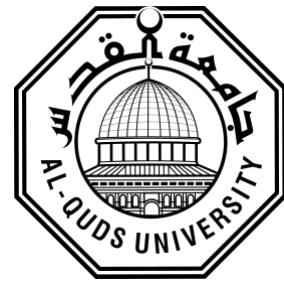


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**Immunological detection of Anti-nuclear antibodies
(ANA) using continuous cell lines and DNA rich non-
pathogenic organism**

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M.Sc. Thesis

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(ANA) using continuous cell line and DNA rich non-
pathogenic organism**

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**A thesis submitted in partial fulfillment of requirements
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Thesis Approval

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Jerusalem – Palestine

1438/2016

Dedication

My thesis is dedicated to

“Mom and Dad”

I am so thankful for their encouragement

And for making me be who I am

Declaration

I Certify that this thesis submitted for the degree of Master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or Institution.

Signed:

A handwritten signature in blue ink, appearing to read "Mai Ibrahim Baker".

—

Mai Ibrahim Baker

Date: 22.12.2016

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Abstract

Antinuclear antibodies are autoantibodies that target intracellular antigens, abnormal level of antinuclear antibodies are found in patients with Systemic Autoimmune Rheumatic Diseases (SARD); are classified into a class of non-organ specific autoimmunity, as a different of autoantibodies attack multiple organ systems. It includes six different types of diseases: Systemic Lupus Erythematosus (SLE), Sjogren Syndrome (SjS), Systemic Sclerosis (SSc), Polymyositis (PM), Dermatomyositis (DM) and Rheumatoid arthritis (RA). The antinuclear antibodies can be detected using serological tests, the antinuclear antibodies test; termed as ANA. Thus, it is considered as a primary test for diagnosing patients with SARD. This test can be done using different techniques, the two most common techniques are: Indirect Immunofluorescence Assay (IIF) and Enzyme Linked Immunosorbent Assay (ELISA). In addition, western blot can be used to characterize the anti-nuclear autoantibodies. The identification of anti-nuclear autoantibodies in patient serum is a key role in SARD diagnosis. Different purified antigens of cells from mice, calves or human carcinoma cells were used in ELISA and western blot, because the specificity of ANA test depends on the quality of antigens used. This study is mainly aimed at developing ANA screening test using continuous THP1 cell lines, non-pathogenic *Leishmania tarentolae* and salmon sperm DNA, because they are suitable in ELISA testing, due to the large nuclei of THP1 cells, and the high rich of DNA of the kinetoplast in *Leishmania tarentolae*. The results gave 100% positivity using THP1, *Leishmania tarentolae* and salmon sperm DNA in ELISA assay. The autoantibodies against dsDNA gave a better result in salmon sperm DNA ELISA than leishmanial and

THP1 extract. However, for the other anti-nuclear autoantibodies, the results of THP1 and Leishmanial extract ELISA were better than salmon sperm DNA ELISA. Furthermore, the results were better in THP1 nuclear extract compared to leishmanial extract. In addition, results showed that different anti-nuclear autoantibodies can be identified using the immunoblotting of THP1 and leishmanial separated proteins, but the more relevant reactive bands appeared in THP1 nuclear extracts.

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Table of abbreviations

Abbreviation	Full word
ANA	Antinuclear Autoantibodies
APS	Ammonium Persulfate
CD	Cluster of Designation
CENP	Centromere P
DM	Dermatomyositis
DMEM	Dulbecco's Modified Eagle's Medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
ENA	Extracatable Nuclear Antigen
FCS	Fetal Calf Serum
IIF	Indirect Immunofluorescent Assay
kDa	Kilo Dalton
MHC	Major Histocompatibility Complex
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PM	Polymyositis
PMSF	Phenylmethylsulfonyl Fluoride
RA	Rheumatoid Arthritis
RPMI	Roswell Park Memorial Institute medium
SARD	Systemic Autoimmune Rheumatic Disease
SDS	Sodium Dodecyl sulfate
SjS	Sjogren Syndrome
SLE	Systemic Lupus Erythematosus
Sm	Smith
SSc	Systemic Sclerosis
TCR	T-Cell Receptor

TEMED

Tetramethylethylenediamine

Introduction

1.1 Immune tolerance

Our bodies are armed with strong immune system that aims to fight and eliminate foreign bodies. In addition, our immune system has the ability to distinguish between self and non-self-antigens. Thus, it only responses to foreign antigens, this is known as immune tolerance.

Our immune system builds this tolerance by process of both central-tolerance and peripheral tolerance (Kyewski & Klein, 2006). In central tolerance, our immune cells; lymphocytes, learn to discriminate between self and non-self during their development, T cells in the thymus and B cells in bone marrow. In thymus, T cells become mature after proliferation, differentiation and selection (Kyewski & Klein, 2006). During early thymocyte development, T cell precursors move from bone marrow to thymus (Schwarz & Bhandoola, 2006), and become double negative cells; these cells neither express CD4 nor CD8 receptors on their surfaces. Then, these cells pass through four stages in order to rearrange their T-cell receptor gene loci. Cells with rearranged T cell receptor (TCR) beta chain are able to proliferate and initiate rearrangement of their TCR Alpha chains, and become double positive cells. This means, the cells have both CD4 and CD8 receptors. Later, they undergo negative and positive selection in thymic cortex (Kyewski & Klein, 2006; Sprent & Kishimoto, 2001).

The double positive cells are exposed to the Major Histocompatibility Complex (MHC) molecules; MHC I and MHC II, in thymic epithelial cells. The double positive cells that don't respond, or that bind with a high affinity to MHC molecules are deleted by negative selection. This means, only double positive cells that bind intermediately to MHC molecules are positively selected and survived. After that, they are presented to self-antigen with MHC molecules in

medulla. This process called, negative selection. Cells that don't respond to self-antigen become mature lymphocytes expressing either CD4 or CD8. However, auto reactive cells that respond to self-antigen are marked for death and removed (Judith A. Owen, 2013; Kyewski & Klein, 2006; Sprent & Kishimoto, 2001).

A few of autoreactive mature T cells can escape to peripheral immune system. By different mechanisms of peripheral tolerance; either by clonal anergy or deletion, these cells are regulated and suppressed by regulatory T cells (Judith A. Owen, 2013; Xing & Hogquist, 2012). The anergy mechanism lead to inactivate the response of auto reactive T cells toward self-antigens, and this happen by blocking of signals induced from CD28 ligation. This state is known as T cell hyporesponsiveness. The other way is by deletion of the autoreactive cells, these cells are died through Fas- mediated apoptosis (Xing & Hogquist, 2012).

The B cell tolerance occurs in bone marrow where B cell precursors undergo steps to become mature B cells. At first, B cell precursors become pre-B cells expressing receptors on their surface. The fate of any B cells that fail to express receptors is death. These pre-B cells that can recognize wide varieties of protein, such as self- antigens are either marked for death or locked away. However, some cells are subjected for receptor editing. Then, they leave bone marrow to enter peripheral system where peripheral B cell tolerance are built. When these naïve B cells become activated and bind to antigens with moderate affinity, they proliferate, and then transform to class switching, somatic hyper mutation or plasma cells. While those cells that bind to antigens with high affinity are killed and removed (Goodnow, Adelstein, & Basten, 1990; Judith A. Owen, 2013).

1.2 Autoimmune disease

The autoimmunity is defined as a disease caused by an unexpected response of immune system against self-components, resulting in severe injury in tissues and organs (Ghazvini, 2010). Thus, it is considered as a chronic disease, and it may cause significant mortality and morbidity (Ghazvini, 2010; Walsh & Rau, 2000). This disease affects about 5%-7% of the population worldwide, 78% of them are women(Smith & Germolec, 1999). The prevalence of this disease is rapidly increasing in the developing countries. In the United States, it mostly affects up to 23 million Americans(Walsh & Rau, 2000). In addition, depending on several studies in low and middle income countries; South Africa, Iran and Philippine, the autoimmune diseases; such as SLE, affects more than 100 million people (Vento & Cainelli, 2016).

There are 80 different types of auto immune disease. Scientists found it difficult to classify autoimmune diseases into groups, as their causative mechanisms are still not fully understood. However, these diseases are traditionally categorized into two groups: organ-specific and systemic autoimmune disease. Organ specific autoimmunity is directed against one specific organ, while in systemic autoimmunity, the immune response is directed against many different organs which is not restricted to one organ (Judith A. Owen, 2013)

The autoimmune diseases are mostly happened when peripheral tolerance is dysregulated in individuals with autoimmune disease prone genotype. In other words, the immune tolerance may collapsed in individuals who are genetically susceptible to autoimmune diseases (Mackay, 2000). Thus, in presence of any triggers such as: environment, sex hormones, neuroendocrine influence or autoantigen, the controlled auto-reactive cells become activated after responding to them. This leads to serious damage to cells and organs achieved by the mechanism of humoral or cell- mediated responses (Smith & Germolec, 1999). In addition, autoimmune disease may also

developed when individual is infected by viral or bacterial disease. In this case, as viral and bacterial agents mimic host tissues, the immune system starts recognizing self-tissues and attacking them (Wucherpfennig & Strominger, 1995). Rheumatic fever is a good example for this type of autoimmune disease where occurs after *streptococcal* infection. These bacterial antigens are mimic the heart muscle. Therefore, the antibodies will response against both *streptococcal* antigens and heart muscle (Denick, 1992).

Furthermore, there are sex differences in autoimmune disease development. Women are susceptible to the autoimmune disease three times more than men (Beeson, 1994). In women, the immune system responses to infection and vaccination is highly activated than men, as it produces more antibodies and T helper 2 cells. Hence, the high level of antibodies increase the risk of developing an autoimmune disease. In addition, the ovarian hormone, estrogen is also known to have the ability to alter the immune response, and result in developing autoimmune disease (Cutolo, Sulli, Seriolo, Accardo, & Masi, 1995; Cutolo, Sulli, & Straub, 2012).

1.3 The systemic autoimmune rheumatic diseases (SARD)

This group includes: Systemic Lupus Erythematosus (SLE), Sjogren Syndrome (SjS), Systemic Sclerosis (SSc), Polymyositis (PM), Dermatomyositis (DM) and Rheumatoid arthritis (RA)(Judith A. Owen, 2013). As previously mentioned, the immune response attack different organs and tissues in these diseases, and the tissue damage caused by auto-antibodies or by accumulation of immune complexes. The diagnosis of systemic autoimmune rheumatic diseases depends on both clinical manifestation and laboratory tests. The most relative serological test for SARD is ANA test (Mahler, Pierangeli, Meroni, & Fritzler, 2014; Pisetsky, 2012).

