سورة طه-الاية 114

Dedication

This work is dedicated to

My parents,

Brother,

Sisters,

Husband,

My lovely daughter,

Friends and

Teachers

To all who has ever taught me anything

Acknowledgments

Firstly, we would like to thank **ALLAH** for giving us health and patience to complete this work. We would like to express our sincere gratitude to our supervisors Prof. Elfadhil A Omer Dr. Mogahid M Elhassan and Dr. Nageeb S Saeed for their continuous support of my PhD research project, for their patience, motivation, enthusiasm, and immense knowledge. Their guidance helped us in all the time of research and writing of this thesis. I also thank Dr. Elhaj Mansoor for his unlimited support and help. Special thanks also extend to my best friend Hiba Khalid for her help and encouragement. We would like to thank all doctors, technicians, officers and everyone in our department for the good time we have with them. Our thanks and appreciations also go to my colleagues in developing the project and the people who have willingly helped us. We would like to express our special gratitude and thanks to all those who gave us such attention and time.

CONTENTS

List of contents

ABSTRACT .....

DEDICATION .....

CONTENTS .....

List of Tables .....

List of Figures .....

ABBREVIATIONS .....

ACKNOWLEDGEMENTS .....

CHAPTER ONE GENERAL INTRODUCTION AND OBJECTIVES 1

1.1 Introduction 2

1.2 Rationale 4

1.3. Objectives of the study 4

1.3.1 General objective *5*

1.3.2 Specific objectives *5*

CHAPTER TWO LITERATURE REVIEW OF HUMAN PULMONARY TUBERCULOSIS 6

2.1 Historical background 7

2.2 Taxonomy and characteristics features of mycobacteria 7

2.3 Evolution of *M. tuberculosis* complex 8

2.4 Epidemiology of the disease 9

2.5 Host genetic susceptibility to tuberculosis 11

2.6 Pathogenesis and host immune response 16

2.7 Diagnosis of tuberculosis 21

2.7.1 Diagnosis of active tuberculosis *21*

2.7.2 Diagnosis of latent infections *22*

2.8 Challenges for Controlling Tuberculosis infection 23

CHAPTER THREE MATERIALS AND METHODS 26

3.1 Study design 27

3.2 Ethical clearance 27

3.3 Study Area, Population and Duration 27

3.4 Dignostic scheme 27

3.4.1 Conventional method for diagnosis 27

3.4.1.1 Collection of Pathological Samples 27

3.4.1.2 Processing of samples……28
3.4.1.3 Decontamination of Sputum Samples……28
3.4.1.4 Identification of *Mycobacterium tuberculosis*(M.*tuberculosis*) Isolates.....28
3.4.2 Cultural Characteristics and Growth Rate Identification……28
3.4.2.1 Pigment Production……29
3.4.3 Biochemical Tests……29
3.4.3.1 Catalase Test……29
3.4.3.2 Detection of Heat-Labile Catalase Test at 68ºC/pH7……30
3.4.3.3 Nitrate Reduction Test……30
3.4.3.4 Para-Nitrobenzoic Acid (PNB) Susceptibility Test……30
3.4.3.5 Thiophen-2-Carboxylic Acid Hydrazide (TCH) Susceptibility Test ……31
3.4.4 Preparation of McFarland Turbidity Solution……31
3.4.5 Preparation of Bacillary Suspension……31
3.4.6 Preparation of drug-containing media……31
3.4.7 Drug susceptibility testing (DST)……32
3.4.8. Quality control of prepared media for drug susceptibility testing……32
3.4.2 Molecular investigation.....32
3.4.2.1 DNA Extraction……32
3.4.2.2 Primers of Insertion Sequence IS6110……32
3.4.2.3 Screening of IFN-γR1 Polymorphisms (PCR-RFLP)……33
3.4.2.4 Screening of IFNγR1 +95 T→C polymorphisms by (RFLP) *33*
3.4.2.5 Screening of IFNγR1 -56 T→ C polymorphisms by (RFLP).....*33*
3.4.2.6 Screening of IFNγR1 295 deletions 12 polymorphisms by (PCR-CTPP)……34
3.4.2.6 DNA Prepration *35*
3.4.2.6.1 Preparation of host immune cells lysate for extraction of genomic IFN-γR1DNA.....*35*
3.4.2.6.2 Protein precipitation in cells lysate.....*35*
3.4.2.6.3 Precipitation of extracted IFN-γR1DNA *35*
3.4.2.6.4 DNA Hydration……36
3.4.3 Polymerase Chain Reaction for Detection IFN-γR1.....*36*
3.4.3.1 Procedure for preparation and running of agarose gel electrophoresis *39*
3.4.4 Preparation of reagents used for detection of interferon gamma (IFN-γ) in human sera 40
3.4.4.1 Preparation of Serum Samples……40
3.4.4.2 Preparation of Wash Buffer ……40
3.4.4.3 Preparation of standard human IFN-γ (Pharm Pak) cytokine……40

