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The Relationship between Infection with *Mycoplasma Pneumoniae* with Rheumatoid Arthritis Disease

A Thesis

Submitted to the Council of College of Medicine ,University of Babylon, in a Partial Fulfillment for the Requirements of the Degree of Doctorate of Philosophy in Medical Microbiology

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بسم الله الرحمن الرحيم

﴿وَعَلَّمَكَ مَا لَمْ تَكُنْ تَعْلَمُ وَكَانَ فَضْلُ اللَّهِ
عَلَيْكَ عَظِيمًا﴾

صدق الله العلي العظيم
(النساء:١١٣)

Supervision Certificate

We certify that this thesis was prepared under our supervision at the Department of Medical Microbiology, College of Medicine, University of Babylon, in a partial fulfillment for the requirements of the degree of Doctorate of philosophy in Medical Microbiology.

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Dedication

To my Master and Mawlaya

Expatriate of Tus and the Genial of Souls

Imam Ali Ibn Musa Al-Ridha

Peace be upon them

WAEL / 2023

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Summary

Rheumatoid arthritis (RA) is an autoimmune disease causing chronic inflammation of the joints. The main cause of rheumatoid arthritis is unclear, but there is a suggestion about the development of rheumatoid arthritis, represented by the several factors: genetic, environmental and microbial infection. So the study focuses on the including human leucocyte antigen (HLA-DRB1) gene variants, influence the susceptibility to RA. The HLA-DRB1 gene is part of a family of genes called human leukocyte antigens (HLA) complex and microbial infection. Therefor this study aims to illustrate the relationship between (RA) and *Mycoplasma pneumoniae*.

The study is performed by (50) clinically confirmed RA cases for different ages, ranging from 25 to 75 years old at the Rheumatology Clinic in Merjan Medical City during the period from February 2022 to October 2022. Moreover, There are (40) apparently healthy subjects were used as a control group.

Anti-*Mycoplasma pneumoniae* IgG-Ab, Vascular Endothelial cell Growth Factor A (VEGF-A), HLA-DRB1 and Interleukin-6 (IL-6) were detected by enzyme-linked immunosorbent assay (ELISA) technique. The digests of restriction polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) were used to study the rs9271366, rs3135388 and rs35445101 HLA-DRB1 gene polymorphisms in RA patients and controls.

The results show that 18:50 (36%) among RA patients has infection with *Mycoplasma pneumoniae.*

The results shown that there is a significant ($P < 0.05$) increase in serum concentration level of VEGF-A in RA patients infected with *Mycoplasma pneumoniae* as compared to healthy individuals, but the results of RA patients with *Mycoplasma pneumoniae* infection and RA patients

without *Mycoplasma pneumoniae* infection were non-significant (P > 0.05).

Also the results are shown that a significant increase in the serum concentration level of HLADRB1 in the RA patients infected with *Mycoplasma pneumoniae* as compared to the RA patients non infected with *Mycoplasma pneumoniae* and healthy individuals (P<0.05).

As for the serum concentration level of IL-6 in the RA patients infected with *Mycoplasma pneumoniae* as compared to RA patients non infected with *Mycoplasma pneumoniae* and healthy individuals (P<0.05) is a significant increase as shown by the results.

In HLA-DRB1 gene, single nucleotide polymorphism (SNP) rs9271366 showed that G allele as dominant pathogenic allele in which the individuals who carry GG and AG genotype have the susceptibility to the rheumatoid arthritis disease 2.57 fold comparing to individuals who carry AA genotype (OR 2.57 CI 95% 1.07-6.17). While SNP rs35445101 showed that A allele as recessive pathogenic allele in which the individuals who carry AA genotype have the susceptibility to the rheumatoid arthritis disease 9.75 fold comparing to individuals that carrying GG and AG genotype (OR 9.75 CI 95% 1.19-79.78).

The results are shown that rs9271366 with RA infection with *Mycoplasma pneumoniae* and RA non-infection with Mycoplasma that G allele represent as dominant pathogenic allele in which the individuals who carry GG and AG genotype have more susceptibility to Mycoplasma infection than subject that carry AA genotype (p = 0.036) and also showed that no association of genotype between rs35445101 and *Mycoplasma pneumoniae* infection (p>0.05).

Finally, it has been shown that the haplotype AA of rs9271366 and rs35445101 respectively identified a protective effect with (OR = 0.24, P = 0.017) comparing to other haplotype also shown that haplotype GA has a significant difference between patients and control (P <0.0001).

List of Abbreviations

Abbreviation	Meaning
ACCP	Anti-cyclic citrullinated peptide
ACP	anti citrullinated protein
ACR	American college of rheumatology
ADS	anti-double strand
AKA	Anti-keratin antibody
APC	Antigen presenting cell
APF	Anti-perinuclear factor
AS	ankylo
BALT	Bronchus-associated lymphoid tissue
bDMARDs	Biological disease modifying anti-rheumatic drugs
BMI	Body mass index
CCL20	C-C motif Chemokine Ligand 20
CCR6	C-C motif Chemokine receptor 6
CD	Cluster of differentiation
CRP	C-reactive protein
DAS28	Disease activity score 28
DMARDs	Disease modifying anti-rheumatic drugs
EA	Early antigen
EDTA	Ethylene diamine tetra acetic acid
EIA	Eenzyme immune assay
ELISA	Enzyme linked immune-sorbent assay
EORA	Early onset rheumatoid arthritis
ESR	Erythrocyte Sedimentation Rate
EtBt	Ethidium Bromide
EULAR	European league against rheumatism

FC	Fragment crystallization
FLS	Fibroblast-like synoviocyte
GCF	Gingival crevicular fluid
gp39	Glucoprotein-39
hFib	Human fibrinogen
HLA	Human leucocyte antigen
HLA-DRB1	Human HLA Class 2 Histocompatibility Antigen, DRB1 Beta Chain
HRP	Horseradish peroxidase
HTLV-1	Human T-lymphocyte virus-1
IC	Immunity complexity
IL	Interleukin
IFN	Interferon
ILD	Interstitial lung disease
IL-β	Interleukin-beta
IM	Infectious mononucleosis
MAb	Monoclonal antibody
MAD	Malondialdehyde
MDR	Multiple drug resistance
MHC	Major histocompatibility complex
μl	Microliter
ml	Milliliter
MMP	Matrix metalloprotease
MP	Mycoplasma pneumoniae
MRI	Magnetic resonance imaging
MTX	Methotrexate
NA	Nucleic antigen
NKc	Natural killer cell

NLR	NOD-like receptor
NSAID	Non-steroid anti-inflammatory drug
OA	Osteoarthritis
PAD	Peptidyl arginine deiminase
PBMCs	Periperial blood mononuclear cells
PCR	Polymerase chain reaction
PCR	Polymerase chain reaction
PD	Periodontitis
PSTAT-1	Phosphorylated signal transducer and activator of Transcription-1
PTPN22	Protein tyrosine phosphate-non receptor 22
RA	Rheumatoid arthritis
RF	Rheumatoid factor
rpm	Round per minute
SD	Standard deviation
SE	Shared epitope
SF	Synovial fluid
SLE	Systemic lupus erythematosus
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical packge for the social sciences
TBE	Trise base boric acid
TCR	T cell receptor
TGF-β1	Transforming growth factor beta 1
Th1	T helper cell-type 1
Th2	T helper cell-type 2
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNFi	Tumor necrosis facror inhibitor

TNF-α	Tumor necrosis factor-alpha
Treg	T regulation cell
UTI	Urinary tract infection
VCA	Viral capsid antigen
VEGF-A	Human Vascular Endothelial cell Growth Factor A
XDR	Extensivly drug resistance
YORA	Young onset rheumatoid arthritis

Table of Contents

List of Tables

List of Figures

CHAPTER ONE

INTRODUCTION
AND
LITERATURE REVIEW

1.1 Introduction

Rheumatoid arthritis (RA) constitutes one of the most common systemic autoimmune diseases characterized by synovial inflammation and joint destruction leading to significant disability and early mortality. People affected by RA account for 0.5–1.0% of adults worldwide (Jang *et al*., 2022).

RA pathogenicity is mostly complicated and have heterogeneous manifestations. Autoantibodies are considered as characteristic of seropositive RA, they are biomarkers of a more erosion disorder. Several changes may occur before clinical initiate of disease, this stage is probably last several years characterized by the appearance of autoantibodies without signs of joint inflammation (Edilova *et al*., 2021).

Genetics and environmental factors have an important role in the development of RA, also some microbes have appeared as an election factor to initiate of RA. Microbiome in different organs of the body like eye, oral, intestine have been embroiled in RA the relationship of RA cause and microbiome still unclear. However, microbial colonies that inhabit the human body has marked influence of the immune system thus it is embroiled in many autoimmune diseases (Jan *et al*., 2022).

HLA associations are widely studied across the populations worldwide and are found to be important in prediction of disease susceptibility resistance and of evolutionary maintenance of genetic diversity. The exponential increase of genomic data has deeply influenced human genetics and has also influenced the way genetic variation in HLA genes is analyzed and interpreted (Xu and Yin, 2019).The range of disease phenotypes that show associations with genetic variants within the MHC is vast spanning autoimmune disorders (e.g., type 1 diabetes, rheumatoid arthritis and psoriasis) response to infections (e.g., outcome of infection by dengue and HIV) neuropsychiatric disorders (e.g., narcolepsy, schizophrenia) and specific forms of cancer (e.g., Hodgkin's lymphoma). In this context the current study examines the association of three globally most frequent HLA SNPs to the occurrence and detection of RA (Carapito *et al*., 2016).

Rheumatoid arthritis (RA) is associated with HLA-DRB1 genes encoding the shared epitope (SE) a 5-amino acid motive. RA is usually preceded by the emergence of anti-citrullinated protein/peptide antibodies (ACPAs). Citrulline is a neutral amino acid resulting from post-translational modification of arginine involved in peptidic bounds (arginyl residue) by PeptidylArginine Deiminases (PADs). ACPAs recognize epitopes from citrullinated human fibrin(ogen) (hFib) and can be specifically detected by the AhFibA assay. Five citrullinated peptides derived from hFib together represent almost all of the epitopes recognized by patients with ACPA-positive RA. The use of antibody fine specificities as markers of clinical phenotypes has become a major challenge (Larid *et al*., 2021)

Mycoplasma pneumoniae is the smallest self-replicating biological system which is a common cause of upper and lower respiratory tract infection also it is the causative agent of wide range of pulmonary and extra-pulmonary manifestations (Shams *et al*., 2019). *M. pneumoniae* are fastidious bacteria. Most of them require cholesterol a unique property among prokaryotes. Because of fastidious nature of *M. pneumoniae* culture methods are relatively insensitive, time consuming, expensive and labour intensive and are successful in only 30-60 per cent of serologically diagnosed cases (Drago, 2019).

Periodontitis (PD) is presently regarded as a risk factor for RA. Porphyromonas gingivalis bacteria were the main cause of PD, this bacterium can be expressed of peptidyl arginine deiminase (PAD), this enzyme participated to the development of RA during the production of anti-cyclic citrullinated peptide antibodies (ACCPA) by stimulating citrullination that consider key to initiate of RA (Beyer *et al*., 2018).

RA effectiveness may be more prevalent in women than men may be a result of female hormones, but the function of these hormones in the progression of RA still indefinite. RA prevalence in female more than male in rate of up 4 until the 50 age, but less of 2 after 60 ages. Furthermore, the top of RA prevalence occurs during the age of 50 years (Alpízar-Rodríguez *et al*., 2017).

Many inflammatory mediators involve TNF-α and interleukins such as (IL- 1, IL-6, IL-17) are released via flow way at arthritis site of the joint which create an environment to the cartilages and bone erosion. Based on that possibility of treatment for RA patients involve monoclonal antibodies incorporation proteins against these molecules (Alunno *et al*., 2017).

Vascular endothelial growth factor (VEGF) a representative angiogenic cytokine can strongly induce endothelial cell activation and proliferation. VEGF and its receptors are highly expressed in RA synovial tissues. Their expression levels parallel the degree of synovial angiogenesis (Liu *et al*., 2022). VEGF concentrations are also increased in both the synovial fluid (SF) and serum of RA patients (Lakshmanan *et al*., 2022).

1.2 Aim of the Study

The present work aimed to study the association between rheumatoid arthritis (RA) with *Mycoplasma pneumoniae* infection.

1.2.1. Objectives

1. Detection of *Mycoplasma pneumoniae* in RA patients.
2. Identification for the role of some immune markers (Anti-Mycoplasma pneuminiae IgG,HLA-DRB1,VEGF-A and IL-6) with the susceptibility to RA.
3. Study the (MHCII) associations with *Mycoplasma pneumoniae*.
4. Study of polymorphism SNPs of rs9271366,rs3135388 and rs35445101 in HLA-DR Genes.

1.3 Literatures Review

1.3.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by joint swelling, joint tenderness and destruction of synovial joints leading to severe disability and premature mortality. Given the presence of autoantibodies such as rheumatoid factor (RF) and anti–citrullinated protein antibody (ACPA) which can precede the clinical manifestation of RA by many years, RA is considered an autoimmune disease. Autoimmunity and the overall systemic and articular inflammatory load drive the destructive progression of the disease. Although structural changes best distinguish RA from other arthritic disorders joint damage is rarely apparent in the very early stages of disease, but rather accumulates consistently over time (Komatsu and Takayanagi, 2022).

Patients with RA live three to 12 years less than the healthy individuals particularly due to cardiovascular disease especially in those with high disease activity. The biologic therapies may extend life in those with RA by reversing progression of atherosclerosis (Chodara *et al*., 2017; Friedewald *et al*., 2010)

The onset of disease is not the similar in all patients, but differs depending on type, number and the pattern of joint involvement (Kobak and Bes, 2014)The disease course may also be various depending on the absence or presence of several variables includes genetic predisposition, swollen joints frequentness , serum autoantibody and severity of the inflammatory process (Gossec *et al*., 2010).

RA is complicated miscellaneous disease occasionally categorized as a syndrome with shared features. The clinical manifestations of RA involve inflammatory mediators with non-equilibrium among pro- and anti-inflammatory cytokines effectiveness this may result immunity complications in many body systems. In RA patient increase serum cytokine level considered as a marker for disease severity and this pathophysiologic involvement of pro-inflammatory cytokines are believed to be implicated in bone and cartilage degradation (Pretorius *et al*., 2017).

Main symptoms of RA are pain, swelling, redness and stiffness, due to inflammation that emerges in the joint. However, the disease symptoms of entire body comprise fatigue, fever, loss of weight, and the appetite lose. Moreover, RA may be a result increase in cardiovascular risk, osteoporosis, and high risk of cancer (Macfarlane *et al*., 2019).

The significant sign of RA is symmetrical inflammatory arthritis. It may appear within small synovial joints and patient have swollen, painful, rigid joints which leads to loss of the joint function and become deformed as a result cartilage erosion (Hansen, 2016).

Early detection and treatment to control inflammation is of critical importance in RA give potency for quick destruction and functional weakness of the joint. The progression of RA and manner of joint involvement differs from patient to patients. However, in most patients (70%) some degree joint destruction in feet and hands can be detected by X ray during first 2 years from onset of disease (AI-Rubaye *et al*., 2017).

1.3.2: Epidemiology

RA is estimated to affect 1.3 million adults in the United States (U.S.), approximately 0.5-1% of the population over the age of 35 years. RA affects females more than males, in a 3:1 ratio, and the onset is mostly seen in the fourth and fifths decades of life (Otón and Carmona, 2019).

Incidence rates of RA are higher in northern Europe and North America compared with southern Europe. In North America and northern Europe RA affects 0.4% to 1% of the population in southern Europe it affects 0.3% to 0.7% of the population. Female to male ratio is 2-3:1 and the prevalence of RA increase with age (Bao et al., 2019; Fiehn et al., 2018; Holroyd et al., 2019; Tuncer et al., 2018).

The prevalence of RA increased yearly from 0.28% in 2009 to 0.32% in 2012 in Korea. The incidence of RA in 2010 was 28.5 per 100,000 person-years (Won *et al*., 2018). There was regional difference in the prevalence of rheumatoid arthritis. The incidence appears to be highest in Pima Indians (5.3%) and Chippewa Indians (6.8%), and lowest in China and Japan (0.2%-0.3%) (Gibofsky, 2012).

The incidence of RA was increasing from 1.60% in 2001 to 3.02% in 2011 and the cumulative risk was 22.74% in Babylon city (Alkazzaz, 2013).

1.3.3 Causes and Risk Factors

The main cause of RA disease is still unclear, but there are several factors that contribute to the development of RA of which is the interaction of genetic and environmental factors, tobacco smoking, microbial infections all these factors increase risk of the RA. In addition to early life exposures such as if mother smoked child has a greater risk of RA (Iqbal and Rattu, 2019).

The exact cause of RA is unknown with many different stimuli having demonstrated an ability to activate the immune and inflammatory process seen in RA. RA may be triggered by exogenous (infective) and/or endogenous stimuli (tissue proteins or immunoglobulin's) in patients with a genetic predisposition. While infectious agents have been suggested as caused agents for RA a single organism has not been identified and it may be numerous agents may be able to trigger an RA in predisposing individuals. Nonetheless, several agents including Gram negative anaerobic bacteria like those found in periodontitis have been implemented in the etiology of RA (Demoruelle *et al*., 2014; Yap *et al*., 2020).

1.3.3.1 Role of the Genetic Factors

Many factors have strongly suggested that genetics are a major effect on the development of RA. These factors involve the general increased prevalence of RA within families leading to estimations of familial risk contribution to seropositive RA of ~40–50% of seropositive RA with strongest risk seen in first degree relatives. In addition genetic factors in RA are suggested by increased prevalence of disease within certain racial groups such as North American Natives who exhibit prevalence rates of RA of 5–7%. The strongest of the genetic risk factors is a set of alleles within the Major Histocompatibility Complex (MHC) that encode amino acid sequences that predict structural similarities in the Human Leukocyte Antigen (HLA) peptide-binding groove and are termed in aggregate the shared epitope (SE) (Deane *et al*., 2017).

RA relation with HLA class II alleles is considered the initial clue for genetic risk factors the HLA genes A, b, C encode to MHC class I with function to present of antigen to CD8 cells while the HLA-DR, -DP, -DQ genes encode to MHC class II which present the antigen to CD4 cells. This means class I and class II molecules encode by HLA genes in one area within chromosome 6 this area with high polymorphism. At the DRB1 locus there are collective peptide sequences shared between variety HLA class II alleles. However, in a variety of populations observe variation that gives susceptibility to RA (Terao *et al*., 2016).

The largest genetic relationship with RA outside the HLA region lies within the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene. PTPN22 encodes lymphoid tyrosine phosphatase which is a negative regulator of signal transduction from the TCR. Mutations in the PTPN22 gene are associated with multiple autoimmune diseases and have been shown to give opposite effects. Many studies investigating the function of this variant have had conflicting results with some suggesting it results in a gain of function (Yarwood *et al*., 2016).

1.3.3.2 Role of The Environmental Factors

Environmental factors have a role in the development of RA. Many pollutions participate to the pathogenicity of diseases involving smoking, microbial infections and exchange in microbiota in predispose person these environmental factors activate have been suggested to stimulate inflammatory mediators at sites of the body such as lung and gingiva breakdown immunity tolerance to self-tissues finally targeted in RA (Cappelli *et al*., 2018).

Smoking and hormones infections involve the inducement of illness in genetically susceptible people. Smoking is suggested to be one of the most environment risk agent related to RA may be fulfill rate of complicated modulation influences on the innate and adaptive immunity (Angelotti *et al*., 2017).

Furthermore, interaction of smoking and genetic factors was examined and their co-relation with RA risk was observed. It was suggested that HLA-DRB1- shared epitope (SE) is strongly associated with increased risk due to smoking and is more evident in seropositive RA. Smokers having two copies of HLA- DRB1-shared epitope (SE) have higher risk of RA than those who never smoke and having no SE allele. A recent study reported that SE-alleles and smoking are associated with both anti-CCP positive and anti-CCP negative RA. Smokers with two copies of the SE allele have higher risk of both ACCP-positive and ACCP–negative RA 36.11- fold and 12.29-fold respectively as compared to nonsmokers not carrying SE alleles (Jalil *et al*., 2016).

1.3.3.3 The Microbial Infections

The gut microbiota produce balance for maintaining the immune response through interaction with epithelial cells in the intestine. Moreover, it is produced short chain fatty acid as metabolic for maintain to tolerance condition. More researches suggest the gut microbes have a relationship with diseases and the host immune system (Bodkhe *et al*., 2019).

Some of joint related molecules among them type II collagen, glycoprotein gp39 showed arthritogenic potentials. In addition in human various infectious factors are related to self-limited reactive arthritis. Molecular mimicry or cross- reactivity between a joint-related molecules or microbes and a self-antigen may lead to auto reactive T-cell clone (Neidhart and Neidhart, 2019).

Furthermore, the change of the gut or oral cavity microorganisms may influence mucosal immunity, inducing aberrant immune response that affect joint in patient with RA. Modern researches suggested that gut dysbiosis participate in the progression of arthritis in the intestine through stimulation of autoimmune T cells. Suggested mechanisms to relate autoimmune cell activation and infection involve mimicry among immune cells and human cell epitopes (Pianta *et al*., 2017).

One of the common causes of atypical pneumonia is *Mycoplasma pneumoniae,* which results in respiratory tract infections. Some studies have indicated that relation of Mycoplasma in the synovial joint with development of RA. However, the decrease of epidemiological searches of autoimmunity diseases like RA may prevent of the expanded dominance of spread infections involving Mycoplasma (Chu *et al*., 2019).

Some researches referred to the infections by microbes such as *Porphyromonas gingivalis*, *Proteus mirabilis*, Epstein-Barr virus, human immunodeficiency virus, Parvovirus, hepatitis virus, herpes virus, human T-lymph-tropic virus-1(HTLV-1), Mycoplasma, *Streptococcus pyogenes*, Salmonella, Mycobacterium and Enterobacterium may increase of development of RA pathogenesis (Li *et al*., 2013).

Although, the pathological role of intestinal microbial has not understood, further investigation will illustrate how the biased microbiome would contribute to the pathophysiology of RA. Interestingly a plenty presence of Prevotella bacterium has been indicated that relationship with existence of shared epitope genotype in RA implication that potential participate of a specific human leukocyte antigen (HLA) genotype in the formation of unique microbiome and also in the susceptibility of autoimmune diseases (Masuko, 2018).

Another site for the presence of microbes associated with RA is the oral cavity the patients with RA mostly associated with periodontitis the bacteria such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis* that inhabit oral cavity, also can be its presence in synovial joint fluid in RA patients. The peptidyl arginine deiminase that produced by *Porphyromonas gingivalis* which induce the citrullination process of protein in gingiva which inflamed this process results systemic production of anti-cyclic citrullinated peptide (ACCP) Antibodies and release Inflammations in inherently sensitively individuals (Vural *et al*., 2020).

All distinguished environmental agents for RA may be interacting with the mucous surface of organ tissues involves oral cavity, lung and gastrointestinal tract that notion indicated to these local cells press on the mucosa result post- translational modulation of peptides implicated in pathogenesis of RA. Some studies have inspected possibility relationship among respiratory viral infections and progression of RA (Joo *et al*., 2019).

The word pneumoniae is an inflammation of one or both lungs parenchyma that is more often but not always caused by infection. The many causes of pneumoniae include bacteria, viruses, fungi and parasites. Bacterial pneumoniae in the COVID-19 patient as it is the major cause of morbidity and mortality according to the new classification of pneumoniae namely categories: Community-Acquired Pneumoniae (CAP), Hospital-Acquired Pneumoniae (HAP), Healthcare-associated (HCAP) and ventilator Associated Pneumoniae (VAP) (Hinkle and Cheever, 2018).

Bacteria in the respiratory tract are categorized into divisions based on the a etiology of typical and atypical organisms. Typical organisms can be cultured on standard media or gram stain, but atypical organisms do not have such properties. Typical pneumoniae is caused by *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenza* and aerobic and anaerobic gram-negative bacteria (Sattar *et al*., 2021).

While atypical bacterial pneumoniae is caused by atypical organisms that are not detectable on gram stain and cannot be cultured using standard methods the most common organisms are *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae* and *Legionella pneumophila*. Atypical bacterial pneumonia generally is characterized by a symptoms complex that includes headache, low-grade fever, cough and malaise. Constitutional symptoms often predominate over respiratory findings. Although, in most cases the presentation can be in the milder spectrum of community-acquired pneumoniae some cases especially if caused by *Legionella pneumophila* may present as severe pneumoniae necessitating intensive care unit admission (Payus *et al*., 2022). Co-infection with SARS-CoV-2 and atypical bacteria has been identified in a few studies .Due to overlapping clinical presentations and image features, it is difficult to distinguish between SARS-CoV-2 and bacteria causing atypical pneumoniae. Besides this it is unclear whether coinfection with atypical bacteria can cause worse clinical outcomes in COVID19 patients (Davis *et al*., 2020).

1.3.3.4 *Mycoplasma pneumonia*e

Mycoplasma pneumoniae is a common pathogen that causes upper and lower respiratory tract infections in people of all ages responsible for up to 40 % of community-acquired pneumonias. *Mycoplasma pneumoniae* produces diseases of varying severity ranging from mild respiratory tract infection to severe atypical pneumoniae (Kumar, 2018). In addition to respiratory tract infection *Mycoplasma pneumoniae* is responsible for extra pulmonary infections such as pharyngitis, encephalitis, Steven –Johnson syndrome, septic, arthritis pericarditis and other manifestations of autoimmune pneumoniae that progress to severe life-threatening pneumonia (Waites *et al*., 2017).

Mycoplasma pneumoniae is a member of the family Mycoplasmataceae and orders Mycoplasmatales which belong to the class mollicutes (Mollis = soft and cutis = skin, in Latin) that comprise four orders, five families, eight genera and about two hundreds know species that have been detected in human (ADIKE, 2018). The Mycoplasmataceae family includes two genera responsible for causing human disease namely the genus Ureaplasma and the genus Mycoplasma which includes several species that have been diagnosed as pathogens to humans (Al-Essa *et al*., 2021).

Mycoplasma species are the smallest free-living organisms. These organisms are unique among prokaryotes in that they lack a cell wall a feature largely responsible for their biological properties such as their lack of a reaction to Gram stain and their lack of susceptibility to many commonly prescribed antimicrobial agents including beta-lactams, Macrolides such as azithromycin and clarithromycin are generally considered to be the agents of the first choice for the treatment of MP infections (Formosa *et al*., 2006).

Mycoplasma pneumoniae possesses a genome smaller than another prokaryotic cell and a single circular chromosome containing 0.58 to 2.2 Mbp a low G+C content (23 to 40 mol%) the smallest genome responsible limits biosynthetic capabilities and responsible for many biological characteristics (Matteau *et al*., 2020).

Mycoplasma pneumoniae bacteria spread from person to person contact by respiratory droplets and is exclusively a human pathogen. It is primarily an extracellular pathogen that has evolved a specialized attachment organelle for close association with host cells. This attachment is critical to the bacteria's survival and ability to infect. The close association between *Mycoplasma pneumoniae* and the host cells prevents the hosts mucociliary clearance mechanisms from removing the bacterium. The bacterium attaches to and damages the respiratory epithelial cells at the base of cilia. This activates the innate immune response and produces a local cytotoxic effect (Jeris, 2019).

1.3.3.4.1 Virulence factors

1.3.3.4.2 Adhesions

Mycoplasma pneumoniae adherence and gliding motility are essential for colonization of the airway mucosa and are mediated by a multifunctional membrane-bound cell extension the terminal or attachment organelle. The terminal organelle is complex with 11 distinct substructures evident by electron cryotomography (Williams *et al*., 2018).

Biochemical and mutant analyses indicate that these adhesion complexes include proteins P1, P40 and P90 which co-localize generally to the terminal organelle and Protein P30 which localizes almost exclusively at the distal end of the terminal organelle is essential for functional P1 adhesion complexes P1 operates directly in receptor binding and gliding. Still, these and other accessory proteins require adherence and gliding motility (Jiang *et al*., 2021).

Thus the spontaneous loss of P40 and P90 or P30 renders P1 nonfunctional and *Mycoplasma pneumoniae* non-adherent, non-motile and virulent. *Mycoplasma pneumoniae* engages sialylated glycoproteins as receptors for adherence to respiratory epithelium and pretreatment of host cells with neuraminidase to remove terminal sialic acid residues inhibits Mycoplasma attachment (Williams *et al*., 2018).

1.3.3.4.3 Community-Acquired Respiratory Distress Syndrome (CARDS) Toxin

Mycoplasma pneumoniae expresses a unique ADP-ribosylating and vacuolating cytotoxic designated community-acquired respiratory distress syndrome (CARDS) toxin. CARDS toxin persists and localizes in the airway in CAP patients. Which homology to the S1 subunit of pertussis toxin, CARDS toxin binds to specific cellular receptors is internalized and induces hyper inflammation, histopathology, mucus hyperplasia and other airway injuries (Ramasamy *et al*., 2018).