1.3.1. The Antinuclear antibodies (ANA)

The antinuclear antibodies are immunoglobulins that encounter against the nuclear and cytoplasmic components (Cabiedes & Nunez-Alvarez, 2010). The presence of ANA in the circulation is not always associated to the autoimmune disease. It is known that the ANA may be present in healthy individuals, but in a low titer. Further, Individuals may show a high titer of ANA in response to infectious disease. However, they are reduced whenever the infectious is resolved (Tan et al., 1997).

In autoimmune diseases, such as systemic autoimmune rheumatic diseases, high levels of autoantibodies are produced to attack nuclear parts inside cells. These antinuclear antibodies include two groups based on their detection in circulation: Autoantibodies against DNA and histone: They are against both single and double stranded DNA, and histones. These autoantibodies are found with a high level in Systemic Lupus Erythematosus (SLE) patients. The other group is autoantibodies against extractable nuclear antigens (ENA) (Cabiedes & Nunez-Alvarez, 2010; Kumar, Bhatia, & Minz, 2009; Mahler, Meroni, Bossuyt, & Fritzler, 2014; Mahler, Pierangeli, et al., 2014; Muro, 2005): Those are extractable antigens from nuclei, including:

- A) Anti-smith (sm) antibodies: The autoantibodies against smith antigen were first described by Tan and Kunkel, in 1966 (Tan & Kunkel, 1966). These autoantibodies are very specific to SLE disease. Their presence with other autoantibodies against dsDNA, nucleosomes and ribosomal P-Proteins considered as pathognomonic for the disease (Gill, Quisel, Rocca, & Walters, 2003). Anyway, they are only found in 20% of SLE patients (Wenzel, Bauer, Bieber, &

Bohm, 2000). The Smith antigens are a small nuclear ribonucleoproteins (snRNPs) present in snRNPs core unit. It is composed of nine polypeptides (B1, B', B3, D1, D2, D3, E, F and G) responsible for splicing of pre-mRNA. Their molecular weight ranges from 9 to 29.5 kDa. Autoantibodies commonly presented against B and D polypeptides (Caponi, Bombardieri, & Migliorini, 1998). The Smith antigens are mimic to proteins found in Epstein-Barr virus. So that, anti-Sm antibodies are directed against Smith antigens as result of Epstein-Barr virus infection (Sundar et al., 2004; Zieve & Khusial, 2003).

- B) Anti-nuclear ribonucleoproteins (nRNP): The antigen of these auto-antibodies are presented in the core unit of snRNPs, U1-nRNP. It contains specific proteins 70K, A and C (Benito-Garcia, Schur, Lahita, & American College of Rheumatology Ad Hoc Committee on Immunologic Testing, 2004). These auto-antibodies can be directed against nRNPs either by the molecular mimicry to Epstein-Barr virus or by nuclear component presentation (Venables, 2006). They are also found in SLE patients with ratio of 30-40% (Tan & Kunkel, 1966). They are detected in SSc and PM/DM diseases as well (Houtman et al., 1985; Houtman et al., 1986).

Anti-SSA/Ro and anti-SSB/La antibodies: They are detected in 30-60% of Sjogren syndrome (Hernandez-Molina, Leal-Alegre, & Michel-Peregrina, 2011; Manoissakls, 2001; Peene, Meheus, Veys, & De Keyser, 2002), and also in 20%-60% of SLE disease (Wenzel et al., 2000; Zieve & Khusial, 2003). The SSA/Ro antigens are ribonucleoprotein consists of RNA molecule and protein with molecular weight of 60 kDa. The SSA/Ro antigen contribute in activating mRNA.

The 60 kDa protein is commonly targeted by anti-SSA/Ro. The antigen of SSB/La is a 48 kDa phosphoprotein. This protein is considered as a helper for enzyme of RNA polymerase III. The anti-SSB/La antibodies are frequently found in parallel with Anti-SSA (Ben-Chetrit, 1993; Hernandez-Molina et al., 2011; Peene et al., 2002).

- C) Anti Scl-70 antibodies: The antigen of this autoantibody is topoisomerase I. It is located inside the nucleolus (Guldner et al., 1986). Further, this enzyme participate in DNA replication and transcription. Its molecular weight 110 kDa. (Guldner et al., 1986). However, in western blot, it was found to have a molecular weight of 70 kDa.(Aeschlimann et al., 1989) The anti Scl-70 antibodies are highly associated with Systemic sclerosis patients (Douvas, Achten, & Tan, 1979).
- D) Anti- Jo-1 antibodies: They are specific marker for polymyositis with ratio of 20-30%. They are less frequently found in dermatomyositis diseases. Anti- Jo-1 antibodies target hisyidyl tRNA synthetase. This enzyme catalyzes tRNA and histidine binding during protein synthesis. The molecular weight of this enzyme is 50 kDa (Mescam-Mancini et al., 2015; Schmidt et al., 2000; Shinjo & Levy-Neto, 2010; Sugie, Tonomura, & Ueno, 2012).

1.3.2. Rheumatoid arthritis (RA)

It is a common chronic inflammatory disease occurs when the auto-reactive T cells attack tissue in joints, result in joint swelling, joint tenderness, and destruction of synovial joints. It may be also attacks bones, cartilages, skin, lungs and kidneys. The chronic case of disease may leads to severe disability and premature mortality (Schumacher, Pessler, & Chen, 2003) . The RA affects individuals during middle age. Further, it affects women two and half times more than

men. In this disease, many auto antibodies are produced, the most common known as rheumatoid factors, class of IgM. They can bind to fragment of Fc region of IgG, forming IgM-IgG complexes which deposit into joints. The deposition of IgG-IgM complex into joints leads to complement cascade activation. It also leads to develop hypersensitive reaction type III which resulting in joint inflammation (Aletaha et al., 2010a).

RA are diagnosed by symptom of patient, and by other tests; such as imaging and serological tests. The X-ray for hands and foots support the diagnosis of RA, as it shows the affected joints. Usually, the specific serology test used for diagnosing RA are Rheumatoid factor (RF) antibody and Anti-Citrullinated Protein Antibody (ACPA). Though patients with RA also show a high level of ANA, they overlap with other autoimmune diseases. Thus, ANA test is not usually done as the test is not specific for RA (Agmon-Levin et al., 2014; Aletaha et al., 2010b; Bas et al., 2002; Schumacher et al., 2003)

1.3.3. Systemic Lupus Erythematosus (SLE)

It is a chronic disease caused by producing of different auto antibodies to a variety of self-antigens. It affects many organs including: skin, joints, kidney, lung, heart, and gastrointestinal tract. There are different and mixed clinical manifestations, depending on the auto antibodies presented in the affected individual. The initial clinical manifestations of disease are: Malar rash, fever, weakness, hair loss, musculoskeletal symptoms, vascular abnormalities, and photosensitivity. In the severe case, other manifestations may include: hemolytic anemia, renal failure, arthritis, inflammatory serositis, lymphopenia, thrombocytopenia, and neurological problems(Judith A. Owen, 2013). In SLE disease, the autoantibodies to dsDNA and small nuclear ribonucleoproteins (snRNPs); such as smith antigen (Sm), are mostly produced. Anyway, the level of anti-Sm antibodies are constant. Thus, they are not associated with disease

activity. In addition, other different autoantibodies are also produced but in a variable level, such as, autoantibodies to histone proteins, RNA binding proteins, and DNA polymerase components. These autoantibodies form immune complexes which resulting in complement activation. It also develop type III hypersensitive reaction causing nephritis, skin lesions and arthritis (Gill et al., 2003; Smith & Germolec, 1999; Tan & Kunkel, 1966; Venables, 2006; Wenzel et al., 2000; Zieve & Khusial, 2003).

The prevalence of SLE is different between genders, ages, races and geographic locations. It affects women ten times more than men, and it usually affects individual between ages of 45-64 (Danchenko, Satia, & Anthony, 2006). In racial tendencies studies, it showed that SLE frequently affects non-Caucasian individuals (Housey et al., 2015; Ward, 2004). For instance, it was reported that the prevalence rates in UK at the Nottingham area were 3.7 in 100,000 men and 45.4 in 100,000 women. In addition, he found that the prevalence is more amongst Afro-Caribbean groups (Hopkinson, Doherty, & Powell, 1993).

The diagnosis of SLE is based on both clinical and laboratory criteria. The ANA test are done to detect the high level of antinuclear antibody to double-stranded DNA antigen (anti-dsDNA), and other autoantibodies with variable level such as anti-Sm, anti-SSA, anti-SSB and anti-nRNPs (Cabiedes & Nunez-Alvarez, 2010; Gill et al., 2003).

1.3.4. Sjögren's Syndrome (SjS):

It is a chronic autoimmune inflammatory disease that affects exocrine glands; commonly lacrimal and salivary glands, leading to their functional impairment which results in keratoconjunctivitis sicca and xerostomia. There are two type of Sjögren's syndrome, Primary and Secondary. The most common type is Primary Sjögren's syndrome which occurs independently. The prevalence of this disease around 1 to 3%. It can affects all of ages and both

sexes, but it mostly affects women with ratio 9:1 of women to men. Secondary Sjögren's syndrome occurs in people who have another rheumatologic disorder, most commonly rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) (Tincani et al., 2013; Voulgarelis & Tzioufas, 2010). Furthermore, patients with SjS have an increased incidence of lymphoma.

The diagnosis of Sjögren's syndrome is highly dependent on clinical manifestation and on autoimmune reactivity such as, the presence of lymphatic infiltrative lesions in the salivary gland. In addition, detection of auto antibodies against Ro(SSA) and La(SSB) in patient serum(Manoissakls, 2001).

1.3.5. Systemic Sclerosis (SSc)

It is a multisystem autoimmune disease of unknown etiology that involves the micro vascular system and connective tissue. It characterized by widespread vascular injury and progressive fibrosis of skin and internal organs which may lead to death (Casale, Buonocore, & Matucci-Cerinic, 1997). Skin thickening is the main symptom of SSc disease, and this due to the increase of collagen production. Other symptoms may include arthritis joint and internal organs. However, the symptoms of disease depend on the form of SSc disease; limited and diffuse cutaneous. In limited SSc disease, it affects face, hands and feet. But the diffuse cutaneous affects many regions of the skin, and internal organs and systems (Silman & Newman, 1996).

