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And Scientific Research
University of Diyala
College of Education for Pure Science
Department of Biology

# Immunogenetic Study on Acute and Chronic Brucellosis in Diyala province

A Thesis

Submitted to the Council of College of Education for Pure Science/ University of Diyala in a Partial Fulfillment for the Requirements for the Degree of Doctorate of Philosophy in Biology

By

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# Dedication

To...

My beloved father who I miss a lot and who would have been proud of me for reaching this achievement, may Allah have mercy on him.

To...

The beating heart ...Her supplication is the secret of my success.. My Dear Mother.

To...

My support and strength ..Loyal partner ... My Husband Basim.

To...

My little princesses .. The flowers of my life ..My lovely daughters.. Jana ,Rayana ,Zuha.

To...

The kind hearts that have always supported me... My Brothers and Sisters.

To...

My teachers, my sincere thanks and appreciation.

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All who supported me.

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# Summary

Brucellosis is caused by bacteria of the genus Brucella, which are small Gram- negative, non-motile, non-spore forming, aerobic, facultative intracellular coccobacilli capable of invading epithelial cells, placental trophoblasts, dendritic cells, and macrophages. Four species infect humans: *B. abortus*, *B. canis*, *B. melitensis*, and *B. suis*. Symptoms include undulant fever, profuse sweating and joint and muscle pain. brucellosis is one of the most common zoonotic diseases worldwide, with endemic disease areas in the Middle East, Central Asia, Africa, and Central and South America. Infection in humans results primarily from contact with infected livestock or consumption of contaminated livestock products; cattle and small ruminants are the primary reservoir hosts.

The current study aimed to determine the most prominent cellular and humoral immunological features of acute and chronic brucellosis (CD3, CD4, CD8, IL-17, IL-18, IFN-γ), and to explore certain host gene polymorphism that may affect susceptibility and virulence of brucellosis.

This cross-sectional study was conducted in Diyala province for the period from February 2020 to Jun 2021. It includes (55) patients who were clinically suspected as having brucellosis and primarily had positive for Rose Bengal agglutination test. The patient's age range were (12-65) years. They were allocated from Baquba Teaching Hospital and Al Battol Teaching Hospital for maternity and children. They were 11 males and 44 females. Additionally, 35 apparently healthy individuals were included as control group. They were 16 males and 19 females. Their ages ranged (14–65) years. The study was approved by the scientific committee in the Directorate of Health/ Diyala as well as by a scientific and ethical committee at the College of Education for Pure Science- University of Diyala. Sociodemographic and clinical information were collected from

each subjects through a special questionnaire preconstructed for this purpose. Human privacy was respected by taken patient's verbal consent. Statistical analysis was done using Statistical Package for Social Science (SPSS) version 27. P value < 0.05 was considered significant.

Both study groups were subjected firstly for detection of serum anti-brucella IgG and IgM antibodies using ELISA technique. Followed by determination of serum (IL-17, IL-18 and IFN-γ) using separated kits of ELISA techniques. Furthermore, assessment of (CD3, CD4 and CD8) using ELISA techniques, and lastly, detection of IL-17, IL-18 and INF-γ genes using Conventional-PCR technique.

The results showed that all patients were positive for Rose Bengal (100%), versus 5.7% of control with a statistically significant difference (P= 0.0001). Anti-IgM Positivity rate among patients was 32.7%, while 5.7% of control were positive with statistically significant difference (P= 0.003). The results found that 43.8% who were positive for IgM were also positive for CD3 versus 17.5% who were positive for IgM but negative for CD3. The difference was statistically significant (P= 0.040).

The anti-brucella IgG positivity rate among patients was 56.4% which was statistically significant compared to control (P= 0.0001). Fever was present in 63.3% of those with positive IgG, which was statistically significant compared to those with no fever (P= 0.0001). Moreover, the results revealed that 56.4% of those with positive anti-brucella IgG had detectable *INF-γ* gene with a statistically insignificant difference (P=0.249).

The CD3 positivity rate among patients was 58.2% versus 28.6% of control with a statistically significant difference (P= 0.006). The results showed that 77.8% who were positive for CD3 were also positive for anti-

brucella IgM, with a statistically significant compared to its counterpart (P= 0.040). The results found that 61.5% who were positive for CD3 were also positive for INF-γ, with a significant difference compared to its counterpart (P=0.036). The CD4 positivity rate among patients was 45.5% while that of the controls was 42.9% with a statistically insignificant difference (P= 0.809). The positivity rate of CD8 among patients was 96.4% while that of the control was 45.7% with a statistically significant difference (P= 0.0001). The results found that the INF-γ positivity rate was significantly associated with the CD8 positivity rate (98.1% Vs 66.7%), (P= 0.005).

Results of interleukins showed that the IL-17 positivity rate among brucella patients was 58.2% while that of the control was 42.9% with a statistically insignificant difference (P= 0.156). The IL-17 positivity rate was insignificantly higher among males versus female (63.6% *Vs* 56.8%), (P= 0.682). The IL-17 positivity rate was significantly higher among those participants with CD4 positive compared to those who were CD4 negative (80.0% *Vs* 40.0%), (P= 0.003). The IL-18 positivity rate among patients was 27(49.1%) which is higher than that of controls 11(31.4%). However, the difference was failed to reach the level of statistically significant (P= 0.098). The IL-18 positivity rate was higher among those participants complaining joint pain compared to their counterpart (55.3% *Vs* 12.5%) with statistically significant difference (P= 0.025). The positivity rate of INF-γ in patient was 94.5% which is highly significant compared to that of control (34.3%), (P= 0.0001). Results also showed that the INF-γ positivity rate was significantly associated with those who consume dairy products compared to their counterpart (98.0% *Vs* 60.0%), (P= 0.0001). The INF-γ positivity rate was significantly higher among those participants with CD3 positive compared to their counterpart (100.0% *Vs* 87.0%), (P= 0.036). Furthermore, the INF-γ positivity was significantly higher among those

with positive CD8 compared to those with negative CD8 (96.2% *Vs* 50.0%) with significant difference (P= 0.005).

The PCR detection rate of the three genes; the IL-*17* gene detection rate among brucella patients was 60% which was significantly higher compared to that of the control 20%, (P= 0.0001). The detection rate of *IL-18* gene among the patients was 72.7% which is also highly significant compared to that of the controls 28.6%, (P= 0.0001). Additionally, the *INF-γ* gene detection rate among the patients was 70.9% which is highly significant as compared to its counterpart in controls 31.4%.

The *IL-17A* genetic sequences in chromosome 6 was analyzed in 51 samples. The alignment results of the 1075 bp samples revealed the presence of seven variations, (Single Nucleotide polymorphism (SNPs), in the analyzed samples in comparison with the referring reference DNA sequences. These seven SNPs were replaced with other nucleotides at positions 215, 219, 341, 356, 537, 603, and 679. Two of these SNPs were detected in three polymorphic patterns; A356G, a homozygous A/A, S55, heterozygous A/G, S49 homozygous G/G. The other five observed nucleic acid substitutions exhibited only two polymorphic forms; a wild type homozygous form and a mutant heterozygous form. These mutations were A215T, A219C, A341G, A537C, and G679A. Furthermore, it was found that four of the detected SNPs were previously known as they were deposited as rs1582236522, rs3819024, rs2275913, and rs8193037 for A341G, A356G, A603G, and G679A respectively. The rs1582236522 SNP is highly recognized in the targeted *IL-17A* gene due to its high frequency (0.5). However, this SNP was not reported in any publications indexed in the dbSNP. Likewise, rs3819024 SNP was also deposited in high frequency (0.33) in the dbSNP. By reviewing the same dbSNP records, it was found that three of our observed SNPs (A215T, A219C, and A537C) were not

deposited previously in the dbSNP, so their novel nucleic acid substitutions were confirmed and validated. The high LD values of the first three SNPs could explained the ability of these SNPs to hasten the development and progression of brucellosis.

The *IL-18* genetic sequences in chromosome 12 which encodes for interleukin 18 protein. The alignment results of the 776 bp samples revealed the presence of three SNPs in the analyzed samples in comparison with the referring reference DNA sequences.. In these detected SNPs, cytosine was replaced with guanine at position 384 (C384G), cytosine was replaced with adenine at 624 (C624A), and adenine was replaced with guanine at position 625 (A625G). Concerning C384G, three patterns of polymorphisms were detected in this locus; homozygous C/C, heterozygous C/G and homozygous G/G patterns. In contrast to both C384G and C624A, only two polymorphic forms were observed in the case of A625G SNP. Thus the 776 bp *IL-18* amplicons, contain only three retrieved SNPs , the LD values of these SNPs were ranged between low and high values.

The *INF-γ* genetic sequences in chromosome 12 encoding *INF-γ* protein ,The alignment results of the 790 bp samples revealed the presence of only one SNP in the analyzed samples in comparison with the referring reference DNA sequences. In this SNP, adenine was replaced with thymine at position 705 in the analyzed locus. This SNP was detected in three patterns, homozygous A/A, heterozygous A/T and homozygous T/T patterns. By reviewing the dbSNP engine, they were found previously deposited . However, it is highly recognized in the targeted *INF-γ* gene due to its high frequency that ranged between 0.25 to 0.35 (https://www.ncbi.nlm.nih.gov/snp/rs2430561).

In conclusions, the current study found that brucellosis elicit a comblex cellular and humoral immune responses and that the single nucleotide polymorphisms in cetain genes co-regulated protective immune parameters, particularly *IL-17* gene may has a role in brucellosis development and severity.

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# *List of Abbreviations*

| Abbreviation | Name |
|---|---|
| APC | Antigen Presenting Cell |
| BCL2A1 | B-Cell Lymphoma 2-related protein A1 |
| BCSP | Brucella Cell Surface Protein |
| CD | Cluster Differential |
| CRP | C-Reactive Protein |
| CTLs | $CD8^+$ cytotoxic T lymphocytes |
| DCs | Dendritic Cells |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| ELISA | Enzyme-Linked ImmunoSorbent Assay |
| ER | Endoplasmic Reticulum |
| GTPase | Guanosine Triphosphatase |
| HLA | Human Leukocyte Antigen |
| HRP | Horse Radish Peroxidase |
| HsP | Heat Shock Protein |
| IFN-γ | Interferon Gamma |
| IL | Interleukin |
| LPS | Lipo- Poly-Saccharide |
| MCP-1 | Monocyte Chemoattractant Protein-1 |
| MHC | Major Histocompatibility Complexes |
| NK | Natural Killer Cell |
| O.D | Optical Density |
| OMPs | Outer Membrane Proteins |
| PCR | Polymerase Chain Reaction |
| Rab | Ras-associated binding proteins |
| RBT | Rose Bengal Test |
| SAT | Serum Agglutination Test |
| SNP | Single Nucleotide Polymorphism |
| T4SS | Type IV Secretion System |

| | |
|---|---|
| TGF | Transforming Growth Factor |
| Th | T-helper |
| TIR | Toll /Interleukine-1 Receptor |
| TMB | Tetramethylbenzidine |
| TNF-a | Tumor Necrosis Factor-alpha |
| Tregs | Regulatory T cells |
| UTRs | Untranslated Regions |

# Chapter One

## 1.1. Introduction:

Brucellosis is one of major bacterial zoonotic infectious diseases. It is worldwide distributed; however, it is rare in most industrialized countries and more common in low-income and middle-income countries (Franc *et al*., 2018). Brucellosis is endemic in Iraq and Middle East countries has the highest reported prevalence of brucellosis in the world (Daood *et al.*, 2020). Actually it is one of the neglected diseases in these areas. Brucella species are gram-negative, nonmotile, non-encapsulated, and facultative intracellular bacteria that can cause acute and chronic zoonotic disease in humans (Sofian *et al.*, 2016). The genus *Brucella* consisted of six recognized species, grouped according to their primary host preferences, that is, *B. abortus* (cattle), *B. melitensis* (sheep and goats), *B. suis* (pigs), *B. ovis* (sheep), *B. canis* (dogs) and *B. neotomae* (wood desert rats). Recent isolates from human (*B. inopinata*), aquatic mammals (*B. pinnipedialis and B. ceti*), and a common vole (*B. microti*) are recognized as new species, bringing the current number to 10 species in the genus (Adem *et al.*, 2021). Domestic and wild animals are reservoirs of the bacteria, which are usually transmitted to humans through the consumption of contaminated unpasteurized dairy products, direct contact with infected animals, and inhalation of infected aerosols (Baldi and Giambartolomei, 2013). In human it mostly affect bones and joints, largely reported in all ages and sexes in high-risk areas (Adetunji *et al*., 2019).

Clinically, there were a wide range of clinical manifestations such as undulant fever, night sweat, malaise, insomnia, arthralgia, nervousness, asthenia, depression (Jafari *et al*., 2015) and sexual impotence (Khan and Zahoor, 2018). Human brucellosis is also known for multiple organ involvement causing encephalitis, meningitis, endocarditis, arthritis,

orchitis, and prostatitis (Samaha *et al.,* 2008). Although non-specific and often misleading clinical presentation of active brucellosis has made it a diagnostic puzzle; however, several laboratory tests had been developed for the diagnosis of brucellosis, mostly relay on anti-brucella IgM and IgG antibodies as well as the molecular methods based on PCR techniques (Alişkan, 2008; Jindan *et al.*, 2019; Tekle *et al.*, 2019; Dal *et al.*, 2019).

*Brucella species* survive within a variety of cells, including macrophages, and spread in mononuclear phagocytes to reticuloendothelial sites (Rasouli *et al.,*2013). Both cell-mediated and humoral immunity are responsible for the clearance of brucella infection (Orozco *et al.,* 2003; Eskandari-Nasab *et al.,* 2013). Host protection against *Brucella species* primarily depends on cell-mediated immunity, involving mainly activated antigen-presenting cells (macrophages, dendritic cells) and CD4+ and CD8+ T-lymphocytes (Eskandari-Nasab *et al.,* 2013). Many Studies have shown that the changes of T lymphocyte are crucial to the interpretation of the clinicopathological features of brucellosis in the process of chronic infection and recurrence (Velasquez *et al.,* 2012; Skendros and Mitroulis, 2012). There are three mechanisms of acquired immunity in Brucella infection: (1) the antimicrobial effect of macrophage induced by interferon-gamma secreted by CD4+, CD8+, γ, and β T lymphocyte (Durward *et al.,* 2012) ; (2) the hosts eliminate the macrophages infected by Brucella through the cytotoxic effect of CD8+, γ, and β T lymphocytes (Durward-Diioia *et al.,* 2015); and (3) the modulating effect of the antibody can enhance the phagocytosis of macrophages (Baldwin and Goenka, 2006). Activated CD8+ T lymphocytes and CD4+ Th1 type immune responses play an important role in the scavenging of intracellular pathogens. *Treg* has the function of inhibiting the proliferation of T lymphocyte and eliminating the pathogen. The changes in the number and function of Treg inhibit the

immune function of the host, which may be one of the mechanisms that leads brucella infection progresses into chronicity (Wang *et al.,* 2014).

Because the cytokine production profile varies in the different stages of brucellosis, the serum levels of cytokines can be used as candidate prognostic markers for human brucellosis. Assessing cytokine levels in patients with acute and chronic brucellosis is not only useful for detecting the immune response, but can also be indicative of the severity of brucellosis (Lin *et al.,* 2020). After entering the human body, Brucella likely activates immune cells and promotes the production of cytokines by T lymphocytes. It found that IL-17, IFN-γ, and TNF-α levels were higher in patients with brucellosis than healthy controls, indicating a strong immune response was occurring. Indeed, previous studies have shown Brucella is effectively cleared through the secretion of IFN-γ, IL-2, and TNF-α by T helper (Th) 1 cells (Giambartolomei *et al.,* 2002; Celli, 2006). Th1 cells play a central role in immunity to brucellosis, beside the strong cooperative role for Th1 and Th17 cells in immunity to brucellosis which is more evident during acute and relapse phases of brucellosis (Rahmanpour *et al*., 2019).

Interleukine-18 plays a protective role against brucellosis (Pasquali *et al.,* 2001) . It has been found that Caspase-1-related inflammasomes are sufficiently activated to induce the secretion of cytokines, such as IFN-γ and IL-18, which are significantly increases to induce cellular immune response (Karaca *et al*., 2019). On the other hand, During Chronic brucellosis reduces lymphocyte proliferation and Th1 cytokine secretion, enhances IL- 5 and TGF-β production, affirming that the immune responses plays a crucial part in the progression and development of chronic diseases (Ghaznavi *et al*., 2017).

Production and release of cytokines rely mostly on human genetic factors, so variations in the regulatory sequences of the cytokine genes can greatly affect the cytokine balance. It is extensively documented the role of cytokines in protection and amelioration of the clinical course of brucellosis (Elfaki *et al*., 2015; Kayhan *et al*., 2016; Lopez-Santiago *et al*., 2019). Some of these cytokines were remain in the body for longer time after treatment (Rasouli *et al.,* 2013; Xu *et al*., 2019) .The connection between cytokine gene polymorphisms and brucellosis disease status was studied in various populations with variable results (Bravo *et al*.,2003; Karaoglan *et al*., 2009; Eskandari-Nasab *et al*., 2013). A few studies report an association between cytokine gene polymorphism and development of brucellosis (Bravo *et al*., 2003; Rezazadeh *et al*., 2006; Rasouli *et al*., 2007; Karaoglan *et al*., 2009). Moreover, the serum levels of IL-6, IFN-gamma and TNF-α were significantly higher in brucellosis patients, but IL-1β, TGF-β1, IL-2, IL-4 and IL-8 levels are insignificantly higher in patient. There was a positive correlation between IFN-γ, TNF-alpha and IL-6 levels with CRP levels. IL-6, IFN- γ and TNF-alpha levels were significantly lowered post-treatment (Lin *et al*., 2020).