1.3.3.4.4 Molecular Mimicry

The term molecular mimicry can be described simply as pathogens sharing a structural relationship with the host are tolerated as self just like constituents of the host. The immune response targets the pathogen-peptide mimicking the hosts self-antigen activating naive auto reactive T-cells specific to the corresponding self-antigen. *Mycoplasma pneumoniae* antigen mimics host cell components. Thus the host immune response induced by the pathogen causes auto-immune responses and injuries to multiple organs. The C-terminal region of the P1 and P30 proteins in *Mycoplasma pneumoniae* show high levels of homology to troponin, cytoskeletal proteins, keratin and fibrinogen of the host. Antibodies produced in response to *Mycoplasma pneumoniae* infections will target various host tissues and form immune complexes which aggravates the autoimmune response leading to inflammatory injuries in the extra-pulmonary tissues (Jiang *et al*., 2021).

1.3.3.4.5 Antigen Variation

The most common strategy pathogenic microorganisms employ to evade immune surveillance and control by the host is antigenic variation. Antigenic variation is successfully employed by various bacterial pathogens including Mycoplasma spp. Antigenic variation leads to continuous alterations or modifications of the surface molecules that are mainly targeted by the host immune systems. Consequently, the humoral (antibody) response generated against the previous old surface molecules cannot effectively recognize and neutralize the modified new molecules allowing the pathogen to persist in the infected host for a prolonged period (Qin *et al*., 2016).

Antigenic variation is also hypothesized to be involved in the repeated epidemics caused by *M. pneumoniae*. This antigenic variation in this species and *M. genitalium* is predicted to be generated through homologous recombination between specific repetitive DNA elements dispersed throughout their genomes. These repetitive elements encode the antigenic surface molecules P1 of *M. pneumonia* (Hsieh *et al*., 2022).

While antigenic variation through homologous recombination may be crucial for the propagation of Mycoplasma species in human populations a major issue for these organisms is the maintenance of the integrity of their genomes particularly because these genomes are much more compact than the genomes of most other bacterial taxa. Interestingly, the DNA repair systems that maintain genome integrity in *M. pneumoniae* and *M. genitalium* may also be involved in the recombination between repetitive DNA elements Consequently, the DNA repair machinery of these organisms may be directly involved in antigenic variation (Hakim *et al*., 2021).

1.3.4 Pathogenesis of *Mycoplasma pneumoniae*

The infection of respiratory tract by *M. pneumoniae* initiates from the close association between the bacteria and the mucosal epithelium that occurs as a result of cytadherence which is considered to be the major virulence factor of *M. pneumoniae* to adhere to the hosts respiratory mucosa and produce local effects that appear as clinical signs of disease (Waites and Taylor-Robinson, 2011).

The cells parasitized by *M. pneumoniae* can exhibit a number of cytopathic effects and impaired metabolism as a result of the local damage. Epithelial cells of the respiratory tract typically lose their cilia, appear vacuolated and show a reduction in oxygen consumption, glucose utilization, amino acid uptake and macromolecular synthesis, finally resulting in exfoliation (Waites and Talkington, 2004).

These subcellular events in the air passage of the lung are translated clinically as the persistent hacking cough typical of *M. pneumoniae* bronchitis and pneumonia. Damage to cells of the upper respiratory tract and the ensuing local inflammatory response is reflected by pharyngitis, hoarseness, coryza, earache, conjunctivitis and cervical adenopathy. Mycoplasmal species that infect humans are primarily mucosal pathogens that cause clinical disease by their deleterious effects on the host-cell epithelium, but this concept has been changing as evidence accumulates that some species can invade eukaryotic cells (Waites *et al*., 2009).

Information obtained from the complete sequencing of the *Mycoplasma pneumoniae* genome showed the elimination of a number of genes essential for independent existence consistent with the life cycle of an intracellular organism (Meseguer *et al*., 2003).

Despite intracellular growth and replication has been described for *Mycoplasma pneumoniae* in vitro. This process has not been proven to occur during natural infections. However, some of the clinical characteristics of *Mycoplasma pneumoniae* infections are consistent with what may be expected from an intracellular pathogen including the establishment of latent or chronic infections limited efficacy of some antimicrobials necessity for prolonged treatment to eradicate infection in some instances and circumvention of the host immune response (Waites and Talkington, 2004).

1.3.5 Immune Response associated with Rheumatoid Arthritis

1.3.5.1. Role of the Innate Immunity

The important role of innate immunity in distinguishing of microbes and its role in inflammatory mediators that occur in autoimmune diseases has been distinctive leads to the best conception of the pathophysiology of diseases. Thus, there is a modification in the natural function of immune systems, Natural killer cells, Dendritic cells and Neutrophil cells are participating in the development of autoimmunity. In addition to Macrophage cells regarded as the more implicated cells in RA because it produce of the TNF-α and this cytokine is essential in pathogenicity of RA (Pabón-Porras *et al*., 2019).

Therefore, the mechanism of innate immune has significant role in RA pathogenesis recent studies show the relation between the expression of interferon (IFN) response genes and severity of arthritis mainly this due to (IFN) activity that associate with high amount of cytokines and chemokine in pre-clinical stage another player in the mechanism of RA pathogenesis by fibroblast-like synoviocyte (FLS) involve in a assortment of other pathological mechanisms such as an immunological response key site to hypoxia that distinguish the micro-milieu of inflammation RA joints (Picerno *et al*., 2015).

However, inadequately organized complement stimulation underlies the pathogenesis of human inflammatory and autoimmune diseases involving RA where the cartilages, bones and synovium are targeted. Recent studies of this disease have confirmed that the autoimmune response develops over time in an asymptomatic preclinical stage that is related with mucosal inflammation. In particularly, experimental models of this disease has proved that each of the three chief complement activation pathways plays a significant role in recognition of damaged joint tissue, although the lectin and magnification pathways show particularly impactful role in the initiation and increase of damage (Holers and Banda, 2018).

1.3.5.2. Role of the Adaptive Immunity

The response of the adaptive immune system in RA patients is slow compared with innate immune response some activated cells that overstep the innate immunity barriers, but the adaptive immune response is specific response and generate speed memory response to later infection for same antigen adaptive immune system comprise cell-mediate response and humoral response which submitted by T cells and B cells (Jansen, 2017).

Future studies using single-cell-based sequencing methodologies and the availability of detailed transcriptional definitions of T cells are underway to facilitate characterization of T cell subset differentiation in the phases of RA development. Understanding those factors that define T cell differentiation in RA or perhaps other cell lineages that could contribute to the evolution of this unique disease might be critically important because the detection of T-reg cells in synovium has also been extensively documented-clearly their functional impact is insufficient or they may have a functional deficit. Clearly the autoantibody production is a hallmark of RA. B cell lineages at each stage of development are detected in synovium, including the presence of mature plasma cells. The synovium provides a cytokine-rich milieu for B cell survival (Firestein and McInnes, 2017).

1.3.5.2.1. Role of T-Cells

Synovial inflammation produces from leukocyte cell infiltration of the synovial compartments, enabled by increased expression of adhesion molecules and chemokine's in the endothelium. Infiltrated cells include granulocytes, monocytes/ macrophages, Natural killer (NK) cells, B cells and T cells (CD4 + CD8 T cells) all contribute in production significant amounts of pro- inflammatory cytokines and chemokine's. T CD4 cells, but not CD8 T cells or B cells are essential to initiate of disease, but not for the inflammatory stage of disease hyper activation of the immune response and the presence of autoantibodies in the synovial micro-environment is sufficient for disease development (Mellado *et al*., 2015).

Cellular and biochemical analyses of human CD4 T cell have revealed abnormal T cell antigen receptor (TCR) signaling in RA patient. Surprisingly CD4 T cell from patient with RA is hypo-responsive to TCR engagement ex *vivo* as evidenced by decrease calcium mobilization and interleukin-2 (IL-2) production. This may be due in part to the reduced TCR and linker of activated T cell expression as well as cellular changes relationship with immune senescence. Although, CD4 T cell from patient with RA seem to have decreased signaling capacity when stimulated in *vitro* they appear to hyper- proliferate during clonal expansion and differentiate into effector cell that drive disease. It is not currently understood how to reconcile these paradoxical observations of diminished TCR signaling in the setting of increased T cell proliferation and effector function, and it is not clear whether this RA T cell phenotype is directly causal in disease pathogenesis or rather results from exposure to chronic inflammation (Ashouri *et al*., 2019).

Th1 cells in response to intracellular pathogen and release high concentration of IFN-γ and TNF. A new important subset of CD4 T helper is Th17 cell, which produce IL-17 cytokine, also chemokine's receptors CCR6 and CCR4 expressed by Th17 and CCL20 as a ligand for CCR6 is expressed at a high degree in inflamed joint (Bazzazi *et al*., 2018).

1.3.5.2.2. Role of B-Cells

B lymphocytes produce auto-reactive pathogenic antibodies such as RF and ACPA which are well established indicators of disease and severity of disease as they enhance tissue damage in a pre-clinical model of autoimmune arthritis. Auto-reactivity to malondialdehyde (MDA) has recently been reported in RA patient and is also related to disease activity and synovial pathogenesis. Moreover, B lymphocyte is antigen-presenting cell for auto-reactive T cell as shown in both murine and human studies. A defect in early B cell tolerance has also been reported in humans with the majority of naive B lymphocyte expressing poly-reactive autoantibodies involving RF and ACPA. These B lymphocytes are resistant to Fas-induced apoptosis and therefore not suppressed by T regulatory cells (Pala *et al*., 2018).

Furthermore, B cell produces pro and anti-inflammatory cytokines in response to certain stimuli. IL10-producing B cells consider a chief subgroup of regulatory B cells (B-regs) that inhibit inflammatory response. B-regs cell play a critical role in the evolution and maintenance of the chronic inflammatory RA. However, the IL-10-producing B-regs doing their immune suppressed influence by inhibiting Th1 and Th17 response impairing antigen presented cell (APC) function and pro-inflammatory cytokine release by monocyte influencing inflammatory cytokine production and inducing differentiation of IL-10- and TGF-β-producing regulatory T cells. B-regs can also contribute to T-regulation cell induction by secreting TGF-β. The multiple B-regs subsets that are induced by different environments and regulate a wide range of function point to their intricate role during the immune response in health and disease (Bankó *et al*., 2017).

1.3.5.2.3. Role of the Immunoglobulin

The auto-antibodies are present in 50-80% of patients with arthritis. Patients with rheumatoid arthritis inflammations cannot backup to normal state due to the T-reg cells decrease in number and low function. Thus, most the patients stay complain from complications both humeral and cellular immunity share in some development of arthritis due to produce of autoantibody. But humeral immune has an extensive role in RA (Derksen *et al*., 2017).

Antibodies in RA represented in auto-antibodies have an important role in different autoimmune inflammatory diseases of which RA when occur defect in ordering the function of the immune system at this time the antibodies will attack self-antigens. Numerous antibodies presence in rheumatoid arthritis of which Anti-cyclic citrullinated peptide antibody (ACCPA) and rheumatoid factors (RF) this auto-antibodies as routine checking in the diagnosis of RA patient. But not each RA patients include RF (Yap *et al*., 2020).

1.3.5.2.3-A. Rheumatoid Factors

Rheumatoid factor (RF) is auto-antibody that directed to the Fc part in IgG. More recently anti-citrullinated protein (ACPAs) as well as anti-carbamylated protein antibodies were presence to be linked with RA. These autoantibodies target Neoepitopes induced in the joints during inflammation by post- translational modification of proteins. In the inflamed RA joints raised efficacy of proteases contributes to damage of tissue by degrading matrix proteins (Falkenburg *et al*., 2017).

RF and ACCPA were involved as serological markers in the RA classification criteria of the new European League Against Rheumatism and American college of Rheumatology (EULAR / ACR) the diagnostic marker that the most common uses of ACCPA test for check antibody against cyclic citrullinated peptide. Many studies suggested that ACCPA was the best indicator for RA identification also best prognostic of cartilage and bone destruction (Elshafie *et al*., 2019).

Some researches show that immunosuppression therapies capable to reduce the RF serum level. However, the medical benefit of RF in observation disease potency and therapy response is restrictive. Gradual reduction in RF level equivalent to the reduction of disease effective in patients that treated with classic modify anti-rheumatic drugs or biologic treatment such as infliximab drug (Rocha *et al*., 2019).

1.3.5.2.3-B. Anti-cyclic Citrullinated Peptide Antibodies

The initial time to appeared of that patient serum with RA have a special antibody to kerato-hyaline molecules in 1964 year. These molecules firstly has termed anti-perinuclear factor (APF) because they stain manner on the cell of the buccal mucosa. In 1979 similar group of antibodies which characterized as anti-keratin antibody (AKA). While, before presence the modern amino acid Citrulline is fundamental for recognizing this antibody group. Citrulline produce from arginine amino acid by predominance through peptidyl arginine deiminase enzyme (Kerkman, 2017).

Numerous antibody-depended tests are used for diagnosis of Anti-cyclic Citrullinated Peptide Antibodies (ACCPA). The majority of the tests use cyclic citrullinated peptides (CCP) depended on the origin of tests and group of peptides these tests can be divided into 3 groups CCP1- CCP3 the tests used variable peptide denoting to found of citrullinate more than a particular citrullinated epitope itself this fundamental for detect of ACCPA which is in agreement with their cross-reactive nature (Trier *et al*., 2019).

One possible mechanism by which ACCPA can facilitate RA events is its ability to form immune complex (IC) which can activate cells. Indeed, several studies have shown that ACCPA Fc tail can stimulate both the expressing cells of the Fcγ-receptor and the complement system. Since citrullinated antigens are expressed in the in *vivo* tissue including the synovial compartment these studies suggest that ACCP-containing immune complex (IC) can induce inflammation in RA. Importantly, other studies have shown that RF can enhance these effects in *vitro* probably by promoting IC formation by multivalent binding. These observations relate the two characteristic serological findings in RA to one pathogenic pathway and are consistent with clinical findings that arthritis is more serious in ACCPA and RF patients (Toes and Pisetsky, 2019).

The presence of ACCPA in a patient with RA defines a distinct clinical phenotype with a tendency toward the development of chronic disease early initiate of erosions more destructive disease and more extra-articular manifestations. ACCPA offer the significant advantage of being stable biomarker even in patient with early RA of whom only 5% experience seroconversion. Whether there is a difference in the inflammatory encumbrance between the subgroups is still disputed, and recent evidence suggests that those with ACCPA-negative RA may have a higher inflammatory encumbrance, at least in the early stage of the disease (Orr *et al*., 2017).

1.3.5.3. Role of the Cytokines

Numerous clinical studies have crystallized and validated the pivotal participation of cytokines in the RA pathogenesis. A varied range of circulating cytokines shows shortly before the initiate of articular disease and especially during the transition from systemic to localized disease. Cytokines regulate cellular phenotype, localization, activation status and longevity in the synovial and lymphoid micro-environments (McInnes *et al*., 2016).

Many researchers indicated that pro-inflammatory cytokines involve tumor necrosis factor TNF-α, interleukins IL-1, IL-6, IL-17 and the mediators produced via a downstream pathway in the arthritic joint constitute the milieu- driving cartilage and bone erosion. Most cytokines act as a factor of cellular recognition, inflammation, immune pathology and organize of immune response (Alunno *et al*., 2017).

Experimental studies on human RA synovial cells have shown that a combination of IL-1β, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) causes a significant a rise in the production of inducible nitric oxide synthase and nitrite. It is established that transforming growth factor-beta (TGF-β) and IL-4 down-regulate induce nitric oxide synthase expression in murine macrophages. It has been proposed that IL-10 is responsible for inducible nitric oxide synthase expression during the pathogenesis of RA (Dey *et al*., 2016).

In intense synovitis there are numerous immune cells producing high amounts of cytokines that causing significant symptoms of RA such as, pain in the joints, swelling, rigidity and stimulate systemically the liver to release acute phase protein. Cytokine reaction is suggested to have the main role in developing by RA and also a modification of intensity period of related inflammation (Baker *et al*., 2013; Ting *et al*., 2018).

Many investigations have shown that IL-6 plays a role in the regulation of both inflammatory and immune response, as well as in hematopoiesis (including B lymphocyte maturation and immunoglobulin production, T lymphocyte maturation and activation) and the induction of acute phase proteins in the liver. IL-6 plays a crucial role in inflammatory conditions and immune-mediated diseases and is an important mediator of RA (Kasama *et al*., 2016).

The significant inflammation of RA arises because of the excess of secretion that encourages cytokines above those of the inhibiting ones. TNF-α is the principal cytokine among the pro-inflammatory cytokines that controls the production of certain inflammatory mediators in the synovial tissue. This is also involves in bone and cartilage degradation through chondrocyte activation and osteoclasts. Certain primary cytokines involved in RA pathogenesis include IL- 1 and IL-6. The main cytokines produced by Th17 cell are IL-17A and IL-17F as pro-inflammatory cytokines are also responsible for the development of chemokine's differentiation of osteoclasts and the release of molecules for cell adhesion (Mateen *et al*., 2019).

1.3.5.3.A. Pro-Inflammatory Cytokines

Interleukin (IL)-1 and tumor necrosis factor alpha (TNF-α) are the more pro- inflammatory cytokines involved in RA. The influx and/or local activation of mononuclear cells and the formation of new blood vessels are main findings in synovial membrane. Differentiation of naive T cells into Th17 cells contributes to synovitis. B cells further the pathogenic process via antigen presentation and autoantibody and cytokine production. Enzymes secreted by synoviocytes and chondrocytes degrade cartilage. The release of cytokines especially TNF-alpha and IL-1 have multiple detrimental effects on cartilage and bone. Pro- inflammatory cytokines act locally, but also have systemic effects such as production of acute-phase proteins, anemia of chronic disease, cardiovascular disease, osteoporosis (Nalbant and Birlik, 2017).

Interferon-gamma (IFN-γ) is one of the cytokines that produced mainly by Th1 and NK cells it has an important role in activation of macrophage and up regulates expression of MHC-II on the antigen presented cells (APCs). Consequently, it promotes the potency of cell to present exogenous antigen to CD4 and CD8 lymphocyte cells. Further, correct of leukocytes track to inflammation site is critical for immune response to be active. This track is controlled by chemokine's a diversity of which can be stimulated by IFN-γ (Schurgers *et al*., 2011).

1.3.5.3.B. Inflammatory Cytokines

Higher amount of cytokines in synovial joint sites of patients compared with in serum of the RA patient which is usually low. Most these cytokines are pro- inflammatory. Many cytokines which causing locally damage of synovial joints also have systemically effects on RA patient are IL-1, IL-6, IL-15, IL-16, IL-17, IL-18, TNF-α, interferon-γ and GM-CSF factor (Venkatesha *et al*., 2014) .

The function in IL-6 pathology has been reported in RA patients there was a propensity for them to increase the level of IL-6 in gingival crevicular fluid (GCF). Therefore, some studies showed higher IL-6 serum values in patients with RA compared to patients with no systemic disease yet comparable to variables of age, gender and smoking status. IL-6 serum rates showing a positive association with RA behavior. These findings indicate that IL-6 developed both systemically and locally may play a role in controlling periodontal inflammation in RA patients. IL-17 has specific importance to the pathogenicity of periodontal inflammations as a result it includes inflammations and antimicrobials immunities. Studies confirm the IL-17 mediates protection against extracellular pathogen simultaneously with IL-22 (Martu *et al*., 2019).

1.3.5.3.C. Anti-Inflammatory Cytokines

Inflammatory regulation and control of the numerous immune cells by produce anti-inflammatory cytokines in most immune reactions in case of rheumatoid arthritis there are disorder between pro and anti- inflammatory cytokine due to insufficient amount of anti-inflammatory cytokines like IL-13, IL-11, IL-10 for regulation activities of pro-inflammatory cytokines (Ridgley *et al*., 2018).

1.4. Association between HLA-system and disease

HLA genes represent the human major histocompatibility complex (MHC) and which are located on a 3.6 Mb region on chromosome 6p21. This region consists of more than 200 highly polymorphic genes that encode for class-I and class-II HLA antigens, as well as many other proteins that have important roles in the immune system MHC region makes up only 0.3% of the genome. Class-I and class-II HLA-encoded molecules are mostly known for their function in antigen presentation. Both HLA classes have important roles in the adaptive immune response and are essential for regulating immunity. HLA class-I molecules are expressed on the surface of all nucleated cells and present peptides that are derived from intracellular proteins, mostly following cell damage or after viral infection to cytotoxic T cells to induce clearance of the damaged or infected cell (Van Drongelen and Holoshitz, 2017).

HLA class-II molecules are typically expressed on the cell surface of antigen presenting cells such as B cells, macrophages and dendritic cells as well as activated T cells. These molecules present peptides derived from the extracellular space to T helper cells which in turn stimulate B cells to produce antibodies towards that specific antigen thereby enabling an antigen specific immune response. Fifty years since the first description of an association between HLA and human disease, HLA molecules have proven to be central to physiology protective immunity and deleterious disease-causing autoimmune reactivity (Dendrou *et al*., 2018)).

In the past, many genetic polymorphisms have been suspected to be associated with diseases. The most extensively studied markers are blood groups, enzymes and serum proteins. The relative risk in all of these reports has been less than 2. One of the most thoroughly investigated associations is that of carcinoma of the stomach with blood group A(Kennedy *et al*., 2017) Analysis of the data on 53,155 patients shows a relative risk of 1.22 indicating that a type A person is only 1.22 times more likely to develop stomach cancer than those who are not type A. In contrast, the association between HLA-B27 and ankylosing spondylitis has a relative risk of 69. There are many diseases in which relative risk value is greater than 2. Thus, the available data on HLA associations with diseases have far surpassed all the existing knowledge in this field (Hiwa *et al*., 2018).

The discoveries of HLA associations with certain diseases have been a major breakthrough in our understanding of the genetics of these diseases. It has been postulated that at least part of the genetic basis of the associated diseases lies in the HLA region of chromosome 6. HLA markers are already providing some useful clinical information regarding the diseases by calculating the relative risk of the disease in association with HLA genotype (Braun, 2019).

1.4:1: Mechanisms of associations HLA-system and disease

1.4.1.1: Involvement of HLA Antigens

There are many suggestions for this involvement as; the HLA antigens may act as receptors to some viruses or bacterial proteins leading to susceptibility to some diseases. Another suggestion is the HLA antigens may have molecular mimicry to some infectious proteins leading to infectious diseases. In other group of non-infectious disease HLA antigens may have similarity to certain hormone receptors so will compete with these receptors for hormone binding leading to disease. In some autoimmune diseases the HLA antigen may act as hapten-carrier creating immune response towards the surface of the cells possessing this specific HLA antigen leading to an immune response directed in part against the tissue. Some HLA antigens may be modified by certain viruses leading to susceptibility to specific viral infections (Kennedy *et al*., 2017).

1.4.1.2: Involvement of HLA Genes

The second group of hypotheses proposes that HLA antigens are not involved in the causation of the disease. They are markers for the "disease susceptibility genes" that are very closely linked with the HLA complex. The observed population associations are due to the existence of linkage disequilibria between the HLA antigens and the alleles for the disease susceptibility genes. Where in disease related to immune response gene (Irg) and immune suppression gene (Isg) it is hypothesized that it exists in the D/DR region of the HLA complex then there will be stronger linkage disequilibria with certain HLA loci. In gens not related to immune response the HLA association may be due to the presence of metabolic gene (gene coded for enzyme or precursor in certain metabolic pathway) in linkage disequilibrium with HLA genes (Braun, 2019).

1.4.2. Association between HLA-system and RA

The importance of inherited risk alleles in the pathogenesis of RA is highlighted by increased concordance for RA in monozygotic compared to dizygotic twins and by the familial clustering. The most important region of the human genome in RA susceptibility is the major histocompatibility complex (MHC) which encodes for genes that are essential to immune responses, notably the HLA-A, HLA-B, HLA-C, and HLA-D proteins. These structures are expressed on the surface of antigen-presenting cells and are required for recognition of peptide antigens by T lymphocytes leading to initiation of immune responses. The RA-associated MHC allele was initially identified as HLA-DR4 and later localized to a five-amino-acid sequence from residues 70–74 of the beta chain of subtypes of HLA-DR4 and selected other DR alleles shared epitope which is located within the MHC peptide-binding cleft (Jan *et al*., 2022).

More lately polymorphisms that rule additional amino acid differences in HLA-DR that are located outward the shared epitope have also been strongly associated with susceptibility to Rheumatoid arthritis (Okada *et al*., 2014; Viatte *et al*., 2013).

The mechanism for predisposition of MHC to RA leftovers to be established while presentation of a pathogenic auto-antigen by RA associated alleles is an gorgeous theory other possibilities exist for example unique pro-inflammatory properties of the shared epitope itself (Holoshitz, 2010; Ling *et al*., 2013).

Genome-wide association studies have recognized more than on hundred other loci that related with susceptibility to RA each of which has a modest effect (Kochi *et al*., 2014). The specific genes related with these loci mostly function in cells that mediate immune responses such as lymphocytes and antigen presenting cells reinforcing the concept that RA is an autoimmune disease. The identified loci associated with RA (both MHC and nonMHC) are more powerfully linked to seropositive RA such as RA in which either rheumatoid factor (RF) or antibodies to citrulline containing proteins (ACPA) are present. PTPN22 (protein – tyrosine phosphatase non-receptor type 22) is the greatest effectuated RA-associated non MHC gene which encrypts a tyrosine phosphatase that is articulated in lymphocytes controls signaling through the T cell receptor for antigen and influences lymphocyte expansion (Stanford and Bottini, 2014).

1.5 Diagnosis of RA

Depending on the extending of the cartilages and swelled joints in 2010 the American college of Rheumatology (ACR) and European League against Rheumatism (EULAR) suggested dividing criteria of RA. Serum level of auto- antibody like rheumatoid factor (RF) and anti-citrullinated proteins auto- antibodies (ACCPA), amount of acute-phase molecules like C reactive proteins (CRP), erythrocyte sedimentation rate (ESR) and period of symptoms. Sensitivity of ACCPA is 60-75% while the RF-positive rate is 60-70% (Mun *et al*., 2018).

1- Clinical features are classified into followed types; Articular class, this type can show nearly in each RA patients with arthritis of the peripheral synovial joint. In some time oligo-arthritis that prevalent in onset of disease. Poly- arthritis prevalence in latter general image of RA disease, additionally, mono- arthritis is uncommon and not include axial in RA excepting cervical spines, clinical articular manifestations include, chronic symmetric polyarthritis, morning stiffness that involve small and large joints. Another type, extra- articular manifestations that involve interstitial lung diseases, coronary heart disease, pericarditis, myopathy and atherosclerosis (Salehi-Abari, 2016).

2- X Ray and magnetic resonance imaging (MRI); The hands and feet are more affected by RA disease therefore, the X- ray perfect for diagnosis, but in the early phases of RA may be no change in the joint, only osteopenia nearby joint with swelling in soft tissues and small space than normal joint. In advanced stages of disease occur erosion in the bones. In addition to there are other medical mechanisms which use in diagnosis of RA such as ultrasound (Dumbreck *et al*., 2015).

3- Serum markers diagnosis; The RF and Anti-CCP are considered two markers for diagnosis of RA, the Anti-CCP have high specificity approximately 95% opposite 85% for RF this important value to progressive of erosive disease however, with high specificity each of the RF and Anti-CCP may be detected in many diseases (Kourilovitch *et al*., 2014).

4- Erythrocyte sedimentation rate (ESR) with C-reactive protein (CRP) have relation to progression of diseases activities and confirm benefit to estimate responses to therapies. Despite, these markers are broadly used to detect disease in RA patient. Generally, ESR is a low sensitivity marker while CPR despite having more sensitivity it presents with normal values in patient those have activity disease and treating with biological treatment. Thus, ESR and CRP not prognostic the later risk of disease development in patients (Hirata *et al*., 2015).

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study Design, Population and Time Frame:

Case control study

2.1.1 Patients

Study population included patients with rheumatoid arthritis (RA) the total of (50) rheumatoid arthritis patients consisting of (12) males and (38) females were involved in this study. The age range was from (25 - 75) years. Patients clinically diagnosed by specialized physician in a Rheumatology department in the Medical Merjan City. Moreover all patients were subjected to laboratory diagnosis: ACCP,RF and ESR. The patients were admitted during the period February 2022 to October 2022. All patients were apparently not suffering from other chronic disease.

2.1.2 Inclusion Criteria for Patients

Inclusion criteria of patient group: patients were diagnosed by a specialized physician depending on clinical finding, and proved by rheumatoid factor (RF), ACCP and ESR tests. All patients were not suffering from any other disease.

2.1.3 Exclusion Criteria for The Patients

Exclusion criteria of RA patients were involved:

- Chronic diseases other than RA.

2.1.4 Control

Apparently healthy Control Included 40 subjects with age from (25 - 75) years, Selected as Age and sex matched with RA group.