SSc disease is a rare disease, its onset is ranging from 30 to 55. It affects most of racial groups in all geographical areas. Anyway, some studies showed that black people at a high risk to develop the disease compared to White people. Furthermore, women from three to four times are more common to have the disease compared to men (Tager & Tikly, 1999).

The diagnosis of systemic sclerosis is based on combination of clinical and laboratory features. Patients with Systemic sclerosis present auto antibodies against a variety of nuclear and cytoplasmic antigens, the unique one is Anti-Scl-70 (Casale et al., 1997).

1.3.6. Polymyositis (PM) and Dermatomyositis (DM)

They are an idiopathic inflammatory myopathies with incidence of 1:100,100 per year. They predominantly affect skeletal muscles, resulting in muscle inflammation and weakness. Other organ systems may be involved such as: skin, cardiac, gastrointestinal, and pulmonary systems(Khan & Christopher-Stine, 2011). PM and DM may occur in isolation or in connection with connective tissue disease or cancer. The cause of disease is not fully understood until now. However, different factors may lead to cause the disease such as: genetic, *Toxoplasma gondii* or *Coxasckie A* virus infection, or stress. The autoantibodies detection help in disease diagnosis. The anti-Jo-1 antibodies are found in PM and DM disease with ratio of 70%. (Hak, de Paepe, de Bleecker, Tak, & de Visser, 2011)

1.4 ANA detection techniques

Different immunological techniques are previously used to detect ANA. The first method is used by Hargraves, in 1947, to detect Lupus Erythematosus (LE) cells in bone marrow in order to diagnose Systemic Lupus Erythematosus (SLE). He gave an evidence that LE cells react with proteins and DNA inside the nucleus. Therefore, this method called Lupus Erythematosus (LE) cell test (Hargraves, Richmond, & Morton, 1948). However, this test also showed a positive results in other autoimmune diseases.

In 1959, Holman explained the phenomenon of LE cell. He showed that LE cells are a macrophages, inside their nuclei inclusion of degraded nuclear fractions (Holman & Deicher,

1959). After that, different techniques were developed. Most common techniques used are Indirect Immunofluorescent assay (IFA) and Enzyme Linked Immunosorbent Assay (ELISA).

1.4.1. Indirect Immunofluorescene assay (IIF)

In 1957, Friou applied the Indirect immunofluorescent assay for ANA detection (Friou, 1958). At that time, it was the only gold standard technique used for SARD diagnosis due to its high sensitivity and specificity (Copple, Sawitzke, Wilson, Tebo, & Hill, 2011; Meroni & Schur, 2010). This technique is performed by incubating the patient serum on slide coated with substrate. After that, a fluorescence tagged anti-immune globulin G antibodies are added to visualize the bounding antibodies to nucleus of substrate used by fluorescence microscope(Meroni & Schur, 2010).

Different substrates were previously used, such as: HeLa cells, tissue sections and chicken erythrocytes. In 1966, Tan modified the IIF technique using a substrate of liver or kidneys of mice (Tan & Kunkel, 1966). In 1975, HEp-2 cells were introduced as a substrate in IIF, and they are most commonly used for ANA detection until now, as they increased the sensitivity of the technique. These are cultured cells originate from human laryngeal squamous cell carcinoma (Buchner, Bryant, Eslami, & Lakos, 2014). They have a large nuclei, high rate of mitosis, and on their surfaces hundred antigens are presented. This increase their ability in detection of different auto antibodies (Muro, 2005).

The evaluating of cell staining results depend on a five major nuclear patterns: homogenous, speckled, centromere, nucleolar and nuclear. These are most commonly recognized patterns detected on Hep-2 substrates (Buchner et al., 2014). The homogenous

pattern is characterized by condensed chromatin of the mitotic cells. This pattern is the result of anti-dsDNA antibodies. In speckled pattern, two patterns are seen, fine and coarse. Fine or diffuse nuclear staining are seen in fine speckled pattern, and it is associated with anti-SSA and anti SS-B. The coarse pattern shows granular nuclear staining, as result of anti-sm and anti-RNP. In centromere pattern, multiple nuclear dot are shown, caused by anti-CENP antibodies. The nucleolar pattern are associated with anti-RNA polymerase III, anti-fibrillarin and anti-Th/To. It is a homogenous or speckled staining of nucleoli. The pattern nuclear is shown as result of anti-sp100.

In 1976, substrate of *crithidia* was used in IFF to detect the anti-dsDNA in sera of SLE patients. This species has a kinetoplast which is contains a large amount of double stranded DNA (Slater, Cameron, & Lessof, 1976).

1.4.2. The enzyme-linked immunosorbent assay (ELISA)

Recently, the enzyme-linked immunosorbent assay (ELISA) has replaced the IFF technique. It is most commonly used in clinical laboratory to detect ANA. This assay is based on antigens extracted from HEp-2 cells, or on recombinant antigens (Buchner et al., 2014; Copple et al., 2011; Emlen & O'Neill, 1997). There are different ELISA kits for ANA detection, and they are differ in sensitivity and specificity. The specificity of ELISAs for ANA test depends on the quality of antigens used. Therefore, it is very important to use the antigen with same sequence and conformation of human antigen (Cabiedes & Nunez-Alvarez, 2010; Copple et al., 2011).

Regarding ANA detection test in clinical laboratories, a screening of ANA test is firstly performed. Then, the positive ANA results are processed for Anti-ENA, or Anti-dsDNA ELISA kits in order to characterize the antinuclear antibodies presented in the patient sera. In screening ANA test, the ELISA wells are coated with a mixture of several nuclear antigens including:

dsDNA, histones, ribosomal P-proteins, nRNP/Sm, Sm, SS-A, SS-B, Scl-70, Jo-1, centromeres. Anti-ENA ELISA is separately coated with specific antigens on the ELISA wells including: nRNP/Sm, Sm, SS-A, SS-B, Scl-70, Jo-1. The other kit of anti-dsDNA, the wells are coated with dsDNA. Hence, ELISA is specific and sensitive technique for ANA screening, and also for ANA characterization (Abeles & Abeles, 2013; Agmon-Levin et al., 2014; Copple et al., 2011; Gill et al., 2003; Kumar et al., 2009; Mahler, Pierangeli, et al., 2014; Meroni & Schur, 2010; Neogi et al., 2010; Pisetsky, 2012).

1.4.3. Western blot assay

This assay is used to characterize the auto antibodies presented in the serum of patient. It is based on the nuclear proteins separation according to their molecular weight by sodium dodecyl sulphate- polyacrylmide gel electrophoresis (SDS-PAGE). The separated proteins are transferred onto a nitrocellulose membrane. Then, it is cut into strips to test the sera after a period of incubation time. This way, the auto antibodies existed in the sera are bound to specific antigens on the membrane. In order to detect the reaction between the antibodies and antigen after adding the second antibody, different methods can be done, most commonly are colorimetric and chemilumilenscence reaction. The positive results are shown as a band on the strip. Further, the band appeared on the strips are either compared to the marker or to the positive controls to identify the autoantibodies (Kumar et al., 2009).

However, previous studies showed that this technique only detects linear epitopes but not the conformational ones. Therefore, it may not detect anti-SSA/Ro and anti-Scl70 (Kumar et al., 2009).

The aim of study

The main aim of this study is to develop an ELISA test for screening systemic autoimmune diseases (SARD) using continuous cell-line culture and high rich DNA cells. The specific objectives are:

- 1- To identify and collect sera from different patients with systemic autoimmune diseases.
- 2- To test THP1 cell lines for their suitability in diagnosis of systemic autoimmune diseases using ELISA test.
- 3-To test the suitability of using Kinetoplast DNA rich non-pathogenic parasite (*Leishmania tarentolae*) for diagnosis of systemic autoimmune diseases using ELISA test.
- 4- To correlate the different obtained ELISA results with the results of immune-blotting assay using different *Leishmania tarentolae* and THP1 cells antigenic extracts.
- 5- To compare the obtained results of both used ELISA systems and immunoblotting with the results obtained using commercial kits for the detection of systemic autoimmune diseases.

Materials & Method

2.1 Sample collection and transportation

47 samples were collected from Al-Makassed hospital laboratory after obtaining approval from the hospital manager. The duration of sample collection was from July, 2013 until September, 2016.

2.2 Data collection

The data of patients who have positive ANA were collected from the archive department at Al-Makassed hospital. The data included age and gender. The patient age was included only for individual, who has a registration number at the hospital.

2.3 Tissue culture

A ready cultured THP1 and *Leishmania tarentolae* cells were used. The cells were counted, and observed under an inverted microscope for next passage preparation. Then, subcultured into several flasks using fresh DMEM and RPMI with 10% FCS, 1mg of L-Glutamine, 10mg/ml of penicillin-streptomycin (Sigma, USA). Media was filtered. For Leishmania subculturing, 1 ml from ready leishmanial culture media was added to 9ml from prepared RPMI media. Then, they were incubated at 26-28 °C. After 3 days, the growth of leishmania was seen under an inverted microscope to see if they need further sub-culturing. If the color was changed into yellow, the sub culturing was done immediately. For THP1 cells, the sub-culturing was done with dilution of 1:1 using DMEM media, then, incubated at 37 °C for 3 days, and monitored under inverted microscope to examine the cell's growth.

2.4 Cell counting

The counting of *Leishmania tarentolae* and THP1 cells was done manually on a hemocytometer slide using an optical microscope. The average cell count was calculated for all cells counted in each square, and multiplied by 10000.

2.5 Extraction of cytoplasmic and nuclear fractions

Regarding nuclear extraction from nucleus of THP1, and cytoplasmic extraction from *Leishmania tarentolae*, the cultured media with total cell count of 10^5 cell/ml were taken into a 50ml sterile falcon tube, centrifuged at 2500 rpm for 10 mins. Pallet was washed using 10 ml 1XPBS-T, and collected at 2500 rpm for 5 mins.