3.4.4.4Evaluation of gamma interferon (IFN-γ) cytokine in sera of infected patients and healthy controls using ELISA……42
3.4.5 Statistical analysis of collected data 42
CHAPTER FOUR RESULTS.....43
4.1 Demographic data/risk factors for enrolled participants.....44
4.2 Conventional identification of *M. tuberculosis* 48
4.2.1 Microscopic examination of M. tuberculosisfrom sputum and culture smears *48*
4.2.2 Culturing of smear-positive sputum samples from.....*49*
4.2.3 Biochemical analysis.....*49*
4.2.3.1 Catalase Assay……...…49
4.2.3.2 Nitrate Reduction Assay……49
4.2.3.3 Para-Nitrobenzoic (PNB) Acid Susceptibility Test……...…49
4.2.3.4 Thiophen 2-Carboxylic Acid Hydrazide (TCH) Susceptibility Test……..….49
4.2.4 Drug susceptibility testing.....*50*
4.3 Genotyping of human genomic IFN-γR1 gene.....50
4.3.1 PCR Results……..50
4.3.2 *Genotyping of PCR-Amplified IFNγR1 DNA Products* *51*
4.4 Tribal origin and other risk factor in association with genetic variations in TB confirmed cases 57
4.5 Evaluation of gamma interferon (IFN-γ) cytokine in sera of infected TB patients and healthy controls using ELISA 62
CHAPTER FIVE DISCUSSION 65
DISCUSSION.....66
CHAPTERR SIX…….....70
6.1 Conclusion……71
6.2 Recommendations……..71
REFERENCES.....72
APPENDICES.....83

List of Table

Table 1	PCR Master Mix for Amplifying Genomic IFN-γR1	37
Table 2	Primers list for amplification of various polymorphic regions alongIFN-γR1	38
Table 3	Gene cycling conditions for PCR amplification of IFN-γR1	39
Table 4	Demographic data and questionnaire for TB infected patient	44
Table 5	Frequency distribution of infected cases according to age grouping	48
Table 6	Frequency distribution of BCG vaccinated cases according to gender	48
Table 7	Genotyping of IFN-γR1 gene extracted from immune cells of healthy human controls at genomic positions (+95, and -56) using PCR-RFLP or at position +295 deletion 12 employing PCR-CTPP method	58
Table 8	Genotyping of IFN-γR1 gene extracted from immune cells of infected TB patients at genomic positions (+95 and -56) using PCR-RFLP or at position +295 deletion 12 employing PCR-CTPP method	60
Table 9	Determination of gamma interferon (IFN-γ) levels in the sera of TB-infected patients as compared to control subjects	63

List of Figures

NO. of figures	figures name	page
Figure 1	Interplay between human innate immune response and T lymphocytes upon infection with mycobacteria and *Salmonella*	13
Figure 2	detection of genetic defects affecting IFN-γand its various components including receptors (IFN-gR1 and 2) and signal transducer and activator of transcription (STAT1)	15
Figure 3	Serial Dilutions of Standard Human Gamma Interferon (IFN-γ)Using 160µl Volumes	41
Figure 4	**1.5%** Agarose gel with positive sputum samples (2-7), while sample 8 appeared free from IS6110 with 123 bp.	50
Figure 5	Agarose gel electrophoresis showing absolute absence of digestion of 170bp amplicon at position +95 promoter of IFN-γR1 gene extracted from immune cells of infected patients and healthy controls and amplified by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method	52
Figure 6	Showing significant differences between +95TT and +95T→C, and the groups	52
Figure 7	Detection of allelic polymorphism at position -56 of *IFNγR1* extracted from immune cells of infected patients and healthy controls and amplified by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP) method	53
Figure 8	Non-Significant Differences of Frequency Distribution Between -56TT Genotype, -56TC and -56CC, and the Groups	54
Figure 9	Detection of allelic polymorphism at position+295 deletion 12 within *IFNγR1* extracted from immune cells of infected patients and healthy controls and amplified by Polymerase Chain Reaction–Confronting Two-Pair primers (PCR-CTPP) method	56
Figure 10	This graph Showing Non-Significant Differences Between +295 Deletion 12, and the Groups	56
Figure 11	Determination of Gamma Interferon (IFN-γ) Levels in Sera of Patients group with Pulmonary Tuberculosis and in sera of control group.	64