2.1.5 Ethical Approval

The study was conducted in accordance with the ethical principles in the Declaration of Helsinki. It was carried out with the patients verbal approval before the sample was taken. The study protocol and patient consent forms were reviewed and approved by Babylon Health Directorate and the committee on publication ethics at the College of Medicine, University of Babylon.

2.1.6.The Study design and Steps of this work

The study design was case-control study. The steps study of the present work was illustrated in figure (2-1).

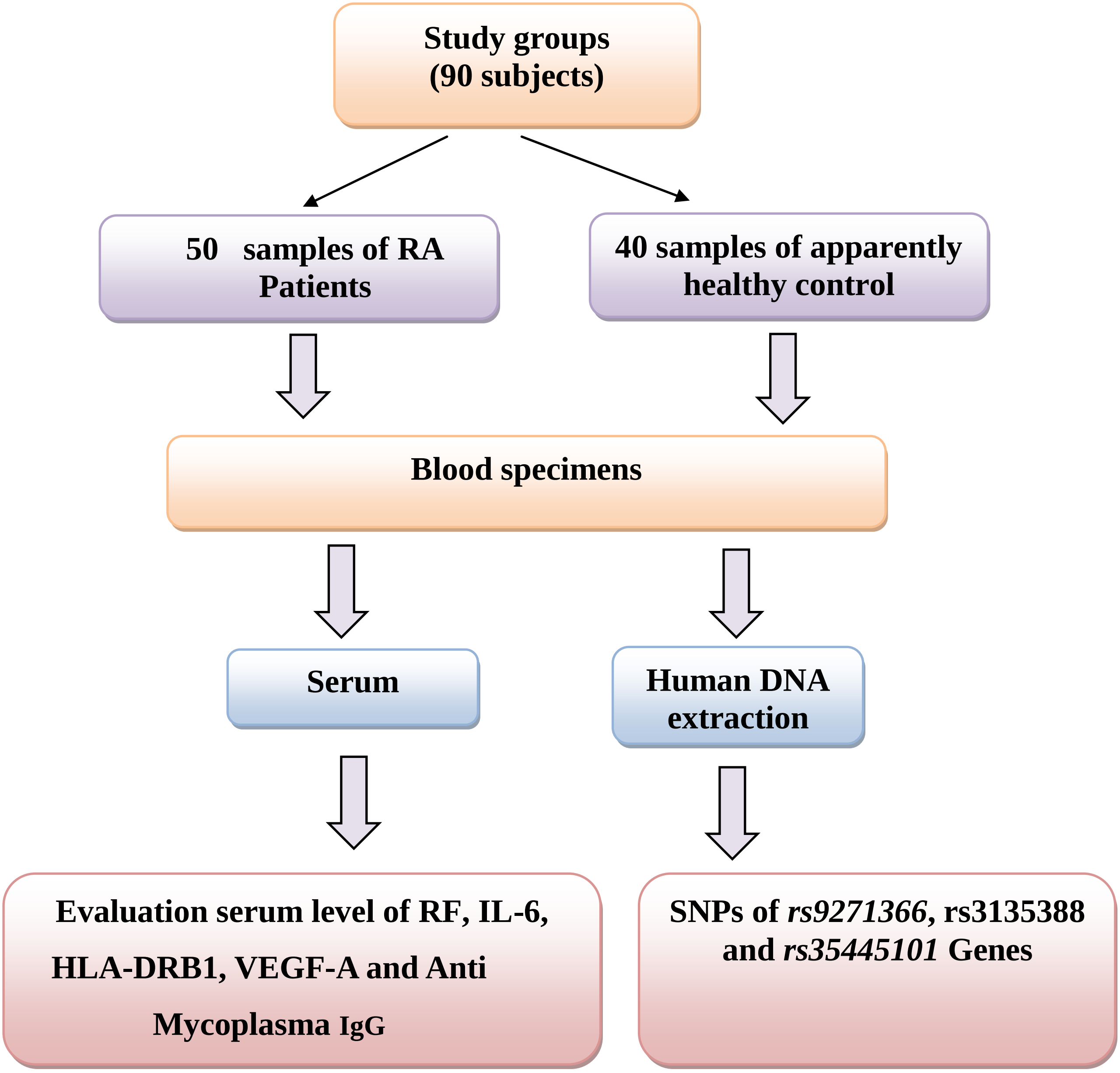


Figure (2-1): Scheme of the steps of the study.

2.2 Collection of Clinical Samples

2.2.1 Sample size

Fisher's formula is used for sample size determination as recommended $\mathbf{n=Z^2P(1-P)/d^2}$ (Pourhoseingholi *et al*., 2013).

Where:

n= sample size.

Z= the corresponding value for the 95% confidence interval which =1.96.

P= the proportion of rheumatoid arthritis (RA) in Babylon province .

So the total sample size required according to the equation was 50 patients.

2.2.2 Data Collection Tools

2.2.2.1 Questionnaires

Included the following information (number, sex, age of patients, length, weight, body mass index, duration of disease, and the RA patient committed to treatment or non-committed to treatment). As shown in (appendix1)

2.2.3 Blood samples

Five ml of freshly venous blood were collected from each participant 2ml of which was kept in EDTA tube and use for extraction of human DNA. The other 3ml of blood kept in the gel tube without anticoagulant until be clotting (Volobaev *et al*., 2021). It was undergone centrifugation at 2500 rpm for 15 minutes then the serum was collected and preserved at -20°C until being used Human Interleukin 6, HLA- DRB1, Human Vascular Endothelial cell Growth Factor A and Anti-*Mycoplasma pneuminae* IgG were estimated by ELISA techniques.

2.3. Materials

2.3.1 Laboratory Equipment

The Laboratory equipment used in this study were listed in the table (2-1).

Table (2-1): Laboratory apparatus and Equipment.

No.	Item	Company	Country
1	Autoclave	Hirayama	Japan
2	Biological safety cabinet	Thermo Scientific	Germany
3	Centrifuge	Hettich	Germany
4	Digital camera	Nikon	USA
5	ELISA reader	Biotec	USA
6	ELISA shaker	Pasture	France
7	ELISA washer	Biotec	USA
8	Horizontal gel electrophoresis	Biorad	Japan
9	Hot plate	Medico	USA
10	Incubator	Incucell	Germany
11	Micropipettes,(2- 20µl), (5-50µl) (20-200µl) , (100-1000µl)	Slamed	Germany
12	Microwave	Argos	Germany
13	Multichannel pipette	Slamed	Germany
14	PCR thermal cycler	Biometra	Germany
15	Printer	Epson	Indonesia
16	Refrigerated centrifuge	Hettich	Germany
17	Refrigerator	Concord	Italy
18	Sensitive digital balance	sartorios	Germany
19	UV- transilluminator	Cleaver	England
20	Vortex shaker	Stuart	Germany
21	Water bath	Grant	England
22	Water distiller	GFL	Germany

2.3.2 Laboratory Instruments

The study required the using of many glassware and disposable materials which listed in the following table (2-2).

Table (2-2): Glassware and plastic equipment

No.	Equipment	Company	Origin
1	Disposable Sterile Syringes,5 ml	Dolphin	Syria
2	EDTA-tubes	Afco	Jordan
3	Ependrof Tubes 1.5 ml	Biobasic	Canada
4	Gel Tube10 ml	AL- Jawaher	Jordan
5	Test tube rack	Himedia	India
6	Tips 200,500,1000µl	BAG Health Care	Germany

2.3.3 Commercial kits and chemical materials

2.3.3.1. Commercial kits

The commercial kits were used in the study are listed in table (2-3).

Table (2-3): Commercial kits used in the study

No.	Kits	Company	Origin
1	Anti-*Mycoplasma pneuminae* IgG	Demeditec	Germany
2	Genomic DNA extraction kit	Promega	USA
3	Human HLA Class 2 Histocompatibility Antigen, DRB1 Beta Chain	BT LAB	China
4	Human Interleukin 6 ELISA Kit	BT LAB	China
5	Human Vascular Endothelial cell Growth Factor A	BT LAB	China
6	PCR master mix	Promega	USA
7	Rheumatoid factor kit	Girona	Spain

2.3.3.2. Chemical Materials

The chemical materials used in this study were listed in table (2-4).

Table (2-4): The Chemical Materials

No.	Material	Company	Origin
1	Absolute Ethanol	Scharlau	Spain
2	Agarose	Pronadisa	Spain
3	Ethidium Bromide	BioBasic	Canada
4	Free nuclease water	Cyntol	Russia
5	Ladder 50bp and 100bp	Promega	USA
6	TBE buffer 5X(Tris base ,boric acid and EDTA)	Thomas Baker	Inda
7	Primers	Macrogen	Korea
8	DNA loading dye	Promega	USA

2.4 Methods

2.4.1 Solutions

2.4.1.1 Phosphate Buffer Solution (PBS) (pH=7.3)

It has been prepared by dissolving one buffered tablet in 100 ml distilled water and sterilized by autoclave. In accordance with the instruction of manufacturer after that was kept at 4^{o}C until used.

2.4.1.2 Ethidium Bromide Solution

It was prepared by dissolving 0.05gm of ethidium bromide in 10 ml distilled water and stored in a dark reagent bottle (Sambrook and Rusell, 2001).

2.4.1.3 Agarose Gel Preparation

The agarose gel was prepared according to the method of Sambrook and Rusell (2001) by adding 1gm agarose to 100ml of 1x TBE buffer. The solution was heated to boiling (using water bath) until all the gel particles dissolved. The solution was allowed to cool down within 50-60^{o}C and mixed with 0.5µg/ml ethidium bromide.

2.4.1.4 TBE buffer

TBE buffer: 27g of Tris base 14 g Boric acid and 1.86 g of EDTA (pH 8) dissolved in 500ml distal water

2.5 Body mass index (BMI)Measurements

BMI was calculated as a continuous variable By measuring heights and weights as weight in kilograms divided by the square of height in meters. BMI values were also categorized into the widely used underweight (<18.5 kg/m^2) normal (18.5–24.9 kg/m^2) overweight (25–29.9 kg/m^2) and obese (≥30 kg/m^2) categories based on the 1998 clinical guidelines (Heart *et al*., 1998).

2.6 Detection of Immunological Markers by ELISA

Serum was used for measurement of rheumatoid factor (RF) by RF agglutination test, While Anti-CCP, anti-*Mycoplasma pneumoniae* IgG antibody, HLA, DRB1, Vascular Endothelial cell Growth Factor A and Interleukin 6 was estimated by ELISA technique.

2.6.1 Detection of RF

RF test was carried out for diagnosis of RA patients a procedure to test listed in following steps according to the instructions of the RF kit (Girona company /Spain).

1. Serum sample was from a centrifuged tube of which 50µ. In addition to, one drop from positive control and negative control in separate circles of slide test.

2. RF-latex reagent were shackled and add one drop to circle with the sample.

3. The drops were mixed by disposable stick and covered the entire circle.

4. The result was read after more than two minutes. Agglutinating occurring in the case of the positive sample.

2.6.2 Estimation of anti-*Mycoplasma pneumoniae* IgG-Ab concentration

2.6.2 -A: Principle

The anti-*Mycoplasma pneumoniae* IgG kit is based on the principle of the enzyme immune-assay. *Mycoplasma pneumoniae* antigen is bounds to the surface of the microtiter strips. Diluted patient serum is pipetted into the wells of the microtiter plate. A binding between the IgG antibody of the serum and the immobilized *Mycoplasma pneumoniae* antigen will takes place. After one hour incubation at room temperature, the plate is rinsed with a diluted wash solution to remove unbound material. Anti-human IgG peroxidase conjugate is added and incubated for 30 minutes after washing the substrate tetramethylbenzidine (TMB) solution is pipetted and incubated for 20 minutes. Inducing the development of blue dye in the wells. The color development is terminated by the stop solution which changes the color from blue to yellow. The resulting dye is measured spectrophotometrically at a wavelength of 450nm. The concentration of IgG antibodies is directly proportional to the intensity of the color.

2.6.2 -B: Assay Procedure

1. One hundred µl were each of the sample and ready to use standers and controls respectively into wells.
2. The plate was covered with plate cover and incubated at room temperature for 60 min.
3. Wells were empty and 300ml of diluted washing solution was washed wells for 3 times.
4. Conjugated 100 µl was added into the wells.
5. Plated was covered and incubated at room temperature for 30 min.

6. Wells of the plate were empty and added 300 ml of diluted washing solution. Repeated 3 times.
7. One hundred µl of substrate was added into the wells.
8. The plate was covered and incubated at room temperature for 20 min.
9. One hundred µl of stop solution was added into each well, to terminate the substrate reaction.
10. Optical density was detected by the automated Mindray ELISA technique at 450nm.
11. Determined infected with *Mycoplasma pneumoniae* according to the value for cut off (0.248) patients with ≥ 0.248 were positive (Cutoff Value = average Negative Control value + 0.15).

2.6.2- C Standard Curve

A standard curve was constructed by plotting the absorbance of each tube on the x-axis against the concentration on the y-axis. The best fit curve was drawn through the points on the graph as shown in figure (2.2).

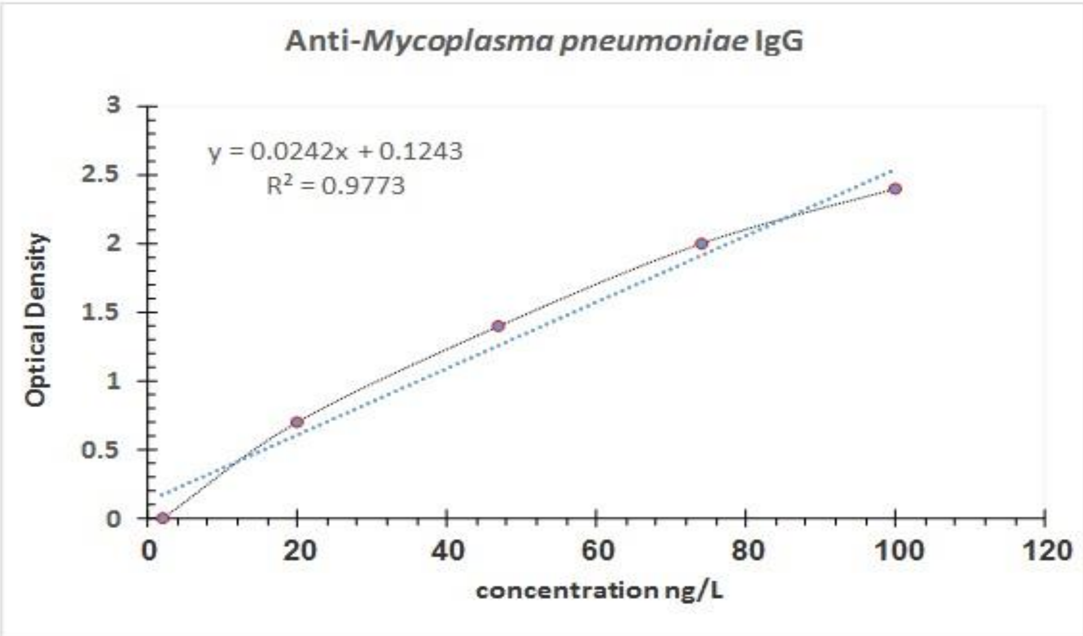


Figure (2-2): Standard curve for Serum anti-*Mycoplasma pneumoniae* IgG-Ab concentration.

2.6.3 Estimation of HLA Class 2 Histocompatibility Antigen, DRB1 Beta Chain

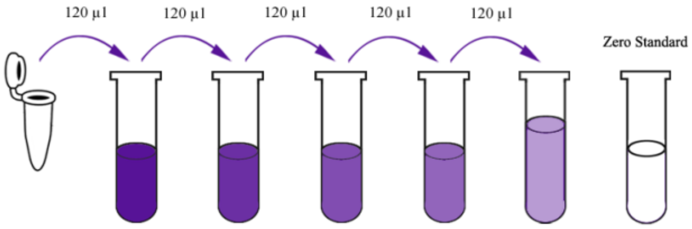
2.6.3-A Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human HLA-DRB1 antibody. HLA-DRB1 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human HLA-DRB1 Antibody is added and binds to HLA-DRB1 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated HLA-DRB1 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human HLA-DRB1. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.6.3-B Reagent Preparation

1. All reagents should be brought to room temperature before use.
2. Standard Reconstitute the 120µl of the standard (9600ng/L) with 120µl of standard diluent to generate a 4800ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (4800ng/L) 1:2 with standard diluent to produce2400ng/L, 1200ng/L, 600ng/L and300ng/L solutions. Standard diluent serves as the zero standard (0 ng/L). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

4800ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
2400ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
1200ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
600ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
300ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
9600ng/L	4800ng/L	2400ng/L	1200ng/L	600ng/L	300ng/L

Figure (2-3) The Diluting of Stock Solution of HLA-DRB1.

3. Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.6.3-C Assay Procedure

1. All reagents, standard solutions and samples prepared as instructed. Brought all reagents to room temperature before use. The assay was performed at room temperature.

2. The number of strips required for the assay were determined. Inserted the strips in the frames for use. The unused strips were stored at 2-8°C.

3. Fifty µl of standard added to standard well. Note: Didn't add biotinylated antibody to standard well because the standard solution contained biotinylated antibody.

4. Forty µl of sample to sample added wells and then added 10µl anti-HLA-DRB1 antibody to sample wells, then added 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mixed well. Covered the plate with a sealer. Incubated 60 minutes at 37°C.

5. The sealer removed and washed the plate 5 times with wash buffer. Soaked wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirated or decanted each well and washed 5 times with wash buffer. Blotted the plate onto paper towels or other absorbent material.

6. Fifty µl of substrate solution A was added to each well and then added 50µl substrate solution B to each well. Incubated plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. 50µl of Stop Solution was added to each well, the blue color changed into yellow immediately.

8. The optical density (OD value) was determined of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.6.3-D Standard Curve

A standard curve was constructed by plotting the absorbance of each tube on the x-axis against the concentration on the y-axis. The best fit curve was drawn through the points on the graph as shown in figure (2.4).

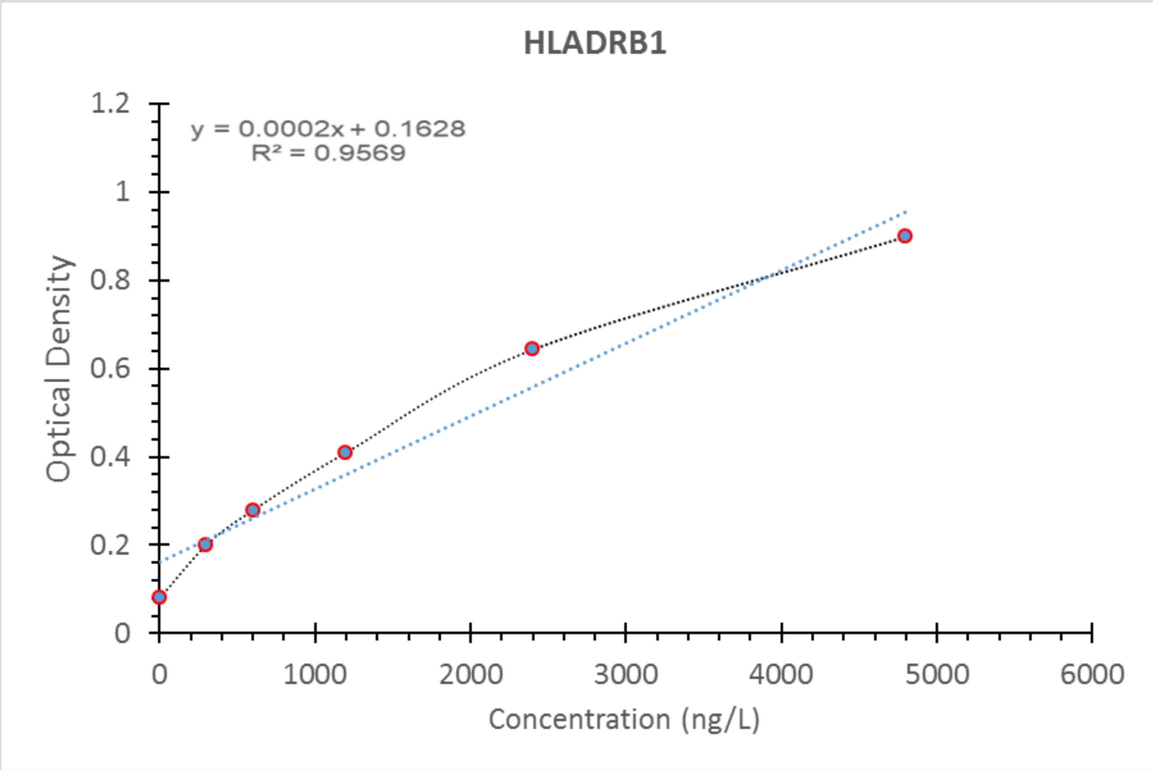


Figure (2-4): Standard curve for Serum HLA-DRB1 concentration.

2.6.4 Estimation of Human Vascular Endothelial cell Growth Factor A

2.6.4 -A Principle

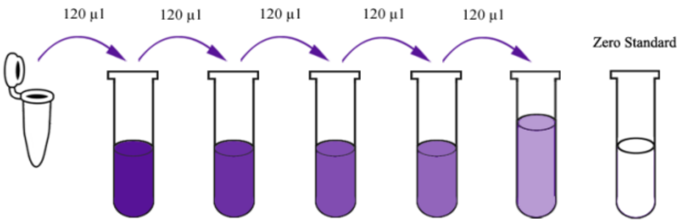
This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human VEGF-A antibody. VEGF-A present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human VEGF-A Antibody is added and binds to VEGF-A in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated VEGF-A antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human VEGF-A. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.6.4 -B Reagent Preparation

1. All reagents should be brought to room temperature before use.

2. Standard Reconstitute the 120µl of the standard (960ng/L) with 120µl of standard diluent to generate a 480ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (480ng/L) 1:2 with standard diluent to produce240ng/L, 120ng/L, 60ng/L and30ng/L solutions. Standard diluent serves as the zero standard (0 ng/L). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

480ng/L	Standard No.5	120µl Original Standard + 120µl Standard Diluent
240ng/L	Standard No.4	120µl Standard No.5 + 120µl Standard Diluent
120ng/L	Standard No.3	120µl Standard No.4 + 120µl Standard Diluent
60ng/L	Standard No.2	120µl Standard No.3 + 120µl Standard Diluent
30ng/L	Standard No.1	120µl Standard No.2 + 120µl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
960ng/L	480ng/L	240ng/L	120ng/L	60ng/L	30ng/L

Figure (2-5) The Diluting of Stock Solution of VEGF-A.

3. Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.6.4- C Assay Procedure

1. All reagents, standard solutions and samples prepared as instructed. Brought all reagents to room temperature before use. The assay was performed at room temperature.

2. The number of strips required for determined the assay. Inserted the strips in the frames for use. The unused strips were stored at 2-8°C.
3. Fifty µl of standard added to standard well. Note: Didn't add biotinylated antibody to standard well because the standard solution contains biotinylated antibody.

4. Forty µl of sample added to sample wells and then added 10µl anti-VEGF-A antibody to sample wells, then added 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well).Mixed well. Covered the plate with a sealer. Incubated 60 minutes at 37°C.

5. The sealer removed and washed the plate 5 times with wash buffer. Soaked wells with 300ul wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirated or decanted each well and washed 5 times with wash buffer. Blotted the plate onto paper towels or other absorbent material.

6. Fifty µl of substrate solution A was added to each well and then added 50µl substrate solution B to each well. Incubated plate covered with a new sealer for 10 minutes at 37°C in the dark.

7. Fifty µl of Stop Solution was added to each well, the blue color will changed into yellow immediately.

8. the optical density (OD value) was determined of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.6.4- D. Standard Curve

A standard curve was constructed by plotting the absorbance of each tube on the x-axis against the concentration on the y-axis. The best fit curve was drawn through the points on the graph as shown in figure (2.6).

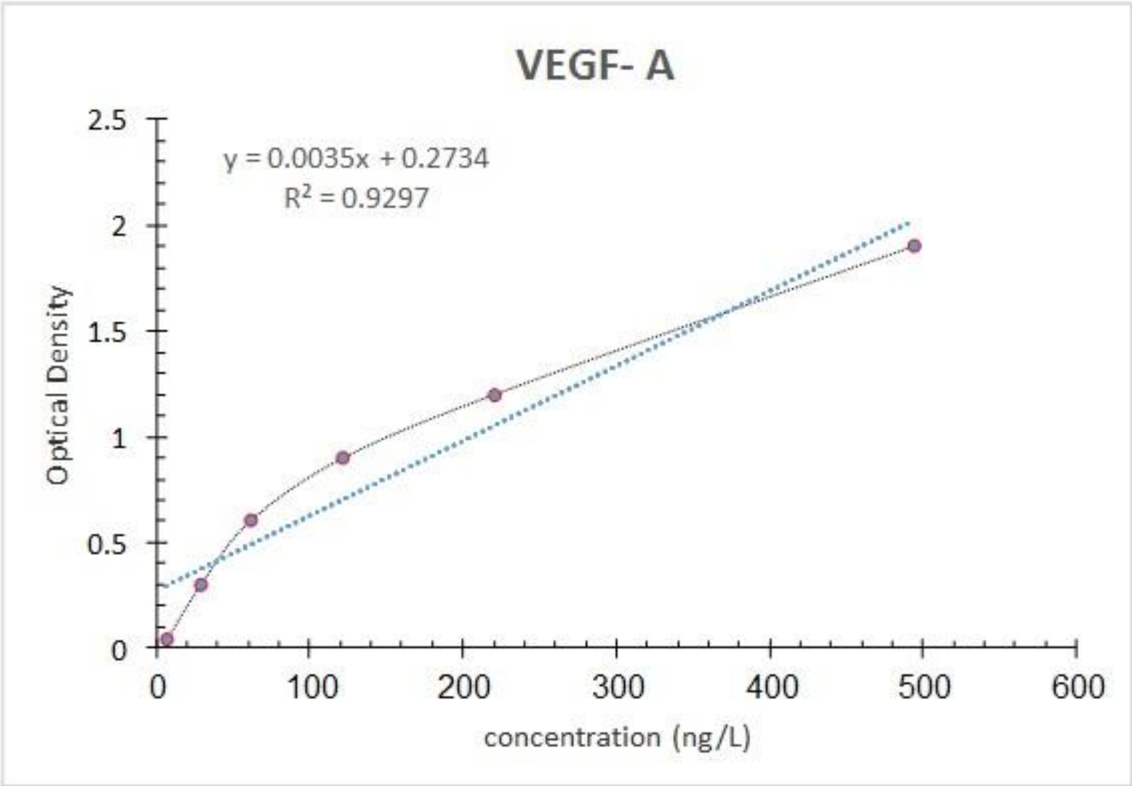


Figure (2-6): Standard curve for Serum Human Vascular Endothelial cell Growth Factor A concentration.

2.6.5 Estimation of Human Interleukin - 6

2.6.5 -A Principle

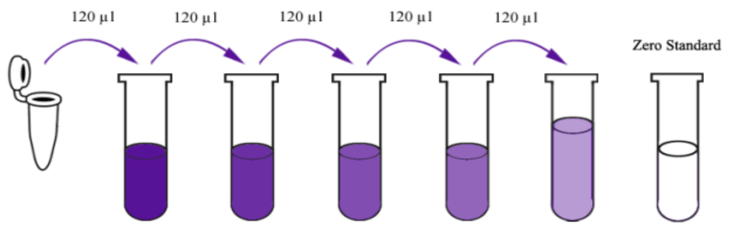
This kit is an Enzyme-Linked Immunosorbent Assay (ELISA). The plate has been pre-coated with Human IL-6 antibody. IL-6 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Human IL-6 Antibody is added and binds to IL-6 in the sample. Then Streptavidin-HRP is added and binds to the Biotinylated IL-6 antibody. After incubation unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human IL-6. The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450 nm.

2.6.5 –B Reagent Preparation

1. All reagents should be brought to room temperature before use.

2. Standard Reconstitute the 120µl of the standard (640ng/L) with 120µl of standard diluent to generate a 320ng/L standard stock solution. Allow the standard to sit for 15 mins with gentle agitation prior to making dilutions. Prepare duplicate standard points by serially diluting the standard stock solution (320ng/L) 1:2 with standard diluent to produce160ng/L, 80ng/L, 40ng/L and20ng/L solutions. Standard diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C and used within one month. Dilution of standard solutions suggested are as follows:

320ng/L	Standard No.5	120μl Original Standard + 120μl Standard Diluent
160ng/L	Standard No.4	120μl Standard No.5 + 120μl Standard Diluent
80ng/L	Standard No.3	120μl Standard No.4 + 120μl Standard Diluent
40ng/L	Standard No.2	120μl Standard No.3 + 120μl Standard Diluent
20ng/L	Standard No.1	120μl Standard No.2 + 120μl Standard Diluent



Standard Concentration	Standard No.5	Standard No.4	Standard No.3	Standard No.2	Standard No.1
640ng/L	320ng/L	160ng/L	80ng/L	40ng/L	20ng/L

Figure (2-7) The Diluting of Stock Solution of IL – 6 .