Cytoplasmic and nuclear extraction was done by adding 400 μ l from cytoplasmic buffer (10mM HEPES, 60 mM KCL, 1mM EDTA, 0.075% (v/v) NP40, 1mM DTT and 1mM PMSF, pH 7.6) to the tube in order to resuspend the pellet. Then, the suspension was incubated on ice for 3 min, centrifuged at 1500 rpm for 4 mins, and the supernatant (cytoplasmic extract) was transferred into a clean tube. Afterward, 400 μ l from cytoplasmic buffer without detergent NP40 was added to wash the pellet, centrifuged again at 1500 rpms for 4 min. After that, 200 ul from nuclear buffer (20 mM Tris NaCl, 1.5 mM MgCl₂, 0.2mM EDTA, 1 mM PMSF, and 25% (v/v) glycerol, pH 8) was added, and 200 μ l 5 M NaCl was also added to adjust the salt concentration. Then, another 200 μ l from nuclear buffer was added. Finally, the extract was incubated on ice for 10 mins, and then, it was mixed using the vortex. The final extracted nuclear, and cytoplasmic fractions were frozen at – 20 °C.

2.6 Salmon sperm DNA preparation

The concentration of salmon sperm DNA solution was prepared to be 0.1% (w/v) in double distilled water.

2.7 Protein and DNA quantitation

The DNA and protein concentration of THP1 nuclear fractions and leishmanial cytoplasmic extracts were measured by spectrophotometer. The optical density was read at 260 nm for DNA quantitation, and at 280 nm for protein quantitation. The concentration of DNA and protein were determined using the following equations:

- For protein: 1 OD at 280nm= 1mg/ml
- For DNA: OD at 260nm * 0.05 ug/ul * DNA dilution

2.8 Enzyme Linked Immunosorbent Assay (ELISA)

Each extract from salmon sperm DNA, *Leishmania tarentolae* and THP1, were coated in different concentration: 10, 50 and 100 ng/ml, into separate polystyrene flat bottom 96 wells ELISA plates. Then, 100 µl from each were coated into separate plates. Kept at 4°C for 3 days, washed three times with 1XPBS with 0.05 Tween-20 solution very well to remove the excess of uncoated antigens. 100 µl from blocking solution (5% FCS-PBS-T) was added for half an hour. Then, first antibody was added, a total of 47 samples and negative control sera. All were tested using extracts from the cells mentioned above. The dilution of sample was 1:100 and 1:200. All samples were tested in duplicates. After two hours of incubation at room temperature, the contents were discarded and wells were washed three time with 1XPBS. 100 µl diluted protein A-HRP (1:6000) was added as second Abs and incubated for 1 hour at room temperature, washed three times with PBS-T. 200 µl of the substrate-chromogen solution (1mg of the O-phenyldiamine to each 1ml pf citrate buffer (PH 4.5) then 4 µl of the substrate H₂O₂ was added to each 10 ml of the citrate buffer containing the chromogen and incubated for 30 minutes at room temperature, color development was measured at 490 nm by using ELISA reader.

2.9 Immunoblotting (Western blot)

The immunoblotting technique was performed according to Towbin et al (Towbin, Staehelin. & Gordon 1979) in order to characterize the autoantibodies found in positive ANA sera. A total number of 23 positive sera were tested against *Leishmania tarentolae* cytoplasmic extract, and 28 positive sera were tested against THP1 nuclear extracts. In addition, 5 negative sera were tested in each trial.

2.9.1. SDS-PAGE Gel preparation

Mini-PROTEAN casting stand and frame (BIO-RAD) were assembled. In order to make 10% resolving gel, 4.7ml of double distilled water were added to a clean tube. Then, 2.5 of 40% polyacrylamide, 2.6 of 1.5M Tris-HCl, pH 8.8 and 100 µl from 10% SDS were also added. 100 µl from Ammonium persulfate (APS) and 10 µl from Tetramethylethylenediamine (TEMED) were the last material added, then all were mixed and poured in the glass plates. The gel were left for about 20-30 min at room temperature to make sure gel had polymerized. Then, 5% stacking gel was prepared by adding 6.2 ml from double distilled water, 1ml of 30% polyacrylamide, 2.6 ml of 0.5 M Tris-HCL pH 6.8, 100 µl from 10% SDS, 100 µl from 10% APS and 10 µl from TEMED. They were mixed and poured on top of resolving gel. The gel was poured until it reached to the end of glass plate, then, comb were inserted, and left for another 20-30 mins.

2.9.2. Sample preparation and loading

Before loading the extracts into the wells of polyacrylamide gel, two volume of extracts were mixed with one volume of loading buffer (Bromophenol 0.004%, 2-mercaptoethanol 10%, Glycerol 20%, SDS 4%, Tris-HCl 0.125). Then, the mixture was heated in a water bath at 95 C for 10 min.

2.9.3. SDS-PAGE Gel Electrophoresis

The gels were soaked in 1X running buffer (SDS 0.1%, Tris-HCl 25mM and glycine 200mM) inside the gel electrophoresis tank. After that, 5 µl from the molecular size marker (New England Biolabs Inc, Beverly, MA, USA) was loaded into one well of each polyacrylamide gel, and 20 µl from the mixture was loaded into each of 7 wells. The electrophoresis was carried out at 50 V for about 1 hour, then the voltage was increased at 100 V for another 2 hours.

2.9.4. Western blotting- Gel transfer

After separation the proteins using SDS-PAGE gel, they were transferred into nitrocellulose filter membrane (Schleicher and Schauell, Dassel, Germany). The polyacrylamide gel was soaked into 1X transfer buffer (0.025 M Tris-HCl, 0.192 M Glycine, and 20% methanol). Then, the gel and the nitrocellulose filter membrane were firmly gathered together inside the gel holder cassette. The gel holder cassette was inserted into Mini Trans-Blot tank filled with 1X transfer buffer (0.025 M Tris-HCl, 0.192 M Glycine, and 20% methanol), then the transfer was carried out at 100 V for one hour. Then, the membrane was stained by Ponceau S (0.1% w/v in 1% v/v acetic acid) (Sigma, Sant Louis, USA) for 1 minute followed by destaining in 1XPBS.

2.9.5. Blocking, first antibody and secondary antibody incubation

Each membrane was cut into strips. These strips were blocked by 1X PBST plus 5%FCS for one hour. The following step, 2ml of 1:200 diluted sera with 1X PBST plus 5% FCS were added to the strips. Then, incubated for 2 hours at room temperature. The strips were washed 3

times using 1X PBST. Secondary antibody (1:4,000 dilution, Protein A) was added to each strip, and incubated for 1 hour. After incubation, strips were washed three times using 1XPBST.

2.9.6. Electrogenerated Chemiluminescence ECL

ECL detection was performed immediately after streptavidin-HRP incubation and washing using EX-ECL detection kit (Biological Industries, Beit Haemek, Israel).

Results

3.1 Pilot study for the previous positive ANA cases:

Records (2012-2015) of Al-Makassed hospital, showed that 2,102 from in and out-patient had done ANA test. Only total of 147 individuals were found to be ANA positive (Table 3.1). From the 147 positive cases; 121 cases were females and it was clearly seen that number of cases in females are always more all over the pilot study survey.

Table 3.1: Total number of ANA diagnosed individuals at Al-Makassad hospital laboratory during (2012-2015).

Year	Number of tested samples			Number of positive cases according to gender		
	Male	Female	Total	Male	Female	Total
2012	204	313	517	6	41	47
2013	204	293	497	6	26	32
2014	247	321	568	8	34	42
2015	200	320	520	6	20	26
Total	855	1,247	2,102	26	121	147

Table 3.2 represents the number of positive cases according to age (only from in-patient hospital clinic); in which it can be seen that most of the diagnosed cases were between (22-30), (31-40), and (41-60) years, while less cases were discovered in early ages or later in life.

Table 3.2: Total number of ANA diagnosed individuals at Al-Makassad hospital laboratory grouped according to age (2012-2015).

Year	Number of positive samples according to age							
	0-5	6-12	13-21	22-30	31-40	41-60	60-80	>80
2012	3	0	0	6	3	10	2	0
2013	5	4	2	8	2	5	5	1
2014	0	0	1	9	6	9	0	0
2015	0	0	5	4	7	8	2	0
Total	8	4	8	27	18	32	9	1

3.2 Optimization of Enzyme linked immunoassay (ELISA):

3.2.1. ELISA based on whole intact cells:

Three different types of cells (THP1, 3T3, and *Leishmania tarentolae* cells) were used in pilot study that aimed to find the most suitable antigen to be used in ANA-ELISA system. At the beginning all these cells were used as intact cells and in a concentration of $10^5/\text{ml}$ in DMEM media for both THP1 and 3T3, while for *Leishmania tarentolae* cells were diluted in RPMI media. Different ELISA plates were coated with 100 μl of each of the three cell type, plates were kept overnight at 4°C . The coated plates were tested with 1:100 and 1:200 diluted sera from previously collected ANA positive samples and with known negative control sera. For the three types of cells only sera samples with high ANA antibody titer gave significant optical density readings compared to negative controls with no superior results concerning the type of the used cells. Figure 2 shows ELISA plate coated with *Leishmania tarentolae* cells and its reactivity against the tested sera. Similar results were obtained for the other cells.

