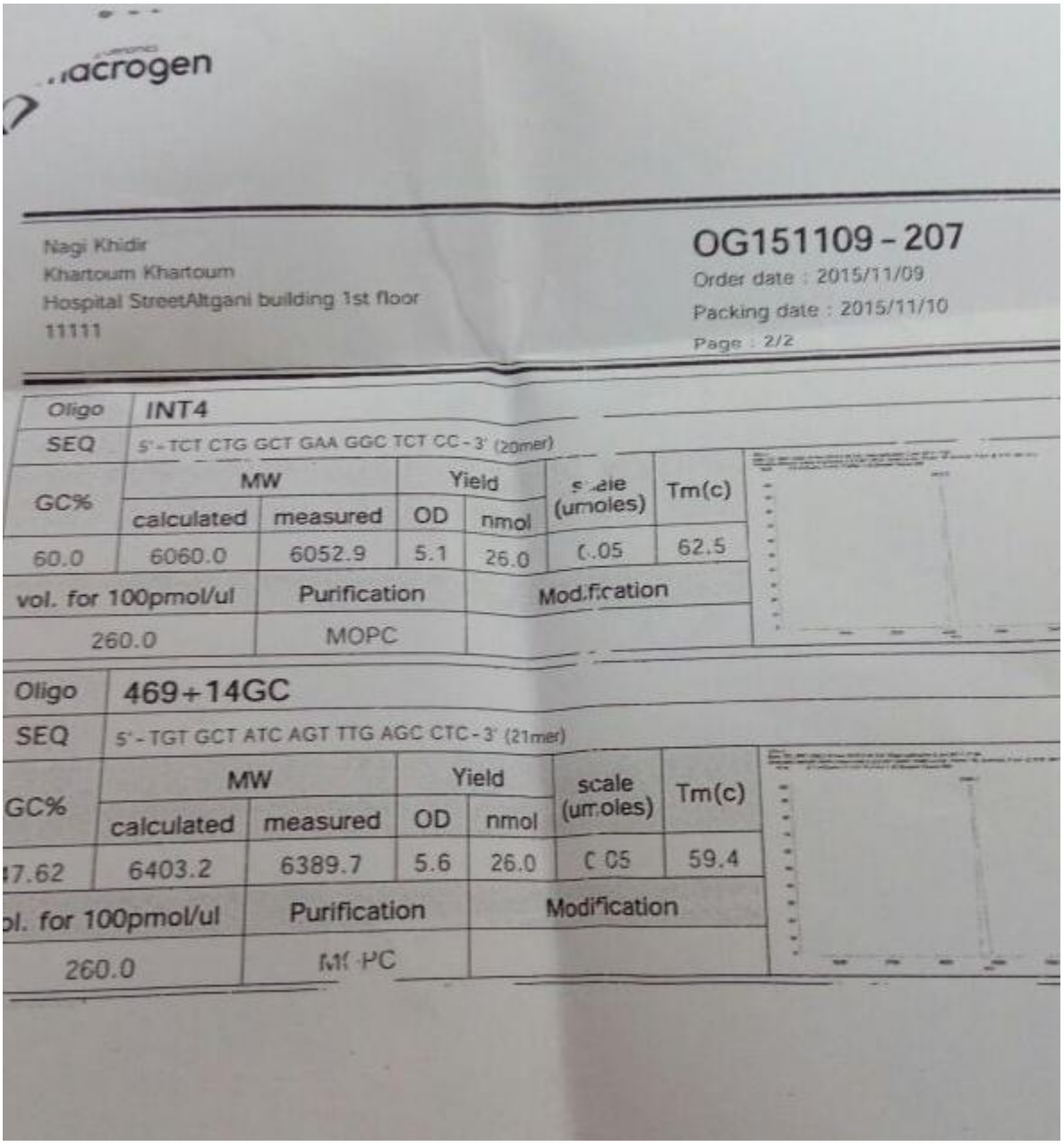
Oligo	D543N					
SEQ	5' - GCA TCT CCC CAA TTC ATG GT - 3' (20mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
50.0	6028.0	6017.8	5.3	26.0	0.05	58.4
vol. for 100pmol/ul	Purification		Modification			
260.0	MOPC					