A growing number of studies report higher prevalence of single nucleotide polymorphisms (SNPs) in the cytokine-encoding genes of patients with brucellosis. These SNPs are possibly the agents responsible for susceptibility to brucellosis and can be important in the clinical course and prognosis of the disease (Davoudi *et al.,* 2006; Zafari *et al.,* 2020). It has been found that several potentially functional single nucleotide polymorphisms (SNPs) have been identified in the human IFN-γ R1 gene promoter and the protective role of TNF-α-857C/T and TNF-α-308 G/A polymorphism against human brucellosis (Ismael *et al*., 2016; Ismael *et al*. 2018; Naseri *et al*., 2019). In regard to IL-17 which is one of the most important inflammatory cytokines that stimulate immunity responses in

humans infected with brucella species and that the induction of SNPs in the IL-17 gene was found to be associated with resistance to brucella infection (Rasouli *et al*., 2013; Keramat *et al*., 2019). For the best of our knowledge, no previous such study on the effect of interleukin genes polymorphism on the brucella infection in Iraq.

## 1.2. Aims of Study:

The current study aimed to:

1. Exploration of the anti-brucella IgM and IgG among patients clinically diagnosed as brucellosis.
2. Detection of certain inflammatory cell markers such as CD3, CD4, and CD8 using ELISA techniques.
3. Detection of certain proinflammatory cytokines like IL-17, IL-18 and IFN-γ and their titers by ELISA techniques.
4. Detection of interleukin 17, IL-18 and IFN-γ genes polymorphism using PCR techniques.
5. Searching for all possible association of these markers that may promote accurate diagnosis of brucellosis.

# Chapter Two

## 2. Literature Review

### 2.1. Brucellosis Preface:

Brucellosis is one of the most frequent bacterial zoonoses spread worldwide. The infection is most often acquired via the consumption of unpasteurized milk and dairy products or direct or indirect contact with infected animals or their excretes (Atluri *et al.*, 2011). The main symptom in animals is abortion, and consequently, the disease was named infectious abortion, contagious abortion, enzootic abortion or Bang's disease. In humans, the cardinal symptom is fever resulting in names like Crimean fever, Mediterranean fever, Rock fever, undulant fever or Malta fever. Finally, the term 'brucellosis' was established to clarify the etiology of a manifold syndrome (Wareth *et al.*, 2020). The genus Brucella encompasses 12 accepted nomo-species. The classical six species are *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis* and *B. neotomae*, which were primarily isolated from bovines, small ruminants, pigs, dogs, sheep and desert wood rats, respectively (Godfroid *et al.*, 2011). Two species of marine origin, *B. pinnipedialis* and *B. ceti*, were isolated from aquatic mammals (Foster *et al.*, 2007). *B. inopinata* and *B. microti* were gained from samples of a breast implant and common voles, respectively (Scholz *et al.*, 2008; Scholz *et al.*, 2010). *B. papionis* was described as eleventh species isolated from baboons (*Papio spp*.) (Whatmore *et al.*, 2014), and *B. vulpis* was isolated from the mandibular lymph nodes of red foxes (Vulpes vulpes) (Hofer *et al.*, 2016).

*Brucellae* are Gram-negative facultative intracellular stealthy pathogens. They can escape recognition of the innate immunity and avoid the intracellular destruction (Martirosyan *et al.*, 2011). The

virulence and pathogenesis of *Brucella species* are mainly associated with their survival and replication inside the host cells, including phagocytic and non-phagocytic cells. Brucellae are closely related, share noticeable similar genomes, but differ in their natural hosts, phenotypes as well as their immunogenic, proteomic and metabolomic properties (Halling *et al.*, 2005; Wang *et al.*, 2012). The mechanism behind host specificity is enigmatic. Host specificity might be caused by species-specific gene inactivation/activation that influences transcriptional regulators and outer membrane proteins (Chain *et al.*, 2005) .

Brucella lacks classical virulence factors and several aspects of its biology e.g., pathogenesis, survival in macrophages, and host specificity, are still not well understood. To explain its virulence and host specificity, a better understanding at the proteome and metabolome level could be helpful (Murugaiyan *et al*., 2020) .

Brucellosis is a systemic disease which can suffer each organ of the body. Incubation period of this infection was between 1 and 3 weeks, but can take several months before demonstrations of disease signs. The most common nonspecific symptoms of brucellosis consist of fever, it can be seen alternatively in 60% of patients both in acute and chronic phases and undulant fever with the same rate in sub-acute patients is recorded (Al-Tawfiq, 2008). Moreover a night sweats, asthenia, insomnia, anorexia and headache are also observable (Bosilkovski *et al.*, 2009) . According to duration and intensity of symptoms, this disease can be divided as: acute period (lower than 2 months), sub-acute (2 months to 1 year), chronic (more than 1 year) (Roshan *et al.*,2014).

**2.2. Epidemiology:-**

Brucellosis is a major zoonotic disease that is widely distributed in humans and animals. The epidemiology of the disease in humans is largely associated with the occurrence of animal brucellosis in livestock and

wildlife (Godfroid *etal.,* 2013) .About 500,000 new cases of human brucellosis are reported each year, making this disease a major health issue in many regions such as Middle Eastern and South-Eastern Asian countries (Dadar *et al.,* 2019 ).

In animals, Brucella infection has deleterious effects on foetal development and reproductive organs, leading to reproductive failure, abortions and infertility (Hald *et al*., 2016). While brucellosis has been successfully eradicated from live-stock in several developed countries, the control of this infection in wildlife remains a perpetual challenge worldwide (Godfroid, 2017). This is especially important as the epidemiological link between livestock and wildlife brucellosis is now well established (OBrien *et al*., 2017; Simpson *et al*., 2018). Several studies in Italy and Spain have reported that wild animals who have close contact with other wildlife species and livestock have a higher prevalence of anti-Brucella antibodies in comparison with the general wildlife population (Muñoz *et al*., 2010; De Massis *et al*., 2019). Hence, animal-to-animal transmission plays a critical role in the epidemiology of *Brucella* infections and stresses the need to determine the contribution of different wildlife species to the risk of spread of this zoonosis. In this respect, it is important to differentiate between a spillover of infection in wildlife contracted from livestock and a sustainable infection, in which case, Brucella spp. maintains itself in wildlife without a source of infection in live- stock or another wildlife species. This kind of information could assist control and prevention programmers knowing whether wild species may potentially contribute to the re-introduction of Brucella infections in livestock (spillback), particularly in regions where brucellosis has been officially eradicated (Godfroid, 2018).

Brucellosis are a matter of concern to economy and public health. Risk factors for human infections by zoonotic pathogens are related to local

disease ecology, including the animal reservoirs. Wildlife can be the source of human disease directly transmitted or indirectly transmitted via domestic animals. Brucellosis is an endemic zoonotic disease in most Middle Eastern countries (Shareef, 2006), and Iraq in the past two decades has been reported for increase in prevalence of human brucellosis (Gwida *et al*., 2010; Zhao *et al*., 2019).

Brucellosis is endemic in Western Asia, India, Middle East, Southern Europe and South America (Mantur and Amarnath ,2008; Franc *et al*., 2018). Study in Iran reported that *B. abortus* biovar 3 is the most prevalent biovar (Dadar *et al*., 2019). Reports of low incidence of brucellosis in endemic areas could be due to either inadequate surveillance or under reporting (McDermott and Arimi ,2002). Brucellosis is mainly caused by *B. abortus* biovar 1 in water buffaloes in parts of Africa, South America, Brazil, Italy, Pakistan and Egypt ( Megid *et al*., 2005; Wareth *et al*., 2014; Ali *et al*., 2017). In Italy, cattle and water buffalos both are affected by *B. abortus* mainly in southern areas (Garofolo *et al*., 2017). Reports of *B. melitensis* infection in cattle are pouring which is a major threat in Kuwait, Sau*di* Arabia, Israel and some southern European countries (Yilma *et al*., 2016). The epidemiology of this disease remains dynamic and unpredictable as several new strains could emerge and present strains could adapt to new animal species as well as changing situations. Disease is rare in children but in endemic areas, such cases have been reported (Caksen *et al*., 2002; Mantur *et al*., 2004).

Brucellosis is endemic among humans and ruminants in Egypt despite the presence of control programs (Abdelbaset *et al*., 2018 ;Hosein *et al*., 2018). The annual incidence of human brucellosis is estimated to be 5 to 12.5 million cases in Egypt (Hull and Schumaker, 2018). Meta-analysis conducted in China to evaluate the prevalence of bovine brucellosis during a ten year period (2008 to 2018) showed that overall

prevalence was 1.9%. Northern China had higher prevalence compared to Southern China, and Jilin province had the highest among the provinces with more than 30% (Ran *et al.*, 2019). Another meta-analysis on animal diseases in North Eastern India showed 17% prevalence of bovine brucellosis (Barman *et al.*, 2020). Though bovine brucellosis was eradicated in Northern part of Ireland in 1980s, it was later reported in 1997 leading to loss of official brucella free status in Northern Ireland. A molecular epidemiological study in Ireland showed that seven clonal complexes were identified based on Multi- locus variable number tandem repeat (VNTR) analysis (MLVA). The study also warranted the use of molecular tools to trace the source of infection (Allen *et al*., 2015).

## 2.3. Clinical Forms of Brucellosis:

### 2.3.1. Acute Brucellosis:

In this form of the disease, most of the patients have the same symptoms including fever, headache, backache, losing weight and anorexia, infection in this form is also the same as febrile influenza. It should be mentioned that the splenomegaly and hepatomegaly in 50-60% of cases are observable (Al Dahouk and Nockler, 2011; Galinska and Zagórski, 2013).

### 2.3.2. Subacute Brucellosis:

This refers to a group of patients who have relapsed because of incomplete or partial antibiotic treatment and patients who have received inappropriate antibiotics because of incorrect diagnosis. The clinical picture is more protean and may be an important cause of fever of unknown origin. The symptoms are generally milder, and localized infection can be seen (Doganay and Aygen, 2003).

### 2.3.3. Chronic Brucellosis:

a) primary

b) Secondary– chronic brucellosis may be both: seropositive, and seronegative (detected by Burnet's reaction, PCR or even the isolation of Brucella rods from human autopsy material), in which there occur: damage to the osteoarticular system of a degenerative character, enlargement of damage to the liver, non-specific neurological symptoms ( Roushan *et al.*, 2014).

## 2.4. Virulence Factors:

The virulence of Brucella species depends on their ability to persist within host cells. Since natural hosts of Brucella almost exclusively shed bacteria in reproductive secretions, *Brucellae* must persist long enough in the host for sexual contact or birth to occur. This allows for transmission to subsequent hosts. Cattle, goats, sheep, and swine, breed only once or twice a year, requiring Brucellae to remain hidden from the host immune system for a substantial length of time. Establishment of persistent infection is dependent on the initial interactions between *Brucella* and the host. Brucellae do not cause overt toxicity, but instead stealthily invade. As a result, Brucellae lack classical bacterial virulence factors including exotoxins, endotoxic LPS, cytolysins, a capsule, functional flagella, fimbriae, plasmids, and inducers of apoptosis (Seleem *et al.*, 2008). Instead, Brucella virulence factors function in three ways: to hide brucellae from immune detection, to protect from any brucellacidal mechanisms employed by the host, and to disrupt the host immune response. Before brucellae even cross host mucosal barriers, in many cases they must survive transit through the stomach. The zoonotic *Brucella species* produce urease, a virulence factor that facilitates the degradation of urea. One product of this reaction is ammonia, which functions to buffer pH and facilitate survival in the acidic environment of the stomach. Interestingly,

*B. ovis* does not express urease, potentially explaining why the primary route of infection for this species is venereal rather than oral (Tsolis *et al*., 2009). Perhaps the best characterized Brucella virulence factor is the type IV secretion system (T4SS) encoded by the *virB* operon. Interestingly, related α-Proteobacteria, which tend to establish chronic infections including *Bartonella, Rickettsia, Wolbachia, Ehrlichia,* and *Anaplasma*, possess orthologous T4SS (Barquero-Calvo *et al*., 2009). Type IV secretion in the context of brucellosis has been reviewed by de Jong and Tsolis (2012). The *virB* genes are conserved among all species of Brucella underscoring their importance for the genus. However, mutant strains lacking a functional T4SS are attenuated *in vitro* in macrophages and *in vivo* in mice and goats. Studies have shown the T4SS to play critical roles in intracellular trafficking and replication of brucellae within macrophages in the time period shortly after infection (Celli *et al*., 2003). The T4SS has also play a vital part in the early stages of placental colonization in cattle (Mol *et al*., 2014). While intense, acute inflammation resulting in abortion is the ultimate outcome of Brucella localization in the pregnant uterus, an anti-inflammatory environment within the placenta is necessary for the initial establishment of infection ( Neta *et al*., 2008). *VirB* mutants are unable to subvert the host inflammatory response in the uterus, suggesting that T4SS effectors not only function in intracellular trafficking of brucellae but also in immune evasion. Another study of *B. microti* has demonstrated that the T4SS is also vital to this non-classical *Brucella species* (Hanna *et al*., 2011).

Other virulence factors of *Brucella species* are BtpA/TcpB and BtpB (Brucella Toll/Interleukin-1 Receptor (TIR)-domain-containing proteins BtpA (also known as TcpB) and BtpB are among such effectors. Although divergent in primary sequence, they interfere with Toll-like receptor (TLR) signaling to inhibit the innate immune responses) (Coronas-Serna *et*

*al.*,2020). These proteins contain Toll/interleukin-1 receptor (TIR) domains and actively inhibit Toll-like receptor (TLR) stimulation by degrading an adaptor molecule in the signaling cascade. One outcome is the inhibition of dendritic cell activation (Atluri *et al.*, 2011). Activated dendritic cells play key roles in bridging innate and adaptive immunity by secreting cytokines and presenting MHC-bound microbial antigens to T lymphocytes. Thus, these virulence factors represent yet another means by which *Brucella* actively interferes with the host immune system. At least *BtpB* may be a T4SS effector as its translocation into macrophages has been shown to be dependent on *virB*. BtpB has also been shown to play an essential role in immune system evasion during brucellae colonization of the bovine trophoblast, indicating that its effects extend beyond macrophages and dendritic cells (Higgins, 2015).

Another essential virulence factor is LPS, and it serves all three necessary functions for brucellae: hiding the organism, protecting from bactericidal mechanisms, and disrupting the immune response (de Bagues Maria-Pilar *et al.*, 2005). Unlike the LPS of *Salmonella* and *Escherichia coli*, Brucella LPS is poorly endotoxic allowing evasion of innate immunity. Similar to other related α-Proteobacteria, brucellae possess LPS with a non-canonical lipid A characterized by very long chain fatty acids (Barquero-Calvo *et al.*, 2009). This LPS structure results in only meager stimulation of TLR4 and weak binding of complement. The result is minimal release of pro-inflammatory cytokines, minimal respiratory burst activity, and limited opsonization of bacteria. The O-side-chain of Brucella LPS protects smooth bacteria from both extracellular and intracellular bactericidal components. Brucellae are resistant to complement, cationic peptides, and neutrophil extracts (Barquero-Calvo *et al.*, 2007). LPS may also play a role in inhibition of apoptosis, induction of IL-10 production, and interference with MHC class II antigen presentation (Forestier *et al.*,

2000; Franco *et al.*, 2007; Skendros *et al.*, 2011). Numerous other putative virulence factors have been described that are beyond the scope of this review. Among these are the BvrS/BvrR two component regulatory system, cyclic β-1,2-glucans, phosphatidylcholine, and unknown inhibitors of apoptosis (Seleem *et al.,* 2008).

**2.5. Intracellular Survival:**

*Brucellae species* have been identified within host macrophages and DCs (Celi,2006). Uptake of *B. suis* and *B. abortus* by macrophages can occur via two different pathways depending on whether bacteria have been opsonized by antibody. Uptake of non-opsonized bacteria, which is thought to occur early during infection in the absence of specific antibody, requires the presence of lipid rafts (Naroeni and Porte, 2002; , Watari *etal.*,2002). Two macrophage receptors have been proposed for Brucella uptake: Class A scavenger receptor (SR-A), which interacts with LPS (Kim *et al.,* 2004), and cellular prion protein (PrPC) which was proposed to bind surface-exposed Hsp60 on *B. abortus* (Watari, 2004).

Although *Brucellae* are able to multiply intracellularly after either route of uptake, the nature of the replicative compartment differs depending on the mode of uptake, with opsonized bacteria replicating in a vacuole that lacks endoplasmic reticulum (ER) markers (Bellaire *etal.*,2005). One reason for this is that the acidic environment provides a signal for induction of the VirB Type IV secretion system (T4SS) (Boschirolit *et al.,* 2002), a virulence factor that is crucial for intracellular replication of *Brucella spp*. in macrophages and DCs (Billard *et al.,* 2008; Salcedo *etal.*,2008).

In macrophages, the brucella-containing vacuole initially fuses with lysosomes after uptake (Starr *et al.,* 2008). This fusion with lysosomes, like acidification, is also important for development of the replicative compartment because blocking the function of the late endosomal GTPase Rab7 with a dominant-negative mutant reduced intracellular replication of

Brucella (Atluri *et al.,* 2011). Therefore acidification of the vacuole to induce expression of the T4SS is one requirement for intracellular replication, but additional signals، or perhaps interaction partners for the T4SS, may be delivered by a limited fusion with lysosomes (Marchesini *etal.,* 2011).

Disruption of the ER exit sites in *B. abortus*–infected macrophages, by blocking the activity of Sar1, prevented the acquisition of ER markers on vacuoles containing Brucella and inhibited intracellular replication (Celli *et al.,* 2005). The *B. melitensis* protein Rab2 interacting conserved protein A (RicA) was shown to be translocated into host cells, where it interacts with the GDP- bound form of Rab2 (De Barsy *et al.,*2011). The strategy of intercepting membrane traffic between the ER and the Golgi apparatus for intracellular replication is one that is shared with a second intracellular pathogen, *Legionella pneumophila.* However, the mechanism of ER targeting by *Brucella spp*. appears to be different from that by *L. pneumophila* (Derre and Isberg, 2004; Kagan *et al.,* 2004).

Despite its ability to multiply to high numbers and acquire a large amount of membrane in infected cells, infection with wild-type *B. abortus* does not lead to cell death. Infected cells undergo normal cell division, showing that basic cellular processes are not affected by brucella infection (Chaves-Olaret *et al.,* 2002).Two observations on Brucella-infected cells yield some insight into possible mechanisms for the infection- induced inhibition of apoptosis. The first is the upregulation of BCL2A1, a member of the BCL2 apoptotic pathway gene family with anti-apoptotic activity. The second observation is that *B. melitensis* infection down-regulates gene expression in mitochondria, which play a central role in induction of apoptosis (He *et al.,* 2006).