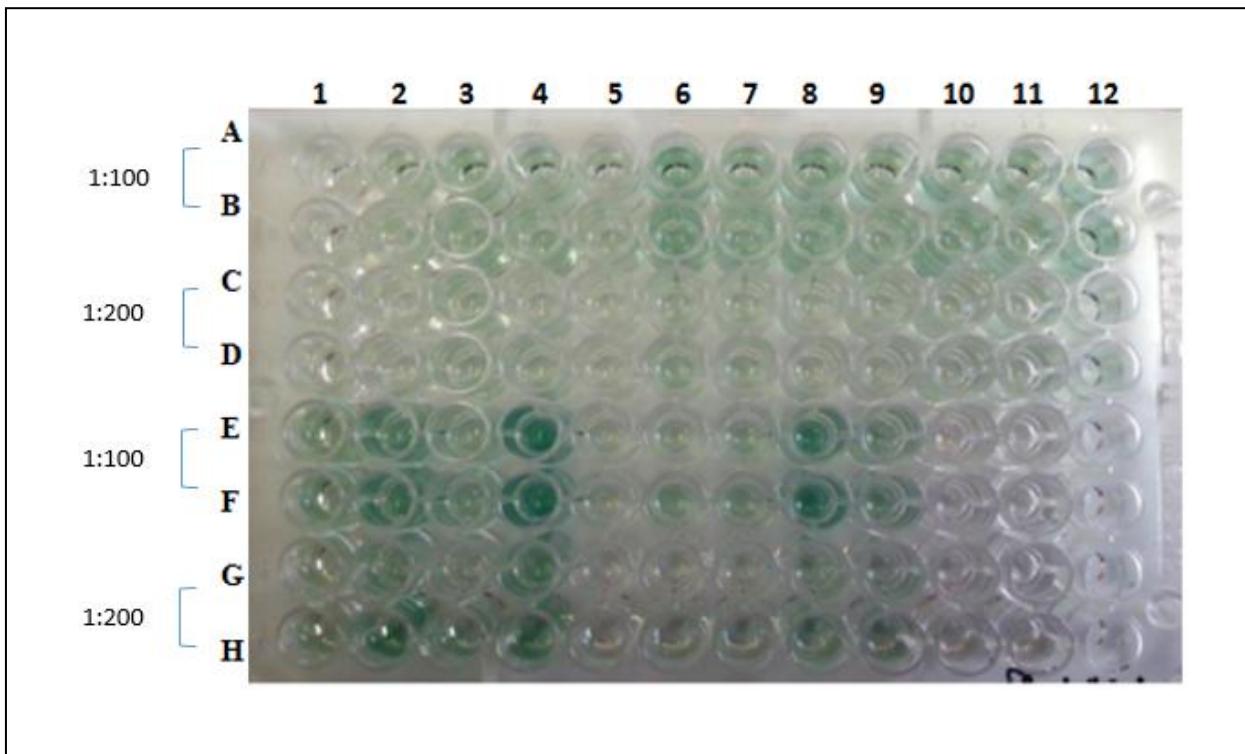


Figure 3.1: ELISA plate coated with *Leishmania tarentolae* cells that was used to test 1:100 and 1:200 diluted ANA negative sera (negative samples 2,3,4,5 (A-D), and other ANA positive sera samples (6-12/A-D), and (1-12/E-H). Blank (1/A-D)

3.2.1. ELISA results using different sources of DNA.

In order to optimize ELISA conditions to follow up the reactivity of ANA positive sera against dsDNA; two different types of DNA were used: 1- DNA that was extracted from cattle liver tissue and 2- Salmon sperm DNA (ssDNA), that was commercially purchased (Sigma, St. Louis, USA). Initially; the two different types of DNA were used in coating ELISA plates in 4 different concentrations (10ng/ml, 50ng/ml, and 100ng/ml). Also two different types and concentrations of second antibodies (anti-human IgG and protein-A) were tested. Coating plates with 100ng/ml of ssDNA combined with protein-A gave the optimal results for performing ANA

ELISA taking into consideration the maximum number of reactivity with different ANA selected positive samples and no reactivity with negative control sera.

3.3 Enzyme linked immunoassay of ANA positive sera against antigenic preparations.

A total number of 47 positive sera samples were obtained from Al-Makassed hospital. The ANA positivity of the obtained samples was confirmed using ANA-screening test (Euroimmun ANA screening kit, Leubeck, Germany). The characterization of auto-antibodies in these individuals was performed using (Euromen ENA profile and Euroimmun anti-dsDNA, Leubeck, Germany) and it was conducted in Al-Makassed laboratories for some samples and the rest of the samples were characterized in Al-Quds University. For the obtained 46 samples; 29 samples were tested against salmon sperm DNA (DNAss), 47 samples were tested against cytoplasm extract from *Leishmania tarentolae* cells, and nuclear extract from THP1 cells.

Figure 3.2 shows the ELISA results of the tested positive sera in two different dilutions (1:100 and 1:200). The obtained ELISA results using DNAss extract as antigen showed 100% positivity in 1:100 antibody titer and 65% positivity using 1:200 antibody titer. Similarly; the ELISA results were found to be positive in all tested samples against *Leishmania tarentolae* cytoplasmic extract and THP1 cells nuclear extracts at 1:100 antibody dilution. While at 1:200 antibody dilution the results were positive for 85% of samples using *Leishmania tarentolae* cytoplasmic extract, and 91% using THP1 cells nuclear extracts.

Table 3.3, show the calculated average results (optical density readings) for 29 serum samples tested against salmon sperm DNA and for 47 sera samples tested separately against *Leishmania tarentolae* cytoplasmic extract and THP1 nuclear extract. Any reading below the

cut-off value was indicated as a negative result. The cut-off value was calculated using the results of negative samples that were run in same ELISA of the indicated antigens.

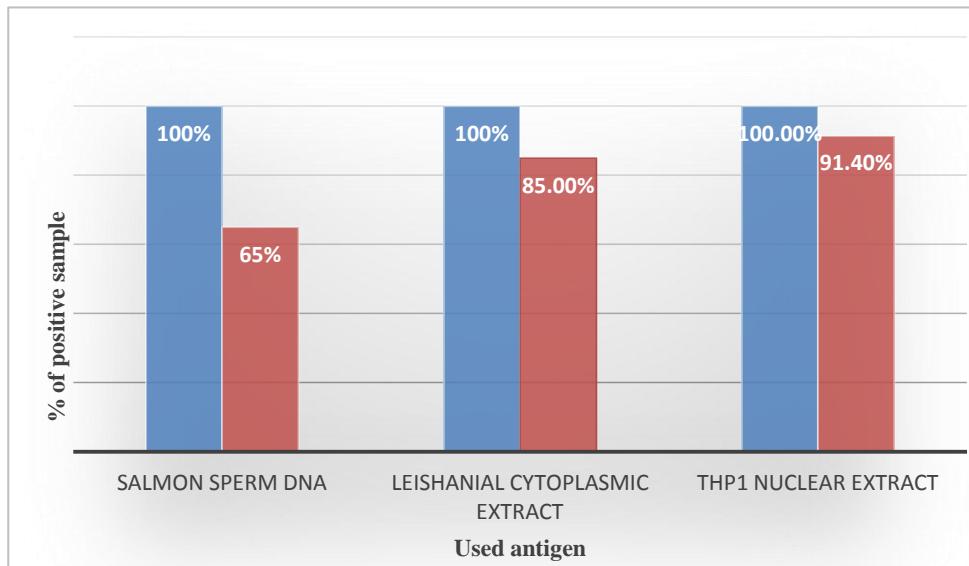


Figure 3.2: The percentages of positive ELISA results of the tested samples using two different antibody titers and against three different antigenic preparations.

Table 3.3: The results of tested ANA positive samples using ELISA against salmon sperm DNA, *Leishmania tarentolae* cytoplasmic extract and THP1 nuclear extract. The results are expressed as spectrophotometric absorbance values

Sample #	Salmon sperm DNA		<i>Leishmania</i> cytoplasmic extract		THP1 nuclear extract		ANA profile
	1:100	1:200	1:100	1:200	1:100	1:200	
Sample1'	Negative	Negative	Negative	Negative	Negative	Negative	ND
Sample2'	Negative	Negative	Negative	Negative	Negative	Negative	ND
Sample3'	Negative	Negative	Negative	Negative	Negative	Negative	ND
Sample4'	Negative	Negative	Negative	Negative	Negative	Negative	ND
Sample5'	Negative	Negative	Negative	Negative	Negative	Negative	ND
Sample6'	Negative	Negative	Negative	Negative	Negative	Negative	ND
Sample7'	Negative	Negative	Negative	Negative	Negative	Negative	ND
1	0.203	Negative	0.5	0.346	0.7645	0.4955	Anti-SSA+SSB
2	ND	ND	0.5	0.3385	0.8765	0.4425	Anti- SSA
3	0.66	0.306	0.399	0.173	0.329	0.1855	Anti-SSA+SSB
4	0.405	0.255	0.4605	0.259	0.4215	0.1965	ND
5	0.692	0.4635	ND	ND	ND	ND	Anti-dsDNA
6	0.7545	0.2315	0.4	0.198	0.3875	0.1375	Anti-SSA+SSB
7	0.6465	0.2695	0.7355	0.3645	0.775	0.648	Anti-SSA+SSB, nRNP
8	0.394	0.159	0.4845	0.199	0.334	0.1795	ENA -ve
9	0.4925	0.1895	0.463	0.1605	0.2365	0.154	Anti-dsDNA
10	0.553	0.2695	0.632	0.314	ND	ND	ENA -ve
11	0.345	0.2415	0.353	0.1975	0.4185	0.219	Anti-nRNP-sm
12	0.204	Negative	0.595	0.2515	0.73	0.5475	Anti-dsDNA
13	ND	ND	0.4305	0.188	0.342	0.218	ND
14	2.5	2.126	1.6755	1.152	1.5	1.0	Anti-dsDNA
15	0.8025	0.2505	0.4585	0.2095	0.527	0.275	Anti-dsDNA + sm
16	0.2625	0.1755	0.3955	0.2895	0.777	0.7135	Anti-nRNP-sm
17	0.2535	0.177	0.682	0.3355	0.787	0.5023	Anti-SSA+SSB
18	0.412	0.259	0.2245	Negative	0.3345	0.173	Anti-SSA+SSB
19	1.6545	0.5815	0.3255	0.162	0.9465	0.39	Anti-dsDNA
20	0.2465	Negative	0.484	0.3375	0.5825	0.446	Anti-SSA+SSB