3. Wash Buffer Dilute 20ml of Wash Buffer Concentrate 25x into deionized or distilled water to yield 500 ml of 1x Wash Buffer. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

2.6.5 –C Assay Procedure

1. All reagents, standard solutions and samples prepared as instructed. Brought all reagents to room temperature before use. The assay was performed at room temperature.
2. The number of strips required for the assay were determined. Inserted the strips in the frames for use. The unused strips were stored at 2-8°C.
3. Fifty µl of standard added to standard well. Note: Didn't add antibody to standard well because the standard solution contained biotinylated antibody.
4. Forty µl of sample added to sample wells and then added 10µl anti-IL-6 antibody to sample wells, then added 50µl streptavidin-HRP to sample wells and standard wells (Not blank control well). Mixed well. Covered the plate with a sealer. Incubated 60 minutes at 37°C.
5. The sealer removed and washed the plate 5 times with wash buffer. Soaked wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash. For automated washing, aspirated or decanted each well and washed 5 times with wash buffer. Blotted the plate onto paper towels or other absorbent material.
6. Fifty µl of substrate solution A was added to each well and then added 50µl substrate solution B to each well. Incubated plate covered with a new sealer for 10 minutes at 37°C in the dark.
7. Fifty µl of Stop Solution was added to each well, the blue color will changed into yellow immediately.
8. The optical density (OD value) was determined of each well immediately using a microplate reader set to 450 nm within 10 minutes after adding the stop solution.

2.6.5 -D. Standard Curve

A standard curve was constructed by plotting the absorbance of each tube on the x-axis against the concentration on the y-axis. The best fit curve was drawn through the points on the graph as shown in figure (2.8).

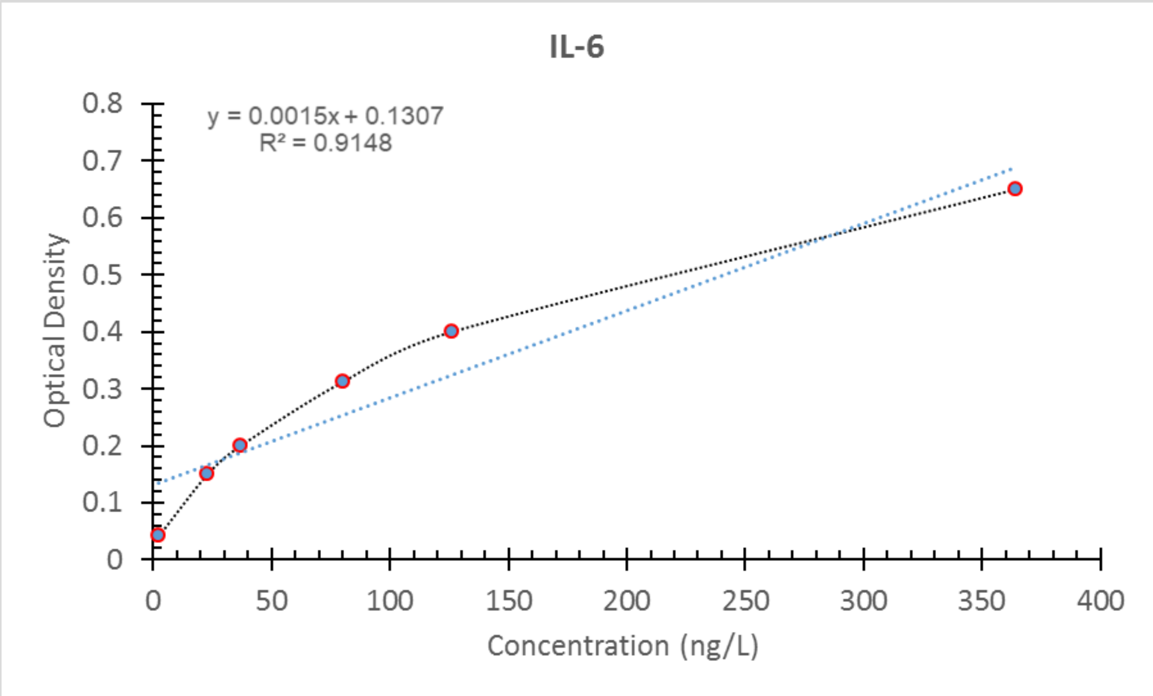


Figure (2- 8): Standard curve for Serum Human Interleukin 6 concentration

2.7 Genotyping assays

2.7.1 Genetic methods design

2.7.1.1 HLA-SNP selection

The studied HLA-Single Nucleotide Polymorphisms (SNPs) were chosen according to HLA-SNPedia as the highest frequency Three SNPs in HLA gene (HLA Allele*Frequencies in World Populations Database search). Table (2-5) illustrates the HLA-SNPs chosen.

Table (2- 5):HLA- Single-Nucleotide Polymorphisms (SNPs) chosen

Site of SNP	SNP	Frequency
HLA-DRB1	rs9271366	0.136
HLADRA	rs3135388	0.087
HLA-DRB1	rs35445101	0.288

2.7.1.2 primer design

The selection of genotyping technique according to (Hashim *and* Al-Shuhaib, 2019).The design of PCR primers according to the protocol of (Hashim *et al*., 2015). It was briefly as follow:

The primers were designed by the aid of NCBI-primer BLAST online software (http://www.ncbi.nlm.nih.gov/tools/primerblast/ index.cgi? LINK _LOC=BlastHome) , at the same time the produced primers was checked for specificity for their target sequences by performing the BLAST against the human genome , then the primers pair was selected according to the demand criteria such as : product length , the similarity of melting temperature , primers length , specificity , etc. The primer ability to form secondary structure was checked by the aid of Oligo Calc online software (http://www.basic. northwestern.edu/ biotools/oligocalc.html) , the primer would be rejected if it had 5 bases or more able to form self-dimerization and/or it had 4 bases able to form hairpin. Each primers pair was checked for dimer formation by the aid of ―Multiple Primer Analyzer‖ online software from Thermo Fisher Scientific Inc.© , the sensitivity of the software was adjusted to the value 2 , the primer pair would be rejected if it made any dimers in this degree of sensitivity .

2.7.1.3 Restriction enzyme selection

The selection of the suitable restriction enzyme was performed by the aid of by Snap Gene viewer software (V6.0.5) , we selected the restriction enzyme according to several criteria such as: the lesser primer mutations needed , the distance of mutation from the variant , compatibility of the produced primers , cost and availability.

Wat Cut online software presentation of the enzyme **MnlI** selection to restrict **rs9271366** SNP. Properties of the accepted primer pair for **rs9271366** SNP is shown in table (2- 6).

Table (2-6): The accepted primer pair for rs9271366 and their properties.

primer	Sequence (5'->3')	Template strand	Length	Tm	GC%	Self com ple men tarity	Self 3' comp lemen tarity	Reference
Forward primer	TTGTGTGGCCA AGACTAG CA	Plus	20	59.53	50.00	6.00	0.00	(Ali *et al.*, 2018)
Reverse primer	TGCCCAAAGTC AGACATC CA	Minus	20	59.23	50.00	5.00	0.00	
Product length	**206**							

The amplicon was cut with **MnlI** restriction enzyme to produce 62 and 144 bp fragment for A allele and 62, 58 and 86 bp fragment for G allele as in figure (2-9)

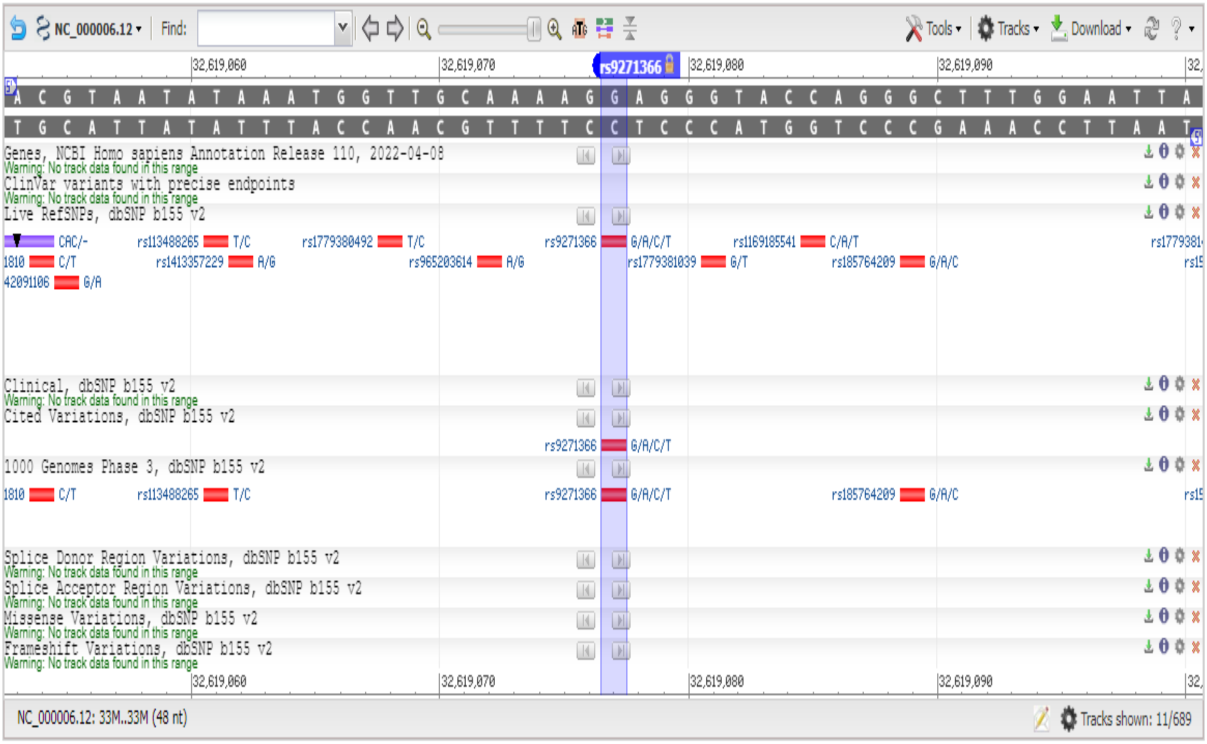


Figure (2-9): The exact position of rs9271366 and primers pair on human genome (NCBI sequence browser).

Wat Cut online software presentation of the enzyme **Bsa29 I** selection to restrict **rs3135388** SNP. Properties of the accepted primer pair for **rs3135388** SNP is shown in table (2-7)

Table (2-7): The accepted primer pair for rs3135388 and their properties

primer	Sequence (5'->3')	Template strand	Length	Tm	GC%	Self complementarity	Self 3' complementarity	Reference
Forward primer	GTAGAGATCTCCCAACAAATCGA	Plus	23	57.74	43.48	8.00	4.00	(Arun, 2016; Benešová *et al*., 2013)
Reverse primer	GAGTCGGTCCTGGGGAATA	Minus	19	57.78	57.89	3.00	2.00	
Product length	**206**							

The amplicon was cut with **Bsa29 I** restriction enzyme to produce 20 and 180 bp fragment for A allele and 206 bp fragment for G allele as in figure (2-10)

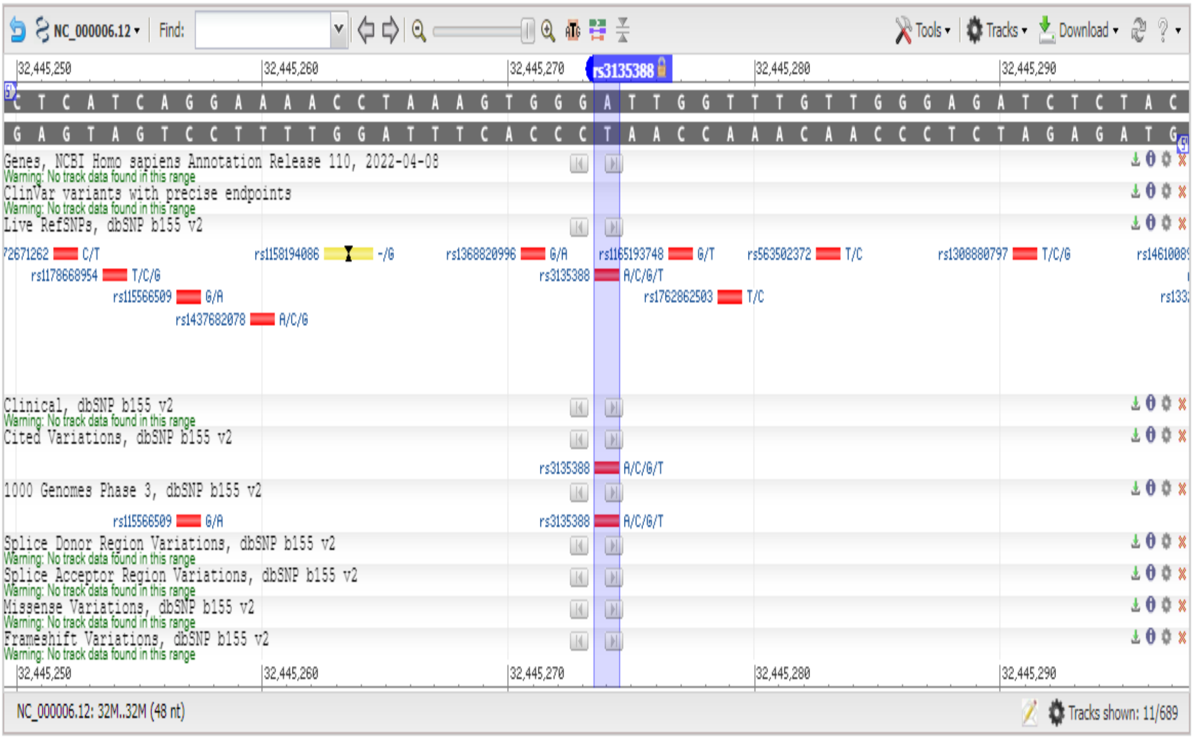


Figure (2-10): The exact position of rs3135388 and primers pair on human genome (NCBI sequence browser).

Wat Cut online software presentation of the enzyme **Pps I** selection to restrict **rs35445101** SNP. Properties of the accepted primer pair for **rs35445101** SNP is shown in table (2-8) as designed by this study.

Table (2-8): The accepted primer pair for rs35445101 and their properties.

primer	Sequence (5'->3')	Template strand	Length	Tm	GC%	Self omplementarity	Self 3' comp lementarity	Reference
Forward primer	GCAGGAAAGCTT TTCATCCTGCAA	Plus	24	62.59	45.83	8.00	3.00	Designed by this study
Reverse primer	CTCCTCTCCACT TAGAAAAGGTGT	Minus	24	59.72	45.83	3.00	0.00	
Product length	**250**							

The amplicon was cut with **Pps I** restriction enzyme to produce 47 and 203 bp fragment for A allele and 47,85 and 118 bp fragment for G allele as in figure (2-11)

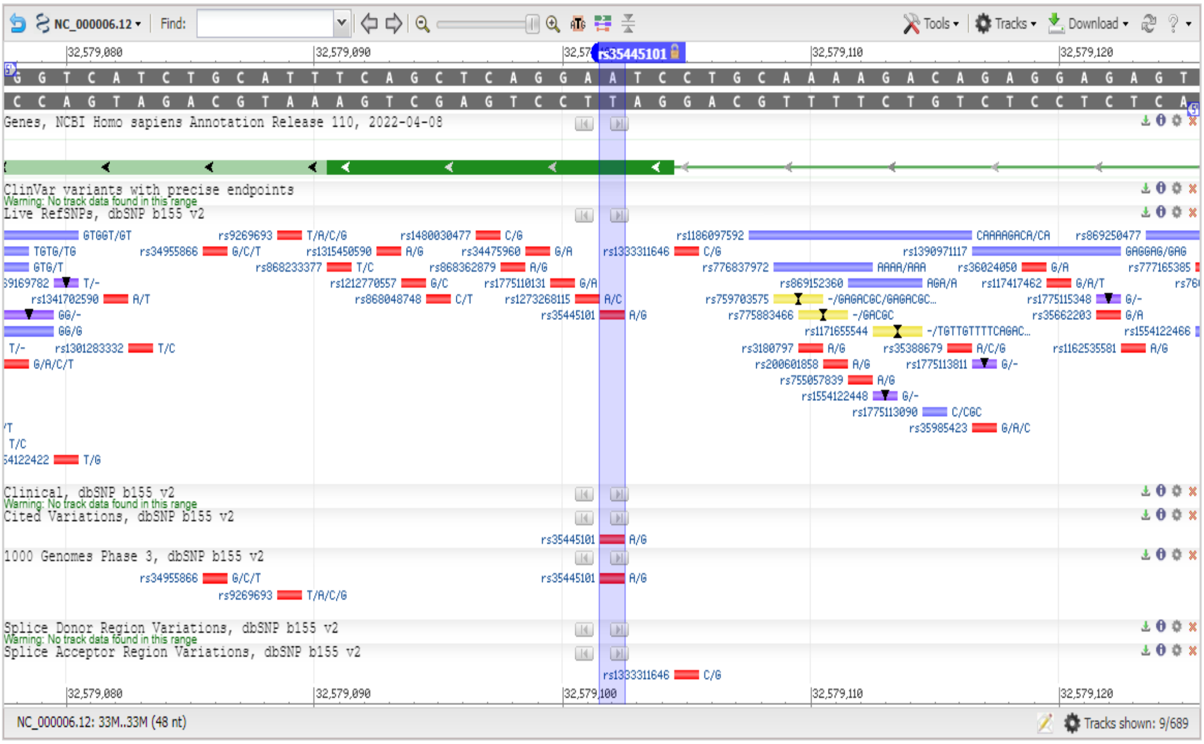


Figure (2-11): The exact position of rs35445101 and primers pair on human genome (NCBI sequence browser).

2.7.1.4 Reconstituting and dilution of primers

The nuclease-free H_2O was added to each primer to obtain a master stock that would be used again to obtain a working stock. The following steps were followed for reconstituting and diluting the primers :

1- The tubes were spin down before opening the caps.

2- The desired amount of free nuclease water was added according to the guidelines of manufacturer to obtain a 100 Pico moles/µl (Master Stock).

3- The tubes was vortex properly for re-suspend the primers evenly.

4- A volume of 10µl of the each primers stock was transferred to a 1.5 ml Eppendorf tube that contains 180µl of sterile, nuclease-free water (Working Stock).

5- The master stock was stored at -20^{o} C.

6- The working stock was stored at -20^{o} C.

7- The working stock was thawed on ice and vortexed before using in PCR and then stored at -20 ^{o}C

2.7.2 Extraction of Genomic DNA from Blood Sample

The Genomic DNA Purification Kit provided by Promega /USA was used to extract genomic DNA from blood samples, and the protocol Isolating Genomic DNA from Whole Blood (300 µl Volume) was followed. The following steps were followed in accordance with the manufacturer's instructions:

1. From Cell Lysis Solution, 900 µl was added to a sterile 1.5 ml microcentrifuge tube.
2. The blood sample gently mixed (collected in an EDTA tube to prevent clotting) until completely mixed, then transferred 300 µl of blood to a tube containing the Cell Lysis Solution and invert the tube six times to mix.
3. To lyse the red blood cells, the mixture was incubated for 10 minutes at room temperature (three times had been inverted during the incubation period). The mixture had been then centrifuged at 13500 rpm for 20 seconds at room temperature.
4. As much of the supernatant as possible was removed and decanted without disturbing the visible white pellet. In the 1.5 ml tube, about 20 µl of residual liquid remained.
5. The white blood cells were resuspended by vortexing the tube vigorously (15 seconds).
6. In the tube containing the resuspended cells, 300 µl Nuclei Lysis Solution was added. To lyse white blood cells, the solution was pipetted 6 times. In this stage, the solution should be very viscous.
7. In the nuclear lysate, 1.5µl RNAse solution was added, the sample was mixed 5 times by inverting, incubated at 37°C for 15 minutes, then cool at room temperature.

8. One hundred microliter of Protein Precipitation Solution has been added to the mixture (nuclear lysate) then vortex vigorously (20 second), after vortexing, small clumps of protein may appear.
9. At room temperature, centrifuged at 13500 rpm for 3 minutes.
10. Transfer the supernatant to a new 1.5 ml microcentrifuge tube containing 300μl of room temperature isopropanol.
11. By inversion, the solution was gently mixed until the white threadlike DNA strands formed a visible mass.
12. Centrifuged at 13500 revolutions per minute for 1 minute at room temperature. DNA was visible in the form of a small white pellet.
13. After decanting the supernatant, one sample volume of 70% ethanol was added to the DNA. After that, gently the tube had been inverted several times to wash the DNA pellet and the microcentrifuge tube's sides. Centrifuged at 13500 rpm for 1 minute at room temperature.
14. By using a sequence pipette tip, the ethanol has been carefully sucked. For 15 minutes, the tube was inverted on a clean absorbent paper to air-dry the pellet.
15. A 100μl DNA rehydration solution was added to the tube which was then incubated to rehydrate the DNA at 65°C for 1 hour. The tube taping mixes the solution regularly.
16. The DNA was kept frozen at -20°C (Hatada *et al*., 1991).

2.7.3 Determination of DNA Concentration and Purity

The DNA quantity and quality was measured by Nano-spectrophotometer (nano-drop) , employing the scanning ability of diode array from 200 to 320 Nm wave length , the absorbance profile then processed and analyzed to determine the DNA quantity and quality by calculating the 260/280 and 260/230 ratios. If a sample showed 260/280 ratio lesser than 1.8 and /or 260/230 ratio lesser than 2, it would be re-extracted (Hashim *and* Al-Shuhaib, 2020).

2.7.4 Agarose Gel Electrophoresis

This process was carried out according to (Lee *and* Bahaman, 2012).

Principle:

Agarose gel electrophoresis is a method of gel electrophoresis used in many scientific fields to separate macromolecules such as DNA fragments in a matrix of 1-2% dissolved agarose in TBE buffer. The DNA fragments such as PCR products were separated by applying an electric field to move the negatively charged DNA molecules through an agarose matrix from negative pole (cathode) to the positive pole (anode) and the DNA fragments were separated by size in the agarose gel matrix. The separated DNA may be viewed with stain, most commonly under UV light, and the DNA fragments can be extracted from the gel with relative ease.

Methods:

1. Agarose gel was prepared by dissolving 2 gm of agarose powder in 100 ml of (1X) TBE buffer in microwave or water bath.
2. Allowed the agarose to cool to 50°C then ethidium bromide (EtBr) was added to a concentration of 0.5 µg/ml and mixed, be careful to add EtBr in over than 50°C because the evaporation of EtBr may be carcinogenic when inhalation.
3. The comb was fixed at one end of the tray for making wells that were used for loading DNA sample then the agarose was powered gently into the tray, and allowed to solidify at room temperature. The comb was then removed gently from the tray.
4. The tray was fixed in an electrophoresis chamber which was filled with (1X) TBE buffer covering the surface of the gel, 5µl of DNA sample of PCR products was transferred gently by micropipette into the signed wells in agarose gel.
5. A volume of 5µl DNA ladder (50-1500 bp) was added to one well in order to estimated measure the size of DNA bands of PCR products.
6. The electric current was applied at 80-100 volt for 20-35 min.
7. The band was observed by a UV light box (UV transilluminator) at a wavelength of 320 nanometers and photographed with an electronic camera system.
8. The PCR product of study samples that give the same band (according to base pair size) that required in the present study was sent for detect DNA sequence.

2.7.5 Photo documentation

Agarose gel was visualized in a UV transilluminator provided with gel documentation unit, agarose gel was placed above the UV transilluminator device, the gel was exposed to UV light and the photos was captured using Nikon digital camera. As shown in (appendix 2)

2.8 Convential Polymerase chain reaction (PCR)

The PCR amplicon that's required for rs9271366, rs3135388 and rs35445101 in current study was amplified by PCR-amplification performed in a programmable thermal cycler gradient PCR system. The amplification of each target region was first optimized by gradient PCR and the best efficient and specific annealing temperature that produce the most efficient , specific product was chosen for further PCR amplification procedure , the latter was performed according to addition the components for amplification of each SNP as in table (2-9)

Table (2-9): The starting PCR reaction ingredients concentration for each optimization processes .

No	Ingredient	Concentration	Added volume
1	Master mix	2.5 x	8 µl
2	DNA	20 ng / µl	2 µl
3	Primers	10 pmol / µl	1 µl for each
5	Molecular grad water		7.5 µl
Total volume			**20** µl

2.8.1 Optimization of PCR Conditions

Optimization of PCR was done by several attempt to detect the best annealing temperature and the best number of amplification cycles. The components of PCR for all the amplified fragments and the optimized PCR programs are stated in table (2-10) and table (2-11) respectively.

Table (2-10): PCR Condition for genotyping of rs9271366 Alleles: G>A, rs3135388 Alleles: A>G and rs35445101 Alleles: A> G

No	Stage	Temperature	Time	Number of cycle
1	Initial denaturation	94 ^{0}C	5 min.	1
2	Denaturation	94 ^{0}C	30 sec.	
3	Annealing	Gradient ± 6^{0}C of the lowest primer Tm	30 sec	35
4	Elongation	72 ^{0}C	30 sec	
5	Final elongation	72 ^{0}C	5 min.	1

After optimization , the best thermo-cycling conditions for rs9271366, rs3135388 and rs35445101 SNPs were listed in table (2-11) these conditions produce the most specific and sufficient PCR product . Then the amplified products were separated by electrophoresis through 2% agarose gel stained with ethidium bromide as shown in (appendix 3).

Table (2-11): Optimized thermo-cycling condition for rs9271366 G>A, rs3135388 A>G and rs35445101 A> G PCR-RFLP genotyping.

<table>
<tr><th>No</th><th>Stage</th><th>Temperature</th><th>Time</th><th>Number of cycle</th></tr>
<tr><td>1</td><td>Initial denaturation</td><td>94 ^{0}C</td><td>5 min.</td><td>1</td></tr>
<tr><td>2</td><td>Denaturation</td><td>94 ^{0}C</td><td>30 sec.</td><td rowspan="3">35</td></tr>
<tr><td>3</td><td>Annealing</td><td>60^{0}C</td><td>30 sec</td></tr>
<tr><td>4</td><td>Elongation</td><td>72 ^{0}C</td><td>30 sec</td></tr>
<tr><td>5</td><td>Final elongation</td><td>72^{0}C</td><td>5 min.</td><td>1</td></tr>
</table>

2.8.2 Restriction Digestion for PCR-RFLP

The restriction reaction carried out by mixing the following :

1- Five ul of PCR product .

2- Two units of the selected restriction enzyme .

3- Restriction buffer 1.5 ul (each restriction enzyme has its restriction buffer supplied by the manufacturer).

4- Bovine serum albumin 0.1 ul.

5- The reaction mixture then completed to 15 ul by molecular grad water.

The reaction mixture incubated in 37 °C water bath for overnight .

2.9 Statistical Analysis

All statistical calculation were performed by the using of SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010, Microsoft Corp. USA). A $P < 0.05$ was considered statistically significant. Chi-square test to assess the categorially association variables and genetic association, according to (Solé *et al*., 2006).

Allele frequencies of genes were calculated by direct gene counting methods, while a significant departure from Hardy-Weinberg (H-W) equilibrium was estimated using H-W calculator for two alleles, which is available free online at:
http://www.had2know.com/academics/hardy-weinbergequilibriumcalculator-3-ale;es.html.

Hardy-Weinberg equilibrium is the expected frequencies of genotypes if mating is non –assortative and there are no mutations from one allele to another. When there are two alleles for a particular gene; A and B, and their respective population frequencies are p and q, then the expected frequencies of the genotypes AA, AB, BB are p2, 2pq and q2, respectively. Significant differences between the observed and expected frequencies are assessed by Pearson‟s Chi-square test it (Graffelman *et al*., 2017).

Haplotype and linkage disequilibrium was analysis by SHEsis Plus (Shen *et al*., 2016).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Age Distribution for Rheumatoid Arthritis Disease

This study included clinically confirmed 50 RA patients and it was found that the number of RA cases have an increase in age groups (49-56) and (57-64) years old which comprise 16 patients (32%) and 13 patients (26%) respectively. Figure (3-1) displayed the age distribution of RA patients.