## 2.6. Risk Factors:

The most important species of Brucella responsible for human disease in order of their significance are *B. melitensis*, *B. abortus*, and *B. suis* (Acha and Szyfres, 2003). Transmission of brucellosis occurs from ingesting (foodborne), directly contacting (penetration through breaks in the epidermis), and inhaling the organism (occupationally exposed). The most common means of exposure is eating contaminated animal products in endemic areas (Olsen and Palmer, 2014). The less common means of infection include person to person (Meltzer *et al*., 2010; Wyatt, 2010), accidental infection with live vaccines (Ashford *et al*., 2004; Strausbaugh and Berkelman, 2003) and through blood donation and tissue transplantation (FAO, 2006). Brucellosis is also one of the most common laboratory-acquired infections. Laboratory workers while handling specimens containing Brucella species may generate aerosol and may results in infection (Noviello *et al*., 2004). In countries where milk and dairy products are always pasteurized before consumption, brucellosis principally affect persons who are in close contact with animals and animal products (occupationally exposed) (Seleem *et al*., 2010). The occupationally exposed vulnerable groups include veterinary doctors, butchers, veterinary technicians, insemination service employees, zoo technicians, farmers working on multi-herd farms, employees of meat and milk processing enterprises (Galinska and Zagórski, 2013). Brucellosis may also occur through international travel to endemic countries. Tourists or business travelers to endemic areas may acquire brucellosis by consumption of unpasteurized milk or dairy products. They may also import contaminated cheese or other dairy products into their countries and infect their families (Godfroid *et al*., 2005; FAO, 2006).

## 2.7. Immunology of Brucellosis:

### 2.7.1. Innate Immunity against Brucella:

The first line of defence, innate immune response, is a non-specific response against pathogens. Autophagy is one of the main elimination mechanisms resulting in efficient degradation of intracellular pathogens (Kuballa *et al.,* 2012). Autophagy is induced via Toll-Like Receptor (TLR) signalling pathway and secretion of Th1 cytokines such as interferon gamma (IFN-γ) (Skendros and Mitroulis, 2012). TLRs are pattern recognition receptors recognizing Pathogen-associated molecular patterns PAMPs such as LPS. The TLR signalling pathway produces pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-12, IL-16, IL- 1b and type I interferon. TLRs bridge the innate and adaptive immune responses by stimulating CD86 and CD80 expression (Iwasaki and Medzhitov, 2004). TLR2, TLR4 and TLR9 are the most important receptors in Brucella recognition (Surendran *et al.,* 2012). TLRs play a critical role in control of Brucella infection by activating host immune responses, recruitment of dendritic cells (DCs) and T-effector cells, and up-regulation of major histocompatibility complexes (MHC) I and II (Kim *etal.*,2013).

Macrophages and DCs have a key role in controlling the initial response against Brucella. Both DCs and macrophages phagocytize the bacteria and promote adaptive immune response by presenting pathogen-derived peptides to naïve T cells. Neutrophils contribute to early stages of innate immune response at the site of infection. During phagocytosis, they kill intracellular pathogens by delivering antimicrobial granules to phagosomes. Upon the fusion of granules, lytic enzymes and reactive oxygen species are released in order to remove Brucella (Nauseef, 2007). Neutrophils are not the preferred niche for Brucella replication but the organisms resist being killed within the neutrophils (Moreno and

Moriyon,2006) . Innate lymphocytes including natural killer (NK) cells, NK T cells and T γδ cells are the junction of innate and adaptive immunity (Skendros and Boura, 2013). They are able to recognize non-peptide antigens without MHC restriction (Dieli *et al.,* 2003). In humans, Brucella-specific phosphoantigens activate Vγ9Vδ2 T cells in the early stage of infection (Amjadi *et al.,* 2019). These cells inhibit Brucella intracellular survival by granule and Fas ligand-mediated cytotoxicity, macrophage activation, IFN-γ production and secretion of granulysin and cathelicidin. Interaction of Fas-FasL leads to the death of infected macrophages without any effect on Brucella viability (Oliaro *et al.,* 2005). It has been demonstrated that Brucella interacts directly with platelets and trigger their activation. Platelets are involved in early infection by promoting Brucella invasion of monocytes/macrophages. They can also build complexes with infected monocytes/macrophages and act as carriers. Moreover, platelets increase the levels of IL-1β, TNF-α, IL-8 and monocyte chemo- attractant protein-1 (MCP-1) and decrease levels of IL-10 during monocyte infection. Platelets can modulate the Brucella-mediated infection of monocytes/ macrophages and promote a pro-inflammatory state, which eventually ends in the resolution of infection (Trotto *et al.,* 2018).

**2.7.2. Adaptive Immunity:**

Adaptive immunity is an antigen-specific response developing after innate immunity to eliminate Brucella and protect the host. After phagocytosis, MHC-I- and II-associated bacterial peptides are presented to T lymphocytes and peptide-MHC complex is recognized by T-cell receptors. Phagocytized bacteria activate antigen- presenting cells (APCs) to secrete IL-12 (Skendros and Boura, 2013), which is required for differentiation of naïve T cells into T helper type 1 (Th1) cells (Manetti *et al.,* 1993). Th1 cells produce IL-2 and IFN-γ and are critical for clearance of Brucella infection (Yingst and Hoover, 2003). Rafiei *et al.* (2006),

showed that a high level of Th1 cells in the early stage of Brucella infection is followed in later stages by Th2 cells, producing IL-4, 5, 10 and 13. In addition to $CD4^+$ T cells, IFN-γ is produced by $CD8^+$, γδ T and NK cells (Yingest and Hoover, 2003). IFN-γ is responsible for the activation of antimicrobial effects in macrophages, increasing the expression of antigen-presenting and co-stimulating molecules on APCs, mobilising cytotoxic T lymphocytes (CTL)-mediated cytotoxicity and apoptosis (Skendros *etal.*, 2011). Thus, IFN-γ is a key cytokine in initiating the immune response against Brucella. Any disturbances of Th1 responses may lead to development of chronic brucellosis and to poor outcome. On the other hand, diminished IFN-γ production with increased in IL-5 and TGF-b production is a pathognomonic picture of development of chronic disease (Ghaznavi- Red *et al.*, 2017).

The IFN-γ gene polymorphisms may be effective in determining the severity of, and the protection against brucellosis. $CD8^+$ CTL take part in the immune response by inducing Fas or perforin-mediated cytotoxicity and IFN-γ secretion (Golding *et al.*, 2001; Durward *et al.*, 2012). B lymphocytes, the key cells in humoral immunity, produce antigen-specific antibodies acting as neutralizers and opsonins. Moreover, antibodies facilitate APC-mediated phagocytosis, activate the complement system and result in antibody-dependent cell-mediated cytotoxicity by macrophages, neutrophils and NK cells (Skendros and Boura, 2013). B cells can be considered as a favourite intracellular niche for Brucella due to their immunoregulatory role (Goneka *etal.*, 2011). Goenka *et al*. hypothesized that the B cells may increase the production of IFN-γ in Brucella infection and clear the infection. They also found that neutralizing IL-10 and transforming growth factor beta (TGF-β) cytokines produced by B cells can positively affect the clearance of infection. The larger amount of TGF-b production in Brucella-infected B cells proves that B cells are related to

chronic brucellosis (Goenka *et al.,* 2012). Some of the outer membrane proteins (OMPs) of Brucella such as OMP25 and OMP31 play key roles in stimulating the immune response. One main antigen involved in this process is GroEL, also known as heat shock protein of 60 kDa (HSP-60), which enhances intracellular survival of bacteria through stimulation of the cell-mediated immune response. The investigation of cytokines profile indicates a Th2-dominant response in both acute and chronic phases of brucellosis. High levels of IL-5 and TGF-β and low levels of IFN-γ were detected. Thus, immune response polarization can determine the progression of brucellosis (Ghaznavi-Red *et al.,* 2017).

Ganji *et al.* (2017), proposed that regulatory T cells (Tregs) with CD25/FoxP3þ phenotype are reduced in chronic compared to acute brucellosis. Reduced number of Tregs leads to T-cell anergy and development of the chronic stage. It has been shown that the number of regulatory T (Treg) cells increase in active human brucellosis and declines after successful treatment (Mr *et al.,* 2016). On the other hand, significant decrease of CD25/FoxP3þ Treg cells leads to induction of anergy due to insufficient costimulatory signals in chronically infected patients (Skendros *et al.,* 2007).

**2.7.3. Cellular Immunity:**

Host protection against *Brucella spp*. depends on cell-mediated immunity, including activated macrophages, dendritic cells and activated T cells. The T-helper 1 (Th1) immune response and the production of IFN-γ from these cells are essential for the clearance of Brucella infection in humans. Kocoglu *et al.* (1986) demonstrated a significant increase in circulating activated T cell numbers (CD3+HLA- DR+) without significant alterations in the subpopulations of peripheral blood lymphocytes of acute brucellosis patients. *Brucella spp*. avoid or manipulate host immunity for their own benefit, and according to our findings, in the case of relapse,

there is a decrease in the frequency of both CD4+ T cells and activated T cells (CD3+HLA-DR+) in the peripheral blood. Chronic brucellosis patients display a defective Th1 response and a decreased percentage of CD4+ T cells expressing CD25 (Kinikli *et al.*,2005; Skendros *et al.*,2008), they hypothesize that the suppression of naïve or activated T cells or a defective Th1 response are either a function of regulatory T cells or suppressor T cells, which leads to the progression of infection both in relapse and chronic brucellosis patients. Corresponding with the phenotypic observations, the limited expansion of CD4+ T cells affects the production of inflammatory cytokines in the Th1 and Th17 T cell groups. However, the significant decrease of IFN-γ and IL-17 levels and the significant increase of IL- 10 in relapse patients suggest a change in the frequency of Treg cells during brucellosis relapse compared with acute brucellosis. Pasquali *et al*. (2010), observed that regulatory T cells suppress the effector functions of CD4+ T cells in infected mice, and they showed that Treg cells allow the expansion of brucellosis. In a clinical investigation, Skendros *et al*. (2008), observed that chronic brucellosis patients retain low percentages of CD4+CD25+ and CD4+CD28+ T lymphocytes after potent stimulation with phytohemagglutinin-cultured peripheral blood cells using *Escherichia coli* lipopolysaccharide (LPS). Accordingly, they concluded that *Brucella spp*. modulate the functional arms of the human immune system.

Interaction between the human immune system and Brucella bacteria is essential for either clearance or survival of the pathogen (Baldwin and Goenka, 2006). Both innate and adaptive immune responses are involved in immunity against Brucella infection (Dornand *et al.,* 2002). Protective immunity to this pathogen is mainly dependent to cell-mediated immune responses so that, the contribution of antigen-presenting cells (macrophages and dendritic cells) and consequently induction of $CD4^+$ and $CD8^+$ T-lymphocytes play crucial roles in these responses (Gorvel, 2008). Two

subsets of $CD4^+$ T cells, Th1 and Th17, are central players of the immune responses to intracellular bacteria (Peck and Mellins, 2010).

The relative importance of CD4+ versus CD8+ T lymphocytes in the control of *Brucella* infection is debated; however, both populations likely play a role in immunity (Ko and Splitter, 2003; Skendros and Boura, 2013). CD8+ T cells function to inhibit *Brucella* replication within macrophages via both IFN-γ production and Fas- or perforin-mediated cytotoxicity. CD8+ T cell numbers seem to increase during certain stages of infection, likely to compensate for lapses in CD4+ T cell responses. This has been observed in Balb/c mice as well as in human patients with chronic brucellosis (Fernandes *et al*., 1996; Skendros *et al*., 2007). Although their numbers increase, CD8+ T may not be able to control infection (Vitry *et al*., 2012). Another study suggests that *B. melitensis* actively inhibits the CD8+ T cell response via secretion of the virulence factor TcpB (Durward *et al*., 2012). This virulence factor was discussed in a previous section in reference to its effects on intracellular trafficking and innate immunity, but has now been indicated in evasion of adaptive immunity as well. TcpB is released by *B. melitensis* within infected macrophages and functions to exclude phosphatidylinositols from the immune synapse formed during macrophageCD8+ T cell binding. This prevents CD8+ T cell mediated killing, but also has effects beyond the immediate survival of *Brucella* within the contacted macrophage. The dampening of the resulting immune response appears to prevent the differentiation of CD8+ effector T cells into a long-lived memory pool. In a Balb/c mouse model, few CD8+ T cells retained a memory cell phenotype during chronic Brucella infection. The few persisting memory cells displayed an exhausted phenotype characterized by deficiencies in IFN-γ, TNF-α, and IL-2 production (Higgins, 2015).

In several models of experimental brucellosis, the protective role of CD8+ CTLs has been demonstrated with substantial evidence. This phenomenon may be presumed to be a compensatory effect for the defective CD4+ T-cell responses which are characteristic of chronic disease. the data from a murine model of chronic brucellosis indicate that CTLs also display a low quality responding capacity, characterized by suppression of Th1 cytokines (IFN-γ, TNF-α and IL-2); this deficit being associated with the *B. melitensis* Toll/IL-1 receptor (TIR)-domain-containing protein (TcpB), which inhibits CD8+ cytotoxic killing of target cells expressing antigenic peptides and constitutes a novel strategy for Brucella to evade adaptive immunity (Durward *et al*., 2012).

CD3+ T cells represent mature lymphocytes, which are the main active cells in cellular immunity, and CD3+ T cells represent the overall level of cellular immunity. Kayhan *et al*. (2016), observed that Patients with acute brucellosis had a significantly higher percentage of CD3+ T lymphocytes compared with healthy individuals and relapse patients ($p<0.001$ and $p=0.01$) respectively.

Celik and Akbulut (2005), who determined that the CD8+ T cell population increases in acute patients compared with healthy subjects. Interestingly, that elevation also occurs in relapse patients. Unfortunately, they do not know whether these CD8+ T cells are functional and whether their expansion is limited or not. Therefore, additional studies are necessary on that case. Beside that; Skendros *et al*. (2007), observed a significant elevation on CD8+ T cell population in chronic brucellosis patients and also relapsed chronic brucellosis patients in comparison to acute brucellosis. They concluded that, elevation of $CD8^+$ T cell population in peripheral blood mononuclear cells of chronic brucellosis and relapsed patients probably compensates ineffectual $CD4^+$T cell response in chronic brucellosis patients.

There was a significant increase in the percentage of $CD4^{+}$/CD25+ T-lymphocytes in acute brucellosis patients compared to controls. On the other hand, a significant decrease in the percentage of CD4+/CD25+ T-lymphocytes was observed in chronic brucellosis group of patients compared to acute brucellosis (Skendros *et al.*, 2008).

**2.8. Measurement of Cytokines:**

Assessing cytokine levels in patients with acute and chronic brucellosis is not only useful for detecting the immune response, but can also be indicative of the severity of brucellosis (Lin *et al.*, 2020). After entering the human body, *Brucella* likely activates immune cells and promotes the production of cytokines by T lymphocytes. it found that IL-17, IFN-γ, and TNF-α levels were higher in patients with brucellosis than healthy controls, indicating a strong immune response was occurring. Indeed, previous studies have shown *Brucella* is effectively cleared through the secretion of IFN-γ, IL-2, and TNF-α by T helper (Th) 1 cells (Giambartolomei *et al.*,2002; Celli ,2006). Interleukin-17 induces IL-12 and IFN-γ production in macrophages and mediates bacterial killing. It also regulates IL-12/Th1 immunity and the host responses to intracellular pathogens. It thus seems that IL-17 may have a main role in protection against Brucella infection (Rasouli *et al.*, 2013). Sofian *et al*. (2016), found that the IL-17 level was significantly higher in the acute brucellosis group compared to controls ($P < 0.01$) and its value decreased in patients at the end of treatment without any significant difference.

Another study showed that acute brucellosis induced a significant increase in IFN-γ and IL-17 levels compared with healthy individuals. Interestingly, IFN- γ and IL-17 levels in relapsed brucellosis patients were significantly lower than in acute brucellosis patients ($p<0.001$). However, IFN-γ levels in the relapsed brucellosis group were significantly higher than in healthy individuals ($p=0.014$) but statistical significance was not

reached for IL-17 levels in relapsed and healthy subjects. Acute brucellosis induced significant elevation of IL-18 level in serum samples in comparison to healthy objects. That elevation reached to 3.2 times in acute brucellosis group in comparison to relapse group ($p<0.001$) (Kayhan *et al*., 2016).

The concentrations of IL-17 and IFN-γ in the brucellosis cases (acute and chronic) were recognizably higher than that in the control group ($p=0.0001$). Moreover, the chronic brucellosis group had lower concentrations of IFN-γ compared to patients suffering from acute brucellosis. No significant difference, on the other hand, was observed between acute and chronic brucellosis patients regarding the level of IL-17 ( Ghaznavi-Rad *et al* .,2017). Because the cytokine production profile varies in the different stages of brucellosis, the serum levels of cytokines can be used as candidate prognostic markers for human brucellosis. High levels of IFN-γ, have been reported in patients with acute or sub-acute brucellosis (Akbulut *et al.,* 2007). For untreated brucellosis patients, Rodríguez-Zapata *et al.* (2010), found significantly higher levels of the IFN-γ, in serum and/or significantly higher percentages of samples with detectable levels than those for healthy controls. Especially striking is the increased presence of high levels of IFN-γ in untreated patients. After treatment, significant reductions and normalization of the percentages of patients with detectable levels of IFN-γ in serum were found.

Keramat *et al.*(2019), found that the serum titers of IL-17 were different in patients and control groups, and this different is significant. The serum levels of cytokines in the patients and control groups were determined by flow cytometric bead array that observed high significantly serum levels of IFN-γ in acute patients compared to under-treatment group and healthy controls as well as in the relapsed patients versus under-treatment patients. Comparison of the serum levels of IL-17A between the

study groups did not show any significant differences (Rahmanpour *et al.*, 2019).