21	0.2195	Negative	0.53	0.211	0.418	0.2185	ENA -ve
22	1.9	0.951	0.39	0.197	0.6075	0.342	Anti-nRNP-sm +SSA
23	0.2	Negative	0.567	0.2325	1.2795	0.389	ENA -ve
24	0.3515	Negative	0.574	0.395	0.5795	0.4255	Anti- Scl-70
25	0.234	Negative	0.3295	0.2605	0.4545	0.34	ENA -ve
26	0.3535	Negative	0.9015	0.2325	1.215	0.5425	Anti- nRNP-sm
27	0.228	Negative	0.27	0.1915	0.2525	0.189	Anti-SSA
28	1.1535	0.26	0.5	0.2755	1.5	0.6755	ENA -ve
29	1.08	0.2445	0.2885	0.1445	0.427	0.2145	Anti-nRNP-sm
30	1.0275	0.27	0.3	0.151	1.1155	0.555	ND
31	0.151	Negative	0.1875	0.155	0.3985	0.3105	ND
32	ND	ND	0.3	0.175	0.479	0.279	Anti-nRNP-sm
33	ND	ND	0.2515	Negative	0.254	Negative	ND
34	ND	ND	0.776	0.247	1.777	1.347	ND
35	ND	ND	0.3195	Negative	0.2485	Negative	Anti-SSA
36	ND	ND	0.368	0.205	0.589	0.2975	Anti-nRNP/sm
37	ND	ND	0.458	0.1765	0.28	0.1965	ND
38	ND	ND	1.208	1.0085	1.641	1.1315	Anti-nRNP/sm
39	ND	ND	0.5475	0.2995	0.667	0.361	Anti-SSA
40	ND	ND	0.289	Negative	0.244	Negative	ND
41	ND	ND	0.2945	Negative	0.7175	0.262	Anti-dsDNA
42	ND	ND	0.282	Negative	0.2465	0.2025	Anti-SSA+SSB
43	ND	ND	0.3465	Negative	0.6425	0.206	ND
44	ND	ND	0.275	0.176	0.2695	Negative	ND
45	ND	ND	0.276	Negative	0.244	Negative	Anti-nRNP/Sm &SSA
46	ND	ND	0.395	0.284	0.3255	0.204	Anti-SSA
47	ND	ND	0.9695	0.834	1.554	1.133	Anti-SSA+SSB

The ELISA results of tested ANA positive serum samples were presented in a separate dot blot figure for each antigen in the ELISA test (Figure 3.3). Higher optical density readings were obtained upon the use of salmon sperm DNA as an antigen compared to other cell extracts at dilution 1:100, and although the readings were still higher in 1:200 dilutions; some samples turned to be negative compared to the same samples that were shown to be positive against the other used *Leishmania tarentolae* cytoplasmic extract and THP1 nuclear extract. The optical density reading of the THP1 nuclear extract were relatively higher than those obtained using *Leishmania tarentolae* cytoplasmic extract, with more positivity upon the use of 1:200 diluted sera samples, a results that reflected more specificity of this antigen in ELISA format approach for the detection of ANA/ENA antibodies. A full discussion of this finding and its future benefits is highlighted in the discussion.

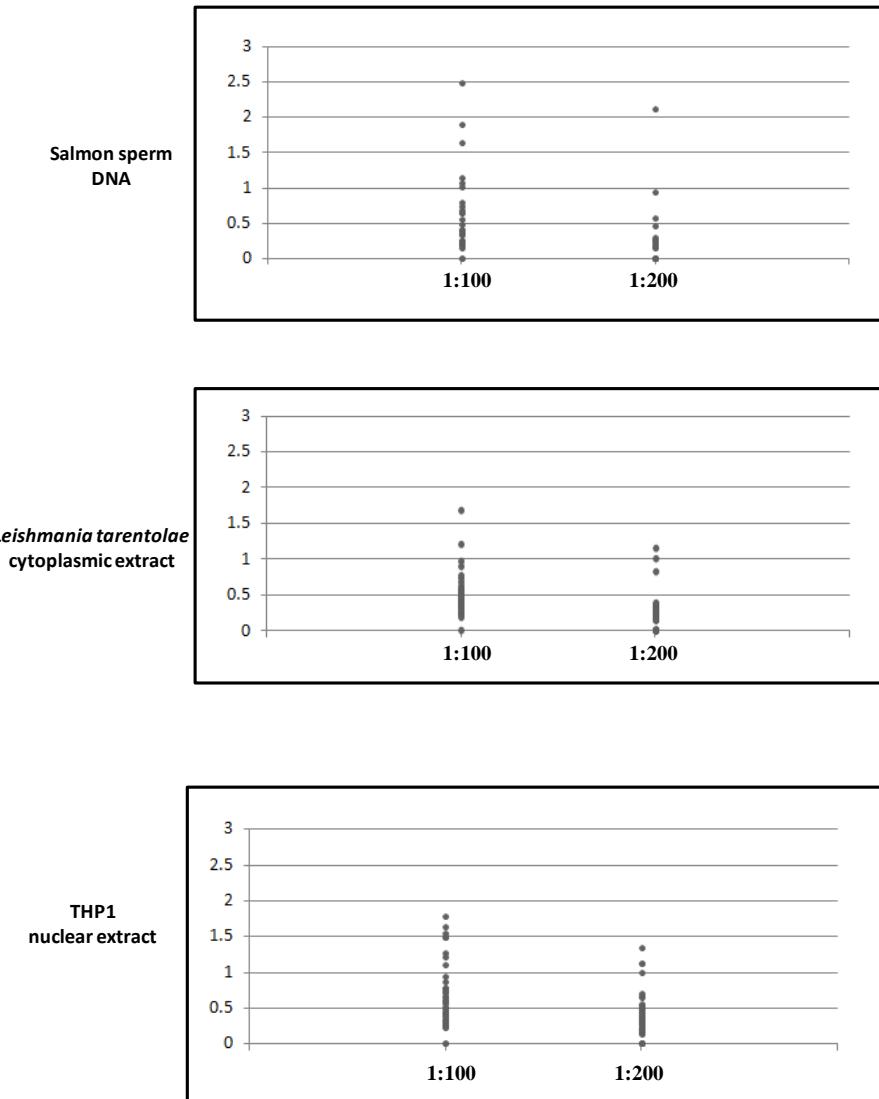


Figure 3.3: The result of tested ANA positive samples in ELISA against salmon sperm DNA, *Leishmania tarentolae* cytoplasmic extract and THP1 nuclear extract.

3.4 Western-Blot analysis of ANA positive sera samples against *Leishmania tarentolae* cytoplasmic extract and THP1 cells nuclear extracts.

The immune-blotting profile of the reactive auto-antibodies found in ANA positive serum were analyzed using Western-Blot method. A total of 23 samples were analyzed against proteins of *Leishmania tarentolae* cytoplasmic extract, and another 29 ANA positive sera samples were analyzed against nuclear extract from THP1 cells. From these samples; 19 different positive sera were shared in this analysis using the tow extracts, and external negative control sera were also included. All tested samples were diluted 1:100 in antibody dilution buffer (PBST-FCS) before analysis.

Figure 3.4 shows the reactivity profile of autoantibodies in the tested ANA positive sera against *Leishmania tarentolae* cytoplasmic proteins. A band with molecular size of 48 kD appeared in some strips, specifically strips number (7, 8, 9, 10, 18, 19, 23, 24 and 25). Strips number: 5, 6, 7 and 19 showed reactivity to a 60 kD band. While strips number 15, 17, 21 and 22, were reactive with a band of 18 kD. Other smaller band size in a range of 9-30 kD were also detected in other strips. The importance of these findings and their correlation to the clinical finding in each of the tested ANA positive sera is shown below.

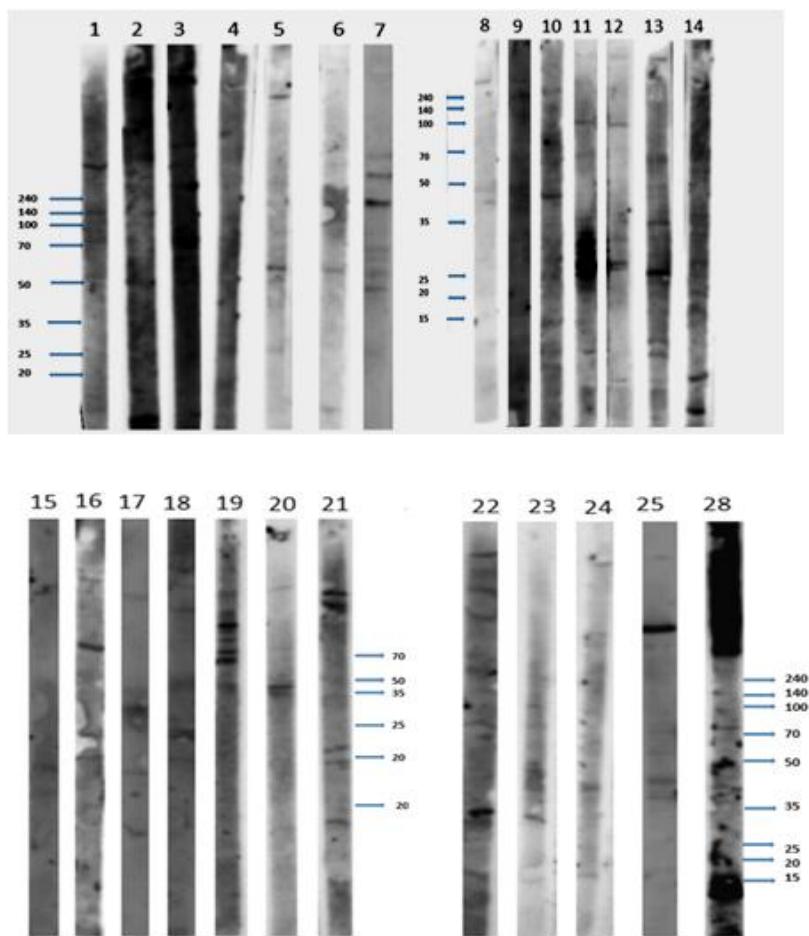


Figure 3.4: Immuno-blotting reactivity of ANA positive sera against SDS-PAGE separated molecules from *Leishmania tarentolae* cytoplasmic extract.

The immune-blotting pattern of the tested ANA positive sera against THP1 nuclear extract proteins is shown in figure 3.5. Most of the strips showed reactivity with different bands in a range of 25 to 70 kDa, although some strips showed no reactive bands (strip number 11,12,19,20 and 21). In details: a band with molecular size of 30 kDa appeared in strip 9, 13 and 16. While 48 kDa band showed in strips numbered: (5c, 11c, 6, 7, 8, 14, 15). 50 kDa band appeared in 4c, 17 and 18, and 60kDa band appeared in 8c, 6, 10 and 17. The strip of 5c and 7c show 25kDa band. 35 kDa band was appeared in strip # 9c. A band with molecular size of 70 kDa appeared on strips 6c and 10c.