Oligo	1627GA					
SEQ	5' - AAC TGT CCC CAC CTA TCC TG - 3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
55.0	5973.0	5983.8	5.2	26.0	0.05	60.5
vol. for 100pmol/ul	Purification		Modification			
260.0	MOPC					

Oligo	3UTR					
SEQ	5' - GCA TCT CCC CAA TTC ATG GT - 3' (20mer)					
GC%	MW		Yield		scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
50.0	6028.0	6043.3	5.3	26.0	0.05	58.4
vol. for 100pmol/ul	Purification		Modification			
260.0	MOPC					

Oligo	1929+55del4					
SEQ	5' - AAC TGT CCC CAC CTA TCC TG - 3' (20mer)					
GC%	MW		Yield		Scale (umoles)	Tm(c)
	calculated	measured	OD	nmol		
55.0	5973.0	5981.6	5.1	26.0	0.05	60.5
vol. for 100pmol/ul	Purification		Modification			
260.0	MOPC					

LIGO



Macrogen

Nagi Khidir
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Hospital StreetAltgani building 1st floor
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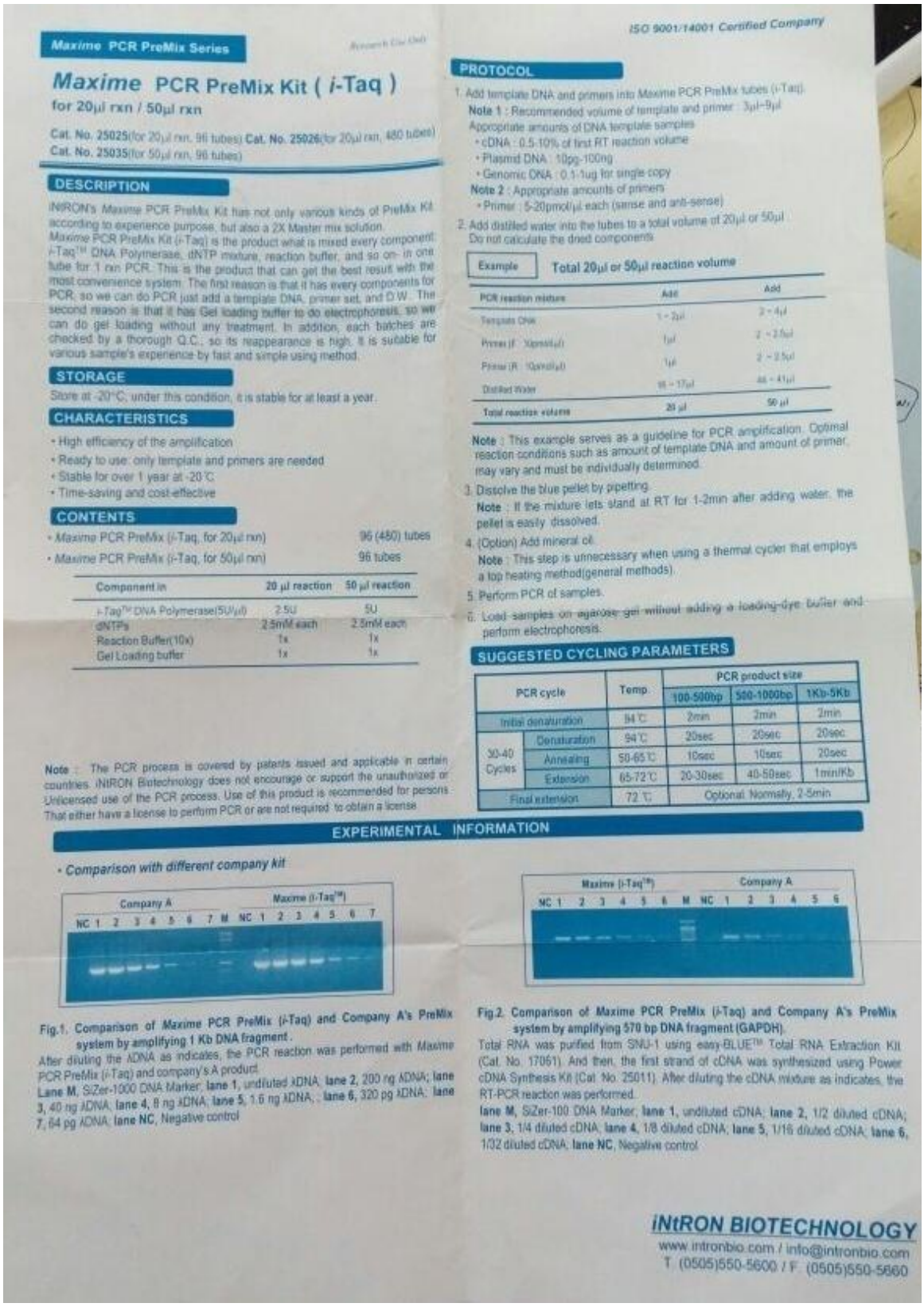
OG151109-207
Order date : 2015/11/09
Packing date : 2015/11/10
Page : 2/2