In a number of studies, involving with acute brucellosis patients, IFN-γ levels were significantly higher in brucellosis patients than they were in the control group (Giambartolomei *et al.*, 2002). Another study observed that mean serum IFN-γ and TNF-α levels in acute brucellosis cases significantly decreased at the end of the treatment and that there was no significant difference between the post-treatment and the control group values (Demirdag *et al.*,2003). Han *et al.* (2017), reported that high levels of the cytokine interferon (IFN-γ) may be a characteristic of brucellosis chronicity. IFN-γ, and IL-18 levels were investigated in the serum samples of each patient and healthy control, significantly increased in patients with acute brucellosis (Karaca *et al.*,2019).

## 2.9. Diagnosis of Human Brucellosis:

Because human brucellosis can affect any organ and body system, the presenting symptoms of the infection are not pathognomonic, and therefore, the disease may be easily confused with other medical conditions (Franco *et al.*,2007). Conversely, over diagnosis of brucellosis may result in untoward drug effects and, no less importantly, in overlooking other serious infectious or noninfectious illnesses. The antibiotic therapy of brucellar infections is also challenging and necessitates prolonged administration of antimicrobial drug combinations that are not routinely prescribed for other infectious diseases (Pappas *et al.*,2005). The correct diagnosis of brucellosis in humans therefore not only is crucial for early and adequate patient management but has also serious public health significance, as it may reveal exposure to sick animals, consumption of contaminated food (especially dairy products), breach of laboratory safety practices, or the intentional release of brucellae as a biological weapon (Yagupsky,2019).

### 2.9.1. Bacteriological Diagnosis of Brucella:

#### 2.9.1.1. Direct Smear Microscopic Examination:

The microorganism can be identified by microscopic examination of stained smear from secretions, fetuses, and exudates like vaginal discharges, placenta, using modified Ziehl- Neelsen (ZN) staining. This can provide a predictive diagnosis of brucellosis, especially with serological support. Brucellae are not a true acid- fast bacillus but show resistant to decolorization by week acids. They seem like short rods or coccobacilli, mostly arranged singly but occasionally in pairs or small groups. They appear as coccobacilli or short rods, usually arranged individually but sometimes in pairs or small groups. To identify and isolate *B. melitensis* accurately it is best to used vaginal swab and milk samples of goats and sheep and culture these samples on culture media Farrell's selective media (Kaltungo *et al.*,2014).

### 2.9.2. Cultural Isolation of Brucella Organism:

*Brucella* may be isolated from the placenta, fetus, vaginal swab, colostrum, milk, and semen, secretion of non lactating udders, the testis and the sites of clinical localization such as hygroma fluids or infected joints. While the microscopy samples include various lymph nodes, spleen, the pregnant or premature post parturient uterus, the udder, and male reproductive organs (Acha and Szyfres., 2003). At the research site, culturing tests are used to diagnose brucellosis. Culturing of *Brucella* from blood is useful in the case of bacteraemia, which does not always exist but culturing milk gives a positive response to show the presence of Brucella. Samples of liver, udder, lymph nodes, spleen, and other organs used for culturing the purpose of brucellosis. Phenotypic characters including CO2 requirement, phage typing, and biochemical tests, are of great deals while using culture techniques for the identification of *Brucella* organisms and other problems in culturing are time-consuming, trained interne and

applications of bio-safety (Ismail. 2019). To culture *Brucella,* broth or agar can prepare from powder media. Solid media, including tryptose agar, trypticase soy agar, and dextrose agar are used to identify and isolate *Brucella* at the primary level. However, species like *B. Hovis* and *B. canis* can be cultured in media by the addition of 5-10 % sterile bovine or equine serum to it (Atlas and Synder, 2015). The optimum pH for the growth of Brucella is 6.6-7.4، whereas, optimum growth temperature ranges from 36 - 38 ◦C (Pacheco-Montealegre *et al.*, 2017). Growth of other microbes and contaminants can be prevented using selective media such as Kuzdas and Morse and Farrell's Morse (Ur Rahman *et al.*, 2019).

Farrell's medium has some drawbacks because some of Brucella strains such as *B. melitensis, B. ovis,* and *B. abortus* cannot show healthy growth. Therefore, Thayer-Martin medium is slightly modified and then used in combination with Farrell's medium to get better growth of these Brucella species ( Padilla *e tal.*,2010).

**2.9.3. Serological Diagnosis:**

Several serodiagnosis methods are found for the determination of brucellosis (Ducrotoy *et al.*, 2016). However, some of the tests are satisfactory sensitive and specific like indirect Enzyme-Linked Immunosorbent Assay (ELISA), competitive Enzyme-Linked Immunosorbent Assay (c- ELISA), Milk ring test, Complement Fixation Test (CFT) and the Fluorescence Polarization Assay (FPA) (Manish *et al.*,2013). In each and every epidemiological situation no single serological test is sufficient, all of which have limitations, particularly when it comes to screening individual animals Fluorescence Polarization Assay (FPA), Complement Fixation Test (CFT) and ELISA are considered more suitable for international trade than Serum Tube Agglutination test (SAT). The Buffered Brucella Antigen Tests (BBATs), i.e., the Rose Bengal Test (RBT) and the Buffered Plate Agglutination test (BPAT), as well as the

ELISA and the FPA, are sufficient screening tests for brucellosis control at the national or local level (Ali *et al.*,2013). If necessary, positive reactions can be retested using an appropriate confirmatory strategy.

**2.9.3.1. Serum Agglutination Test:**

This test is based on the reactivity of antibodies against the smooth lipopolysaccharide of *Brucella.* Excess of antibodies resulting in false negative reaction due to prozone effect can be overcome by applying a serial dilution of 1:2 through 1:64 of the serum samples thus increasing the test specificity (Afify *et al*., 2013). The test is performed at or near neutral pH, which makes it more efficient in detecting IgM antibody. Hence, it is best used to detect acute infections. It is less effective for IgG, resulting in low assay specificity, due to this fact, the SAT, despite being sensitive, is generally not used as a single test, but rather it is used in combination with other tests. Other short comings of the test include false positive and false negative results (Poiester *et al*., 2010). For this reason, the test is only suitable for herd testing, rather than for testing individual animals. Furthermore, the presence of post-vaccinal antibody can confuse the results. The SAT does not detect antibodies to *B. canis* and *B. ovis* because these rough strains of the organism do not have O- polysaccharide (OPS) on their surfaces (Poiester *et al*., 2010).

**2.9.3.2. Serum Agglutination Test with Ethylene-Diamino -Tetracetic Acid (SAT-EDTA):**

An adaptation of the SAT, which involves the addition of EDTA, has proven to significantly increase the test specificity by reducing the chances of cross-reactions, it can, therefore, be used as an alternative to routine SAT (Nasir *et al.*, 2005; Kaltungo *et al*., 2013). It is postulated that is the mechanism by which EDTA reduces non- specificity, by it eliminates attachment of immunoglobulins to the *Brucella* cell wall via the Fc piece

(Neilsen and Yu, 2010). This test works on the principle that the pH of the serum is altered to the isoelectric point of IgM to prevent its agglutination.

**2.9.3.3. Coombs Wright Test:**

In chronic Brucellosis, agglutinin IgG is re placed by non-agglutinin IgG, which can be measured only by Coombs Wright. In addition, this test can also measure IgM antibody and should be ordered in the state of negative wright test (presence of only non- agglutinin IgG) in highly suspected cases. In acute cases, STA and Coombs Wright tests must have different titer simultaneously (due to presence of both agglutinin and non-agglutinin antibodies; negative Coombs Wright and positive STA), while in chronic Brucellosis the same titer is usually expected (both tests are positive). Therefore, if only IgM antibody exists in the individual, the same titer of Wright and Coombs without positive 2- ME test may lead to misdiagnosis and over- treatment (Solís García del Pozo *et al.*, 2014).

**2.9.3.4. The 2-Mercaptoethanol Test:**

The 2-MET are two forms that use either 2- mercaptoethanol or dithiothreitol (Gupte and Kaur.,2015). Dithiothreitol has recommended, because of the toxicity of 2- mercaptoethanol. The disulfide of IgM is being condensed to the manometric molecule and unable to agglutination essentially calculate IgG unable to agglutinate. However, IgG can also be decreased in the procedure, providing false-negative results. Though in general, reduction of IgM increases specificity (Nielsen, 2002). The test not suggested for the global trade due to not eradication vaccinal antibodies. The 2-MET is, however, used prominently for national control and eradication programs (de Glanville *et al.*, 2017).

**2.9.3.5. Buffered Plate Agglutination Test (BPAT):**

The BPAT is also a spot agglutination test where a stained *B. abortu*s antigen is used. Two staining solutions are required: brilliant green (2 gram/100 milliliter) and crystal violet (1 g/100 mL) mixed together in

equal volumes to prepare a stained-cell suspension with a blue–green color. This antigen is mixed on a plate with serum and any resulting agglutination signifies a positive reaction (OIE, 2016).

**2.9.3.6. Rose Bengal Agglutination Test (RBT):**

The RBT is a simple method of diagnosis and is the most widely used for the serological diagnosis of brucellosis in sheep and goat. RBT is performed with a stained *B. abortus* suspension at pH of 3.6-3.7. RBT consists of a simple spot agglutination test where drops of rose Bengal-stained antigen and serum are mixed on a plate and any resulting agglutination signifies a positive reaction (Saavedra *et al.*, 2019). The RBT is an affordable, quick, simple and efficient screening test and is used as a diagnostic test for screening individual animals and herds, as well as in humans. This test was found efficient in diagnosis of the acute human brucellosis and still is used in the diagnosis of chronic cases. Normally, results are obtained in few minutes (Teng *et al.*, 2017).

Although rapid and excellent for screening, this test is not reliable for vaccinated animals, because it can generate false positives due to its high sensitivity (Smirnova *et al.*, 2013). It is conventional that RBT have little specificity in animals and humans that are already immunized with *Brucella* strain 19. Therefore, a positive blood sample should be confirmed by definitive test. In fact, in most countries, the RBT is mostly used as a screening test, followed by the compliment fixation test (CFT) as a confirmatory test for diagnosis of brucellosis (Khan *et al.*, 2017).

**2.9.3.7. Compliment Fixation Test (CFT):**

Complement fixation tests The IgM isotypes incompletely damaged during the inactivation process, so the CFT test mostly detects the IgG isotypes antibody. After the IgM type, the antibodies IgG1 types usually appear. The SAT and CFT best performed the control and surveillance of the disease. The test indicates an association with the recovery of Brucella

from artificial recovery or naturally infected animals. Although the test is rapid and precise, it does not permit differentiation between antibodies due to infection from vaccinal antibodies (Getachew *et al.*,2016). Other hurdles consist of a high figure of reagents and controls required to perform the test. Moreover, each time the assay is set up, a high number of titrations are necessary, and an explanation of the results is subjective due to variation in procedures (Manual, 2009). The complement fixation test may give false adverse reactions because the antibodies of IgG2 type obstruct the complement fixation. Despite all these problems, the complement fixation test is broadly used analysis because it is a most acceptable and specific serological test for the diagnosis of brucellosis, so it is a suggested test for international trade (Manual, 2012).

**2.9.3.8. Immunochromatographic Lateral Flow Assay (ILFA):**

Immunochromatographic lateral flow assay is a simplified version of the ELISA for the detection of Brucella-specific IgM and IgG antibodies. ILFA is capable of identifying acute, persistent, and relapsing infections. It can also be used to monitor treatment. The sensitivity and specificity of ILFA to detect Brucella IgM and IgG in comparison with ELISA or CFT reported 96% and 99%, respectively (Etemadi *et al.*,2019). Therefore, ILFA for both Brucella IGM and IgG antibodies is a suitable method for endemic areas with limited resources (Gupte and Kaur, 2016).

**2.9.3.9. Fluorescence Polarization Assay (FPA):**

Fluorescence Polarization Assay (FPA) is a homogeneous immunoassay, which is single-step assay that do not require repeated washing steps to remove unbound reactants as with conventional primary binding assay. This technique works on the principle of excitation of fluorescent molecules using polarized light to emit it, the emission of light in the solution is inversely proportional to the rotation speed of the molecules. This speed is associated with the viscosity of the solution,

temperature and gas constant, and molecular volume (Jolley and Nasir, 2003). In the serology of brucellosis, a component of O- polysaccharide (OPS) of smaller molecular weight is labeled with fluorescein isothiocyanate to use as an antigen. In different samples of serum, milk, and blood if antibodies are present, they are rotated at a reduced rate because of presences of antibodies (O'Grady *et al.*,2014).

### 2.9.4. Brucellin Allergic Skin Test (BAST):

The skin Brucellin test is an allergic test that measures Brucella unique cellular immune response. BAST based on a delayed-type hyper-sensitivity reaction with a maximum sensitivity at 72 hours post-inoculation. This delayed type of hyper- sensitivity reaction is measured at the site of inoculation by the increase in skin thickness. The test is specific to complement serological tests for the diagnosis of bovine brucellosis, and thus decrease the figure of false-positive reactions significantly by distinguishing brucellosis from other cross-reacting organisms (Manish *et al.,* 2013). The test is more specific to RBPT and CFT in conditions of its specificity (exceeding 99%). The skin test is highly specific, but its weak sensitivity makes it a good herd test, but not an individual certification test. Thus, it is often suggested for use at the herd level as a positive test in unvaccinated animals (Zamri-Saad and Kamarudin , 2016) .

### 2.9.5. Interferon-Gamma (IFN-γ) Release Assay:

The IFN-γ release assay involves stimulation of lymphocytes in whole blood with a suitable antigen such as brucellin. The resulting IFN-γ production is detected through a capture ELISA (OIE, 2018). This is a relatively sophisticated assay that is performed by mixing heparinized blood with brucellin (phosphate buffered saline is used as a negative control) followed by incubation. IFN-γ (an important cytokine in the response against Brucella) is then measured using an ELISA. The method was developed in an attempt to find alternative methods to identify FPSR

animals. However, when studied in *Yersinia enterocolitica* O:9 and *B. abortus* infected cattle, it fails to provide satisfactory discrimination (Ducrotoy *et al*., 2016).

### 2.9.6. Enzyme Linked Immunosorbent Assay (ELISA):

The enzyme linked immunosorbent assay (ELISA) is known as a sensitive and rapid method for diagnosis of brucellosis. Detection of specific immunoglobulin by a single, simple and rapid test is a major advantage with ELISA (Osoba *et al.*,2001; Pappas *et al.*,2005). In addition to benefit of ELISA in diagnosis of brucellosis in endemic area, it could be useful as a screening test in areas with low incidence of disease (Karplus *et al.*,2007). Blood samples are obtained from all patients and controls and checked for Brucella IgM and IgG antibodies by ELISA test using commercially available ELISA kits according to the standard procedure. In one study by Memish *et al.* (2002), the sensitivity of SAT for diagnosis of brucellosis was similar to combination of IgM and IgG ELISA test . In another study, Ciffici *et al*. (2005), found the sensitivity 94.3%, 97.1%, and 71.4% for SAT, ELISA IgG and ELISA IgM, respectively. Hasibi *et al*. (2013), investigated the accuracy of ELISA test for diagnosis of human brucellosis and determined the optimal cut-off value for ELISA results. ELISA IgG test was more reliable than ELISA IgM test in diagnosis of human brucellosis. Using a cut-off of 10 IU/ml and 50 IU/ml had most sensitivity (92.9%) and most specificity (100%) for ELISA IgG test.

### 2.9.7. Molecular Techniques:

The molecular procedure often based on PCR amplification is dominantly used for identification and typing to reduce the problem and hurdles of micro- biological testing (Minda and Gezahegne,2016). DNA extraction is an initial and essential step of PCR as its feature has a considerable impact on method sensitivity (Moussa *et al.*,2011). Initially, for bacterial determination, PCR has been developed (Boschiroli *et*

*al.*,2002). Also, these processes are applied for the identification of brucellosis in humans and animals' clinical samples. The use of a single pair of primer act to the bacterial DNA sequence, such as 16 S-23s RNA operon, 15711 or BCSP31 genes with PCR is a reliable technique for the detection of brucellosis (Godfroid *et al.*,2010). Using a mixture of some primer's pairs for magnification of cell surface protein (BCSP31), outer membrane proteins *OMP2B, OMP31* genes, encoding the external membrane proteins. It is easy to detect the four Brucella species: *B. melitensis, B. suis*, *B. abortus* and *B. canis*. The mixture of seven PCR reactions is another to allocate favoritism between brucella six species. PCR techniques used for the detection of some *Brucella abortus* biovars, which differentiated between S19 and RB51 strain of *B. abortus* and allowed for vaccination against pathogenic strain (Yu and Nilsen ,2010).

**2.9.7.1. Conventional PCR:**

Several PCR tests are increasingly used for the direct identification of human brucellosis because of their high specificity and sensitivity when compared to serological and blood culture methods, respectively (Araj ,2010; Scholz and Vergnaud , 2013; Rodríguez-Lázaro *et al*., 2017). One of the most important characteristics of the PCR methods that highlight its value is the ability to confirm the diagnosis of acute brucellosis (Asaad and Alqahtani, 2012). Numerous target genes have been selected for identification of the genus *Brucella*, including the 16S rRNA (Nimri, 2003), insertion element IS711 (Osterman and Moriyon, 2006; Cloeckaert *et al*., 2000), the 16S-23S internal transcribed spacer region (ITS), the region of coding sequence for recA protein (Scholz *et al*., 2008), outer membrane protein coding for *omp2* (omp2a, omp2b) gene (Bardenstein *et al*., 2002; Barua *et al*., 2016), and the coding sequence for genus-specific PCR assays that target the *Brucella* cell surface protein (*bcsp31*) gene associated with a 31 kDa immunogenic outer membrane protein conserved

among all *Brucella species*. (Ohtsuki *et al*., 2008; Bounaadja *et al.,* 2009; Sanjuan-Jimenez *et al*., 2017).