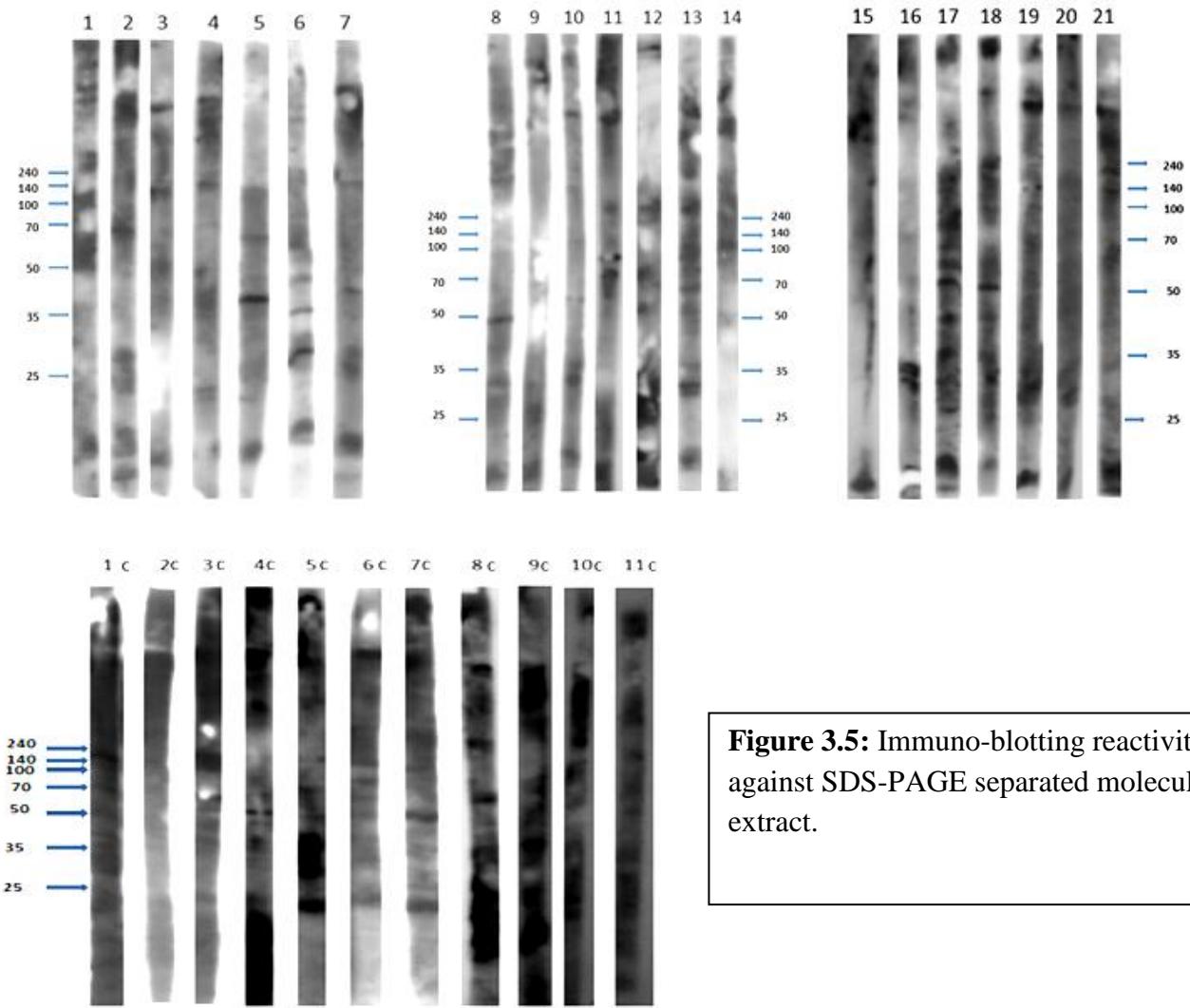


Figure 3.5: Immuno-blotting reactivity of ANA positive sera against SDS-PAGE separated molecules from THP1 nuclear extract.

3.5 Similarity between ENA profiles and immuno-blotting results of the tested ANA positive sera:

The ENA profile test for the selected ANA positive sera samples was determined using commercial kit (Euroimmun, Leubeck, Germany), the main purpose of this test was to determine the type of autoantibodies in patients sera. The tested antigens in this kit were extracted from calf thymus, and include 6 different antigens: nRNP/sm, sm, SS-A, SSB, Scl-70 and Jo-1. The molecular weight of nRNPs and sm are ranging from 9 to 29.5 kDa. While SS-A, SSB, Scl-70 and Jo-1 have molecular weights of 60, 48, 70 and 50 kDa respectively.

Table 3.4 shows the combined results of 23 positive ANA sera in terms of ENA profile, anti-dsDNA test, results of leishmanial ELISA extract, and together with the obtained immunoblotting banding pattern. As shown, Most of these tested positive sera and in a dilution of 1:100 were positive in ELISA testing against leishmanial extracts and all were reactive upon immunoblotting analysis. However, not all of these bands were related to ENA profile.

Sample # 6, strip# 6 gave a band with molecular size of 60 kDa which is similar to SSA antigen molecular weight. As shown in table 3.4, sample 6 has a profile of anti-SSA and anti-SSB autoantibodies. In addition, the 60 kDa band with another band sized 48 kDa were seen in strip # 7, sample #7. The 48 kDa band is predicted to be SSB antigen, as the sample #7 also showed anti-SSA and anti-SSB. Similarly, samples number 18, strip number 18, also showed 48 kDa band. Other samples, such as # 15, 18 and 22 they showed a band size close to 18 and 20 kDa, and their profile was found to be anti-sm or anti-nRNP/sm autoantibodies, so, it is expected that these bands were related to nRNP and sm antigens.

Table 3.4: Summary of the immuno-blotting results of the ANA positive sera reacted against *Leishmania tarentolae* cytoplasmic extract and their correlation to the found ENA profile results.

strip #	Sample #	Band MW (kDa)	ENA profile and dsDNA test	Leishmanial extract ELISA result
1	-	-ve	ND	-ve
2	-	-ve	ND	-ve
3	-	-ve	ND	-ve
4	4	30, 18	ND	+ve
5	5	30, 60	Anti-dsDNA	+ve
6	6	9. 60	Anti-SSA&SSB	+ve
7	7	48, 60	Anti-SSA&SSB Anti-nRNP/sm	+ve
8	8	48	ENA -ve	+ve
9	9	9, 49	Anti-dsDNA	+ve
10	10	15, 48	ENA -ve	+ve
11	11	30	Anti-nRNP/sm	+ve
12	12	27, 100	Anti-dsDNA	+ve
13	13	27,35, 70	ND	+ve
14	14	38,27	Anti-dsDNA	+ve
15	15	18	Anti-dsDNA Anti-sm	+ve
16	16	20, 80	Anti-nRNP/sm	+ve
17	17	18, 30	Anti-SSA&SSB	+ve
18	18	48	Anti-SSA&SSB	+ve
19	19	48 60	Ant-dsDNA	+ve
20	21	48	ENA -ve	+ve
21	22	18, 100	Ant-nRNP/sm Anti-SSA	+ve
22	23	35, 18	ENA -ve	+ve
23	24	35, 48	Anti-Scl70	+ve
24	25	48	ENA -ve	+ve
25	26	48	Anti-nRNP/sm	+ve
26	36	15	Anti-nRNP/sm	+ve

Similarly, the result of 24 positive ANA sera samples, summarized in table 3.5 taking into account the ENA profile and the obtained immunoblotting results against THP1 nuclear extract in correlation to ELISA results. However, 5 samples did not show any reactive bands. Samples (Sample #/strip#) 1/6, 2/7, 3/8, 7/5c, 17/14 and 18/15 showed a band with size 48 kDa, and these samples at least were positive for anti-SSB. Sample #1 strip# 6 also showed a 60kDa band, as well as samples (sample/strip): 6/10 and 20/17. The 60 kD band is expected to be for SSA antigen, as these samples showed a positive result for this antigen. However, sample/strip 6/10 and 20/17 showed another band sized 50 kDa, that indicated the presence of SSB antigen. Other samples that show positive reaction in ENA profile for nRNPs/sm or for sm antigens, showed a band ranging from 25 to 35 kD, such as (sample #/strip#): 15/13, 7/5c, 16/9c. The sample # 24/ strip# 10c showed a band with size 70 kDa. Based on ENA profile results, this sample was positive for anti-Scl70. Thus, this band was predicted to be antigen Scl70.

Table 3.5: Summary of the immuno-blotting results of the ANA positive sera reacted against THP1 nuclear extract and their correlation to the found ENA profile results.

strip #	Sample #	Band MW (kDa)	ENA profile and dsDNA test	THP1 extract ELISA (1:100)
1	-	-ve	ND	-ve
2	-	-ve	ND	-ve
3	-	-ve	ND	-ve
4	-	-ve	ND	-ve
5	-	-ve	ND	-ve
6	1	6,18, 48,60	Anti-SSA&SSB	+ve
7	2	48	Anti-SSB	+ve
8	3	48	Anti-SSA&SSB	+ve
9	5	30	Anti-dsDNA	+ve
10	6	60	Anti-SSA&SSB	+ve
11	12	-ve	Anti-dsDNA	+ve
12	14	-ve	Anti-dsDNA	+ve
13	15	30	Anti-dsDNA Anti-sm	+ve
14	17	48	Anti-SSA&SSB	+ve
15	18	48	Anti-SSA&SSB	+ve
16	19	30	Anti-dsDNA	+ve
17	20	52, 60	Anti-SSA&SSB	+ve
18	22	50	Anti-snRNP/sm Anti-SSA	+ve
19	23	-ve	ENA -ve	+ve
20	26	-ve	Anti-nRNP/sm	+ve
21	28	-ve	ND	+ve
22-4c	4	50	ND	+ve
23-5c	7	25, 48	Anti-SSA&SSB Anti-nRNP/sm	+ve
24-6c	8	70	ENA -ve	+ve
25-7c	9	25	Anti-dsDNA	+ve
26-8c	10	60	ENA -ve	+ve

27-9c	16	35	Anti nRNP/sm	+ve
28-10c	24	70	Anti-Scl-70	+ve
29-11c	25	48	ENA -ve	+ve

A comparison between the immunoblotting banding pattern obtained by the use of leishmanial cytoplasmic and THP1 nuclear extracts is summarized in table 3.6, in which the results of 19 shared sera samples were analyzed by all tests. As it can be seen from this table, only 6 samples gave a shared banding pattern upon the use of leishmanial and THP1 extracts in immunoblotting analysis. From these samples, only the result of 3 samples were related to ENA profile; for example, sample number 6 showed a band sized 60 kDa in both leishmanial and THP1 extract strips, and they also showed a positive reaction for SSA antigen. While in sample 7 and 18, they gave a positive result for a band size of 48 kDa in both extracts, leishmanial and THP1, which was the same molecular weight for SSB antigen.