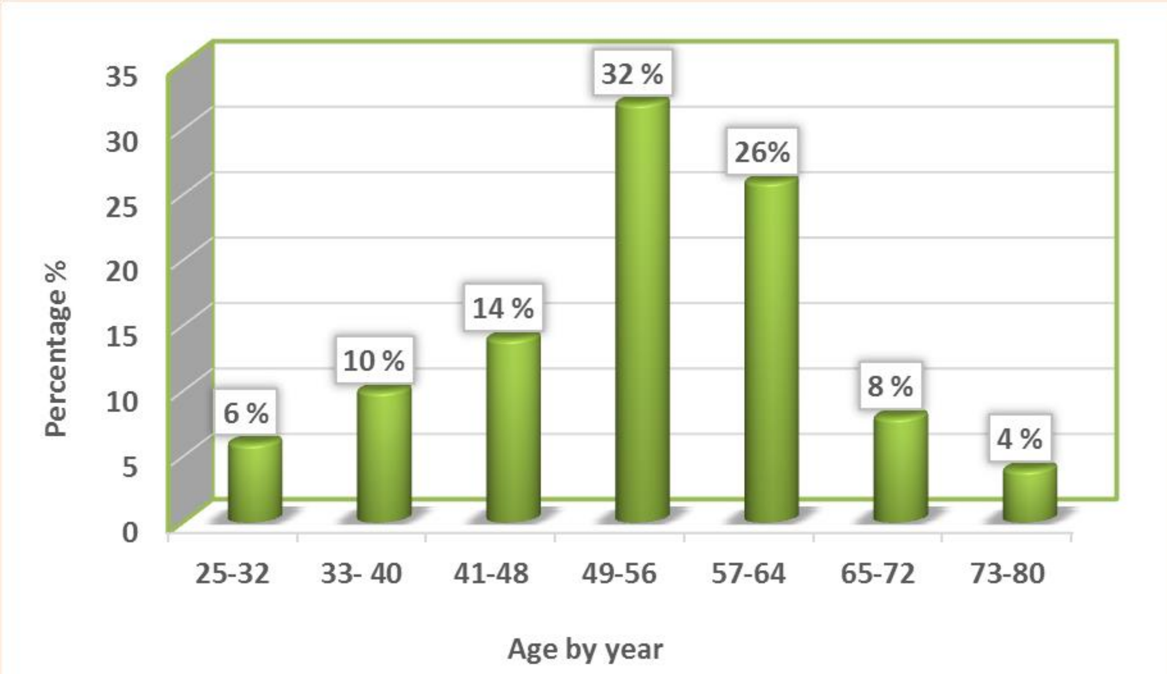


Figure (3-1): Distribution of RA patients according to age.

The result consistent with (Alpay-Kanıtez *et al*., 2019; J. Wang *et al*., 2022) who found the RA with top occurrence at 40-60 years, also this result is consistent with Hussein,(2017) who indicate that the RA highest prevalence within age > 50 years (52%). Arnold *et al*., (2014) showed in study 1809 patients, of which (24.4%) were of the young patients (49.7%) of the middle age (42-63) years while (25.9%) were of the old age (64-88) years. Mangnus *et al*., (2015) referred to the relationship between age and acuteness of joint erosion. The patients have 1.026-fold more joint erosion per year increase in age. Tesser *et al*., (2019) showed in study 592 RA patients, 515 patients were less than 65 years and 77 patients were more than 65 years.

The most common infections are upper respiratory infections to patients less than 65 years (13.2%) and (11.8%) were in patients more than 65 years.Boeters *et al*., (2017) refered to a change point at age 60 years a similar result was obtained (P < 0.001) for an relation between ACPA existence and age of onset in patients aged > 60 years.

3.2 Sex Distribution for Rheumatoid Arthritis Disease

The females were more affected 38 (76%) in RA disease than male 12(24%) (figure 3-2).

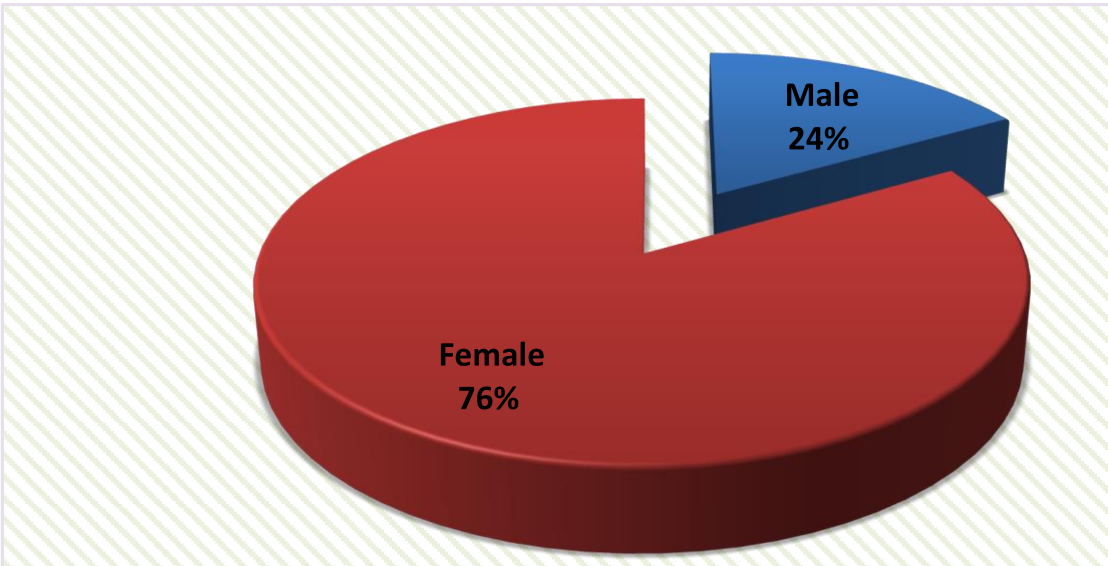


Figure 3-2: Distribution of RA patients according to Sex.

The result of the present study indicated that RA is more common in females than males may due to effect of some hormones or may be the influence of genetic factors. Immunization studies suggested that females produce a higher titer of antibodies than males in fact females in general tend to mount more vigorous immune responses this is particularly apparent in young females as well as woman tend to have higher levels of CD4+T cells and significantly higher levels of serum IgM. This result in agreement with

(Al_Badran *et al*., 2022) who showed from 2014 to the end of 2019 the total number of outpatient visits to the Rheumatology Clinic of Marjan Teaching Hospital was 97372 with an average of 19474 visits per year. During this period approximately 1814 patients developed RA, 1599 women (88%) and 237 men (13%). The number of patients was 201 (11%) in 2014, 360 (19.8%) in 2015, 280 (15.4%) in 2016, 250 (13.7%) in 2017, 414 (22.8%) in 2018 most recently 319 (17%) in 2019. This result in agreement with (van Vollenhoven, 2009) indicated that the females represent major cases of RA illustrative that over representation of females was not understandable, but may involve genetic (X-linked) and hormone factors. Othman *et al*., (2016) who indicated that the RA influence is more prevalent in females (82.5%) than male (17.5%), also this result agreed with (Metli *et al*., 2015) who showed the number the RA patients was 55 females (84.6%) and 10 males (15.4%) patients. Hunter *et al*., (2017) showed the comorbidity 33% of the patients with no variation among females and males. At baseline there is similarity in disease and function features. Coffey *et al*., (2019) showed 148 females and 66 males in a study of 214 RA patients. The mean of the female age was 53.6 years, while 59.1 years were for men. Percentages of females and males had more than 10 joints included at the time the 2010 criteria were non-significant and similarities (82% vs 85%). But in females the joint swelling to more than 10 joints were significant and longer than males (median 65 vs 0).Kawatkar *et al*., (2019) showed an incidence of RA which was a higher rate in females [48% of the 100,000 person] in 1995 while incidence of RA in 2014 was more in females [83% of the 100,000 persons] compared with the males was [27% of the 100,000 person]. That was shown no variation in the patients with RA and control groups in terms of sex and age. Also, the current study is agreed with other study carried out in Iraq which was found a high percentage of females patients with RA (Ataee *et al*., 2015).

3.3 Duration of Rheumatoid Arthritis Disease

Most RA patients have a duration of disease of (1-4) and (5-8) years 20 (40%) and 12 (24%) for each group (table 3-1).

Table(3-1): Duration of RA disease.

Group	Duration of RA disease (Year)	Number of RA patients	Percentage of RA patients
A	1-4	20	40%
B	5-8	12	24%
C	9-12	9	18%
D	13-16	4	8%
E	17-20	4	8%
F	21-24	1	2%
Total		50	100%

The result of the current work refers the number of patients with RA was more in the last years than previous years that means the RA more incidence in the present time than past time. This result in agreement with (Nair *et al*., 2019) who showed the incidence of RA disease demonstrated variation within the study period ranging from 33 per 100,000 to 73 per 100,000 while the prevalence of RA increased over time from 482 per 100,000 in 2001-2002 to 683 per 100,000 in 2014–2015. Ramos-Remus *et al*., (2015) refer in this study which include 279 RA patients the mean duration of RA patients was 10 years. Also this result agrees with (Alsubaie *et al*., 2018) who indicated that the RA duration was less from six months to more than ten years; only 5(2.5%) their duration of disease was least than six months while the duration from six months to two years was 35(17.5%) from two years to five years was 42(21%) from five years to nine years was 51(25%) and from ten and more was 67(33.5%).Horiuchi *et al*., (2017) shows the onset of disease with young onset RA (YORA) patients were 32-58 years and early onset RA (EORA) patients were 60-83 years EORA duration of disease was 1-6 years while in YORA was 1-13 years. Kumagai *et al*.,

(2019) indicated that the significant variation among 70 EORA patients and 190 YORA patients for duration of disease and age the average of the duration for EORA and YORA was 6.66 and 14.7 years and the average age was 73.8 and 57.8 years. Vos *et al.*, (2017) showed the duration of disease was less two years for RA patients were (76.9%) positive for Anti-CCP2 and Anti-CCP3 antibodies while duration of disease were among two and five years in RA patients (72.2%) positive for anti-CCP2 and (77.8%) for anti-CCP3. But in RA patients with duration more than five years were (88.0%) positive for anti-CCP2 and (80.0%) positive for anti-CCP3.

3.4 Treatment of Rheumatoid Arthritis

The result of the current study indicated 42(84%) of the RA patients were using MTX and biological treatment. Moreover, all *Mycoplasma pneumonia* diagnosed were among 42 RA patients whose use RA treatment. This result indicated that the bacterial infection was more common in RA patients committed to treatment than non-committed to treatment (Figure 3-3).

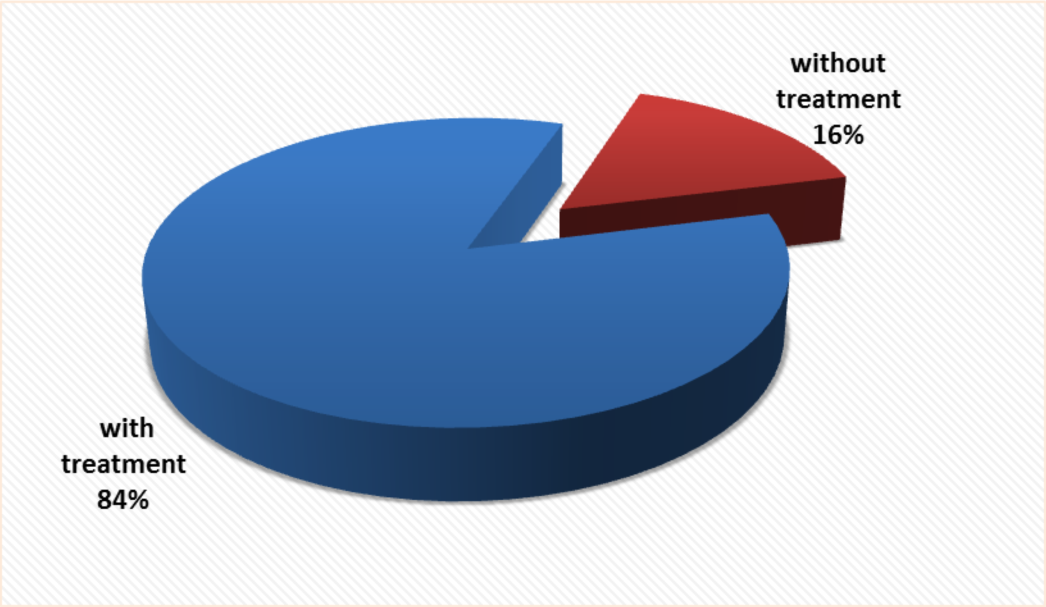


Figure 3-3: RA patients committed to treatment and non-committed to treatment.

The result was agreed with (Jani *et al*., 2019) who refered that the TNFα treatment to be related to severe infections compare with conventional synthetic disease modifying anti- rheumatic drugs (csDMARDs) with the passage of time differ increased risk in onset 6-12 months of therapies 31% higher risk of infections in dose biologic treated patient compare with csDMARDs. Kawashima *et al*., (2017) pointed out an estimation of long dated use of biologic treatment in oldest RA patients > 65 years with an incidence of risky infections who needed to hospitalize among biologics (64 patients) and non-biologics group (119 patients) observe non-significant variation in age (73.7±51 vs 73.7±5.8 years) sex (females; 78.1 vs 69.7%). The patient numbers with risky infections were (≥1) and were non-significant variation among both groups. Also non-significant variability in the incidence rate of risky infections was 8.0[(95 percent CI) 4.7-13.5] and [6.3 (95 percent CI) 4.1- 9.5] per 100 person-years of the community of biologics and non-biologics respectively. Grøn *et al*., (2019) showed 456 risky infections in patients with RA who were treated during one year with (130 Abatacept, 206 rituximab, 120 tocilizumab), 299 of which were within first six months of therapies. The infections appears in patients treated with rituximab then the abatacept and tocilizumab during the first one year in Denmark.

3.5 Level of Anti-*Mycoplasma pneumoniae* IgG-Ab in RA Patients and Control group

3.5.1 Overall Level of anti-*Mycoplasma pneumoniae* IgG -Ab

The results showed significant (P-value < 0.05) increase in the serum concentration level of anti-*Mycoplasma pneumoniae* IgG antibodies in the RA patients infected with *Mycoplasma pneumoniae* as compared to the RA patients non infected with *Mycoplasma pneumoniae* and healthy individuals (table 3-2).

Table 3-2: Numbers of patients and level of anti-*Mycoplasma pneumoniae* IgG -Ab in RA patients and control group.

Study group	Anti-Mycoplasma IgG -Ab (ng/l)			
	Male	Female	Mean± Std. Deviation	P value
RA patients with Mycoplasma infection (18)	3	15	0.45384 ± 0.0718	*<0.000
RA patients without Mycoplasma infection (32)	9	23	0.14162± 0.0115	
Control (40)	13	27	0.12205 ± 0.0121	

***significant comparison (*P.* value < 0.05) among all groups.**

In this study, anti-*Mycoplasma pneumoniae* IgG antibody was studied as a marker for chronic and previous infection (Abbas *et al*., 2014). This result agreed with (Baseman and Tully, 1997) who showed the *Mycoplasma pneumoniae* may be included adhesion molecules that have sequences homology to mammals molecules this molecular mimicry may produce auto-reactive antibody. Also the results agreed with (Ramirez *et al*., 2005) who showed higher level of antibody against *Mycoplasma pneumoniae* in 78 patients with RA (P<0.001) compared with 156 healthy control group.

Sahebari *et al*., (2018) use Real time PCR for detecting *Mycoplasma pneumoniae* in patients with RA 2% of *Mycoplasma pneumoniae* was in RA patients synovial fluid. There is positive *Mycoplasma pneumonia* for three patients 77% of RA were females. Chu *et al*., (2019) elucidate the estimated risk of developing RA related to infections of *Mycoplasma pneumoniae,* his study results showed 3- 1-fold more RA risk (95% CI = 1.16-7.99) between the *Mycoplasma pneumoniae* group aged 65 and older patients diagnosed with *Mycoplasma pneumoniae* were also observed to have a prominent risk of developing RA in the first 2 years compared with the general population especially in 2 age groups (≤19 years and 65 years).Shams *et al*., (2019) showed the presence (11.1%) of the Mycoplasma DNA by PCR technique were out of 99 arthritis samples these patient samples were collected from patient's synovial fluid. Haier *et al*., (1999) showed 18 (53%) Mycoplasma spp DNA were out of 28 RA patients other 13 RA patients were recorded negative result (46.4%). *Mycoplasma pneumoniae* was (5/28) while the other species were *Mycoplasma fermentans* (8/28), *Mycoplasma hominis* (6/28) and *Mycoplasma penetrans.* Moreover, (Abbas *et al*., 2019) showed *Mycoplasma pneumoniae* had a role in inflammatory autoimmune diseases (67.7%) of the anti-*Mycoplasma pneumoniae* IgG antibody out of 64 patients with SLE were seropositive compared control group was not detected antibody. Schwab *et al*., (1993) indicated that superantigens have been role in pathogenesis of the autoimmune diseases arthritis development was involved of the *Mycoplasma arthritis* mitogen and toxic shock syndrome toxin. Kurata *et al*., (2014) showed *Mycoplasma pneumoniae* antigens (50 µg protein/ml), promoted proliferation of lymphocytes without IL-6 and TGF-β1 also antigens of Mycoplasma have a role to promote production of the IL-10, IL-17 and IL-6. Tobón *et al*., (2010) refered that many microorganisms were involved in progression of RA. *Mycoplasma spp*, *Escherichia coli*, *Proteus miribilis* and *Porphyromonas gingivalis* were implicated in the development of RA. Gilroy *et al*., (2001) noticed *Mycoplasma fermentans* DNA in 24 samples were of arthritis patients, synovial fluid were positive 6 (17%) of 35 patients with RA, 11(25%) of 44 patients were detected with seronegative arthritis. While 4 (17%) of 24 patients were with psoriatic arthritis. In comparing, non-positive synovial fluid samples from 19 patients were with osteoarthritis. Nir-Paz *et al*., (2006) reported that *Mycoplasma pneumoniae* may be imprisoned in the synovium of joints causing no symptoms. The inflammatory cause is still ideational. However, *Mycoplasma pneumoniae* may be involved. It would be difficult to detect by conventional means, since antibodies are likely to be present anyway. Johnson *et al*., (2007) showed that molecular PCR of 16S rRNA for detecting *Mycoplasma pneumoniae* in synovial fluid of the arthritis patients were 19 (79%) out of 24 RA patients, and 8 (80%) out of 10 Osteoarthritis (OA) patients.

3.6 Age Distribution for *Mycoplasma pneumoniae*

This study included clinically confirmed 18 MP patients and it was found that the number of MP cases have an increase in age group (49-56) years old which comprised (8) patientsout of 18 (44.44%). Figure (3-4) displayed the age distribution of *Mycoplasma pneumoniae* patients. The results were analyzed in two difference age groups patients and control. The mean age of patients group was 41.95± 13.414 years old while the mean age of control was 43.36± 14.039 years old.

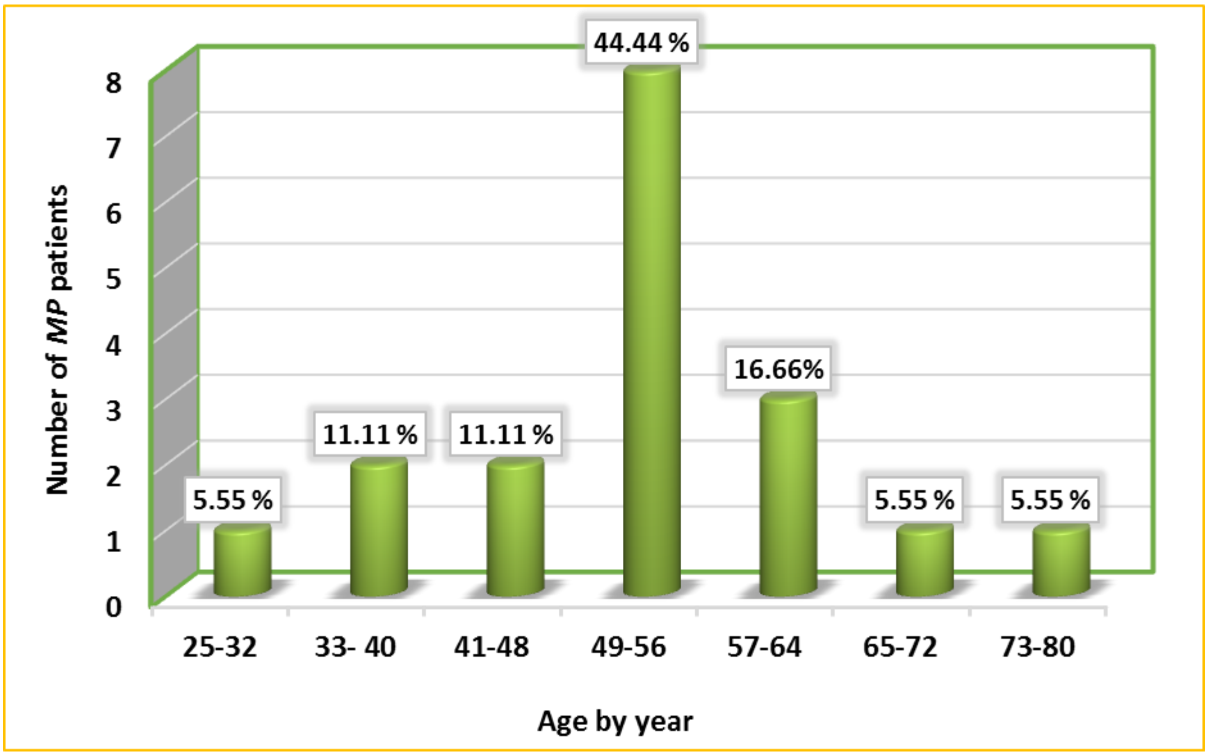


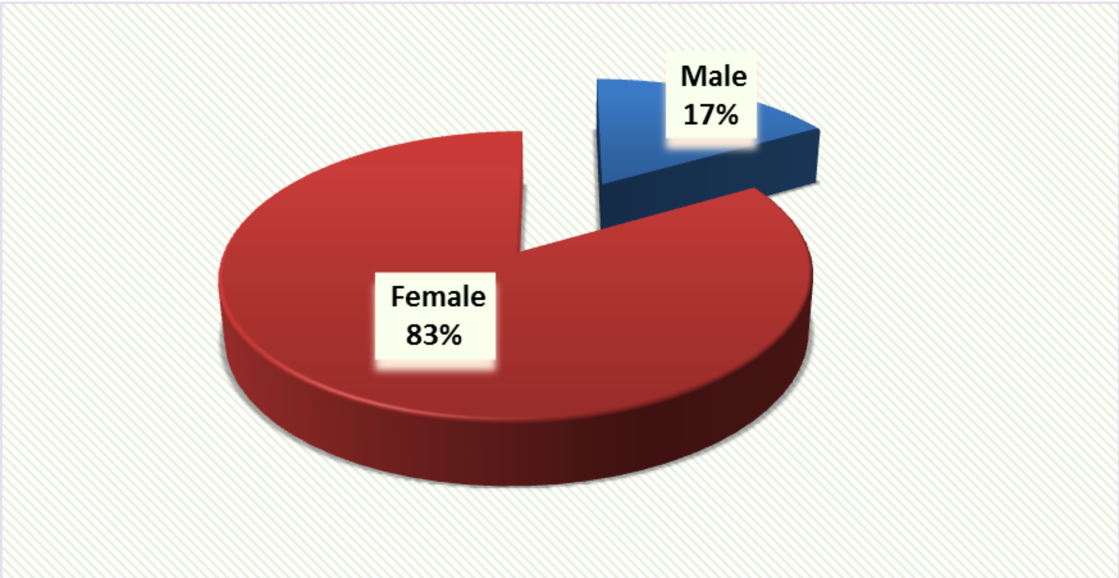
Figure (3-4): Distribution of *Mycoplasma pneumoniae* infection according to age.

There was no statistical difference in mean age between patients and control group ($p > 0.05$) This finding is required to prove age matching between control group and patients group for such a case control study.

This result was agreed with (AL-Baderi and Al-Azawi, 2022) who indicated that the *Mycoplasma pneumoniae* highest prevalence within age > 40 years old (56%).

3.7 Sex Distribution for *Mycoplasma pneumoniae*

The sex distribution of anti-*Mycoplasma pneumonia* -IgG -Ab in 50 RA patients was, females 15 (83%) were more affected than males 3 (17%). Figure (3-5).



Figure(3-5): Sex distribution of anti-*Mycoplasma pneumoniae* IgG-Ab in RA patients.

The results of the present study indicated that the females were more susceptible to *Mycoplasma pneumoniae* infection than males. This result agreed with Ataee *et al*., (2015) who showed the females 79 (60.3%) more than males 52 (39.7%) of 131 samples from RA patients with *Mycoplasma pneumoniae* and *Mycoplasma hominis* were detected by PCR in synovial fluid.

3.8 Cytokine profile of RA patients infected with *M.pneumoniae*

3.8.1 Interleukin-6 Level in serum of RA Patients and Control.

The results were shown that there was significant increase in the serum concentration level of IL-6 in the RA patients infected with *Mycoplasma pneumoniae* (**102.3433** ± **16.91405** ng/l) as compared to the RA patients non infected with *Mycoplasma pneumoniae (***85.9953** ± **13.75934** ng/l**)** and healthy individuals (**44.2393** ± **8.16846** ng/l) (table 3-3).

Table 3-3: IL6 concentration between RA patients with Mycoplasma infection and RA patients without Mycoplasma infection and control group.

Study group	IL6 (ng/l)		
	No	Mean ± Std. Deviation	P. Value
RA patients with Mycoplasma infection	18	102.3433 ± 16.91405	*<0.000
RA patients without Mycoplasma infection	32	85.9953 ± 13.75934	
Control	40	44.2393 ± 8.16846	

***significant comparison (P. Value < 0.05) among all groups.**

This results were agreed with Jarlborg and Gabay, (2022) study that reported a significantly increased levels of serum IL-6 in cases of RA compared with healthy individuals.

Levels of cytokine were significantly related with the disease activity in a cross-sectional analysis at baseline. Whereas IL-6 levels were related with CRP levels in Cohort study that measures cytokine levels in cases with high DAS activity and showed that when IL-6 is decrease the symptoms are improved. The result suggest that IL-6 family cytokines might be involved in the pathogenesis of RA and that levels of IL-6 family cytokines might reflect the activity of the disease (Chung *et al*., 2011).

Other opinion showed that serum levels of IL-6 and IL-11 were significantly elevated in patients with RA compared to healthy control. As shown in previous studies these results supports the hypothesis that IL-6 family cytokines are involved in the pathogenesis of RA(Narazaki *et al*., 2017).

Activated macrophages secrete IL-6, IL-1β and IL-23 cytokines that potentiates Th17 cell development thus enforcing chronic inflammatory response within RA inflammatory joints. Recent data in mice suggested that Th17 may also increase germinal center B cells to produce higher amounts of autoantibodies (Boniface *et al*., 2013).

After the revolution of TNF blockers that licensed to treat RA patients and from the observations of animal study IL-6 serum levels reduced by TNF blocker suggesting a present of interrelationship between these cytokines. They reported that IL-6 binds either to the receptor of membrane bound IL-6 or to a soluble receptor. IL-6 when binds to the soluble receptor it is not neutralized rather it can continue to signal via gp130. Antibodies of IL-6 receptor can inhibit this signaling process. Humanized antibody such as tocilizumab that binds both to soluble and to membrane-bound IL-6 receptor can blocks the receptor complex and lead to prevent the IL-6 trans membrane signaling. They were concluded that IL-6 is a key pro-inflammatory cytokine and is mainly included in the pathogenesis of RA (Yuan *et al*., 2022) and this is in agreement with present study suggestion.

IL-6 plays important role in regulating immune functions. It is involved in the infection process of MP and plays an important role in the pathogenesis of MP (He *et al*., 2018).

One study suggested that IL-6 is associated with the severity of MP. IL-27 is another important cytokine which is firmly associated with IL-6. It can induce the secretion of IL-6 (Guzzo *et al*., 2012) and can also block the activity of IL-6 by its subunit of IL-27 p28 which plays dual roles of proinflammation and anti-inflammation (Stumhofer *et al*., 2010).

IL-6 is associated with the severity of MP, But no reports about IL-6 relating to MP DNA loads and MP infection types in patients with *MP* have been found. IL-6 is an important cytokine that has dual functions in the process of inflammation (Akdis *et al*., 2016). It is mainly secreted by Th2 cells and can promote the secretion of protective antibodies to extracellular microbial pathogens. IL-6 plays an important role in the pathogenesis of *Mycoplasma pneumoniae* (Zhao *et al*., 2020).

L-6 is a pro-inflammatory cytokine that can induce joint damage through several signaling pathways. Tocilizumab and sarilumab were developed in the 2010s to inhibit the IL-6 receptor. Abatacept approved in 2005 is a T-cell modulator that has been shown to be efficacious in slowing RA progression and joint damage (Thomas *et al*., 2023)

3.8.2. Vascular Endothelial Growth Factor (VEGF-A) Level in Serum of RA Patients and Control

The results were shown that there is significant increase in serum concentration level of VEGF-A in RA patients infected with *Mycoplasma pneumoniae* (**81.3939** ± **14.79369** ng/l) as compared to healthy individuals (**48.1068** ± **5.10710** ng/l), but the results of RA patients with *Mycoplasma pneumoniae* infection and RA patients without *Mycoplasma pneumoniae* infection were non-significant (P > 0.05) (table 3-4).