In 1988, the gene encoding for the membrane protein of 31 kDa that was specific for *B. abortus* has been cloned by Mayfield *et al*. (1988). Few years later, Baily and his coworker designed a 223-bp fragment of this gene which was conserved in all species and biovars of the genus *Brucella* in order to evaluate *Brucella* cultures obtained from clinical samples via PCR methods (Dadar *et al*., 2019). Another study showed that the amplification of the design primers, according to species-specific polymorphism in the rRNA 16S-23S spacer region, consistently produced five bands characteristic of the Brucella spp. These bands were common to all 25 examined strains. Since then, this method has been applied for the identification of Brucella strains at the genus level, while carbohydrate profiling was capable to differentiate *B. canis* from the other *Brucella spp*. (Dadar *et al*., 2019 B). Mitka *et al*.(2007), have performed four different PCR assays for the diagnosis of acute and relapsing forms of brucellosis with (I) Brucella cell surface protein (bcsp31) (223-bp-sequence of the conserved gene bcsp31 encoding the immunogenic outer membrane protein of 31 kDa); (II) a 193-bp sequence of the gene omp2 encoding a 26 kDa outer membrane protein of *B. abortus* in all Brucella species; (III) a 282-bp sequence of different region of the gene omp2; and (IV) a 1029-bp sequence of bp26 gene of *B. melitensis* 16 M (also named Omp28), which is identified as an immunodominant antigen. All four tests showed excellent specificity (100%) and high diagnostic sensitivity ranging from 95.5% to 100% in acute infections, depending on the type of specimen and the PCR assay as the I and III PCRs appeared to be slightly more sensitive than II and IV PCRs. In addition, PCRs III and IV have been particularly suggested for the diagnosis of acute brucellosis because of their high detection limits and sensitivity (Mitka *et al*., 2007).

**2.9.7.2. Real Time PCR:**

The real-time PCR technique is more sensitive, specific, reproducible, and rapid than the conventional PCR. The quantitative real-time (qRT)-PCR allows both detection and quantification of the PCR product in real-time, while it is synthesized (Garibyan *et al.*,2013). Real-time PCR can be used for the rapid diagnosis of chronic, but serologically positive, brucellosis and acute brucellosis when serum and blood samples of known clinical presentations are investigated (Wang *et al.*,2014). These assays are developed targeting the 16S - 23S ITS region, IS711 element, and *omp25*, *omp31*, and *bcsp31* genes (Kattar *et al.*,2007; Queipo- Ortuno *et al.*,2008; Zhang *et al.*,2013).

The *bcsp31* gene target can be recommended for the detection of bacteria at the genus level. Species-specific identification confirming the primary diagnosis by a second gene target such as IS711 can be done (Colmenero *et al.*,2011; Bounaadja *et al.*,2009). Several multiplex real-time PCR approaches are developed for the simultaneous detection of *Brucella spp*. and *Mycobacterium tuberculosis* complex (MTC). These techniques amplify the *bcsp31, IS711*, and *omp2a* genes for the detection of *Brucella spp*. and target the *senX3-regX3*, *IS6110*, and *cfp31* genes for the identification of the MTC (Colmenero *et al.*,2010). Sanjuan-Jimenez *et al.* (2013), evaluated three molecular targets (IS711, bcsp31, and omp2a) of Brucella and three targets of MTC (IS6110, cfp31, and senX3- regX3) for their simultaneous detection by a multiplex real- time PCR.

**2.9.7.3. Multiplex PCR:**

Lübeck *et al.* (2004) developed a multiplex PCR test for Brucella diagnostics based on the perosamine synthetase gene (Lübeck *et al.*, 2004).Multiplex PCR tests have been used in numerous studies for the diagnosis of human brucellosis (Wang *et al.*, 2014A). In another study, El Kholy and his coworkers also reported a multiplex PCR test via two types

of primers (B4/B5 and JPF/JPR) for the diagnosis of active human brucellosis in Egypt. The authors demonstrated the high accuracy, sensitivity, and specificity of this method as a promising alternative to culture tests for the diagnosis of human brucellosis. (El Kholy *et al*., 2009).In addition, a multiplex PCR test can be applied to simultaneously identify different Brucella spp. present in human samples. For example, a multiplex PCR assay was developed to detect four important species of the genus *Brucella* in a single test. For this purpose, four pairs of primers targeting *bcsp31, omp2b, omp2a,* and *omp31* genes specific to each *Brucella spp*. have been amplified allowing the accurate detection and differentiation of Brucella species (Imaoka *et al*., 2007).

Multiplex PCR appeared to be an accurate and rapid method to trace sources of infection and could be of great value for the early detection of human brucellosis. Concerning Brucella spp., AMOS-PCR (*B. abortus, B. melitensis, B. ovis*, and *B. suis*-PCR) was the first species-specific multiplex PCR which is based on the polymorphism arising from species-specific localization of the insertion sequence IS`711 in the Brucella chromosome and used to identify and differentiate *B. abortus* bv 1, 2, and 4, *B. melitensis, B. ovis,* and *B. suis* bv 1(Dadar *et al.*,2019). Another multiplex PCR assay called Bruce-ladder multiplex PCR assay has been reported for diagnosis of Brucella even at the biovar level through the combination of various primers. For instance, a 19-primer multiplex PCR was able to simultaneously detect *B. microti, B. ceti, B. pinnipedialis,* and *B. neotomae* as well as *B. abortus* biovars 1, 2, and 4 from biovars 3, 5, 6, 9 (Huber *et al*., 2009).

**2.9.7.4. Multiple Locus Variable-Number Tandem Repeat Analysis (MLVA) and Multi Locus Sequence Typing (MLST):**

Multiple Locus Variable-Number Tandem Repeat Analysis **(**MLVA) is based on the analysis of variable number-tandem repeats (VNTR), which exists in bacterial genomes and seems to be highly discriminatory markers, even when the pathogens investigated belong to monomorphic species with high similarity, such as *Brucella spp*. (DNA– DNA homology >90%) (AI Dahouk *et al*., 2007; Kiliç *et al*., 2011). The most used MLVA genotyping system for brucellosis is MLVA-16، originally developed by Le Flèche *et al*. (2006), and modified by Al Dahouk *et al*.(2007), which consists of 16 genetic markers (MLVA-16). Based on simple PCR reactions, MLVA-16 is accessible and the number of alleles (PCR amplicons) can be analyzed by simple agarose gel electrophoresis or automatic high-throughput procedures. MLVA is a reliable method of monitoring phylogeny of the lineage and the regional and temporal distribution of the disease. Results are strengthened when a larger number of strains are included in the analysis ( Ficht, 2010; Ferreira *et al*., 2017).

According to required test specificity, MLVA can be performed using the 16 loci (MLVA-16; panels 1 and 2 markers), using 11 loci (MLVA-11; panels 1 and 2A markers) or using eight loci (MLVA-8; panel 1 markers) (Le Flèche *et al*., 2006; Ferreira *et al*., 2017). Results showed that the most prevalent MLVA genotype is typically *B. melitensis* biovar 3, frequently isolated in humans and common in the East Mediterranean region (Kilic *et al.*, 2011). The same biovar 3 *B. melitensis* was also found in a human epidemiological study in China (Xiao *et al*., 2015), with genotypic profiles from different countries, such as Israel, Iraq, Lebanon and Syria. These results highlight the importance of quarantine rules, suggesting that poor importation quarantine policies may account for a set of *B. melitensis* infections.

The MLVA-13Bc assay was developed and validated using a combination of 13 VNTRs specifically designed for genotyping *B. canis* strains, with high discriminatory power (Yang *et al.*, 2017). The MLVA assay is rapid, highly discriminatory, and reproducible within human brucella isolates (Saavedra *etal.*, 2019). Multi Locus Sequence Typing (MLST) has been used to identify the species and genotypes of these Brucella isolates (Ma *et al.*, 2016). MLST is a DNA sequence-based typing method for many different bacterial species to differentiate strains and identify clonal lineages.

To improve the resolution of MLST for Brucella, an extended MLST (EMLST) by increasing the sequencing length was described by Chen *et al.* (2011), Through analysis of a large number of sequence data was possible to improve resolution of MLST. These authors found that the EMLST method could increase the length by about 50%. With the increased sequences, more alleles and STs were identified, and the genotyping resolution of the MLST was greatly improved.

**2.9.8. Luminex xMAP Technology**:

Luminex xMAP system is a multiplexed microsphere-based suspension array platform capable of analyzing and reporting up to 100 different reactions in a single reaction vessel by performing discrete assays on the surface of colour-coded beads known as microspheres, which are then read in a compact analyzer (Dunbar, 2006). This technology may be used for high-throughput nucleic acid detection methods. For that purpose, DNA probes to detect PCR amplicons are covalently coupled to the microspheres (Dunbar *et al.*, 2003). The Luminex® xMAP™ System has the advantage that they allow for simultaneous detection of multiple nucleic acid sequences in a single reaction vessel which reduces time, labour and cost as compared to single-reaction-based detection methods.

Pfefer *et al*. (2018), developed a multiplexed assay called Luminex bead-based suspension array for detection and identification of the most common Brucella species (*B. abortus, B. melitensis, B. suis, B. suis bv5, B.canis, B. ovis, B. pinnipedialis, and B. neotomae*) as well as the *Brucella* genus level. The work demonstrated overall excellent accuracy for all strains tested and the platform also allows for flexibility in assay design to easily add more *Brucella* species and to attain excellent target accuracy due to the customization of both primers and probe.

**2.10. IL-17 and Gene Polymorphisms:**

Cytokines of the IL-17 family are crucial components of the inflammatory response, and they are essential for normal host immune responses in humans. IL-17 cytokines are produced by a vast array of cell types, and act on a multitude of cellular targets (Amatya *et al.*,2017; Brembilla *et al.*,2018; McGeachy *et al.*, 2019), eventually inducing production of pro-inflammatory cytokines, chemokines, and prostaglandins. Cytokines of the IL-17 family exert non-redundant, and even opposing functions to promote elimination of intruders, and tissue reconstitution. They are also involved in many human pathologies including inflammatory immune mediated diseases and cancer (Bie *et al.*, 2017; Nies and Panzer, 2020).

Cytokines within the IL-17 family share 16-50% amino acid identity with IL-17A, with IL-17F being the most similar (50 %) and IL-17E the most divergent (16%). The similarity between IL- 17 cytokines is higher in the C terminus and in five spatially and IL-17E the most divergent (16%). The similarity between IL- 17 cytokines is higher in the C terminus and in five spatially conserved cysteine residues. N terminus sequences of IL-17B, IL-17C, and IL-17E are substantially different from those found in IL-17A and IL-17F because of a longer extension of the former three proteins

(Gerhardt *et al.*,2009), suggesting that the N terminus is involved in receptor specificity (Starnes *et al.*,2002).

The IL-17 coding gene is located on chromosome 6p12 and consists of three exons (Chen *et al.*,2006). Interleukin-17 induces IL-12 and IFN-γ production in macrophages and mediates bacterial killing. It also regulates IL-12/Th1 immunity and the host responses to intracellular pathogens. It thus seems that IL-17 may have a main role in protection against Brucella infection (Rasouli *et al.*,2013).Genetic variation in IL-17 has been studied in various diseases, including pulmonary tuberculosis, hepatitis B virus, leishmaniosis, autoimmune disorders (such as multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus gastric), breast cancer and gastric cancer (Ren *et al.*,2017; Sadighi *et al.*,2014).

**2.11. Interleukin 18 and Gene Polymorphisms:**

The gene encoding IL-18 is located on chromosomes 11 in humans. It contains 7 exons with two distinct promoters on exon 1 and 2, including an interferon consensus sequence binding protein and a PU.1 binding sites (Nakanishi *et al*., 2001). Interleukin-18 (IL-18), a member of the IL-1 family of cytokine, was initially termed as IFN-gamma inducing factor and was later defined as a pro-inflammatory cytokine with the ability to induce IFN-γ (Dinarello, 2001). IL-18 encodes a 193 amino acid precursor, first synthesized as an inactive 24-kDa protein without a signal peptide, and is predominantly localized in the cytoplasm (Arend *et al*., 2008; Carta *et al*., 2013; Dinarello ,2018). The IL-18 precursor was primarily found to be expressed at high levels in Kupffer cells, Subsequent reports demonstrated that similar to other members of the IL-1 family such as IL-1α and IL-33 but not IL-1β, IL-18 is constitutively expressed in most cell types including human peripheral blood mononuclear cells (PBMCs), macrophages, dendritic cells (DCs) (Chen *et al*., 2013), osteoblasts, epithelial cells, chondrocytes, and epidermal keratinocytes, thereby suggesting its vital

pathophysiological role in health and disease (Gerdes *et al*., 2002; Sanders and Mishra, 2016).

Interleukine-18 plays a protective role against brucellosis (Pasquali *et al.,*2001) and some infectious diseases (Netea *et al.,*2000). Taking into consideration the important function of IL-18 in stimulation of type 1 immune response which determines the protective immunity against Brucella infection, the suitable level of this cytokine sounds to be crucial in outcome of the disease.

IL-18 gene is placed on the long arm of chromosome 11 and six single nucleotide polymorphisms (SNPs) have been recognized in the potentially functional region of its gene such as the coding sequence, Exon–intron junctions, promoters and untranslated regions (UTRs). Taking into account the involvement of these polymorphisms in altering IL-18 level (Folwaczny *et al.,*2005).

**2.12. Interferon Gamma (IFN-γ) and Gene Polymorphisms:**

Among the pathophysiological manifestations of brucellosis being arthritis, endocarditis and meningitis in humans, and spontaneous abortion in cattle (Ko *et al.,* 2002). Brucella organisms invade cells of the reticuloendothelial system and can be sequestered in macrophages at specific locations within the body, such as the spleen, brain, joints, heart, liver and bone marrow (Fisht, 2003). Both cell-mediated immunity and humoral responses are responsible for the clearance of Brucella infection (Eskandari-Nasab *et al.,* 2013). Brucella antigens induce production of Th1 cytokines in humans and the Th1 immune response has been proven to be essential for the clearance of Brucella infection.

IFN- γ is a critical cytokine for host control of Brucella and a key mediator in conferring protection against Brucella infection both *in vivo* and *in vitro* (Fernandez-Lago *et al.,* 2005). *In vitro* studies have shown that activation of macrophages with IFN-γ results in fewer intracellular *B.*

*abortus* organisms; *in vivo* studies have also supported a role for IFN-γ in control of this infection (Rodriguez-Zapata *et al*., 2010). Patients with acute brucellosis display a Th1 type response with cell proliferation and production of IFN- γ, IL-2 and IL-12 (Eskandari-Nasab *et al.,* 2013), crucial lymphokines in the regulation and generation of the immune response (Giambartolomei *et al.,* 2004).

Interferon gamma polymorphism at the +874 A/T position parallel several studies on brucellosis (Bravo *et al.*,2003; Rasouil and Kiany,2007) pulmonary tuberculosis (Hashemi *et al.*,2011), HIV-1/AIDS infection (Sobti *et al.*,2010), retinochoroiditis toxoplasmosis (Albuquerque *et al.*,2009), intrauterine HBV infection (Yu *et al.*,2006), American tegumentary leishmaniasis (Matos *et al.*,2007), chronic hepatitis C (Dia *et al.*,2006) and chronic hepatitis B and C virus coinfection (Gao *et al.*,2009).

**2.13. Vaccination:**

The best way of prevention, control and eradication of brucellosis is by vaccination of all susceptible animals at risk and elimination of positive animals in endemic areas (Briones *et al*., 2001). Vaccination of cattle in endemic areas is utmost essential (Beauvais *et al*., 2016). Vaccination plays a crucial role in the management of the disease in animals and humans. The most common *Brucella spp*., strain 19, RB51 and Rev1 are widely used as vaccine strains to protect against Brucella infection and related abortions in livestock ( Jezi *et al*., 2019).

The Rev1 vaccine is not recommended for administration during pregnancy as this vaccine may resulting in abortions. Moreover, the antibody response to vaccination interferes in diagnosis of natural infection. the *B. melitensis* Rev1 vaccine is used for small ruminants; However, the Rev1 vaccine can be adapted for cattle vaccination in areas endemic for *B. melitensis* (Beauvais *et al*., 2016).The economic analysis showed that a vaccination program covering the vaccination with S19

vaccine in 90% of the replacement heifers of 3–8 months of age provides excellent economic returns in a brucellosis vaccination program in bovines (Alves *et al*., 2015). *B. abortus* S19Dper vaccine, was found to be safe, immunogenic and also has the potential to be used as DIVA strategy vaccine for prevention and control of bovine brucellosis (Lalsiamthara *et al*., 2015).

Various recombinant proteins like Omp16, Adk, SecB, were studied for their potential to be utilized as vaccine against brucellosis (Alizadeh *et al*., 2019; Huy *et al.*, 2020). Subunit vaccines employing different proteins, like BP26, Omp25, L7/L12, were found to be safer but the protection level was less. *B. abortus* vaccine should also be able to give cross-protection against *B. melitensis*. Besides conventional vaccines, new DNA vaccines and multivalent fusion DNA vaccine have also been developed (CDC, 2017). They were found safe with good efficacy on laboratory and clinical trials. It increases IgM, IgG, IgG2a, and enhanced cell-mediated immune response evidenced as high IFN-γ and lymphoproliferative response of splenocytes (Gomez *et al*., 2017).

Although, vaccination against brucellosis might be the most appropriate disease control measure but the persistence of pathogenic brucella spp. Therefore, the development of novel vaccines that is highly effective and safe under field conditions may significantly impact its prevalence worldwide (Olsen and Stoffregen, 2005).

# Chapter Three

## 3. Materials and Methods

### 3.1 Equipment and Instruments:

The equipments and instruments were used in the study are listed in Table (3.1).

**Table (3.1): Equipment and instruments were used in the study.**

| Item | Company | Origin |
|---|---|---|
| Incubator | Thermaks | Germany |
| Centrifuge | Hettich | Germany |
| Centrifuge | Fisher Scientific | USA |
| Micro spin Centrifuge | My Fugene | China |
| ELISA washer | Biotek | USA |
| ELISA reader | Biotek | USA |
| Refrigerator (4°C) and freezer (-20°C) | TEKA | Spain |
| Water bath | Wisebath daihan | Korea |
| Microwave | Funai | Taiwan |
| Freezer | Royal | Japan |
| Disposable Syringes | A.D.Medical Device | UAE |
| Vacuum EDTA tubes | AFICO – DISP | Jordan |
| Micropipette | Human | Germany |
| Microwave Oven | GOSONIC | China |
| Gel Imaging System | Major Science | Taiwan |
| OWL Electrophoresis System | Thermo | USA |
| Quantus Fluorometer | Promega | USA |
| Thermal Cycler | Bio Rad | USA |
| Vortex | Quality Lab System | England |

## 3.2. Appliances:

The appliances were used in the study are listed in Table (3.2).