Table 3.6: The correlation results between *Leishmania tarentolae* and THP1 immunoblotting and ENA profile testing

Number	Sample #	Bands in strip of <i>Leishmania tarentolae</i> (MW in kDa)	Bands in strip of THP1 (MW in kDa)	Autoantibodies profile
1	5	30, 60	30	Anti-dsDNA
2	9	9, 49	25	Anti-dsDNA
3	12	27, 100	-ve	Anti-dsDNA
4	14	38,27	-ve	Anti-dsDNA
5	19	48, 60	30	Anti-dsDNA
6	15	18	30	Anti-dsDNA Anti-sm
7	6	9, 60	60	Anti-SSA&SSB
8	17	18, 30	48	Anti-SSA&SSB
9	18	48	48	Ant-SSA &SSB
10	7	48, 60	25, 48	Anti-SSA&SSB Anti-nRNP/sm
11	22	18, 100	50	Anti-snRNP/sm Anti-SSA
12	26	48	-ve	Anti-nRNP/sm
13	16	20, 80	35	Anti nRNP/sm
14	24	35,48	70	Anti-Scl70
15	8	48	70	ENA -ve
16	23	35,18	-ve	ENA -ve
17	10	15, 48	60	ENA -ve
18	25	48	48	ENA -ve
19	4	30, 18	50	ND

Discussion

The anti-nuclear autoantibodies are self-reactive antibodies that attack different self-nuclear antigens. They are known to be presented in patients with autoimmune disorders as result of peripheral immune tolerance breakdown. This may be due to different triggered caused by genetic or any other factors such as environment and hormones. Patients with Systemic Autoimmune Rheumatic Disease (SARD) are suspected to have more than one anti-nuclear autoantibodies targeting different organs in the body, leading to severe injuries in tissues and organs. Women are known to be more susceptible to develop Systemic Autoimmune Rheumatic Disease (SARD). For example, it was reported that Systemic Lupus Erythematosus (SLE) affects women ten times more than men (Danchenko et al., 2006). In other autoimmune disease, a different study reported that 9 women to 1 man are affected with sjogren syndrome (Manoissakls, 2001). In this pilot archive data regarding diagnosed patients with positive ANA at Al-Makassed laboratory hospital during the years (2012-2015), it was found that women were more affected than men in terms of autoimmune disorder. Different studies explained the possible reasons for sex difference in autoimmune disorder development. For example, the immune system of women produce more antibodies and T helper 2 cells against external foreign antigens. Thus, the high level of antibodies increases the risk of developing an autoimmune disease. Furthermore, estrogen is known to be capable of altering the immune response and developing autoimmune disease (Beeson, 1994; Cutolo et al., 2012).

Autoimmune disease can be developed at any age (Manuel J., Alberto & Gladis 2012). However, the age onset of autoimmune disease depend on the type of the disease. For example, In SLE disease, 56% of patients start to show symptoms between ages 16 and 55, while 35% of them show symptoms before age 16, and after age 55. However, In Sjögren's syndrome, patients between ages 45 and 50 are more susceptible to have the disease. Another autoimmune disease can develop between ages 30 and 50, such as rheumatoid arthritis. In this study for positive ANA cases during (2012-2015), table 3.2. It was also found that autoimmune disease strikes all ages. However, most of the diagnosed cases were between (22-30), (31-40), and (41-60) years, while less cases were discovered in early ages or later in life.

For the antinuclear autoantibodies detection and identification, the Indirect Immuno-fluorescent technique (IIF), and Enzyme Linked Immunosorbent assay (ELISA) which are still in use for that purpose. In addition, Western blot technique is also performed to identify ANA in patient sera. In these techniques, different antigens were used from cells of liver, kidney, or thymus that are taken from either mice or calves. Scientists tend to target these organs because their cells have many large nucleus. Recently, the most cells used in IFF and ELISA are HEp-2 cells, which are originated from human laryngeal squamous cell carcinoma. They show a high rate of division in culture, also their cells have large nuclei. In addition, hundreds of antigens existed on their surfaces. So that, they increase the sensitivity and specificity of the ANA test in both IIF and ELISA.

In this study, 47 positive ANA sera samples were collected from Al-Makassed hospital laboratory. The sera samples were tested using the commercial kit of ANA screening, and ENA profile (Euroimmun, Leubeck, Germany). The ANA screening test helps only to determine if the sera sample contained anti-nuclear autoantibodies or not. If the result was positive, the sample

must be followed by another test, known as anti-ENA profile test. The main aim of this test is to identify the type of the anti-nuclear autoantibodies in the patient's serum. These kits use purified antigens from calf thymus. The purified antigen of dsDNA, histones, nRNP/S, Sm, SSA, SSB, Scl-70, Jo-1, ribosomal-P proteins, and centromeres are prepared as a mixture to be coated in the ANA screening ELISA test. However, in anti-ENA profile test, only 6 antigens are coated separately. The 6 antigens are nRNP/Sm, Sm, SS-A, SS-B, Scl-70, and Jo-1. Furthermore, an additional test is required when the result of anti-ENA profile is negative which is known as anti-dsDNA test. Hence, as these antigens are the most common antigens targeted by the anti-nuclear autoantibodies in SARD disease, most of clinical laboratories use the above mentioned kits and follow the steps of ANA testing, in order to give a proper diagnosis for the profile of anti-nuclear autoantibodies.

Going deeply to discuss more about the main aim of this research, and discussing more about the result of ELISA tests: The antigenic preparation from salmon sperm DNA, THP1 cell and *Leishmania tarentolae* were used in ELISA, in order to develop an ELISA ANA screening test. Because the THP1 cells are human leukemia monocyte cell lines having unipolar nuclei, and the *Leishmania tarentolae* are non-pathogenic cells that are known to have a kinetoplast in the cytoplasmic region; which contains a network of circular DNA molecules, it was assumed that these cells will be suitable in ELISA for ANA screening. The results of ELISA assays using different antigenic preparations from the above indicated cells revealed that the use of salmon sperm DNA in ELISA assay gave higher OD readings compared to result of THP1 and leishmanial extract ELISA assays. It was clear from table 3.3 which represents ELISA reading results that these values were for sera samples with anti-dsDNA autoantibodies profile. Furthermore, the result of THP1 and leishmanial ELISA test, revealed that THP1 nuclear extract

was better than leishmanial extract for ANA screening test. Taken together, it is concluded that salmon sperm DNA is more suitable for anti-dsDNA ELISA assay. In addition, The THP1 nuclear extract is more appropriate for ANA screening test than leishmanial extract. It is also concluded that the correspondence between the anti-nuclear autoantibodies and the conformational structure of THP1 nuclear antigens are greater than leishmanial extract, and this maybe because THP1 cells were taken from human.

Furthermore, immunoblotting technique to characterize the anti-nuclear autoantibodies using the leishmanial cytoplasmic extract and THP1 nuclear extract was applied. The immunoblotting results gave different bands on strips of both leishmanial and THP1. Some of the obtained bands were related to the anti-ENA profile, regarding the expected different antigens molecular weights. However, the results of THP1 were more related to anti-nuclear autoantibodies profile than leishmanial extracts. As most of anti-ENA profiles were anti-SSA and anti-SSB, most bands appeared were in a size of 48 kDa or 60 kDa. On the other hand, samples with both anti-SSA and anti-SSB profile, they mostly showed only one reactive band, either correlated with anti-SSA or with anti-SSB. Moreover, different bands appeared with size of 9-30 kDa. These bands may relate to nRNPs and sm proteins, or to centromere, histones and ribosomal proteins P.

In conclusion, salmon sperm DNA, *Leishmania tarentolae* and THP1 protein extracts can be used as a screening antinuclear antibodies test depending on the results of ELISA and immunoblotting. However, more confirmed positive ANA using these different antigenic extracts must be tested for further confirmation, and to find a good correlation between the immunoblotting and ENA test. Furthermore, the immunoflourecent assay must be used to

identify the location in the THP1 and *Leishmania tarentolae* for antinuclear antibodies binding sites.

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الكشف عن المضادات المختلفة ل البروتينات المتواجدة في الانوية باستخدام خلايا مزروعة وخلايا الليشمانيا.

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الملخص

الأجسام المضادة للبروتينات وللمادة الوراثية داخل النواة تكون متواجدة بمستوى غير طبيعي عند المرضى الذين يعانون من أمراض الروماتيزم المناعة الذاتية. يمكن الكشف عن هذه الأجسام المضادة باستخدام المصل من المريض لعمل فحص يدعى ب (ANA) . هناك عدة تقنيات للكشف عن تلك الأجسام المضادة، الأكثر شيوعا هي Indirect Immunofluorescence و Enzyme Linked Immunosorbent Assay و كذلك هناك تقنية أخرى تدعى ب western blot لها القدرة على الكشف عن تلك الأجسام المضادة . لقد استخدمنا في هذه الدراسة مستخلصات من أنوبيه خلايا تدعى ب THP1 وكذلك استخدمنا مستخلصات السيتوبلازم من خلايا الليشمانيا. بالإضافة الى المادة الوراثية من Salmon sperm . فان تلك المستخلصات تم استخدامها في تقنية Enzyme Linked Immunosorbent Assay .لقد أظهرت النتائج ان تلك المستخلصات جيدة للكشف عن الأجسام المضادة للأنوية. ولكن المادة الوراثية من Salmon sperm كانت افضل شيء للكشف عن الأجسام المضادة للمادة الوراثية وكانت مستخلصات خلايا THP1 افضل من الليشمانيا للكشف عن الأجسام المضادة المختلفة الأخرى. بالنسبة لتقنية western blot فان نتائجها أظهرت العديد من الحزم البروتينية ولكن هذه النتائج ليست مرتبطة في نتائج الأجسام المضادة. فان خلايا THP1 هي كانت ايضا افضل في هذه التقنية من خلايا الليشمانيا.