Table(3- 4): VEGF-A concentration between RA patients with Mycoplasma infection and RA patients without Mycoplasma infection and control group.

Study group	VEGFA (ng/l)		
	No	Mean ± Std. Deviation	P. Value
RA patients with Mycoplasma infection	18	81.3939 ± 14.79369	<0.000
Control	40	48.1068 ± 5.10710	
RA patients with Mycoplasma infection	18	81.3939 ± 14.79369	<0.911
RA patients without Mycoplasma infection	32	80.9641 ± 11.85173	

Angiogenesis is a prominent feature of rheumatoid synovitis. Formation of new blood vessels permits a supply of nutrients and oxygen to the augmented inflammatory cell mass and so contributes to perpetuation of joint disease. Vascular endothelial growth factor (VEGF) is a potent endothelial cell specific growth factor that is up regulated by pro-inflammatory cytokines and by hypoxia. This results was agreed with Taylor, (2002) who showed serum VEGF concentrations were elevated in rheumatoid arthritis (RA) and correlate with disease activity. VEGF is a dimeric glycoprotein that induces the proliferation and migration of endothelial cells to form new blood vessels and which increases vascular permeability. VEGF plays important roles during wound healing, embryonic development, the growths of certain solid tumors and during ascites formation (Kowanetz and Ferrara, 2006).

VEGF induces pro-inflammatory changes in chronic inflammation such as leukocyte accumulation collagen deposition and blood vessel alterations. Increased vascularity is one of the key features of RA synovitis since active angiogenesis leads to joint invasion, destruction and pain in the pathogenesis of chronic arthritis. It has been demonstrated that VEGF is a key factor in the formation and maintenance of pannus and is abundantly expressed in the synovial fluid and serum of RA patients (Paradowska-Gorycka *et al*., 2016; Sakalyte *et al*., 2022).

A number of inflammatory cell types participate in maintaining a mutually activating network in RA joints which leads to the establishment of a self-perpetuating cycle of autoimmunity. It has been documented that VEGF activates endothelial cells to produce chemokine's such as MCP-1 and IL-8 which may recruit monocytes around endothelial cells in synovial membranes where newly employed macrophages in addition to resident synoviocytes can produce TNF-α and IL-6 when stimulated by VEGF or via cell contact with activated endothelial cells (Konisti *et al*., 2013; Sakalyte *et al*., 2022).TNF-α and IL-6 in turn further enhance the capacities of macrophages and synoviocytes to secrete VEGF and stimulate endothelial cells to induce cell contact mediated macrophage activation which generates a positive feedback loop. Thus VEGF may serve as a functional bridge between endothelial cells and macrophages synoviocytes (Cha *et al*., 2007).

3.8.3 HLAD-RB1 Level in Serum of RA Patients and Control

The results were show that there was significant increase in the serum concentration level of HLAD-RB1 in the RA patients infected with *Mycoplasma pneumoniae* (**748.1100** ± **62.47271** ng/l) as compared to the RA patients non infected with *Mycoplasma pneumoniae* (**502.2916** ± **48.55055** ng/l**)** and healthy individuals (**398.4232** ± **4.11670** ng/l) table (3-5).

Table(3-5): HLA-DRB1 concentration between RA patients with Mycoplasma infection and RA patients without Mycoplasma infection and control group.

Study group	HLADRB1 (ng/l)		
	No	Mean ± Std. Deviation	P. Value
RA patients with Mycoplasma infection	18	748.1100 ± 62.47271	*<0.000
RA patients without Mycoplasma infection	32	502.2916 ± 48.55055	
Control	40	398.4232 ± 26.03628	

***significant comparison (P. Value < 0.05) among all groups.**

These results were agreed with Verbruggen *et al*., (2000) who showed that the mean HLA-DRB1 serum level in RA patients was significantly increased with 277±19 ng/ml compared to 142±13 ng/ml of healthy controls (P<0.001).

This result demonstrated that without *Mycoplasma* spp. form an construct decrease HLA-DRB1 expression in patients. During activation of T-cells, CD4+ T-cells interact with human leukocyte antigen (HLA) or major histocompatibility class II (MHC-II) molecules as well as co-stimulating molecules that are expressed on the surface of APC (Podojil and Miller, 2009).

The presence of *Mycoplasma pneumoniae* leads to the regulation of MHC class II antigen presentation on the cell surface. The role of CD4+ T-cells in RA chronic inflammation is also supported by its association with the particular MHC-II, HLA-DRB in the third hyper variable region which is the similar to amino acid with DR4. This interaction then leads to a more aggressive form of RA (Cope *et al*., 2007; Yap *et al*., 2018).

In recent studies, the occurrence of HLA-DRB1 risk alleles predisposes the formation of N-glycosylation sites in (anti-citrullinated protein antibodies) ACPA-IgG. Still, this predisposition's precise mechanism is unknown (Kissel *et al*., 2019). Nevertheless, due to the fact that HLA-DR (encoded by HLA-DRB1 gene) molecules are necessary for the activation of CD4+ T cell, we assume that it is likely that HLA-DRB1 risk alleles induce somatic hypermutation of ACPA leading to their pathogenicity (Wysocki *et al*., 2020).

This leads to the activation of complex immune responses and specific anti-citrullinated protein antibodies (ACPA) generation found in approximately 75% of RA patients (Van Venrooij *et al*., 2008).

T cell mediated immune responses are generally considered important for protection against Mycoplasmas causing local respiratory infections (Maes *et al*., 2021). T cells are key in the regulation of immune responses and have a critical impact on the development of Mycoplasma induced pneumonia (Dobbs *et al*., 2009). Cellular immune responses appear to be mainly responsible for intracellular infection clearance.

Rheumatoid arthritis (RA) is associated with HLA-DRB1 genes encoding the shared epitope (SE) a 5-amino acid motive. RA is usually preceded by the emergence of anti-citrullinated protein/peptide antibodies (ACPAs). Citrulline is a neutral amino acid resulting from post-translational modification of arginine involved in peptidic bounds (arginyl residue) by

PeptidylArginine Deiminases (PADs). ACPAs recognize epitopes from citrullinated human fibrin(ogen) (hFib) and can be specifically detected by the AhFibA assay. Five citrullinated peptides derived from hFib together represent almost all of the epitopes recognized by patients with ACPA-positive RA. The use of antibody fine specificities as markers of clinical phenotypes has become a major challenge (Larid *et al*., 2021).

The shared epitope (a five-amino acid motif encompassing positions 70 to 74 of the HLA-DRB1 chain) encoded in the major histocompatibility complex (MHC) is present in approximately 70% of patients with ACPA-positive RA (Huizinga *et al*., 2005; Larid *et al*., 2021).

It is thought that citrullinated auto antigen epitopes bind to HLA-DRB1 that contain the SE and are presented to $CD4^+$ T cells which contribute to autoimmunity (Scally *et al*., 2013). Moreover, SE is an important risk factor for severe bone destructive disease (Gorman *et al*., 2004).
study we showed that the frequency of HLA-DRB1 alleles was significantly increased in Hungarian RA patients compared to healthy subjects (31.3% vs. 10.9%, $P < 0.05$)

Anti-cyclic citrullinated peptide has been identified as a potential diagnostic and prognostic marker of RA. Citrulline is an unusual amino acid resulting from an enzymatically modified arginine residue present on certain human proteins. The presence of anti-CCP antibodies is highly specific and sensitive for RA (Sakurai *et al*., 2018). Determination of anti-CCP helps to distinguish RA from other arthropathies; furthermore, as a prognostic marker it may predict persistent erosive more aggressive synovitis (Gautam *et al*., 2020).

The major histocompatibility complex is encoded in human leukocyte antigen gene clusters on chromosome 6. Among MHC class II molecules various HLA-DR alleles have been associated with susceptibility to RA in several racial groups. In addition some HLA-DRB1 alleles have also been related to the severity and outcome of RA (Terao *et al*., 2019). These disease-associated HLA molecules share a common amino acid sequence in the third hyper variable region of the beta chain of the HLA-DR molecule (HLA-DRB1) hence the term “shared epitope”. SE is a sequence of five amino acids. Among the SE variants the QKRAA, QRRAA and RRRAA motifs have been described within various DRB1 variants (J. Guo *et al*., 2019).

There may be an association between SE positivity and the production of anti-CCP antibody. The presence of one or two shared epitope alleles has been associated with anti-CCP antibody positivity (Bolotin *et al*., 2015).

Moreover, unfavorable disease progression has been related to Anti-CCP production and SE positivity. HLA-DR3 has been related to anti-CCP-negative disease. However, there is little information available regarding possible associations between serum Anti-CCP antibody levels and HLA-DRB1 expression. In the present study we investigated associations of SE positivity with Anti-CCP positivity and serum levels. This is the first report on the possible relationship between SE and Anti-CCP in Hungarian RA patients.(Raslan *et al*., 2020)

3.9 Relation between *M.pneumoniae* infection and RA disease

The results were shown that there was 18:50 (36%) of the RA patients that infected with *M.pneumonia*e. They were divided into 3 males and 15 females depending on titer of anti-*M. pneumoniae* IgG. In this study, it was found that there was a positive relationship between infection with Mycoplasma and Rheumatoid arthritis disease. figure (3-6).

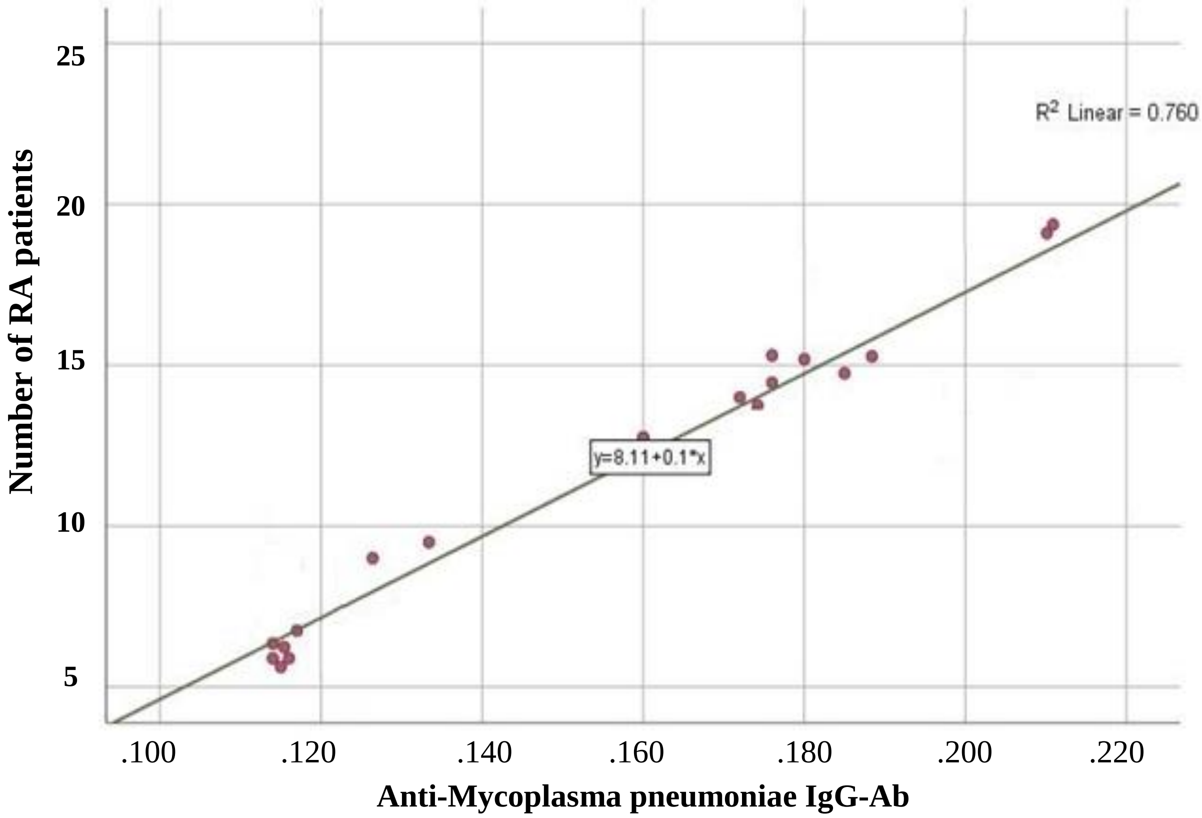


Figure 3-6: Relation between *M.pneumoniae* infection and RA disease.

The *M.pneumoniae* infection may be cofactors in the production of anti-double strand antibody (ADS) and anti citrullinated protein antibody (ACP) autoantibodies after infection and immune stimulation against this bacterium. The result refered to the highest infection rate among patients have ADS or ACP autoantibodies, Moreover most positive cases were with high titer (200 to 300). This referred to the relationship between *M.*

pneumoniae infection and formation of these autoantibodies. One of the results of other study was significant increase of IL-17 in patients with *M. pneumoniae* which has clear role in the rheumatoid arthritis as recorded by (Boniface *et al*., 2013) compared to healthy individual arthritic patients present significantly higher serum IL-17 levels. Accordingly many mouse models of arthritis enforce Th17/IL-17 role during the pathogenesis of RA. Beside IL-17, Th17 cells infiltrated the joints produce IL-22, IFN-γ and GM-CSF that are able to activate bone cells synoviocytes as well as cells infiltrated the joints such as macrophages. Activation of these cells results in the production of other pro-inflammatory cytokines, lysing enzymes and chemokine resulting in increasing migration of immune cells (Robert *and* Miossec, 2019).

Activated macrophages secrete IL-6, IL-1β and IL-23, cytokines that potentiates Th17 cell development, thus enforcing chronic inflammatory response within RA inflammatory joints. Recent data in mice suggest that Th17 may also increase germinal center B cells to produce higher amounts of autoantibodies (Peters *et al*., 2011). While TNF-α would be involved during early RA progression, IL-17 would rather contribute to the chronicity and late pathogenic responses, data accumulated over the years suggest the involvement of the IL-17/Th17 pathway during all progression stages of autoimmune arthritis and related inflammation (Luo *et al*., 2021).The patients in other studies with ANA and RF autoantibodies reveal low infection rate 29% and 15.78% respectively and low effect on its production.

Molecular mimicry in which infection by particular microbes is associated with the subsequent development of specific autoimmune diseases (Ayyez *et al*., 2014). Cytadherence is considered to be the initial step in the virulent process of pathogenic Mycoplasma. *M. pneumoniae* has adhisins that have an extensive sequence homology to mammalian structures. This molecular mimicry could generate immune autoreactivity (Pellefigues, 2020).

In present study there was significant differences at $P < 0.05$ in the prevalence of the serum antibodies (IgG) to *M. pneumoniae* among patients have ADS and ACP autoantibodies as compared with patients having another autoantibodies including Antinuclear Antibody (ANA) and RF. Indicating that patients who have had infection with these microorganism have more risk to produce ADS and ACP autoantibodies. This goes in agreement with (Ramirez *et al*., 2005) when thus reveal an association between the variable Antibodies against *M. pneumoniae* and rheumatoid arthritis(RA).This indicate that people who have had contact with this bacterium are about twice as likely suffering from RA in which ACPA have proved to be powerful biomarkers that allow the diagnosis of rheumatoid arthritis (RA) to be made at a very early stage (Aletaha *et al*., 2010) .

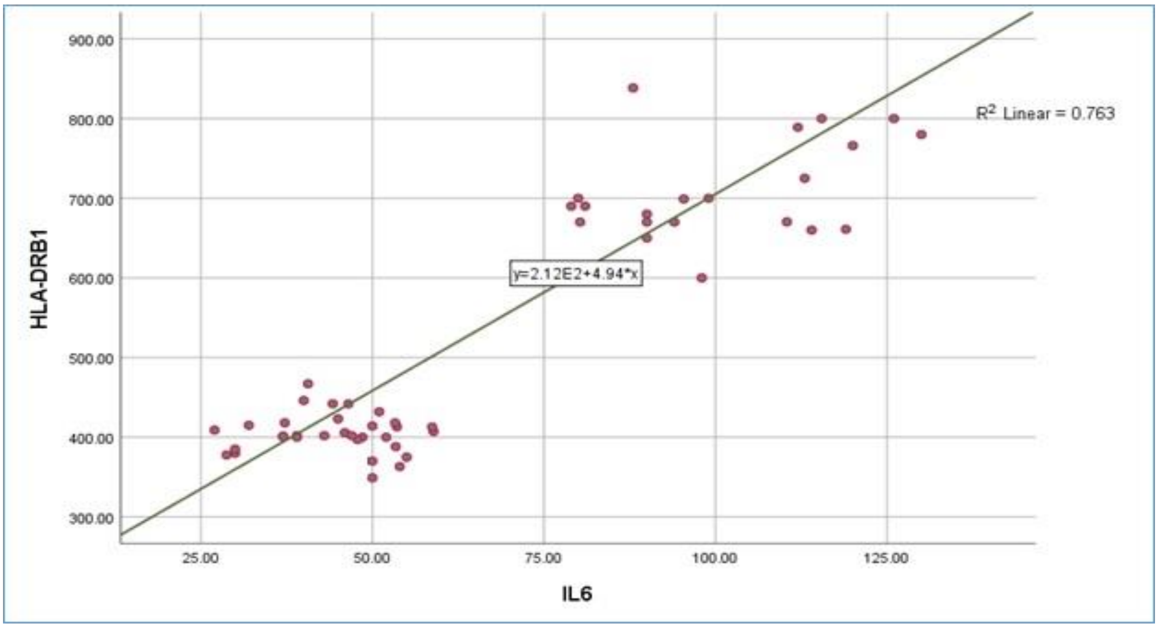
Although pathogenic mechanisms have not been established in autoimmune diseases mycoplasmal infections seem to play an important but not a well understood role in these diseases. Several characteristics of Mycoplasmas make them attractive as agents that may be responsible for triggering autoimmune responses. First, during their intracellular replication and release from host cells, mycoplasmas can capture antigens from the host cell surface and incorporate them into their cell membranes. This can lead to immune responses against these antigens and possibly autoimmune reactions. Second, mycoplasmal antigens can mimic host antigens and trigger immune responses against these antigens with resulting cross reactivity against host antigens. Third, Mycoplasmas can cause apoptosis of host cells with subsequent release of normal host antigens (De Luca *and* Shoenfeld, 2019).

Antinuclear antibody (ANA) is one of the autoantibodies which is occasionally detected in sera of patients with *Mycoplasma pneumoniae* (Andrade *et al*., 2022).

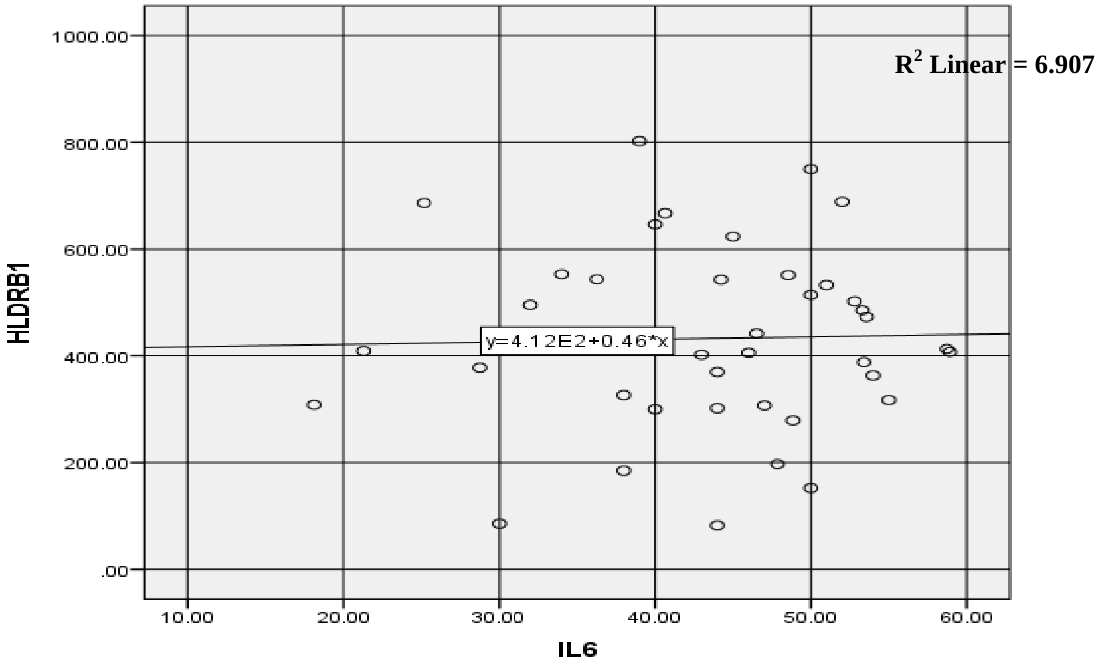
Pashnina *et al*., (2021) searched for the presence of ANA in a total of 22 serum samples collected from 11 patients who were diagnosed to have pneumonia caused by *Mycoplasma pneumoniae* depending on the culture results during the acute phase of the disease. Only one sample was found to be positive for ANA and the second serum sample of the same patient was found to be negative for ANA. The reason why the second serum sample of the patient in this study was negative for ANA may be the reversible polyclonal B cell activation. The results of these studies refer to *Mycoplasma pneumoniae* infection may be one of predisposing factors to production of ANA (Chen and Li, 2022).

3.10 Correlation of HLA-DRB1 and IL-6 for RA patients

The logistic regression between HLA-DR and IL6 in RA patients, show figure (3-7) . that positive correlation between them ,whereas the logistic regression for control is illustrated in figure (3-8)



Figure(3.7): Correlation of HLA-DRB1 and IL-6 for RA patients.



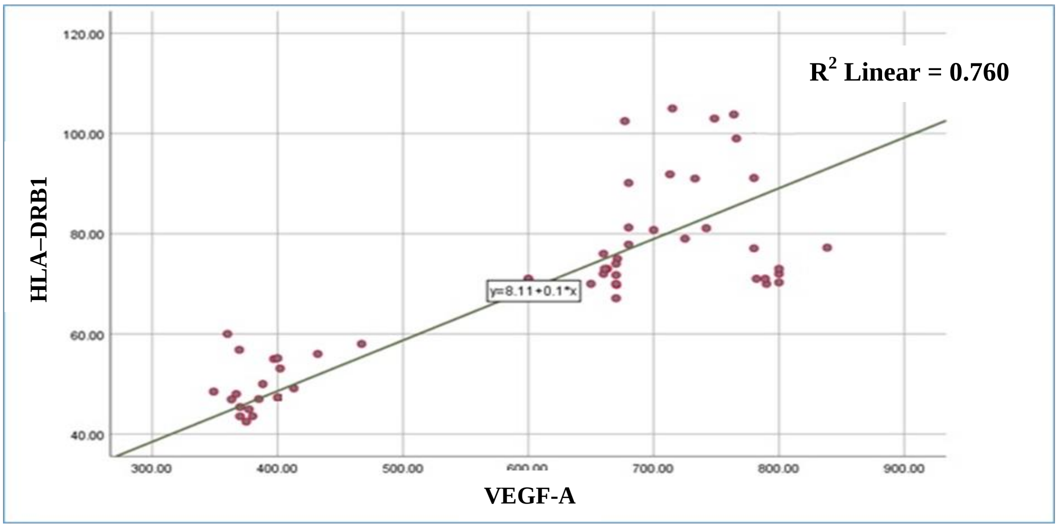
Figure(3.8): Correlation of HLA-DRB1 and IL-6 for control.

This may be attributed to the fact that increased HLA-DRB1 lead to increase in expression of IL6 while HLA-DRB1 was associated with causes serious immune problems in the hosts defenses (Gouel-Chéron *et al*., 2012).

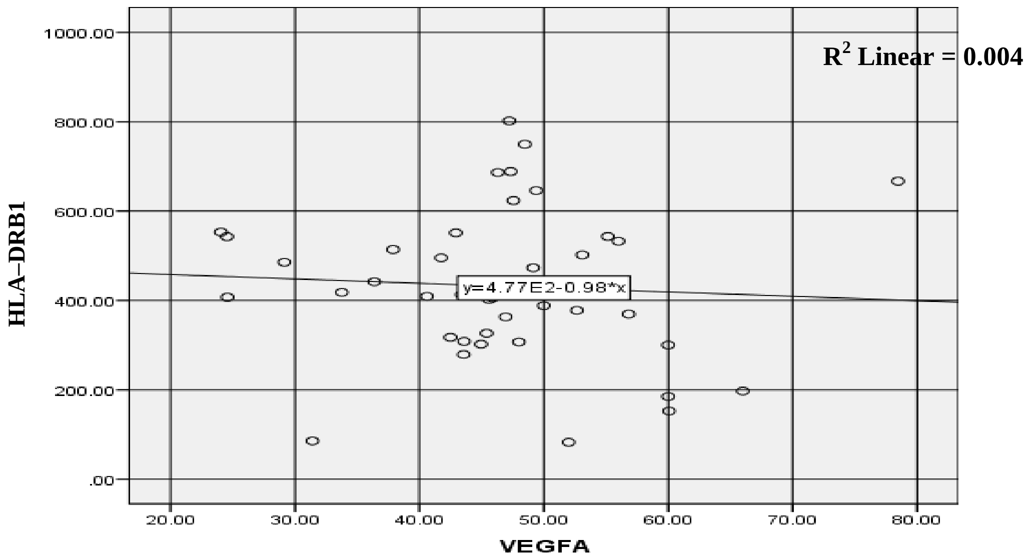
The main culprit of RA auto reactive B-cell also play role in autoantibody production T-cell activation and proinflammatory cytokine production. This B-cell dependent T-cell activation is via expression of co-stimulatory molecules local synthesis of cytokines such as IL-6,TNF-α ,IL12,IL23 and IL-1α (Nagafuchi *et al*., 2016).

3.11 Correlation of HLA-DRB1 and VEGF-A for RA patients

The logistic regression between HLA-DR and VEGF in RA patients, show figure (3-9) . That positive correlation between them ,whereas the logistic regression for control is illustrated in figure (3-10).



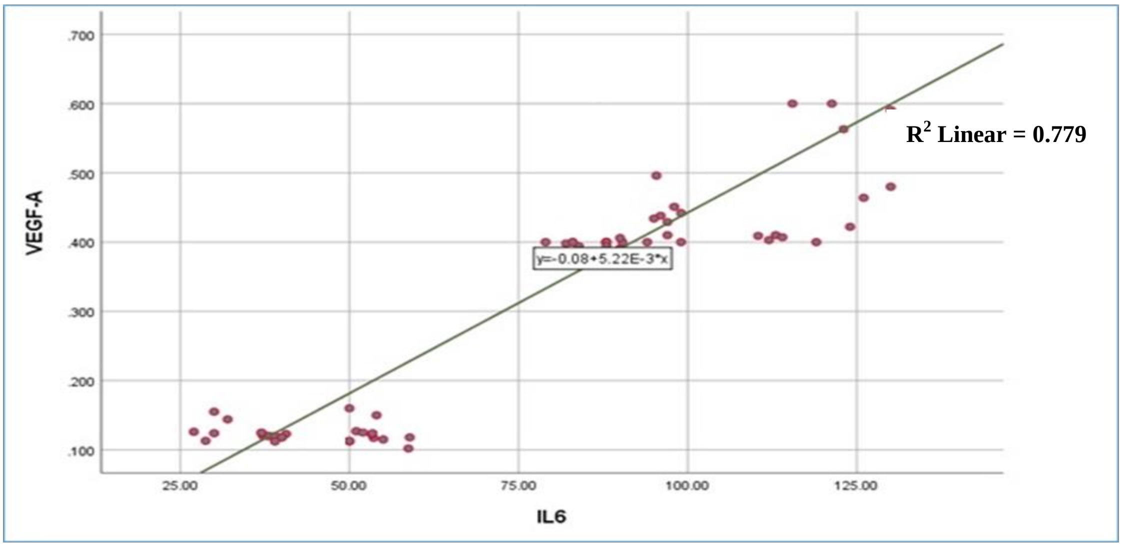
Figure(3.9): Correlation of HLA-DRB1 and VEGF-A for RA patients.