**Table (3.2): Appliances were used in the study.**

| Item | Company | Origin |
|---|---|---|
| 1.5ml, 0.5ml and 0.2ml Tube | JET BIOFIL | Singapore |
| Gel tube | AFICO- DISP | Jordan |
| Tips | Gilson | China |
| Tips –sterile | Bioneer | Korea |
| Cotton | Dunya | Iraq |
| Gloves | Nitrile | Malaysia |

## 3.3. Laboratory Kit:

The following laboratory kits used throughout this study were listed in Table (3.3).

**Table (3.3): Laboratory Kits used in the current study.**

| Laboratory Kit | Company | Origin |
|---|---|---|
| Rose Bengal Kit | Spinreact | Spain |
| Brucella abortus IgM ELISA Kit | Demeditec | Germany |
| Brucella abortus IgG ELISA Kit | Demeditec | Germany |
| IL-17 ELISA Kit | Mybiosource | USA |
| IL- 18 ELISA Kit | Mybiosource | USA |
| INF-gamma ELISA Kit | Mybiosource | USA |
| CD3 ELISA Kit | Mybiosource | USA |
| CD4 ELISA Kit | Mybiosource | USA |
| CD8 ELISA Kit | Mybiosource | USA |
| ReliaPrep™ Blood gDNA Miniprep System | Promega | USA |

## 3.4. Chemicals:

The following chemical powder or solutions were used throughout this study are illustrated in table (3.4).

**Table (3.4): Chemicals powders or solutions used in the study.**

| Chemicals | Company | Origin |
|---|---|---|
| Agarose | Promega | USA |
| Ethidium Bromide Solution (10mg/ml) | Promega | USA |
| GoTag Green Master Mix | Promega | USA |
| 100 bp DNA ladder | Promega | USA |
| Nuclease Free Water | Promega | USA |
| TAE 40X | Promega | USA |
| Quantifluor dsDNA System | Promega | USA |

## 3.5. Primers:

The following primers (company it was Macrogen and origin Korea) were used throughout this study are illustrated in Table (3.5).

**Table (3.5): primers used in the study**

| Primer name | Seq. | Annealing Temp. (°C) | Product size (bp) |
|---|---|---|---|
| *IL-17-2-F | 5`-GTCTCCTCTCCTTTCTAGTT-3` | 55 | 1075 |
| IL17-2-R | 5`-ACACTTCTTGTGTGGTTTAG-3` | | |
| **IL-18 –F | 5`-CTCACACTGAGAACTCCTATTC-3` | 60 | 776 |
| IL18-R | 5`-GTTCAGCAGCCATCTTTATTC-3` | | |
| ***INF-γ –F | 5`-CTTGCTGTAGCATCTCTGATAG-3` | 60 | 790 |

| | | | |
|---|---|---|---|
| **INF-γ-R** | 5`-TGCCTTCCTGTAGGGTATT-3` | | |

(*Rasouli *etal.*,2013; Keramat *etal.*,2019; **Zafari *etal.*, 2020; ***Eskandari-Nasab and Moghadampour, 2018).

## 3.6. Study Subjects:

This cross sectional study was conducted in Baquba- province of Diyala during the period from February 2020 to Jun 2021. The study include (55) Iraqi patients who were clinically suspected as having brucellosis and primarily had positive result by Rose Bengal agglutination test, their ages range (12-65 years). They were allocated from Baquba Teaching Hospital and Al Battool Teaching Hospital. They were (11 males and 44 females). Additionally, 35 apparently healthy individuals were included as control group. They were (16 males and 19 females). Their ages range (14–65) years. The study was approved by the scientific committee in the Directorate of Health /Diyala as well as by a scientific and ethical committees in the College of Education for Pure Science-University of Diyala. Sociodemographic and clinical information was collected from each subjects through a special questionnaire preconstructed for this purpose (appendix I). Human privacy was respected by taken patient's consent.

Both study groups were subjected firstly to Rose Bengal agglutination test, followed by detection of serum anti-brucella IgG and IgM antibodies using ELISA technique. The third step was determination of serum IL-17, IL-18 and INF-gamma using separated kits of ELISA techniques. The fourth step was the estimation of CD3, CD4 and CD8, also using ELISA techniques. While the final step includes detection of IL-17, IL-18 and INF-γ genes using Conventional-PCR technique.

## 3.7. Collection and Preparation of Samples:

About (6-7)ml of venous blood sample were collected from each study and control groups. (2) milliliters of whole blood were placed in EDTA tube and storage in -20°C until use to detecting single nucleotide polymorphisms SNP. The remaining blood sample was divided into two gel tube and left for 15 minutes in room temperature to allow clotting, then the clotted blood samples were centrifuged at 3000 rotation /minutes (RPM) for 10 minutes. The sera were collected and distributed in Eppendorf tubes, one tube was used directly for performing the Rose Bengal test. while other tubes were stored in -20°C until use to evaluate IgG, IgM, CD3, CD4 CD8, IL17, IL-18, INF-γ markers.

## 3.8 Parameters and Methodology of Study:

### 3.8.1. Initial Brucella Serodiagnosis:

#### 3.8.1.1. Serum Rose Bengal Test:

**A. Principle of the Method:**

The Rose Bengal (Spinreact-Spain) is a slide agglutination test for the qualitative and semi-quantitative detection of antibodies anti-Brucella in human serum. The stained bacterial suspension agglutinates when mixed with samples containing specific IgG or IgM antibodies present in the patient sample.

**B. Test procedure:**

**1.** The Reagents and samples were allowed to reach room temperature. The sensitivity of the test may be reduced at low temperatures.

**2.** Fifty Microliter**s** of the sample and one drop of each positive and negative controls were placed into separate circles on the slide test.

**3.** The Rose Bengal reagent was mixed vigorously or on a vortex mixer before using and one drop was added next to the sample to be tested.

**4.** The drops were mixed with a stirrer and it was spread them over the entire surface of the circle. Different stirrers was used for each sample.

**5.** The slide was placed on a mechanical rotator at 80-100 r.p.m. for 4 minutes. False positive results were appear if the test was read later than two minutes.

**C. Reading and Interpretation:**

Microscopically examined for the presence or absence of visual agglutination immediately after removing the slide from the rotator. The presence of agglutination indicated the presence of antibody anti- brucella concentration equal or greater than 25 IU/mL.

### 3.8.2. Determination of Anti-brucella Antibodies:

#### 3.8.2.1. *Brucella abortus* IgM by ELISA:

**A. Principle of the test:**

The Brucella IgM antibody test kit (Demeditec- Germany) is based on the principle of the Enzyme Immunoassay (EIA). Brucella antigen was bounded on the surface of the microtiter wells. Diluted patient serum or ready-to-use standards were added into the wells of the microtiter plate. A binding between the IgM antibodies of the serum and the immobilized Brucella antigen was takes place. After a one hour incubation at room temperature, the plate was rinsed with diluted wash solution, in order to remove unbound material. Then ready to-use anti-human-IgM peroxidase conjugate was added and incubated for 30 minutes. After a further washing step, the substrate (TMB) solution was added and incubated for 20 minutes, inducing the development of a blue dye in the wells. The color development was terminated by the addition of a stop solution, which changes the color from blue to yellow. The resulting dye was measured spectrophotometrically at the wavelength of 450 nm. The concentration of the IgM antibodies was directly proportional to the intensity of the color.

**B. Assay procedure:**

**1.** A series of dilution was performed for the positive control: the first dilution was made by adding 100 Microliters of sample diluent and numbered on tube A. the second tube contained 90 Microliters of sample diluent and 10 Microliters of the positive control was added, and a dilution series was completed for the positive control, as shown in the following table (3.6).

**Table (3.6): Serial dilutions of the positive control.**

| Tube number | Dilution |
|---|---|
| 1.Tube A 0% | 100 Microliters sample diluent. |
| 2.Tube B 10% | 90 Microliters sample diluent +10 Microliters positive control. |
| 3.Tube C 20% | 80 Microliters sample diluent + 20 Microliters positive control. |
| 4.Tube D 40% | 60 Microliters sample diluent +40 Microliters positive control. |
| 5.Tube E 60% | 40 Microliters sample diluent +60 Microliters positive control. |
| 6.Tube F 80% | 20 Microliters sample diluent + 80 Microliters positive control. |
| 7.Tube G 100% | 100 Microliters positive control. |

2. A sufficient amount of microtiter wells for the standards, controls and samples as well as for a substrate blank were prepared.

3. One hundred microliters of each of the diluted (1:101) samples and ready- to- use standards and controls respectively were added into the wells. One well was left empty for the substrate blank.

4. The plate was covered with re-usable plate cover and incubated at room temperature for 60 minutes.
5. The wells of the plate were aspirated and 300 microliters of diluted washing solution were added. This procedure was repeated totally three times. Rests of the washing buffer were afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.
6. One hundred microliters were added each of ready-to-use conjugate into the wells. One well was left empty for the substrate blank.
7. The plate was covered with the re-usable plate cover and incubated at room temperature for 30 minutes.
8. The wells of the plate were aspirated and 300 microliters of diluted washing solution were added. This procedure was repeated totally three times. Rests of the washing buffer were afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.
9. One hundred microliters were added each of the ready-to-use substrate into the wells. Also the substrate blank was pipetted.
10. The plate was covered with the re-usable plate cover and incubated at room temperature for 20 minutes in the dark (e. g. drawer).
11. To terminate the substrate reaction, 100 microliters were added each of the ready-to-use stop solution into the wells. Also was added to the substrate blank.
12. After thorough mixing and wiping the bottom of the plate, the reading was performed of the absorption at 450 nm (optionally reference wavelength of 620 nm).

**C. Qualitative Evaluation:**

The calculated optical density for the patient sera, were compared with value for the cut-off standard. If the value of the sample was higher, the result was positive, and if a value below the cut-off standard, the result was negative.

**D. Quantitative Evaluation:**

For a quantitative determination the absorptions of the positive control were graphically drawing point to point against their concentrations. From the resulting reference curve the concentration values for each patient and control samples were be extracted in relation to their absorption using a computerized Microsoft program.

**3.8.2.2. *Brucella abortus* IgG Determination:**

**A. Principle of the test**

The Brucella IgG antibody test kit (Demeditec- Germany) is based on the principle of the Enzyme Immunoassay (EIA). Brucella antigen was bounded on the surface of the microtiter wells. Diluted patient serum or ready-to-use standards are pipetted into the wells of the microtiter plate. A binding between the IgG antibodies of the serum and the immobilized Brucella antigen takes place. After a one hour incubation at room temperature, the plate was rinsed with diluted wash solution, in order to remove unbound material. Then ready to-use anti-human-IgG peroxidase conjugate was added and incubated for 30 minutes. After a further washing step, the substrate (TMB) solution was added and incubated for 20 minutes, inducing the development of a blue color in the wells. The color development was terminated by the addition of a stop solution, which changes the color from blue to yellow. The resulting cooler was measured spectrophotometrically at the wavelength of 450 nm. The concentration of the IgG antibodies was directly proportional to the intensity of the color.

**B. Assay procedure:**

**1.** A series of dilutions were performed for the positive control: the first dilution was made by adding (100) microliters of sample diluent and numbered on tube A. the second tube contained (90) microliters of sample diluent and (10) microliters of the positive control was added, Then a

dilution series was completed for the positive control, as shown in the following table (3.7).

**Table (3.7): Serial dilutions of the positive control.**

| Tube number | Dilution |
|---|---|
| 1.Tube A 0% | 100 Microliters sample diluent. |
| 2.Tube B 10% | 90 Microliters sample diluent +10 Microliters positive control. |
| 3.Tube C 20% | 80 Microliters sample diluent + 20 Microliters positive control. |
| 4.Tube D 40% | 60 Microliters sample diluent +40 Microliters positive control. |
| 5.Tube E 60% | 40 Microliters sample diluent +60 Microliters positive control. |
| 6.Tube F 80% | 20 Microliters sample diluent + 80 Microliters positive control. |
| 7.Tube G 100% | 100 Microliters positive control. |

2. A sufficient amount of microtiter wells for the standards, controls and samples as well as for a substrate blank were prepared.

3. One hundred microliters each of the diluted (1:101) samples and ready-to- use standards and controls respectively were added into the wells. One well was left empty for the substrate blank.

4. The plate was covered with re-usable plate cover and incubated at room temperature for 60 minutes.

5. The wells of the plate were aspirated and (300) microliters of diluted washing solution were added. This procedure was repeated totally three times. Rests of the washing buffer were afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.

6. One hundred microliters were added each of ready-to-use conjugate into the wells. One well was left empty for the substrate blank.

7. The plate was covered with the re-usable plate cover and incubated at room temperature for 30 minutes.
8. The wells of the plate were aspirated and (300) microliters of diluted washing solution were added. This procedure was repeated totally three times. Rests of the washing buffer were afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.
9. One hundred microliters were added each of the ready-to-use substrate into the wells. Also the substrate blank was pipetted.
10. The plate was covered with the re-usable plate cover and incubated at room temperature for 20 minutes in the dark (e.g. drawer).
11. To terminate the substrate reaction, (100) microliters were added each of the ready-to-use stop solution into the wells. Also was added to the substrate blank.
12. After thorough mixing and wiping the bottom of the plate, performed the reading of the absorption at 450 nm (optionally reference wavelength of 620 nm).

**C. Qualitative Evaluation:**

The calculated optical density for the patient sera were compared with value for the cut-off standard. If the value of the sample was higher, the result was positive, and if a value below the cut-off standard, the result was negative.

**D. Quantitative Determination:**

For a quantitative determination of the absorptions of the positive control were graphically drawing point to point against their concentrations. From the resulting reference curve the concentration values for each patient and control samples were be extracted in relation to their absorption using a computerized Microsoft program.

### 3.8.2.3 Determination of CD3:

**A. Principle of the Test:**

This kit (Mybiosource-USA) is intended to be used for determination the level of CD3 (hereafter termed "analyte") in undiluted original human body fluids, tissue homogenates, secretions or feces samples.

B. **Material Supplied:**

The contents of the CD3 determination kits were listed in table (3.8).

**Table (3.8): contents of the CD3 determination kit (Mybiosource-USA).**

| Items | Materials | 48 Tests | 96 Tests |
|---|---|---|---|
| 1 | Microelisa Stripplate | 12×4 Strips | 12×8 Strips |
| 2 | Standards×6 vials | 0.5ml×6 vials | 0.5ml×6 vials |
| 3 | Sample Diluent | 3.0ml | 6.0ml |
| 4 | HRP-Conjugate Reagent | 5.0ml | 10.0ml |
| 5 | 20×Wash Solution | 15m | 25ml |
| 6 | Stop solution | 3.0ml | 6.0ml |
| 7 | Chromogen Solution A | 3.0ml | 6.0ml |
| 8 | Chromogen Solution B | 3.0ml | 6.0ml |
| 9 | Closure Plate Membrane | 2 | 2 |
| 10 | Sealed Bags | 1 | 1 |
| 11 | Instruction | 1 | 1 |

**C. Assay procedures:**

**1.** The plate, and all reagents and samples were allowed to come to room temperature (18C°-25 C°) naturally for 30 minutes before starting assay procedures.

2. The plate was removed from the foil pouch.

3. Blank wells, standard wells and sample wells were specified.

4. Nothing were added to blank well, (50) microliters from standard (S1,S2 ,S3,S4,S5, S6) were added corresponding to standard wells.
5. Fifty microliters from sample were added to every sample well.
6. One hundred microliters of HRP-conjugate reagent were added to each well, covered with a closure plate membrane and incubated for 60 minutes at 37°C.
7. All wells were washed (including all blank wells) 4 times.
8. Fifty microliters of chromogen solution A were added to every well, followed by fifty microliters of chromogen solution A.
10. The plate was mixed gently and incubated for 15 minutes at 37C°. The plate was protect from light.
11. Fifty microliters stop solution was added to every well.
12. The Optical density (O.D) was read at 450 nm using an ELISA reader within 15 minutes after adding the stop solution.

D. **Calculation of Results:**

**1.** Average the duplicate readings for each standard and sample to subtract average optical density of the Blank/Control (OD0).

| **Concentration** | **Blank** | **$S_1$** | **$S_2$** | **$S_3$** | **$S_4$** | **$S_5$** | **$S_6$** |
|---|---|---|---|---|---|---|---|
| Mean O.D.(450 nm) | $OD_0$ | $OD_1$ | $OD_2$ | $OD_3$ | $OD_4$ | $OD_5$ | $OD_6$ |

2. The professional curve fitting software was used to make a standard curve and calculate the level of the analyte.
3. The process of calculating the positive and negative values for patients and healthy people was done by calculating the value of the mean of the healthy people and comparing it with all the values of the concentrations, if the concentration value is higher than the mean average the result was considered positive, but if it was less than the result was considered negative.

### 3.8.2.4. Determination of CD4:

**A. Principle of the Test:**

This kit (Mybiosource-USA) used double-sandwich ELISA technique. The pre-coated antibody is human CD4 monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labeled. Samples and biotin labeling antibody were added into ELISA plate wells and washed out with PBS or TBS. Then Avidin-peroxidase conjugates were added to ELISA wells in order; TMB substrate was used for coloring after reactant thoroughly washed out by PBS or TBS. TMB turned into blue in peroxidase catalytic and finally turned into yellow under the action of acidic stopping solution. The color depth and the testing factors in samples are positively correlated.

**B. Kit Composition:**

Table (3.9) showed the contents of the CD4 determination kit (Mybiosource-USA).