APPENDICES

Appendix 1	structure of interferon gamma receptor 1 protein involving location of a premature stop codon due to mutation (Panel A) and its receptor complex (Panel B)	84
Appendix 2	schematic flow pathway of the *M. tuberculosis* bacilli showing stepwise loss of DNA defining certain lineages	85
Appendix 3	Preparation of Ziehl-Neelsen Staining and Procedure a. 13% Fuchsin	86
Appendix 4	Preparation of Culturing Media	86
Appendix 5	Preparation of Working Solutions	87
Appendix 6	Significant Frequency of Distribution of Genetic Polymorphism +95T→C According to Infected and Control Groups	88
Appendix 7	Frequency Distribution of -56T→C According to Groups	88
Appendix 8	Frequency Distribution of +295 Deletion 12According to Groups	89

ABBREVIATION

1	AFB	Acid fast bacillus
2	BC	Before Christmas
3	BCG	Bacillus-Calmette Guerin
4	C	Cytosine
5	DNA	Deoxyribonucleic acid
6	DOTS	Directly Observed Short Course Therapy
7	EDTA	Ethyl Diamine tetra acetic acid
8	EMRO	Eastern Mediterranean Regional Office of the World Health Organization
9	ELISA	Enzyme-Linked immune absorbent assay
10	G	Guanine
11	gyrA	Gene encodes the subunit A protein of DNA gyrase
12	gyrB	Gene encodes the subunit B protein of DNA gyrase
13	HLA	Human leukocyte Antigens
14	HIV,	Human Immunodeficiency Virus
15	hsp65	Heat shock protein 65
16	IFN-ϒ	Interferon Gamma
17	IFN-ϒR1	Interferon Gamma Receptor1gene
18	IFN-ϒR2	Interferon Gamma Receptor 2gene
19	Ig	Immunoglobulin
20	IL-12	Interleukin 12
21	IL-23	Interleukin-23
22	I NOS	Inducible Nitrous Oxide Syntheses
23	Kat G	Gene encodes catalase-peroxidase enzyme
24	LJ	Lowenstein-Jensen medium
25	LM	Lipomannan
26	ManLAM	Mannose-capped Lipoarabinomannan
27	MHC	Major histocompatibility complex
28	Min	minute(s)
29	MDR-TB	Multi-drug resistant tuberculosis
30	mRNA	Massenger ribonucleic acid
31	MSMD	Mendelian susceptibility to mycobacterial disease
32	Mtb	*Mycobacterium tuberculosis*
33	MTBC	*Mycobaterium tuberculosis* Complex
34	NK	Natural Killer cells
35	NTM	Non-tuberculous mycobacteria
36	PBMC	Peripheral Blood Mononuclear Cells
37	PCR	Polymerase Chain Reaction
38	PID_s	Primary Immunodeficiency Diseases
39	PPD	Purified Protein Derivative
40	RBC_s	Red Blood Cells
41	RD	region of difference

42	RNA	Ribonucleic acid
43	RNIs	Reactive Nitrogen Intermediates
44	Sec	Second
45	SNP	single nucleotide polymorphisms
46	STAT1	signal transducer and activator of transcription
47	SOD	Superoxide dismutase
48	T	Thymine
49	TAE	Tri-acetate ethyl diamine tetracetic acid buffer
50	TB	Tuberculosis
51	TbD1	*M. tuberculosis* specific deletion 1
52	TMB	3, 3', 5, 5'-tetramethylbenzidinechromogenic buffer
53	TBM	Tuberculosis meningitis
54	Th1	T-helper 1
55	TNF-α	Tumour necrosis factor alpha
56	TST	Tuberculin Skin Test
57	UV	Ultra violet
58	XDR-TB	Extensively Drug Resistant tuberculosis
59	WHO	World Health Organization
60	ZN	Ziehl-Neelsen