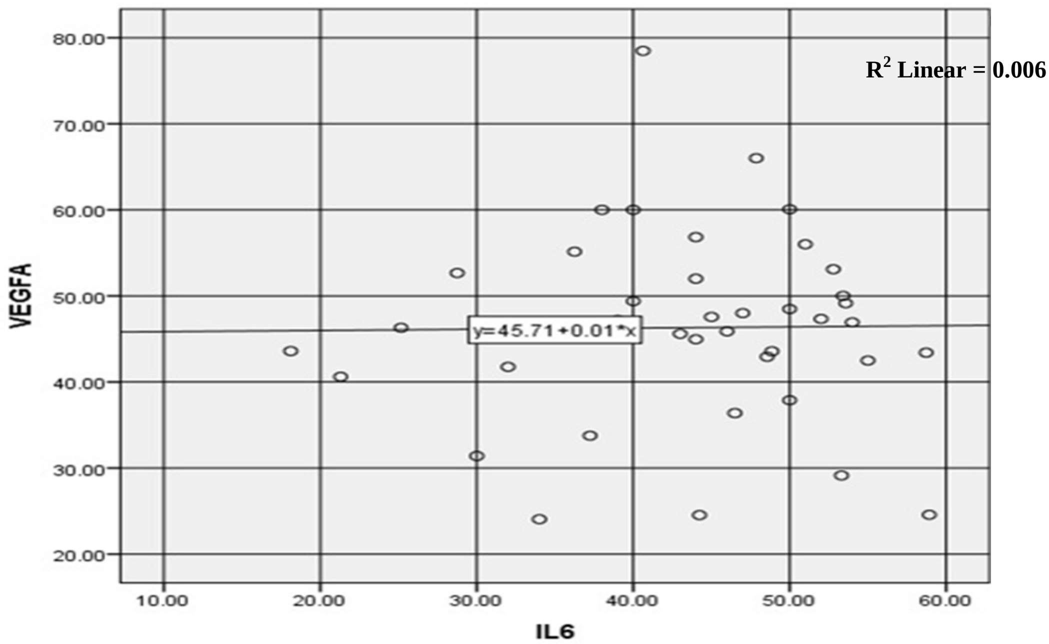
Figure(3.10): Correlation of HLA-DRB1 and VEGF-A for control.

3.12 Correlation of VEGF-A and IL-6 for RA patients

The logistic regression between VEGF-A and IL-6 in RA patients, show figure (3-11). That positive correlation between them ,whereas the logistic regression for control is illustrated in figure (3-12)



Figure(3.11): Correlation of VEGF-A and IL-6 for RA patients.



Figure(3.12): Correlation of VEGF-A and IL-6 for control.

These results suggest a role for IL-6 in regulating VEGF expression in platelets and their precursors. They also confirm the role of circulating platelets in the storage of VEGF and the increased VEGF load of platelets in RA patients**.**

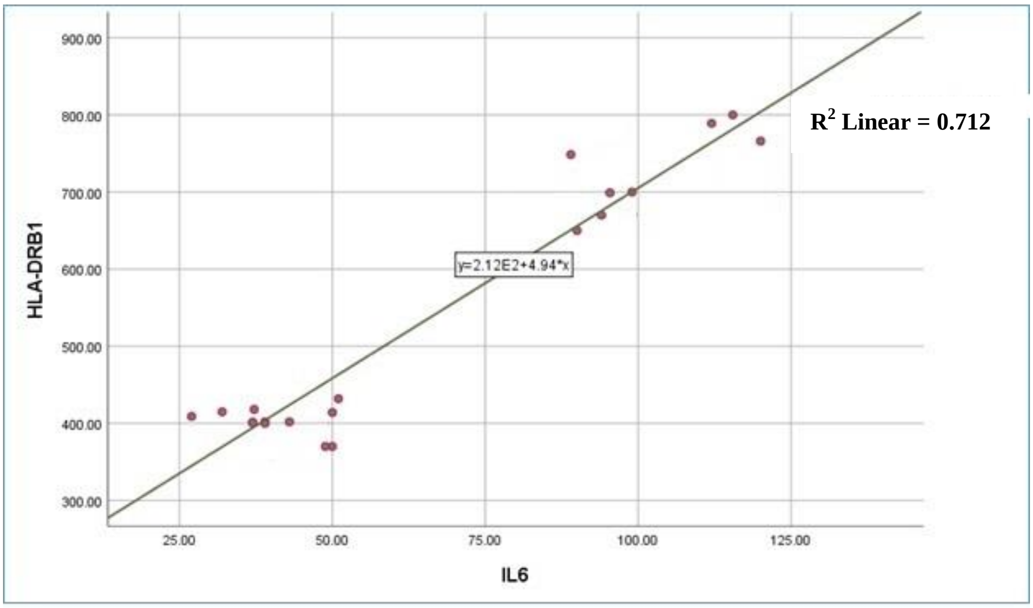
Serum IL-6 levels correlate with VEGF-A and the number of circulating platelets in RA patients with elevated platelet counts. Serum IL-6 is the most discriminative factor separating the healthy controls and RA patients. Several studies have shown that IL-6 might contribute to disease progression, particularly in patients with more advanced breast cancer (Oranskiy *et al*., 2012).

IL-6 production is induced by hypoxia and results in an increase of VEGF mRNA in various cell lines (Cohen *et al*., 1996). Interleukin 6 not only up regulates VEGF expression it increases aromatase activity in both stromal cells and tumor cells and promotes cell migration and inhibits apoptosis (Chen *et al*., 2017).

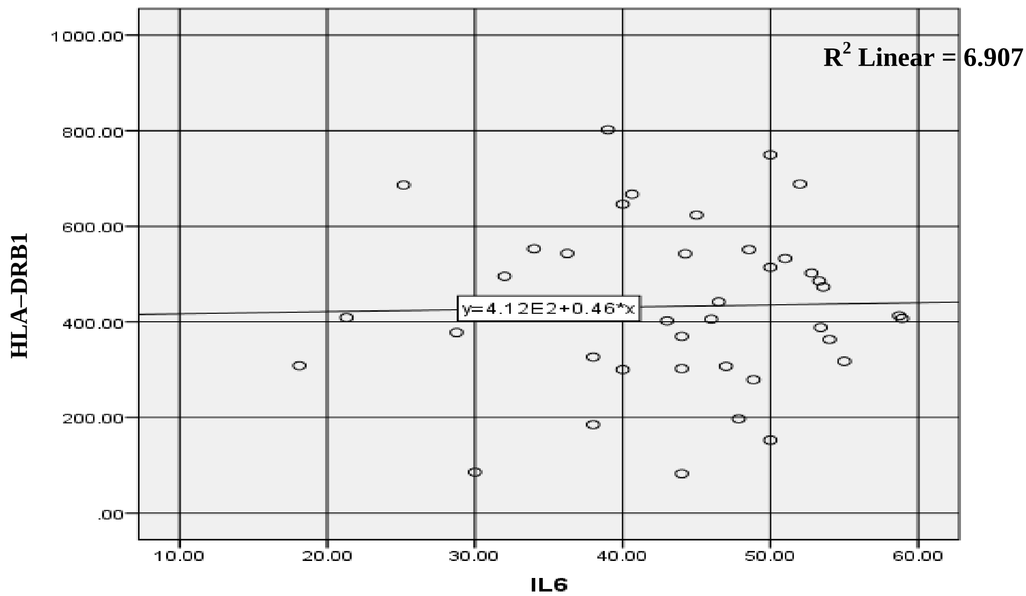
High plasma VEGF levels have been detected in RA patients. Interleukin 6 might influence related angiogenesis in several ways it stimulates megakaryocytes known to produce VEGF (Gedvilaite *et al*., 2019).

3.13 Correlation of HLA-DRB1 and IL-6 for RA patients infection with *M. pneumoniae*

The logistic regression between HLA-DRB1 and IL-6 in RA patients infection with Mycoplasma show figure (3-13) . That positive correlation between them whereas the logistic regression for control is illustrated in figure (3-14)



Figure(3.13): Correlation of HLA-DRB1 and IL-6 for RA patients with *Mycoplasma pneumoniae* infection.



Figure(3.14): Correlation of HLA-DRB1 and IL-6 for control.

The presence of *Mycoplasma pneumoniae* leads to the regulation of MHC class II antigen presentation on the cell surface. The role of CD4+ T-cells in RA chronic inflammation is also supported by its association with the particular MHC-II, HLA-DRB in the third hyper variable region which is the similar to amino acid with DR4. This interaction then leads to a more aggressive form of RA (Cope *et al*., 2007; Yap *et al*., 2018).

HLA-DR (encoded by HLA-DRB1 gene) molecules are necessary for the activation of CD4+ T cell we assume that it is likely that HLA-DRB1 risk alleles induce somatic hyper mutation of ACPA leading to their pathogenicity (Wysocki *et al*., 2020).

The activation of CD4+ T cell that lead to activated macrophages to secrete IL-6, IL-1β and IL-23 cytokines that potentiates Th17 cell development thus enforcing chronic inflammatory response within RA inflammatory joints. Recent data in mice suggest that Th17 may also increase germinal center B cells to produce higher amounts of autoantibodies (Boniface *et al*., 2013).

3.14 Association of HLA-DR gene polymorphism with RA

3.14.1 Association of rs9271366 gene polymorphism with RA

The allelic association analysis as listed in table (3.6) show that there is no significant ($p > 0.05$) differences in allele frequencies between patients and control.

Table (3.6): Allelic frequencies and association of rs9271366 with RA

Allele	Control (No.40)		Patient (No.50)		OR (95% CI)	P. Value
	Count	Proportion	Count	Proportion		
A	59	0.74	62	0.62	1.72 (0.91-3.27)	0.0966
G	21	0.26	38	0.38	0.58 (0.31-1.103)	

On the other hand cases group showed a significant deviation from Hardy –Weinberg comparing to control group which showed no deviation from Hardy –Weinberg ($P < 0.05$) as in table (3.7). This indicates that A allele a protective factor in patients with (OR 1.72 CI 95% 0.91-3.27) and that allele G considered a risk factor in healthy subjects with (OR 0.58 CI 95% 0.31-1.103).

Table (3.7): rs9271366 exact test for Hardy-Weinberg equilibrium.

Allele Frequency	AA	AG	GG	A	G	P. Value
All subjects	34	53	3	121	59	0.0016
Control	20	19	1	59	21	0.24
Case	14	34	2	62	38	0.0025

The results shows that G allele as dominant pathogenic allele in which the individual that carry GG and AG genotype have the susceptibility to the disease 2.57 fold comparing to individual that carry AA genotype (OR 2.57 CI 95% 1.07-6.17).Furthermore the genetic association of rs9271366 genotype with RA was analysis under different model inherit as listed in table (3.8) and figure (3.15).

Table (3-8) Genotype frequency and association under different model of inheritance for rs9271366 with RA.

Model	Genotype	Control	Case	OR (95% CI)	P. Value
Codominant	A/A	20 (50%)	14 (28%)	1.00	0.1
	A/G	19 (47.5%)	34 (68%)	2.56 (1.06-6.19)	
	G/G	1 (2.5%)	2 (4%)	2.86 (0.24-4.66)	
Dominant	A/A	20 (50%)	14 (28%)	1.00	0.032
	A/G-G/G	20 (50%)	36 (72%)	2.57 (1.07-6.17)	
Recessive	A/A-A/G	39 (97.5%)	48 (96%)	1.00	0.69
	G/G	1 (2.5%)	2 (4%)	1.62 (0.14-8.60)	
Over dominant	A/A-G/G	21 (52.5%)	16 (32%)	1.00	0.049
	A/G	19 (47.5%)	34 (68%)	2.35 (0.99-5.55)	

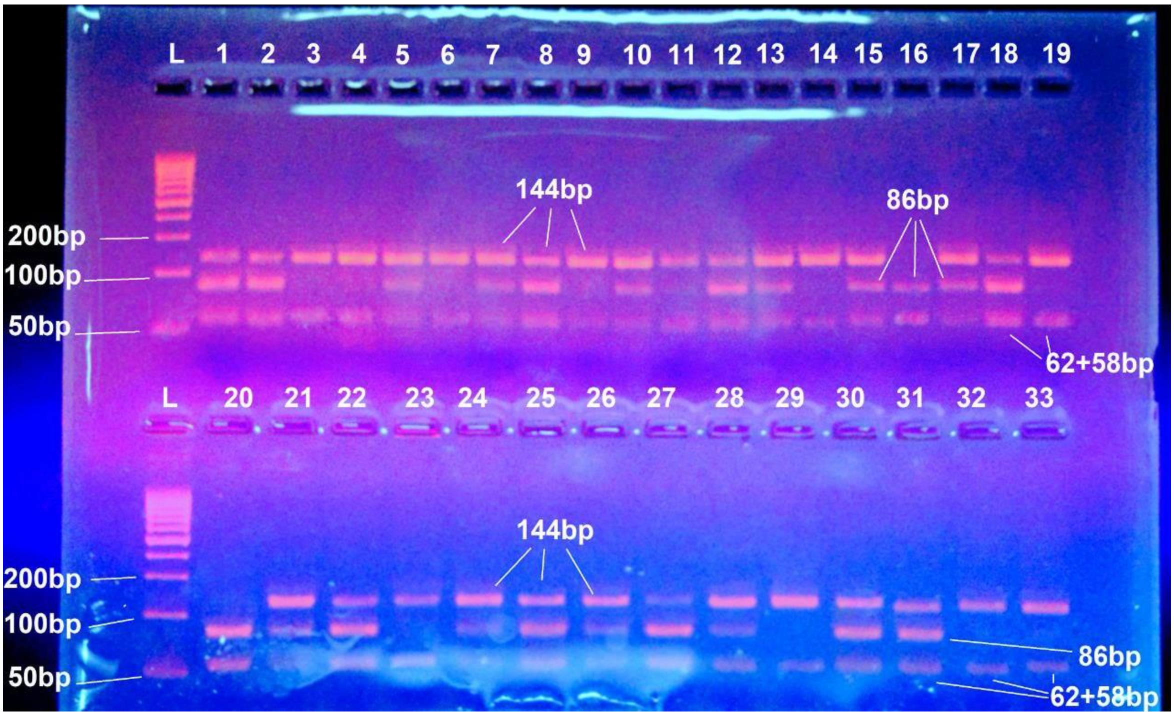


Figure (3-15): Genotyping of rs9271366 by PCR-RFLP technique , lanes L DNA ladder ;lanes 3,4,6,9,11,14,19,23,29,32 and 33 AA genotype;lanes1,2,5,7,8,10,12,13,15,17,18,21,22,24,25,26,27, 28,30 and 31 AG genotype ; lanes 16 and 20 GG genotype .

Liu *et al*., (2019) who showed the SNP rs9271366 (tag SNP for HLA-DRB1*15:01) confers the highest risk for Systemic lupus erythematosus (SLE) among the 13 MHC gene alleles that display association with SLE (p = 8.748E-10; OR = 3.5). Among the 26 non-MHC gene alleles analyzed.

Zúñiga *et al*., (2013) who showed that the SNP rs9271366 (OR = 2.06, 95% CI: 1.71–2.50) the highest risk for Systemic lupus, HLA-DRB1 the main and major association for autoimmune disease in Europeans and in most populations.

Williams *et al*., (2016) who found four independent SNPs in the MHC region associated with risk of SLE in African-American women. The most strongly associated SNP with SLE was rs9271366 near the HLA-DRB1 gene. Conditional haplotype analysis revealed three other SNPs (rs204890, rs2071349 and rs2844580) that were associated with SLE independent of the rs9271366 SNP. A genotype score combining the four newly identified SNPs showed an additive risk according to the number of high-risk alleles.

3.14.2 Association of rs35445101 gene polymorphism with RA

The allelic association analysis as listed in table (3.9) show that there is no significant ($p > 0.05$) differences in allele frequencies between case and control.

Table (3.9): Allelic frequencies and association of rs35445101 with RA

Allele	Control (No.40)		Case (No.50)		OR (95% CI)	P. Value
	Count	Proportion	Count	Proportion		
G	62	0.78	78	0.78	1.03 (0.51-2.09)	0.9361
A	18	0.22	22	0.22	0.97 (0.48-1.97)	

On the other hand cases group showed a significant deviation from Hardy Weinberg comparing to control group which showed no deviation from Hardy Weinberg ($P < 0.05$) as in table (3.10). This indicates that G allele a protective factor in patients with (OR 1.03 CI 95% 0.51-2.09) and that allele A considered a risk factor in healthy subjects with (0R 0.97 CI 95% 0.48-1.97).

Table (3.10): rs35445101 exact test for Hardy-Weinberg equilibrium.

Allele Frequency	GG	AG	AA	G	A	P. Value
All subjects	61	18	11	140	40	0.00018
Control	23	16	1	62	18	0.65
Case	38	2	10	78	22	0.0001

The results shows that A allele as recessive pathogenic allele in which the individual that carry AA genotype have the susceptibility to the disease 9.75 fold comparing to individual that carry GG and AG genotype (OR 2.57 CI 95% 1.19-79.78).Furthermore the genetic association of rs35445101 genotype with RA was analysis under different model inherit as listed in table (3.11) and figure (3.16)

Table (3-11): Genotype frequency and association under different model of inheritance for rs35445101 with RA.

Model	Genotype	Control	Case	OR (95% CI)	P. Value
Codominant	G/G	23 (57.5%)	38 (76%)	1.00	0.0001
	A/G	16 (40%)	2 (4%)	0.08 (0.02-0.36)	
	A/A	1 (2.5%)	10 (20%)	6.05 (0.73-50.40)	
Dominant	G/G	23 (57.5%)	38 (76%)	1.00	0.062
	A/G-A/A	17 (42.5%)	12 (24%)	0.43 (0.17-1.05)	
Recessive	G/G-A/G	39 (97.5%)	40 (80%)	1.00	0.0064
	A/A	1 (2.5%)	10 (20%)	9.75 (1.19-79.78)	
Over dominant	G/G-A/A	24 (60%)	48 (96%)	1.00	0.0001
	A/G	16 (40%)	2 (4%)	0.06 (0.01-0.29)	

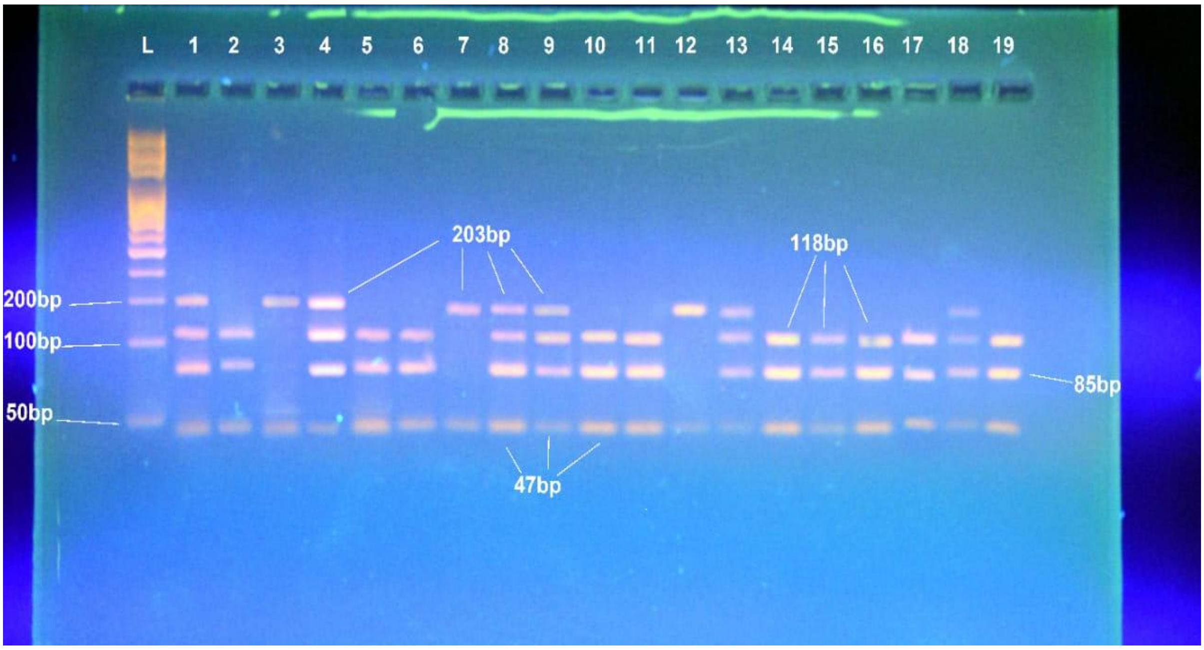


Figure (3.16): Genotyping of rs35445101 by PCR-RFLP technique , lanes L DNA ladder ; lanes 3,7 and 12 AA genotype ; lanes 1,4,8,9,13 and 18 AG genotype ; lanes 2,5,6,10,11,14,15,16,17 and 19 GG genotype.

The HLA system is composed of a family of class I and class II genes within major histocompatibility complex on chromosome 6 (Han *et al*., 2015). DRB1 is a member of HLA class II genes that is expressed in immune cells and functions as a key regulator of immune response to foreign antigens, as well as a discriminator of self from non-self-antigens (Kummee *et al*., 2007). From a genetic perspective a growing number of association studies have implicated the HLAD-RB1 genetic alterations in a broad range of immune mediated diseases including RA and HCC (Zong *et al*., 2017).

The strongest mechanism involved the modulation of major histocompatibility complex class II, DPB 1 (human leukocyte antigen [HLA]-DPB1-rs1042153) major histocompatibility complex class II, DQ beta 1 (HLA-DQB1- rs1130399, HLA-DQB1-rs1049056, HLA-DQB1-rs1049059, and HLA-DQB1-rs1049060) and major histocompatibility complex class II, DR beta 1 (HLA-DRB1-rs35445101) consequently affecting their regulatory roles in all the candidate pathways except the hematopoietic cell lineage pathway. Previous studies have demonstrated that HLA-DPB1 polymorphisms were significantly associated with the risk of HBV infection susceptibility (Wu *and* Qin, 2013).

3.14.3 Association of rs3135388 gene polymorphism with RA

Result show that A allelic frequency of rs3135388 in Babylon population is lesser than 1% in which all studied samples (90) show GG genotype only for this we exclude the rs3135388 from farther genetic association analysis . as in figure (3.17)

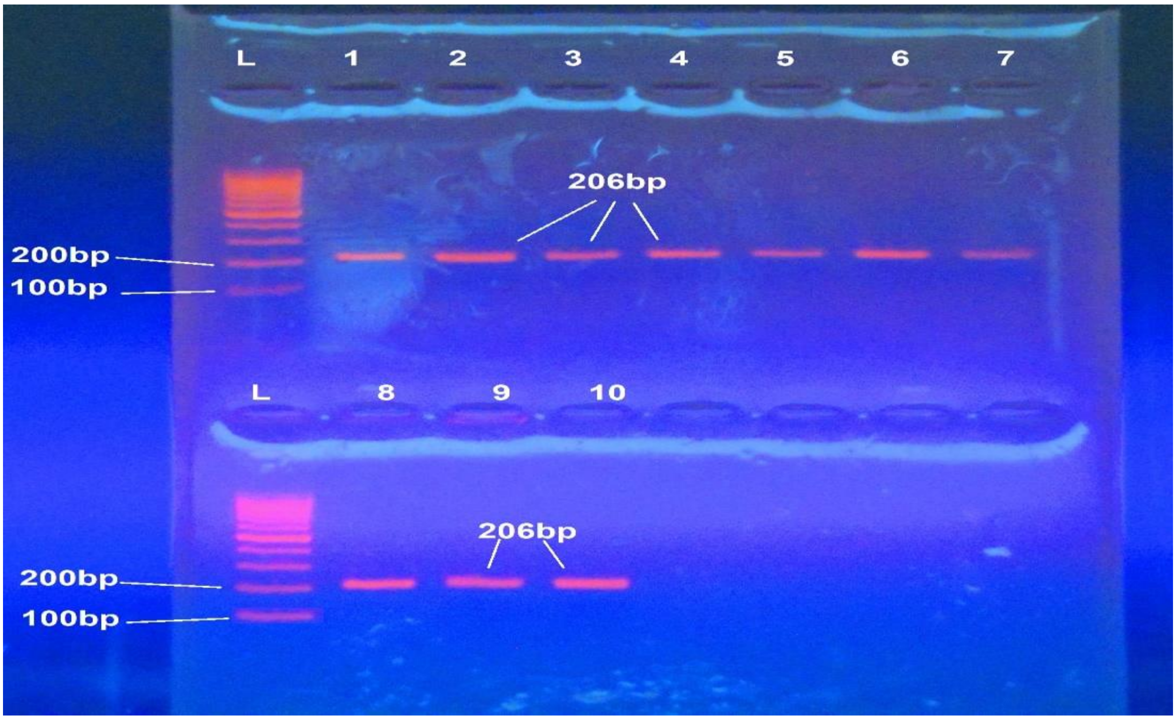


Figure (3.17) : Genotyping of rs3135388 by PCR-RFLP technique , lanes L DNA ladder ; other lanes GG genotype.

The global allelic frequency of rs3135388 show a huge variation among the different population Asia and Africa record the lowest frequency 1% and 3% respectively while the Europe record the highest frequency 12% figure (3-18) .This rare cases in could be explained negative result obtained in this study regarding rs3135388 (Ghorbani, 2022).

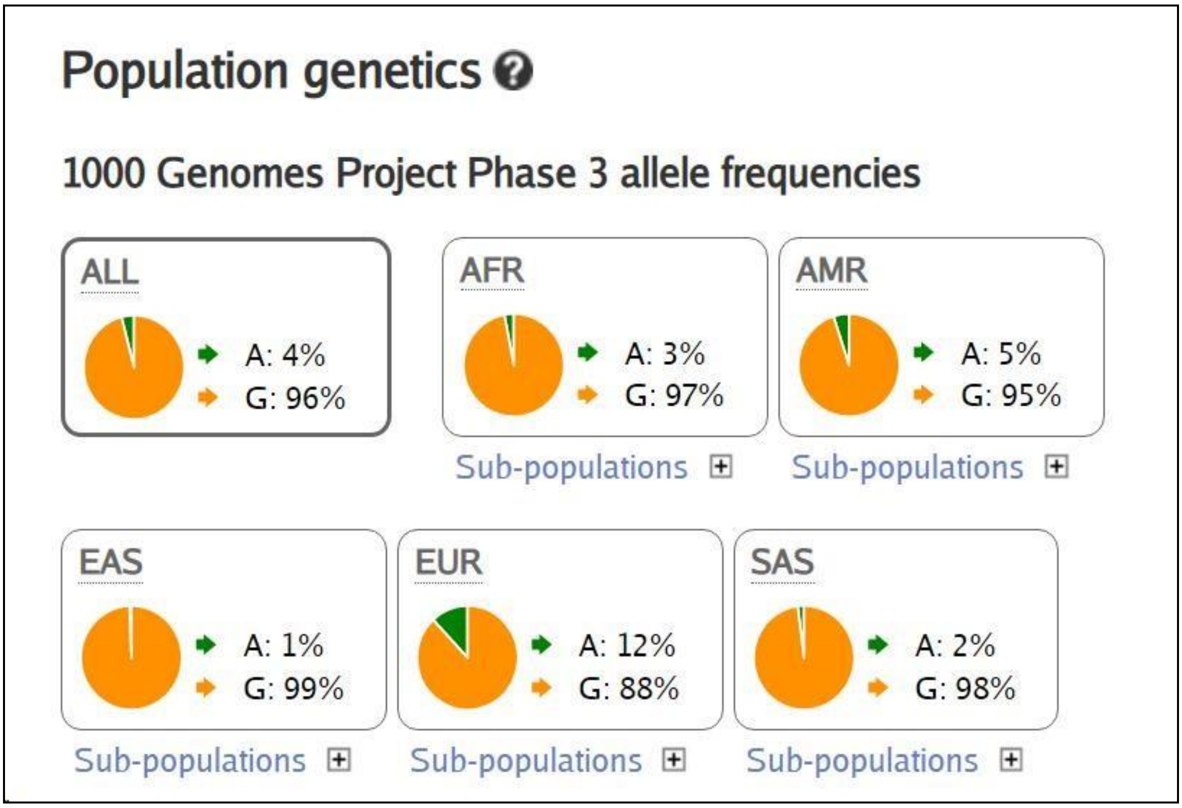


Figure (3.18):Global allelic frequency of rs3135388 (Karakaya *et al*., 2019).

3.15 Association of *M.pneumoniae* infection with HLADR gene polymorphism in RA patient

3.15.1 Association of rs9271366 with RA patient infected with *M.pneumoniae*

The allelic association analysis as listed in table (3.12) show that there is no significant ($p > 0.05$) differences in allele frequencies between RA patient with Mycoplasma infection and RA patient without Mycoplasma infection.

Table (3.12): Allelic frequencies and Association of rs9271366 with RA infection with *M.pneumoniae* and RA non-infection with *M.pneumoniae*.

Allele	RA non infected		RA infected		OR (95% CI)	P. Value
	Count	Proportion	Count	Proportion		
A	43	0.67	19	0.53	0.55 (0.24-1.26)	0.1562
G	21	0.33	17	0.47	1.83 (0.79-4.23)	

The results shows that G allele represent as dominant pathogenic allele in which the individual that carry GG and AG genotype have more susceptibility to Mycoplasma infection than subject that carry AA genotype ($p = 0.036$).Furthermore the genetic association of rs9271366 genotype with RA infection with Mycoplasma was analysis under different model inherit as listed in table (3.13) .

Table (3-13): Genotype frequency and association under different model of inheritance for rs9271366 with RA infection with *M.pneumoniae*.