**Table (3.9): Contents of CD4 determination kit.**

| Name | 96 Tests | 48 tests | Storage |
|---|---|---|---|
| 1. Antibody precoated plate | 8x12 | 8x6 | 4/-20 $^0$C |
| 2.Human CD4 Standards | 2 vials | 2 vials | 4/-20 $^0$C |
| 3.Biotinylatedantibody (1:100) | 1 vial | 1 vial | 4/-20 $^0$C |
| 4.Enzymeconjugate (1:100) | 1 vial | 1 vial | 4/-20 $^0$C |
| 5. Enzyme diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 6. antibody diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 7. Standard diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 8. Sample diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 9. Washing buffer (1:25) | 1 vial | 1 vial | 4/-20 $^0$C |
| 10. Colour Reagent A | 1 vial | 1 vial | 4/-20 $^0$C |
| 11.Colour Reagent B | 1 vial | 1 vial | 4/-20 $^0$C |

| | | | |
|---|---|---|---|
| 12. Colour Reagent C | 1 vial | 1 vial | 4/-20 $^{0}$C |
| 13. Instruction | 1 set | 1 set | Room temperature |

**C. Test Preparation:**

1. The ELISA Kit was removed of refrigerator 20 minutes in advance and take was tested when it was balanced to room temperature.
2. The concentrated washing solution was diluted with double distilled water (1:25).
3. For preparation of human CD4 standard sample: 1.0 ml diluent were added into human CD4 lyophilized standard sample and was kept it for 30 minutes. After the sample was completely dissolved, was mixed it slightly and labeled on the tube then take dilutions as needed. (It is recommended to using following concentration value to standard curve: 20,10, 5, 2.5, 1.25, 0.625, 0.312ng/ml).
4. Legend of standard sample dilution method: 7 clean tubes were taken and labeled with 1, 2, 3 , 4, 5 , 6 , 7 , respectively. 300 microliters standard sample diluent were added into each tube. 300 microliters diluent were taken from tube 1 to tube 2 and mixed well. Further 300 microliters diluent were taken from tube 2 to tube 3, and mixed well. Steps were repeated above up to tube 7. Standard sample dilution in tube 8 is negative control.
5. Biotinylated human CD4 antibody liquid: Referring to needed amount, antibody diluent were employed to dilute the concentrated biotinylated antibody (1:100) to form biotinylated antibody liquid. The preparation was done 30 minutes in advance, and it was only used on that day.
6. Enzyme-conjugate liquid: Referring to needed amount, the concentrated enzyme-conjugate was diluted by enzyme-conjugate diluent (1:100) to

form enzyme-conjugate liquid. The preparation was done 30 minutes in advance, and it's only for use on that day.

7. Colour Reagent liquid: color reagent liquid was prepared 30 minutes in advance with color reagent A and colour reagent B by the proportion of 9:1.

**D. Test Procedures:**

**1.** The needed strips were taken out from zip lock bag for balanced to room temperature. The unused strips and desiccant were put back into the sealed aluminum foil bag at 2-8 for storage.

2. The samples or different concentration of human CD4 standard samples were added to corresponding wells (100 Microliters for each well),0 ng/ml well was filled with standard diluent. The reaction wells were sealed with adhesive tapes and was putted in incubator at 37 for 90 minutes.

3.The biotinylated human CD4 antibody liquid was prepare 30minutes in advance.

4. The ELISA plate was washed 3 times.

5. The biotinylated human CD4 antibody liquid was added to each well (100 Microliters for each). Reaction wells were seal with adhesive tapes and was putted in incubator at 37 for 60 ℃ minutes.

6. Enzyme-conjugate liquid was prepare 30minutes in advance.

7. The ELISA plate was washed 3 times.

8. Enzyme-conjugate liquid was added to each well except blank wells (100 Microliters for each). The reaction wells were seal with adhesive tapes and putted in incubator at 37 for 30 minutes.

9. The ELISA plate was washed 5 times.

10. 100 Microliters color reagent liquid were added to individual well (also into blank well) and was put in dark incubator at 37ºC . When color for high concentration of standard curve became darker and color gradient was appears, the hatched was stopped.

11. One hundred microliters color reagent C were added to individual well (also into blank well) and Mixed well. The OD was read at（450nm）.

**E. Result Determination:**

1. The professional curve fitting software was used to make a standard curve and calculate the level of the analyte.

3. The process of calculating the positive and negative values for patients and healthy people was done by calculating the value of the mean of the healthy people and comparing it with all the values of the concentrations, if the concentration value is higher than the mean average the result was considered positive, but if it was less than the result was considered negative.

**3.8.2.5. Determination of CD8:**

**A. Principle of the Test:**

This (Mybiosource-USA) used double-sandwich ELISA technique. The pre-coated antibody is human CD8 monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labeled. Samples and biotin labeling antibody were added into ELISA plate wells and washed out with PBS or TBS. Then Avidin-peroxidase conjugates were added to ELISA wells in order; TMB substrate was used for coloring after reactant thoroughly washed out by PBS or TBS. TMB turned into blue in peroxidase catalytic and finally turned into yellow under the action of acid. The color depth and the testing factors in samples are positively correlated.

**B. Kit Composition:**

Table (3.10) showed the contents of the CD8 determination kit (Mybiosource-USA).

**Table (3.10): Contents of CD8 determination laboratory kit.**

| Name | 96 Tests | 48 tests | Storage |
|---|---|---|---|
| 1. Antibody precoated plate | 8x12 | 8x6 | 4/-20 $^{0}$C |
| 2.Human CD8 Standards | 2 vials | 2 vials | 4/-20 $^{0}$C |

| | | | |
|---|---|---|---|
| 3.Biotinylatedantibody (1:100) | 1 vial | 1 vial | 4/-20 $^0$C |
| 4.Enzymeconjugate (1:100) | 1 vial | 1 vial | 4/-20 $^0$C |
| 5. Enzyme diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 6. antibody diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 7. Standard diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 8. Sample diluent | 1 vial | 1 vial | 4/-20 $^0$C |
| 9. Washing buffer (1:25) | 1 vial | 1 vial | 4/-20 $^0$C |
| 10. Colour Reagent A | 1 vial | 1 vial | 4/-20 $^0$C |
| 11.Colour Reagent B | 1 vial | 1 vial | 4/-20 $^0$C |
| 12. Colour Reagent C | 1 vial | 1 vial | 4/-20 $^0$C |
| 13. Instruction | 1 set | 1 set | Room temperature |

**C. Test Preparation:**

1. The ELISA Kit was removed of refrigerator 20 minutes in advance and take was tested when it balanced to room temperature.
2. The concentrated washing solution was diluted with double distilled water (1:25).
3. Human CD8 standard sample: 1.0 ml standard diluent were added into human CD8 lyophilized standard sample and was kept it for 30 minutes. After the sample was completely dissolved was mixed it slightly and mark labeled on the tube, then take dilution as needed. (It is recommended to using following concentration value to standard curve: 20,10, 5, 2.5, 1.25, 0.625, 0.312ng/ml).
4. Legend of standard sample dilution method: Take 7 clean tubes and label them with 1,2 ,3 ,4 ,5 ,6 ,7 , 8 respectively. 300 microliters standard sample diluent were added into each tube. 300 microliters diluent were taken from tube 1 to tube 2 and mixed well. Further 300 microliters diluent were taken from tube 2 to tube 3 , and were mixed well. Steps

were repeated above up to tube 7. Standard sample dilution in tube 8 is negative control.

5. Biotinylated human CD8 antibody liquid: Referring to needed amount, employed antibody diluent to dilute the concentrated biotinylated antibody (1:100) to form biotinylated antibody liquid. The preparation was done 30 minutes in advance, and it was only for use on that day.
6. Enzyme-conjugate liquid: Referring to needed amount, the concentrated enzyme-conjugate was diluted by enzyme-conjugate diluent (1:100) to form enzyme-conjugate liquid. The preparation was done 30 minutes in advance, and it was only for use on that day.
7. Color reagent liquid: color reagent liquid was prepared 30 minutes in advance with color reagent A and color reagent B by the proportion of 9:1.

**D. Test Procedures:**

1. The needed strips were taken from zip lock bag which balanced to room temperature. The unused strips and desiccant were put back into the sealed aluminum foil bag at 2-8 ℃ for storage.
2. The samples or different concentration of human CD8 standard samples were added to corresponding wells (100 Microliters for each well), 0ng/ml well was filled with standard diluent. The reaction well were seal with adhesive tapes and was putted in incubator at 37 ℃ for 90 min.
3. The biotinylated human CD8 antibody liquid was prepare 30miutesn in advance.
4. The ELISA plate was washed 2 times
5. The biotinylated human CD8 antibody liquid was added to each well (100 Microliters for each). Reaction wells were seal with adhesive tapes and putted in incubator at 37 ℃ for 60 ℃ minutes.
6. Enzyme-conjugate liquid was prepared 30 minutes in advance.
7. The ELIA plate was washed 3 times.

8. The Enzyme-conjugate liquid was added to each well except blank wells (100 Microliters for each). The reaction wells were seal with adhesive tapes and putted in incubator at 37℃ for 30 minutes.
9. The ELISA plate was washed 5 times.
10. One hundred microliters color reagent liquid were added to individual well (also into blank well), put in dark incubator at 37℃. When color for high concentration of standard curve become darker and color gradient was appears, the hatched was stopped.
11. One hundred microliters color reagent C were added to individual well (also into blank well) and Mixed welland the OD was read at（450 nm）.

**E. Result determination:**

1. The professional curve fitting software was used to make a standard curve and calculate the level of the analyte.
3. The process of calculating the positive and negative values for patients and healthy people was done by calculating the value of the mean of the healthy people and comparing it with all the values of the concentrations, if the concentration value is higher than the mean average the result was considered positive, but if it was less than the result was considered negative.

### 3.8.2.6. Determination of Human IL-17:

**A. Principle of the Test:**

This kit (Mybiosource –USA) was based on sandwich enzyme-linked immune-sorbent assay technology. Capture antibody was pre-coated onto 96-well plates. The biotin conjugated antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color

product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the target amount of sample captured in plate. The O.D. absorbance was read at 450 nm in a microplate reader, and then the concentration of target was calculated.

**B. The Kit Components:**

Table (3.11) revealed the components of the IL-17 determination laboratory kit used in the present study.

**Table (3.11): Contents of IL-17 determination laboratory kit.**

| Item | Specifications (48T/96T) | Storage |
|---|---|---|
| ELISA Microplate (Dismountable) | 8×6 /8×12 | 4°C/-20°C |
| Lyophilized Standard | 1 vial / 2 vial | 4°C/-20°C |
| Sample/Standard Dilution Buffer | 10 ml /20 ml | 4°C |
| Biotin-labeled Antibody (Conc.) | 60 ul /120ul | 4°C (protect from light) |
| Antibody Dilution Buffer | 5ml /10ml | 4°C |
| HRP-Streptavidin Conjugate (SABC) | 60ul /120ul | 4°C (protect from light) |
| SABC Dilution Buffer | 5ml /10ml | 4°C |
| TMB Substrate | 5ml /10ml | 4°C (protect from light) |
| Stop Solution | 5ml /10ml | 4°C |
| Wash Buffer(25X) | 15ml /30ml | 4°C |
| Plate Sealer | 3/5pieces | |
| Product Description | 1 copy | |

**C. Test preparation:**

1. All reagents and samples were kept for room temperature for 20 minutes before use.

2. A 30 milliliters concentrated wash buffer were diluted into 750 milliliters wash buffer with deionized or distilled water.
3. One milliliter sample dilution buffer was added into one standard tube (labeled as zero tube), the tube was kept at room temperature for 10 minutes and then mixed them thoroughly.
4. Seven Eppendorf tubes were labeled with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. 0.3 milliliters was added of the sample dilution buffer into each tube. 0.3 milliliters was added of the above standard solution (from zero tube) into first tube and was mix them thoroughly. 0.3milliliters were transferred from first tube to second tube and was mixed them thoroughly. 0.3 milliliters were transferred from second tube to third tube and mixed them thoroughly, and so on. Sample dilution buffer was also used for the blank control.
5. Preparation of Biotin-labeled antibody working solution: It was prepared it within 1 hour before experiment. The required total volume of the working solution was calculated: 0.1milliters / well × quantity of wells. (Allow 0.1-0.2 ml more than the total volume.). The Biotin-detection antibody was diluted with antibody dilution Buffer at 1:100 and was mix them thoroughly. (i.e. one microliter Biotin-labeled antibody was added into 99 microliters antibody dilution buffer).
6. Preparation of HRP-Streptavidin Conjugate (SABC) working solution: It was prepared it within 30 minutes before experiment. The required total volume of the working solution was calculated: 0.1ml / well × quantity of wells. (Allow 0.1-0.2 milliliters more than the total volume), the SABC was diluted with SABC dilution buffer at 1:100 and were mix them thoroughly. (i.e. one microliter of SABC was added into 99 microliters of SABC dilution buffer.

**D. Assay Procedure:**

1. Standard, test samples (diluted at least1/2 with sample dilution buffer), control (blank) wells were set on the pre-coated plate respectively, and then, records their positions. Plate was washed 2 times before adding standard, sample and control (blank) well
2. Preparation of standards: One 100 microliters were aliquot of zero tube, first tube, second tube, third tube, fourth tube, fifth tube, sixth tube and sample dilution buffer (blank) into the standard wells.
3. Addition of sample: One 100 microliters were added of properly diluted sample into test sample wells.
4. The plate was sealed and incubated with a cover and incubate at 37°C for 90 minutes.
5. The cover was removed and discard the plate content, and plate was washed 2 times with wash buffer.
6. Biotin-labeled antibody: One 100 microliters Biotin-labeled antibody working solution were added into above wells (standard, test sample and blank wells). The solution was added at the bottom of each well without touching the sidewall, the plate was covered and incubate at 37°C for 60 minutes.
7. The cover was removed, and plate were washed 3 times with wash buffer, and the wash buffer was stayed in the wells for 1-2 minute each time.
8. HRP-Streptavidin Conjugate (SABC): One 100 microliters of SABC working solution were added into each well, the plate was covered and incubate at 37°C for 30 minutes.
9. The cover was removed and plate were washed 5 times with wash buffer, and the wash buffer was stayed in the wells for 1-2 minute each time.

10. TMB Substrate: 90 microliters TMB Substrate were added into each well, the plate was covered and incubate at 37°C in dark within 10-20 minutes.
11. Stopping solution: 50 microliters stop solution were added into each well. The color was turn yellow immediately. The adding order of stop solution should be as the same as the TMB substrate solution.
12. OD Measurement: the O.D. absorbance was read at 450 nm in microplate reader immediately after adding the stop solution. Regarding calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank well). The standard curve was plotted as the relative O.D.450 of each standard solution (Y) versus the respective concentration of the standard solution (X). The target concentration of the samples were interpolated from the standard curve. It was use professional software to do this calculation.
13. The process of calculating the positive and negative values for patients and healthy people was done by calculating the value of the mean of the healthy people and comparing it with all the values of the concentrations, if the concentration value is higher than the mean average the result was considered positive, but if it was less than the result was considered negative.

**3.8.2.7. Determination of Human IL-18:**

**A. Principle of the Test:**

This kit (Mybiosource – USA) was based on sandwich enzyme-linked immune-sorbent assay technology. Capture antibody was pre-coated onto 96-well plates. The biotin conjugated antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and washed with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP

enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stopping solution. The density of yellow is proportional to the target amount of sample captured in plate. The O.D. absorbance was read at 450 nm in a microplate reader, and then the concentration of target was calculated.

**B. Kit Components:**

Table (3.12) revealed the components of the IL-17 determination laboratory kit used in the present study.

**Table (3.12): Contents of IL-18 determination laboratory kit.**

| Item | Specifications (48T/96T) | Storage |
|---|---|---|
| ELISA Microplate (Dismountable) | 8×6/8×12 | 4°C/-20°C |
| Lyophilized Standard | 1vial/2 vial | 4°C/-20°C |
| Sample/Standard Dilution Buffer | 10ml/20ml | 4°C |
| Biotin-labeled Antibody (Concentrated) | 60ul/120ul | 4°C (protect from light) |
| Antibody Dilution Buffer | 5ml/10ml | 4°C |
| HRP-Streptavidin Conjugate (SABC) | 60ul/120ul | 4°C (protect from light) |
| SABC Dilution Buffer | 5ml/10ml | 4°C |
| TMB Substrate | 5ml/10ml | 4°C (protect from light) |
| Stop Solution | 5ml/10ml | 4°C |
| Wash Buffer(25X) | 15ml/30ml | 4°C |
| Plate Sealer | 3/5pieces | |
| Product Description | 1 copy | |

**C. Reagent Preparation:**

1. All reagents and samples were kept in room temperature for 20 minutes before use.

2. Three hundred milliliters concentrated wash buffer were diluted into 750 milliliters wash buffer with deionized or distilled water.
3. One milliners sample dilution buffer was added into one standard tube (labeled as zero tube), the tube was kept at room temperature for 10 minutes and was mixed them thoroughly.
4. Seven Eppendorf tubes were labeled with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. 0.3 milliners of the sample dilution buffer were added into each tube. 0.3 milliners were added of the above standard solution (from zero tube) into first tube and mix them thoroughly. 0.3 milliners were transferred from first tube to second tube and was mixed them thoroughly. 0.3 milliners were transferred from second tube to third tube and was mixed them thoroughly, and so on. Sample dilution duffer was used for the blank control.
5. Preparation of Biotin-labeled antibody working solution: It was prepare within 1 hour before experiment. Required total volume of the working solution was calculated: 0.1milliliters / well × quantity of wells. (Allow 0.1-0.2 milliliters more than the total volume.). The Biotin-detection antibody was diluted with Antibody Dilution Buffer at 1:100 and was mixed them thoroughly. (i.e. One microliters Biotin-labeled antibody was added into 99 microliters antibody dilution buffer.).
6. Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution: It was prepare within 30 minutes before experiment. The required total volume of the working solution was calculated: 0.1 milliliters / well × quantity of wells. (Allow 0.1-0.2 milliliters more than the total volume.) The SABC was diluted with SABC Dilution Buffer at 1:100 and were mix them thoroughly. (i.e. one microliters of SABC was added into 99 microliters of SABC dilution buffer.)

**D. Assay Procedure:**

1. Standard, test samples (diluted at least1/2 with sample dilution buffer), control (blank) wells were set on the pre-coated plate respectively, and then, records their positions. Plate was washed 2 times before adding standard, sample and control (blank) wells.