Oligo	INT4						
SEQ	5'-TCT CTG GCT GAA GGC TCT CC-3' (20mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
60.0	6060.0	6052.9	5.1	26.0	0.05	62.5	
vol. for 100pmol/ul	Purification		Modification				
260.0	MOPC						

Oligo	469+14GC						
SEQ	5'-TGT GCT ATC AGT TTG AGC CTC-3' (21mer)						
GC%	MW		Yield		scale (umoles)	Tm(c)	
	calculated	measured	OD	nmol			
47.62	6403.2	6389.7	5.6	26.0	0.05	59.4	
ol. for 100pmol/ul	Purification		Modification				
260.0	MOPC						

6.2.5. Appendix5:

Maxime PCR PreMix (iNtRON Technology, Korea)



Maxime PCR PreMix Series

ISO 9001:14001 Certified Company

Maxime PCR PreMix Kit (i-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) Cat. No. 25026(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's Maxime PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (i-Taq) is the product what is mixed every component i-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C, under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20°C
- Time-saving and cost-effective

CONTENTS

- Maxime PCR PreMix (i-Taq, for 20μl rxn) 96 (480) tubes
- Maxime PCR PreMix (i-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license

PROTOCOL

1. Add template DNA and primers into Maxime PCR PreMix tubes (i-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl
   Do not calculate the dried components

Example **Total 20μl or 50μl reaction volume**

PCR reaction mixture	Add	Add
Template DNA	1 ~ 2μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	20 μl	50 μl

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94°C	2min	2min	2min
30-40 Cycles	Denaturation	94°C	20sec	20sec	20sec
	Annealing	50-65°C	10sec	10sec	20sec
	Extension	65-72°C	20-30sec	40-50sec	1min/Kb
Final extension		72°C	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- *Comparison with different company kit*

Fig.1. Comparison of Maxime PCR PreMix (i-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.
After diluting the λDNA as indicates, the PCR reaction was performed with Maxime PCR PreMix (i-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1**, undiluted λDNA; **lane 2**, 200 ng λDNA; **lane 3**, 40 ng λDNA; **lane 4**, 8 ng λDNA; **lane 5**, 1.6 ng λDNA, ; **lane 6**, 320 pg λDNA; **lane 7**, 64 pg λDNA; **lane NC**, Negative control

Fig.2. Comparison of Maxime PCR PreMix (i-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1**, undiluted cDNA; **lane 2**, 1/2 diluted cDNA; **lane 3**, 1/4 diluted cDNA; **lane 4**, 1/8 diluted cDNA; **lane 5**, 1/16 diluted cDNA; **lane 6**, 1/32 diluted cDNA; **lane NC**, Negative control

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6.2.6. Appendix 6:

Map of River Nile State, Sudan.