Model	Genotype	RA non infected	RA infected	OR (95% CI)	P. Value
Codominant	A/A	12 (37.5%)	2 (11.1%)	1.00	0.11
	A/G	19 (59.4%)	15 (83.3%)	4.74 (0.92-24.49)	
	G/G	1 (3.1%)	1 (5.6%)	6.00 (0.26-140.05)	
Dominant	A/A	12 (37.5%)	2 (11.1%)	1.00	0.036
	A/G-G/G	20 (62.5%)	16 (88.9%)	4.80 (0.94-24.62)	
Recessive	A/A-A/G	31 (96.9%)	17 (94.4%)	1.00	0.68
	G/G	1 (3.1%)	1 (5.6%)	1.82 (0.11-31.03)	
Overdominant	A/A-G/G	13 (40.6%)	3 (16.7%)	1.00	0.072
	A/G	19 (59.4%)	15 (83.3%)	3.42 (0.82-14.24)	

3.15.2 Association of rs35445101 with RA patient infected with *M.pneumoniae*

The allelic association analysis as listed in table (3.14) show that there is no significant ($p > 0.05$) differences in allele frequencies between RA patient with Mycoplasma infection and RA patient without Mycoplasma infection.

Table (3.14): Allelic frequencies and Association of rs35445101 with RA *M.pneumoniae* infection and RA without *M.pneumoniae* infection.

Allele	RA non infected		RA infected		OR (95% CI)	P. Value
	Count	Proportion	Count	Proportion		
G	50	0.78	28	0.78	0.98 (0.37-2.62)	0.9679
A	14	0.22	8	0.22	0.02 (0.38-2.73)	

The results showed no association genotype between rs35445101 and *Mycoplasma pneumonia* infection ($p > 0.05$) as show in table (3.15).

Table (3-15) Genotype frequency and association under different model of inheritance for rs35445101 with RA infection with *M.pneumoniae*.

Model	Genotype	RA non infected	RA infected	OR (95% CI)	P. Value
Codominant	G/G	24 (75%)	14 (77.8%)	1.00	0.39
	A/G	2 (6.2%)	0 (0%)	0.00 (0.00-NA)	
	A/A	6 (18.8%)	4 (22.2%)	1.14 (0.27-4.76)	
Dominant	G/G	24 (75%)	14 (77.8%)	1.00	0.82
	A/G-A/A	8 (25%)	4 (22.2%)	0.86 (0.22-3.37)	
Recessive	G/G-A/G	26 (81.2%)	14 (77.8%)	1.00	0.77
	A/A	6 (18.8%)	4 (22.2%)	1.24 (0.30-5.13)	
Over dominant	G/G-A/A	30 (93.8%)	18 (100%)	1.00	0.18
	A/G	2 (6.2%)	0 (0%)	0.00 (0.00-NA)	

3.16 Haplotype association of rs9271366 and rs35445101 with RA

The results showed that the haplotype AA of **rs9271366** and **rs35445101** respectively show a protective effect with (OR=0.24, P = 0.017) comparing to other haplotype also the results showed that haplotype GA has a significant difference between patients and control the table (3.16) show the haplotype association.

Table (3-16): Haplotype association of rs9271366 and rs35445101 with RA

	rs9271366	rs35445101	Total	control	patient	OR (95% CI)	P. Value
1	A	G	0.5264	0.5127	0.4909	1.00	---
2	G	G	0.2514	0.2623	0.2891	1.57 (0.67 - 3.67)	0.3
3	A	A	0.1458	0.2248	0.1291	0.24 (0.08 - 0.76)	0.017
4	G	A	0.0764	0	0.0909	NA [NA~NA]	0.0001

3.17 Linkage Disequilibrium between rs9271366 and rs35445101 with RA

The results of Linkage Disequilibrium analysis between rs9271366 and rs35445101 show a week linkage value (D`= 0.38).This is agree with that HLA gene always have a high rate of rearrangement the figure (3.) explain the haploplot between rs9271366 and rs35445101.

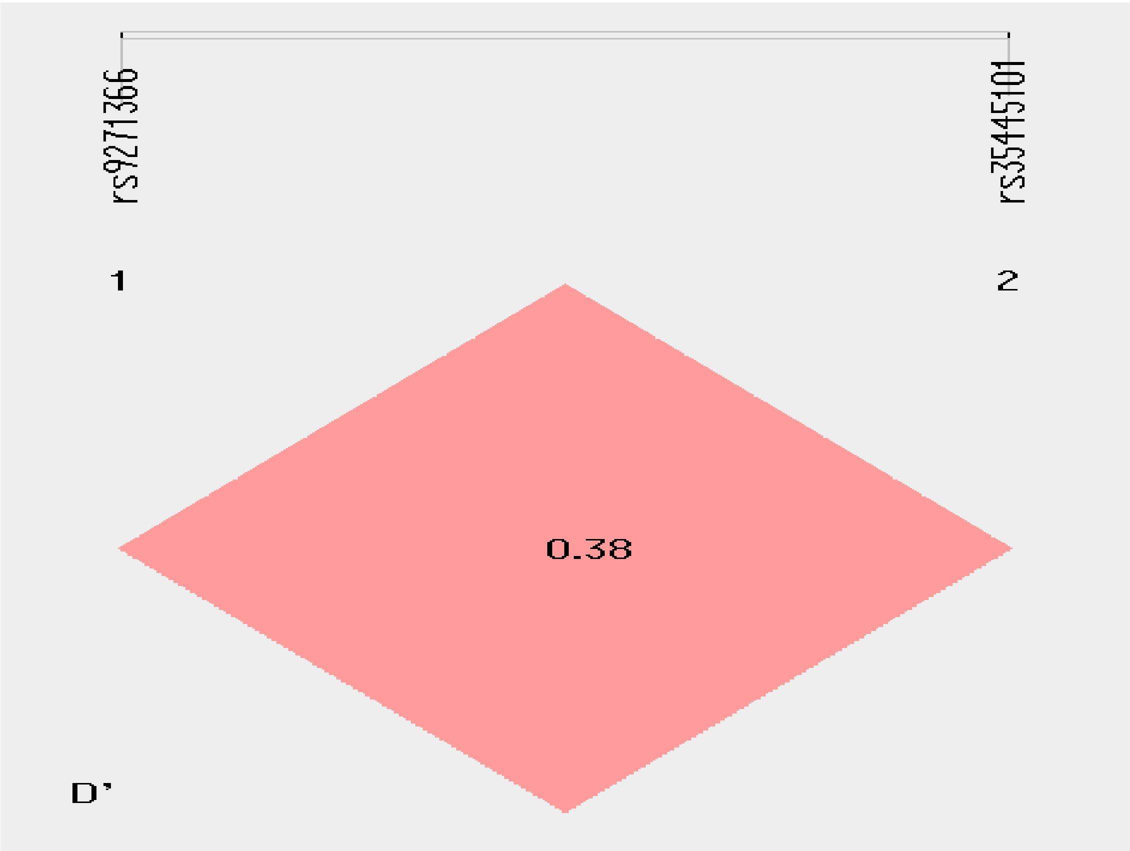


Figure (3.19) : Haploplot of Linkage Disequilibrium of rs9271366 and rs35445101 the value represent D`.

The major histocompatibility complex (MHC) system is the most polymorphic gene cluster of the mammal genome. In humans, this is a genomic locus extended to approximately 3.6 Mb located on the short arm of chromosome 6 (6p21.3) and it is known as the HLA system. The fact that there are genes inherited in close association with HLA alleles contributes to reinforce the idea that many genes are involved in the development of autoimmune diseases and explains how the positivity of a single polymorphism is not enough to develop a particular disease. Therefore, due to the linkage disequilibrium (LD) the concept of the extended MHC (xMHC) has been proposed, which is a genomic region of about 7.6 Mb including the classical MHC locus (Horton *et al*., 2004). The HLA encodes mostly immune associated genes distributed in three regions: class I, class II, and class III which are related by the structure and function of the proteins that they encode (Kaufman *et al*., 1984). The main function of HLA gene products is the presentation of antigens to the immune cells. For instance class I gene products (HLA-A, B, and C) are in charge of presenting endogenous peptides as well as peptides which are products of intracellular infections. In contrast, the class II-coded molecules (HLA-DR, DP, and DQ) present exogenous peptides such as bacterial antigens which are the result of antigen processing. Finally, the class III region encodes immune regulatory molecules e.g., tumor necrosis factor (TNF), C4 of complement system, and heat-shock proteins (Shiina *et al*., 2009). From the evolutionary point of view the HLA system is highly conserved as its proper function is crucial for the immune response against pathogens. The proteins codified in this region regulate and interact with other proteins which can be codified within the same region or not. This system must ensure the correct recognition and presentation of a tremendous diversity of pathogens. Therefore, even if these proteins are highly conserved their genes are also the most polymorphic in particular those codifying for HLA class I and II. This variability is represented in a huge number of alleles which cause slight variations in the structure of the proteins giving to a single individual the capability to react against different pathogens conserving their essential structure and the function (Trowsdale and Knight, 2013). According to the nomenclature for factors of the HLA system (Marsh *et al*., 2010) and HLA databases the number of alleles reported for human HLA is 14473 (Robinson *et al*., 2015). The comparison between the genomic sequences of class I and class II alleles shows that mutations in particular nucleotide substitutions are concentrated in the exons that encode areas which directly interact with the pathogen molecules and with the T cell receptor (TCR) (Shiina *et al*., 2009). Indeed the number of different alleles in HLA class I is 10730 while for class II is 3743(Wysocki *et al*., 2020).

In rheumatoid arthritis (RA) HLA is the most important genetic risk factor of which the HLA-DRβ1 allele is the most important. HLA-DRβ1 alleles especially DR4 and DR1, encode the HLA-DRβ chain. HLA-DRβ contains 5-amino-acid sequence motifs (positions 70–74 share 5-amino-acid sequences namely QKRAA, QRRAA and RRRAA) which are called shared epitopes (SEs) and are closely related to RA susceptibility (Kim *et al*., 2015).

Anticitrullinated protein antibodies (ACPAs) are important RA disease markers. Patients carrying HLA-DRβ1 alleles with SEs tend to be susceptible to ACPA-positive RA and those not carrying HLA alleles with SEs tend to be susceptible to ACPA-negative RA. In SE-positive RA patients the ACPA titer in patients with the DRβ1*04 allele is higher than that in patients with the DRβ1*01 allele (Holoshitz *et al*., 2013). HLA- DRβ1 encoding SEs not only increase the risk of RA but also increase the risk of early disease onset more severe bone erosion and ACPA. Moreover, the correlations between SEs and RA and between SEs and disease severity depend on the gene dose. Carrying 2 alleles encoding SEs is associated with a much higher risk of joint damage than is carrying 1 allele. In addition the DRβ chains encoded by the alleles DRβ1*01:03, DRβ1*13:01, DRβ1*13:02 and DRβ1*04:02 have an opposite protective effect in RA (van der Woude *et al*., 2010). However, some scholars question the theory that SE-expressing HLA-DR molecules of RA patients present specific antigens because comparative analysis found that the eluted peptide antigens of different HLA-DR molecules expressing SEs have almost no overlapping similar fragments (Scholz *et al*., 2016).

Monocytes play an important role in the invasion and immunity to resist pathogens. The main functions of monocytes antigen expression and cytokine production are mediated by certain surface molecules from major histocompatibility complex (MHC) classII such as monocyte human leukocyte antigen (HLA-DR). HLA-DR allows antigen presentation to T cells and is crucial for the initiation of the cascade of the immune response. Hence HLA-DR expression suggests the antigen processing and presentation capability of the expanded subset (Wang *et al*., 2012).While the symptoms of *MP* pneumonia are correlated with the induction of pro-inflammatory cytokines (Fan *et al*., 2016).

Specific T cell epitopes are located in P1 protein on *MP*. This could result in T cell changes after *MP* infection. Activated T cells are divided into three phases early (CD69+), middle (CD25+) and late [human leukocyte antigen D-related (HLA-DR+)] phase by surface biomarkers (Guo *et al*., 2015).

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

1. *Mycoplasma pneumoniae* infection is associated with RA disease.
2. The females are more affected to infection with *Mycoplasma pneumoniae* among RA patients.
3. The HLA-DRβ1 in RA patients infected with *Mycoplasma pneumoniae* was significant (P<0.05) as compared to the RA patients non infected with *Mycoplasma pneumoniae* and controls.
4. The VEGF-A in RA patients was significant (P<0.05) Compared with healthy controls.
5. The IL-6 in RA patients infected with *Mycoplasma pneumoniae* was a significant (P<0.05) as compared to the RA patients non infected with *Mycoplasma pneumoniae* and controls.
6. The levels of HLA-DRB1, VEGF-A and IL6 were significantly correlated among RA patients.
7. rs9271366 was significantly associated with RA and G allele represent as dominant pathogenic allele in which the individual that carry GG and AG genotype have more susceptibility to Mycoplasma infection than subjects that carry AA genotype (p= 0.036).
8. rs35445101 was significantly associated with RA where the A allele represented a pathogenic recessive allele, but it didn't show any allelic or genotype that was significantly associated with Mycoplasma infection (P >0.05).
9. The haplotype AA of rs9271366 and rs35445101 respectively showed a protective effect with (OR= 0.24,P = 0.017) comparing to other haplotype.
10. The haplotype GA has a significant difference between patients and control (P <0.0001).

Recommendations

1- Studying of the relationship between microbial infections as a possible cause for RA and other types of autoimmune diseases for example SLE and diabetes type 1.

2- Early detection of microbial diagnostic screening tests in the individuals with family history of RA.

3- Determine the genotyping of genes in the present study in other provinces in Iraq to give a complete picture about genotype distribution in Iraq population regarding RA disease.

4- Consider the use of genetic analysis as an alarm parameter to change life style for predisposing individuals.

5- Study clinical significant of cytokines other than IL-6 in RA patients .

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Appendix

Case information sheet

Appendix 1

1- Name Number

2- Address

3- Age years.

4- Gender: Male Female

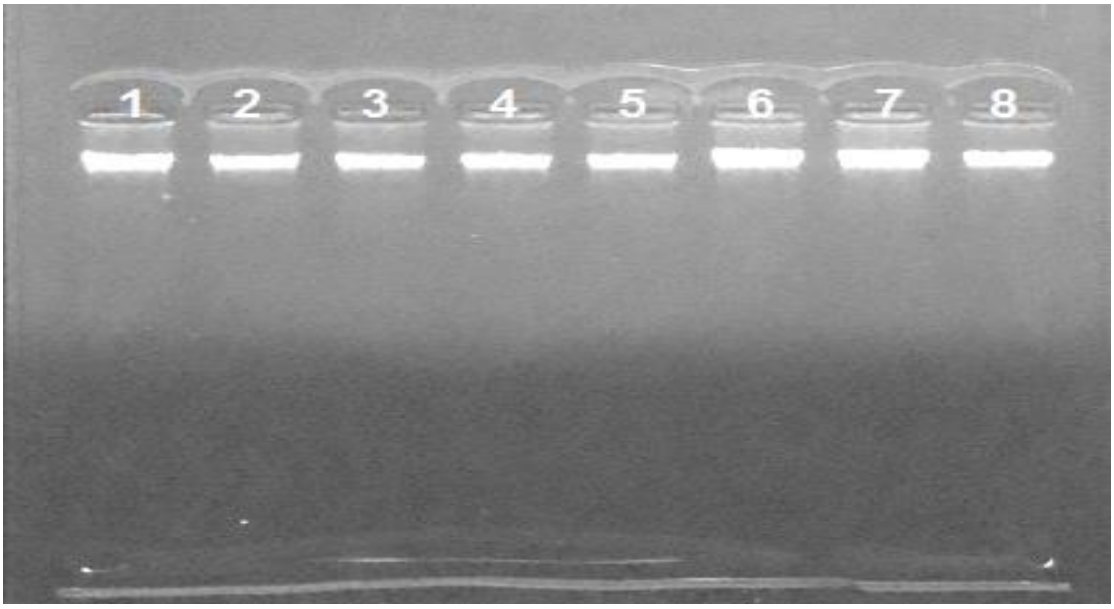
5- BMI: Length Weight

6- Chronic other current disease : Yes NO

7- Duration of onset : Treatment type

8- Criteria for diagnosis

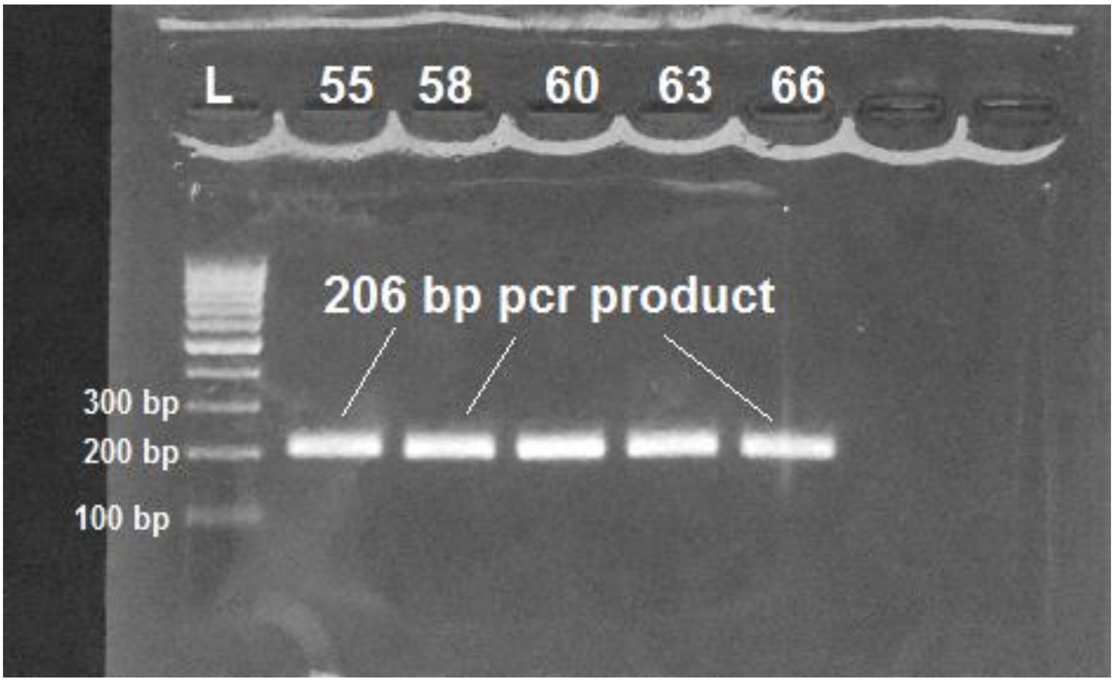
Appendix 2



DNA Extraction Product from Whole Blood. Lane(1-6) DNA Extracted from RA Patients, and Lane (7-8) DNA Extracted from Controls.

Appendix 3

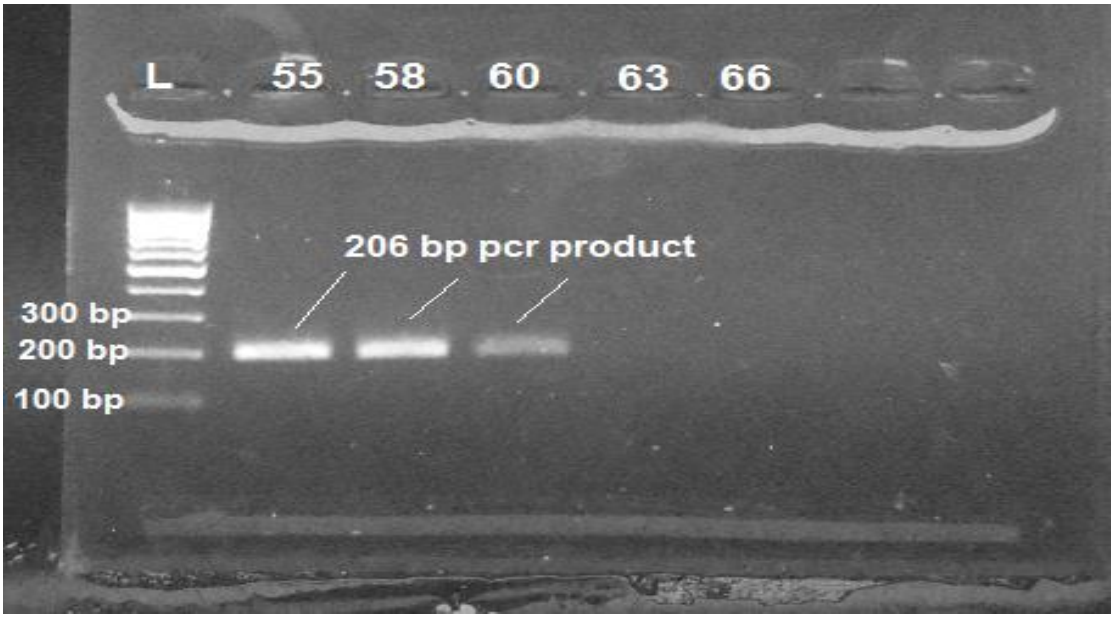
-A-



Gel electrophoresis of gradient PCR product (Rs9271366) , lane L:DNA ladder, other lane represent different annealing temperatures as indicated on each lane.

Appendix 3

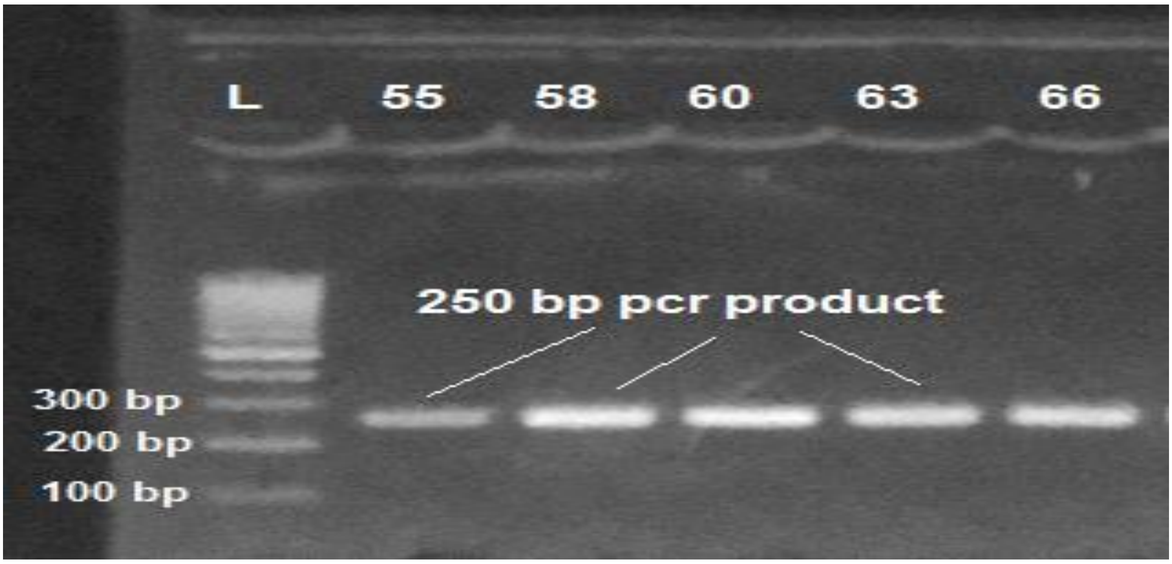
-B-



Gel electrophoresis of gradient PCR product (Rs3135388), lane L:DNA ladder, other lane represent different annealing temperatures as indicated on each lane.

Appendix 3

-C-



Gel electrophoresis of gradient PCR product (Rs35445101), lane L:DNA ladder, other lane represent different annealing temperatures as indicated on each lane.

الخلاصة:

التهاب المفاصل الرثوي هو مرض مناعي يسبب التهاب مزمن في المفاصل و السبب الرئيسي لالتهاب المفاصل الرثوي متمثل في عدة عوامل منها الوراثية والبيئية وكذلك الاصابات الميكروبية . لذا تركزت الدراسة على المتغيرات الجينية بما في ذلك مستضد كريات الدم البيضاء البشرية (HLA-DRB1) والتي تؤثر على قابلية الاصابة بمرض التهاب المفاصل الرثوي. ان جين (HLA-DRB1) هو جزء من عائلة من الجينات التي تسمى مستضدات كريات الدم البيضاء البشرية ، لذلك هدفت الدراسة الى توضيح العلاقة بين الاصابة بالمفطرة الرئوية والتهاب المفاصل الرثوي.

تم تطبيق هذه الدراسة على (٥٠) حالة مشخصة سريرياً لمرضى التهاب المفاصل الرثوي حيث تتراوح اعمارهم بين (٢٥-٧٥) عام في العيادة الاستشارية لأمراض المفاصل في مدينة مرجان الطبية خلال المدة من شهر شباط ٢٠٢٢ لغاية شهر تشرين الاول ٢٠٢٢ وكذلك (٤٠) شخص كمجموعة ضابطة (اصحاء ظاهرياً).

تم الكشف عن مضادات المفطرة الرئوية (IgG-Ab) وعامل نمو الخلايا البطانية الوعائية نوع (أ) (VEGF-A) ومستضد كريات الدم البيضاء البشرية (HLA-DRB1) وكذلك الانترلوكين (IL-6) بواسطة تقنية مقايسة الممتز المناعي المرتبط بالأنزيم (ELISA) . تم استخدام الانزيمات القاطعة من خلال تقنية PCR-RFLP لدراسة تعدد الاشكال الجيني rs9271366 و rs3135388 و rs35445101 لدى مرضى التهاب المفاصل الرثوي.

اظهرت النتائج ان نسبة الاصابة ٥٠:١٨ (٣٦%) من مرضى التهاب المفاصل الرثوي لديهم اصابة بالمفطرة الرئوية.

اظهرت النتائج ان هنالك فروقات ذات دلالة معنوية ($P<0.05$) لمستوى عامل نمو الخلايا البطانية نوع (أ) لدى مرضى التهاب المفاصل الرثوي المصابين بالمفطرة الرئوية مقارنة بالمجموعة الضابطة اما مرضى التهاب المفاصل الرثوي المصابين بالمفطرة الرئوية ومرضى المفاصل الرثوي غير المصابين بالمفطرة الرئوية لم تظهر النتائج دلالة معنوية ($P >0.05$).

اظهرت النتائج ان هنالك زيادة معنوية ذات دلالة احصائية ($P<0.05$) في مستوى تركيز (HLA-DRB1) في مصل الدم لدى مرضى التهاب المفاصل الرثوي المصابين بالمفطرة الرئوية مقارنة بمرضى التهاب المفاصل الرثوي غير المصابين بالمفطرة الرئوية والمجموعة الضابطة.

اما بالنسبة لمستوى تركيز (IL-6) في مصل دم مرضى التهاب المفاصل الرثوي المصابين بالمفطرة الرئوية مقارنة بالمرضى من غير المصابين بالمفطرة الرئوية والمجموعة الضابطة فقد اظهرت النتائج وجود دلالة احصائية معنوية ($P<0.05$) .

اظهرت النتائج في النمط الجيني rs9271366 ان الاليل G هو اليل ممرض سائد حيث يكون الاشخاص الذين لديهم النمط الجيني GG,AG لهم القابلية على الاصابة بالمرض (٢,٥٧) ضعف مقارنة بالأشخاص الذين لديهم النمط AA (OR: 2.57 CI 95% 1.07- 6.17) بينما في النمط الجيني rs35445101 ظهر (A) كاليل ممرض متنحي حيث يكون الاشخاص الذين لديهم النمط الجيني AA القابلية للإصابة بالمرض (٩.٧٥) اضعاف مقارنة بالأشخاص الذين لديهم النمط الجيني GG,AG (OR: 9.75 CI 95% 1.19 -79.78) .

اظهرت النتائج النمط الجيني rs9271366 لدى مرضى التهاب المفاصل الرثوي المصابين بالمفطرة الرئوية ومرضى التهاب المفاصل من غير المصابين بالمفطرة الرئوية ان G اليل هو اليل ممرض سائد حيث ان الاشخاص الحاملين للنمط الجيني AG,GG اكثر عرضة للإصابة بالمفطرة الرئوية من الاشخاص الحاملين للنمط الجيني AA ($P= 0.036$) كما اظهرت النتائج عدم وجود علاقة بين النمط الجيني rs35445101 والاصابة بالمفطرة الرئوية ($P>0.05$).

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامـــعة بابـــــل

كلية الطب

العلاقة بين المفطرات الرئوية والإصابة بإلتهاب المفاصل الرثوي

اطروحة مقدمة الى

مجلس كلية الطب – جامعة بابل

كجزء من متطلبات نيل درجة دكتوراه فلسفة

في علم الاحياء المجهرية الطبية

من قبل

وائـل رشيـد عبيـد الفتـلاوي

بكالوريوس علوم حياة / جامعة بابل – ١٩٩٩

ماجستير احياء مجهرية طبية / جامعة بابل – ٢٠١٨

بإشراف

أ.د.محمـد عبد كاظم السعدي   **أ.د.عادل حسن علي اكبر**

١٤٤٤ هـ   **٢٠٢٣ م**