ABSTRACT

Tuberculosis (TB) is a killing infectious bacterial disease with socioeconomic and grave public health implications. Approximately two thirds of global population infected with *M. tuberculosis* and only 10% of individuals develop clinical disease. Sudan is one of the few countries which suffer from high burden of disease accounting for 209 cases/100,000 population. A number of host genetic factors including gamma interferon influence disease susceptibility. The cytokine mediates immunity for control of progressive infection. Thus, mutations within gamma interferon receptor 1 (*IFN-γ*R1) result in increased susceptibility to pulmonary TB (PTB). This study was carried out in Khartoum state during the period from January 2015to December 2016 to improve detection of *Mycobacterium tuberculosis* in Sudanese with symptoms of tuberculosis infection using different conventional and advanced diagnostic techniques. One hundred specimens of blood and sputum were collected from different hospitals in Khartoum State including Abu Anja Hospital, Tropical Disease Teaching Hospital, Elasha'ab Teaching Hospital, Umdrman Hospital and Police Hospital. By using Polymerase Chain Reaction Restriction Fragment Length Polymorphism technique was adopted to detect mutation in genes. This study showed that the gene extracted from immune cells of infected TB patient at genomic position +95, _56 using PCR-RFLP or at position +295deletion 12 employing PCR-CTPP method. There is no mutation in position +95, in_56 there is 23 mutations was statistically insignificant(*p*-value=0.771) and at position +295deletion 12 there is 10 mutations found its non-significant (*p*-value=0.343).

Research findings revealed that the four-demographic data gender, BCG vaccination, socioeconomic status and smoking were significant (*P*-value=0.000)

associated with increased risk of novel development of pulmonary tuberculosis (PTB).

In conclusion, this study was designed to determine the role of three polymorphisms located within the promoter region of *IFN-γR1* gene in triggering development of TB among Sudanese patients. Collected data showed that the tested polymorphisms have potential link in increasing risk of developing TB among Sudanese patients in position _56 and +295deletion 12 but in +95 there is no risk.

الخلاصة

الدرن (السل) مرض بكتيري معدي له تأثير علي الصحه العامه في العالم. ثلثين من سكان العالم تقريبا مصابين بالسل وفقط 10% يتحول الى مرض .السودان واحد من البلدان القليلة التي تعاني من انتشار المرض بحساب 209 حاله لكل100.000 من السكان. هناك عدد من العوامل الجينية متضمنةجاما انترفيرون تؤثر على ارتفاع الاصابه بالمرض.السايتوكاينس (البروتين) يتوسط الحصانة للسيطرة على العدوى التقدمية وبالتالي الطفرات الجينيه داخل المستقبلات لجاما انترفيرون1تعمل على زيادة الاصابه بمرض السل الرئوي .

اجريت هذه الدراسة بولاية الخرطوم في الفترة من يناير 2015 الى ديسمبر 2016 للكشف عن دور المستقبلات لجين انترفيرون جاما في زياده نسبه الاصابه بمرض الدرن تم جمع العينات من اشخاص يعانون من اعراض مرض الدرن بأستخدام وسائل تشخيص تقليدية ومتطورة مختلفة. مائة من عينات الدم والبلغم تم جمعها من مستشفيات مختلفة بولاية الخرطوم وتتضمن مستشفى ابو عنجه ، مستشفى ام درمان، مستشفى الشعب التعليمي، مستشفي طب المناطق الحارة و مستشفى الشرطة.

تمت عملية ازالة التلوث من جميع العينات ومن ثم تم تزريعها بوسط لونستن جونسن بناء على طريقة بتروف المعدلة ، تم اعداد مسحه من كل عينة وتم صبغهما بصبغة زيل نلسون ، تم استخلاص الحمض النووي ومن ثم تم اجراء تقنية تفاعل البلمرة المتسلسل للكشف وجود الطفرة الجينية.

اظهرت الدراسة بان الجين المستخلص من الخلايا المناعيه للاشخاص المصابين بمرض الدرن في الموقع +95 حيث لم نجد اي طفره جينيه ،اما بالنسبه للموقع - 56 كانت هناك 23 طفره حيث كانت نسبتها وهي غير مؤثرة (p-value=0.771) .بالتحليل الاحصائ . وبالنسبة للموقع 295+حذف 12بنسبة . وهي غير مؤثرة أيضاً (p-value=0.343)

اظهرت النتائج هناك اربعة من البيانات السكانية وهي العمر والمستوى المعيشي والتدخين والتطعيم ضد السل ,كان لها تاثيركبير في انتشار المرض.

نستخلص من هذه الدراسه التي صممت لتحديد دور ثلاثه من الطفرات الجينية الموجودة على جين مستقبل انترفيرون جاما في تطورالاصابة بمرض السل الرئوي لدى المرضى السودانيين .المعلومات التي تم تجميعها اظهرت الطفرات المختبرة لها علاقه في زيادة انتشار وتطور المرض في كل من الطفرتين 56 - و 295+حذف 12وفي المقابل لم نحصل على اي طفره في الجين 95+.