2. Preparation of Standards: One 100 microliters were aliquot of zero tube, first tube, second tube, third tube, fourth tube, fifth tube, sixth tube and sample dilution buffer (blank) into the standard wells.

3. One 100 microliters of properly diluted sample were added into test sample wells.

4. The plate was sealed with a cover and incubate at 37°C for 90 minutes.

5. The cover was removed and discard the plate content, and plate was washed 2 times with wash buffer.

6. One100 microliters Biotin-labeled antibody working solution were added into above wells (standard, test sample and blank wells). The solution was added at the bottom of each well without touching the sidewall, the plate was covered and incubate at 37°C for 60 minutes.

7. The cover was removed, and the plate was washed 3 times with wash buffer, and the wash buffer was left stayed in the wells for 1-2 minute each time.

8. One 100 microliters of SABC working solution were added into each well, the plate was covered and incubate at 37°C for 30 minutes.

9. The cover was removed and plate were washed 5 times with wash buffer, and the wash buffer was stayed in the wells for 1-2 minute each time.

10. Ninty microliters TMB Substrate were added into each well, the plate was covered and incubate at 37°C in dark within 10-20 minutes.

11. Fifty microliters of stopping solution were added into each well. The color was turn yellow immediately. The adding order of stop solution should be as the same as the TMB Substrate Solution.

12. The O.D. absorbance was read at 450 nm in microplate Reader immediately after adding the stop solution. Regarding calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank well). The standard curve was plotted as the relative O.D.450 of each standard solution (Y) versus the respective concentration of the standard solution (X). The target concentration of the samples were interpolated from the standard curve. It was use some professional software to do this calculation.

**3.8.2.8. Determination of Human IFN γ:**

**A. Principle of the Assay:**

This kit (Mybiosource – USA) was based on sandwich enzyme-linked immune-sorbent assay technology. Anti- IFN-γ antibody was pre-coated onto 96-well plates. The biotin conjugated anti- IFN-γ antibody was used as detection antibodies. The standards, test samples and biotin conjugated detection antibody were added to the wells subsequently, and wash with wash buffer. HRP-Streptavidin was added and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the IFN-γ amount of sample captured in plate. The O.D. absorbance was read at 450 nm in a microplate reader, and then the concentration of IFN-γ was be calculated.

**B. Kit Components:**

Table (3.13) revealed the components of the IFN-γ determination laboratory kit used in the present study.

**Table (3.13): Contents of IFN-γ determination laboratory kit.**

| Item | Specifications (48T/96T) | Storage |
|---|---|---|
| Micro ELISA Plate (Dismountable) | 8 ×6 or 8×12 | 4°C/-20°C |
| Lyophilized Standard | 1 vial or 2 vial | 4°C/-20°C |
| Sample / Standard dilution buffer | 10ml/20ml | 4°C |
| Biotin- detection antibody (Concentrated) | 60ul/120ul | 4°C |
| Antibody dilution buffer | 5ml/10ml | 4°C |
| HRP-Streptavidin Conjugate(SABC) | 60ul/120ul | 4°C (shading light) |
| SABC dilution buffer | 5ml/10ml | 4°C |
| TMB substrate | 5ml/10ml | 4°C (shading light) |
| Stop solution | 5ml/10ml | 4°C |
| Wash buffer (25X) | 15ml/30ml | 4°C |
| Plate Sealer | 3/5pieces | |
| Product Description | 1 copy | |

**C. Reagent Preparation:**

1. All reagents and samples were kept in room temperature before use.
2. Thirty milliliters concentrated wash buffer were diluted into 750 milliliters of wash buffer with deionized or distilled water.
3. One milliliters sample dilution buffer was added into one standard tube (labeled as zero tube), the tube was kept at room temperature for 10 minutes and mixed them thoroughly.
4. Seven Eppendorf tubes were labeled with 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and blank respectively. 0.3 milliliters of the sample dilution buffer were added into each tube. 0.3mmilliliter were added of the above standard solution (from zero tube) into first tube and mix them thoroughly. 0.3

milliliters were transferred from first tube to second tube and was mixed them thoroughly. 0.3 milliliters were transferred from second tube to third tube and mixed them thoroughly, and so on. Sample dilution buffer was used for the blank control.

5. Preparation of Biotin-labeled Antibody Working Solution: It was prepared within 1 hour before experiment. The required total volume of the working solution was calculated: 0.1milliliter / well × quantity of wells. (Allow 0.1-0.2 milliters more than the total volume). The Biotin-detection antibody was diluted with antibody dilution Buffer at 1:100 and was mixed them thoroughly. (i.e. 1Microliters Biotin-labeled antibody was added into 99Microliters antibody dilution buffer.).
6. Preparation of HRP-Streptavidin Conjugate (SABC) Working Solution: It was prepare within 30 minutes before experiment. The required total volume of the working solution was calculated: 0.1milliliters / well × quantity of wells. (Allow 0.1-0.2 milliliters more than the total volume.). The SABC was diluted with SABC dilution buffer at 1:100 and were mix them thoroughly. (i.e. One microliters of SABC was added into 99 microliters of SABC dilution buffer.)

**D. Assay Procedure:**

1. Standard, test samples (diluted at least1/2 with sample dilution buffer), control (blank) wells were set on the pre-coated plate respectively, and then, records their positions. The plate was washed 2 times before adding standard, sample and control (blank) wells.
2. Preparation of Standards: One100 microliters were aliquot of zero tube, first tube, second tube, 3third tube, fourth tube, fifth tube, sixth tube and sample dilution buffer (blank) into the standard wells.
3. One 100 microliters of properly diluted sample were added into test sample wells.
4. The plate was sealed with a cover and incubate at 37°C for 90 minutes.

5. The cover was removed and discard the plate content, then the plate was washed 2 times with wash buffer.
6. One100 microliters Biotin-labeled antibody working solution were added into above wells (standard, test sample and blank wells). The solution was added at the bottom of each well without touching the sidewall, the plate was covered and incubate at 37°C for 60 minutes.
7. The cover was removed, and the plate was washed 3 times with wash buffer, and the wash buffer was stayed in the wells for 1-2 minute each time.
8. One 100 microliters of SABC working solution were added into each well, the plate was covered and incubate at 37°C for 30 minutes.
9. The cover was removed and the plate were washed 5 times with wash buffer, and the wash buffer was stayed in the wells for 1-2 minute each time.
10. Ninty microliters TMB substrate were added into each well, the plate was covered and incubate at 37°C in dark within 10-20 minutes.
11. Fifty microliters stopping solution were added into each well. The color was turn yellow immediately. The adding order of stopping solution should be as the same as the TMB substrate solution.
12. The O.D. absorbance was read at 450 nm in microplate reader immediately after adding the stopping solution. Regarding calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of blank well). The standard curve was plotted as the relative O.D.450 of each standard solution (Y) versus the respective concentration of the standard solution (X). The target concentration of the samples were interpolated from the standard curve. It was used some professional software to do this calculation.

### 3.8.3. Cytokine Polymorphism Parameters:

#### 3.8.3.1. Selection of Specific Sequence for IL-17, IL-18, and INF-γ Genes.

This study focused on specific sequences for each IL-17, 1L-18, INF-γ genes. Several researchers focused on studying relation of the sequencing in (3) genes and many illnesses such as brucellosis. The IL-17 coding gene is located on chromosome 6p12 and consists of three exons (Chen *et al.,* 2006), Genetic variation in IL-17 has been studied in various disease, including pulmonary tuberculosis, leishmaniosis, rheumatoid arthritis, breast cancer and gastric cancer (Sadighi *et al.*, 2014; Zhang *etal*., 2008).

IFN-gamma at the +874 position several studies on brucellosis (Rasouli and Kiany., 2007), chronic hepatitis C (Dai *etal.*,2006), HIV-1/AIDS infection (Sobti *etal.*,2010). IL-18 plays a protective role against brucellosis (Pasquali *et al.*, 2002) and some infectious diseases (Stuyt *et al.*, 2004). Taking into consideration the important function of IL-18 in stimulation of type 1 immune response which determines the protective immunity against Brucella infection, the suitable level of this cytokine sounds to be crucial in outcome of the disease. IL-18 gene is placed on the long arm of chromosome 11 and six single nucleotide polymorphisms (SNPs) have been recognized in the potentially functional region of its gene (Folwaczyn *et al.*,2005), such as the coding sequence, Exon–intron junctions, promoters and untranslated regions (UTRs). Taking into account the involvement of these polymorphisms in altering IL-18 level.

#### 3.8.3.2. DNA Extraction from Whole Blood:

Genomic DNA was isolated from blood sample according to the protocol ReliaPrep™ Blood gDNA Miniprep System, (Promega, USA) as the following steps:

• Blood sample was thoroughly mixed for 15 minutes in rotisserie shaker at room temperature.

• For each 1.5 milliliters microcentrifuge tube 20 microliters of Proteinase K (PK) Solution was dispensed then 200 microliters of blood was added and briefly mixed • For cells lysis 200 microliters of cell lysis buffer (CLD) was added to the tube and mixed by vortex for 10 seconds.

• All mixes were incubated in water bath at 56°C for 30 minutes.

• While the blood sample was incubated, a ReliaPrep™ Binding Column was placed into an empty collection tube.

• After incubation, the tube removed from water bath and 250 microliters of binding buffer (BBA) was added and mixed by vortex for 10 seconds

• All the tube contents were transferred to the ReliaPrep™ Binding Column and centrifuged for 3 minutes at 12000 rpm.

• The collection tube containing flow through was removed and discarded.

• The binding column was placed into a fresh collection tube.

• For column washing 500 microliters of column wash solution (CWD) was added to the column and centrifuged for 3 minutes at maximum speed, the flow through was discarded and this step was repeated three time.

• After washing step, the column was placed in a clean 1.5 milliliters micro centrifuge tube and 100µl of Nuclease-Free water was added to the column.

• After 5 minutes, the 1.5ml tube with column centrifuge for 5 minutes at 5000rpm.

• After centrifuge, the ReliaPrep™ Binding column was discarded and eluate saved.

**3.8.3.3. Quantitation of DNA:**

Quantus Fluorometer was used to detect the concentration of extracted DNA in order to detect the quality of samples for downstream applications. For 1 microliter of DNA, 199 microliters of diluted

Quantifluor dye was mixed. After 5 minutes incubation at room temperature, DNA concentration values were detected.

**3.8.3.4. Primer Preparation**:

The following primers were used for molecular detection of IL-17, IL-18 and INF-γ genes in blood samples collected from patients suspected as having brucellosis as well as from healthy controls.

**Table (3.14): Primers of IL-17, IL-18 and INF-γ genes.**

| Primer Name | Vol. of nuclease free water (µl) | Concentration (pmol/µl) |
|---|---|---|
| IL17-2-F | 300 | 100 |
| IL17-2-R | 300 | 100 |
| IL18-F | 300 | 100 |
| IL18-R | 300 | 100 |
| INF-F | 300 | 100 |
| INF-R | 300 | 100 |

These primers were supplied by Macrogen company in a lyophilized form. lyophilized primers were dissolved in a nuclease free water to give a final concentration of 100 pmol/microliters as a stock solution. A working solution of these primers was prepared by adding 10 microliters of primer stock solution (stored at freezer -20 $^{0}$C) to 90 microliters of nuclease free water to obtain a working primer solution of 10 pmol/microliters.

**3.8.3.5. Primer Optimization:**

To examine the optimum annealing temperature of primer, the DNA template was amplified with the same primer pair, (Forward) and (Reverse), at annealing temperatures of 55, 58, 60, 63 and 65°C. PCR amplifications were performed with 20 microliters volumes containing 10 microliters GoTaq Green Master Mix (2X); microliters for each primer (10pmol); 6 microliters nuclease free water and 2 microliters of template

DNA. PCR cycling was performed with PCR Express (Thermal Cycler 1 (Veriti), Thermo Fisher Scientific, USA) with the following temperature program: denatured at 94 $^{o}$C for 4 min followed by 30 cycles of denaturation at 94°C for 30 seconds; annealing at 55, 58, 60, 63 or 65°C for 30 seconds; and extension at 72°C for 30 seconds. A final extension incubation of 7 minutes at 72$^{o}$C was included, followed by a 10 minutes incubation at 4$^{o}$C to stop the reactions.

**3.8.3.6. Reaction Setup and Thermal Cycling Protocol:**

**A. PCR Component Calculation:**

Table (3.15) showed PCR component calculation used in the present study.

**Table (3.15): PCR component calculation.**

| | | | | |
|---|---|---|---|---|
| No. of Reaction | 50 | rxn | Annealing temperature of primers | 55, 60C$^{o}$ |
| Reaction Volume /run | 25 | µl | No. of Primers | 3 |
| Safety Margin | 5 | % | No. of PCR Cycles | 30 |

**Table (3.16): Reaction mixture.**

| **Master mix components** | **Stock** | **Unit** | **Final** | **Unit** | **Volume 1 Sample** |
|---|---|---|---|---|---|
| | | | | | |
| Master Mix | 2 | X | 1 | X | 12.5 |
| Forward primer | 10 | µM | 1 | µM | 1 |
| Reverse primer | 10 | µM | 1 | µM | 1 |
| Nuclease Free Water | | | | | 7.5 |
| DNA | | ng/µl | | ng/µl | 3 |
| Total volume | | | | | 25 |
| Aliquot per single rxn | 23µl of Master mix per tube and add 2 µl of Template | | | | |

**B. PCR Program:**

Table (3.17) showed the PCR steps and temperatures, time and cycle of each step used in PCR program that used in the present study.

**Table (3.17): PCR program steps.**

| Steps | ℃ | m: s | Cycle |
|---|---|---|---|
| Initial Denaturation | 95 | 05:00 | 1 |
| Denaturation | 95 | 00:30 | 30 |
| Annealing | 55 OR 60 | 00:30 | |
| Extension | 72 | 01:00 | |
| Final extension | 72 | 07:00 | 1 |
| Hold | 10 | 10:00 | |

**3.8.3.7. Agarose Gel Electrophoresis:**

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplicon. PCR was completely dependable on the extracted DNA criteria.

**A. Solutions**

1 X TAE buffer, DNA ladder marker, ethidium bromide (10 milligrams / milliliter).

**B. Preparation of Agarose**

• One 100 milliliters of 1X TAE was poured into a flask.

• 1.5 grams (for 1.5%) agarose was added to the buffer.

• The solution was heated to boiling (using microwave) until all the gel particles were dissolved.

• One microliters of ethidium bromide (10 milligrams / milliliter) was added to the agarose.

• The agarose was stirred in order to get mixed and to avoid bubbles.

• The solution was left to cool down at 50-60C°.

C. **Casting of the Horizontal Agarose Gel:**

The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed, and the gel was placed in the gel electrophoresis tank. The tank was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 millimeters over the surface of the gel.

**D. PCR Products Loading:**

For PCR product, 5 microliters were directly loaded to well. Electrical power was turned on at 100 volt/ 50 mAmp for 75minutes. DNA moves from cathode to plus anode poles. The ethidium bromide-stained bands in gel were visualized using Gel imaging system.

**3.8.3.8 Standard Sequencing:**

PCR product were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software.

**3.8.3.9. Sequencing Methods:**

**3.8.3.9.1. *DNA Sequencing of PCR Amplicons*:**

The resolved PCR amplicons were commercially sequenced from one direction, forward direction, according to instruction manuals of the sequencing company (Macrogen Inc. Geumchen, Seoul, South Korea). Only clear chromatographs obtained from ABI (Applied Biosystem) sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local samples with the retrieved DNA sequences, the virtual positions, and other details of the retrieved PCR fragments were identified.

**3.8.3.9.2. Interpretation of Sequencing Data**:

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

**3.8.3.9.3. Checking the Novelty of SNPs**:

The observed SNPs were submitted to the dbSNP database to check their novelty. Each particular SNP position was checked in its corresponding reference genome to assess whether it was previously deposited in the dbSNP server. As well, more data were data concerned each individual SNP was revealed with regard to its frequency, and its importance in literature was also considered.

**3.8.3.11. Construction of Linkage Disequilibrium Plot:**

Linkage disequilibrium (or LD), was utilized to analyze the deposited SNPs in the studied loci having multiple polymorphic sites. LD is a measure of the non-random association of alleles at two or more loci that descended from a single and ancestral chromosome. The commonly used summaries D' and $r^2$ have been calculated. D' is the difference between the observed and the expected frequency of a given haplotype. If two loci are independent (*i.e.* in linkage equilibrium and therefore not co-inherited at all), the *D'* value will be 0. The $r^2$ value is the correlation between a pair of loci. It varies from 0 (loci are in complete linkage equilibrium) to 1 (loci are in complete linkage disequilibrium and therefore co-inherited). The precise position occupied by the observed variations within the amplified fragments was located according to their positions in the PCR amplicons. Linkage data were analyzed by the haploview

software, version 4.2 (https://www.broadinstitute.org/haploview/haploview). LD values were estimated using squared allele frequency correlations ($r^2$) between the pairs of loci. The loci were considered to be exhibit significant LD when $P < 0.001$, the rest of $r^2$ values was not considered as informative. After selecting populations to view LD data, the uploaded linkage data, as well as the locus information file of the fragment of interest, were analyzed appropriately.

**3.8.3.10. Statistical Analysis:**

Analysis of data was carried out using the available statistical package of SPSS-26 (Statistical Packages for Social Sciences- version 26). Data were presented in simple measures of frequency, percentage, mean, standard deviation, and range (minimum-maximum values). The significance of difference of different means (quantitative data) were tested using Students-t-test for difference between two independent means. The significance of difference of different percentages (qualitative data) were tested using Pearson Chi-square test ($\chi^2$-test) with application of Yate's correction or Fisher Exact test whenever applicable. Statistical significance was considered whenever the P value was equal or less than 0.05. (Daniel, 2010; Stellman,2010).

# Chapter Four

# 4. Results and Discussions

Results presented in this chapter were based on statistical analysis of data accumulated throughout the study period which was extended from February 2020 to Jun 2021.

## 4.1. Study groups:

### 4.1.1. Age categories:

A total of 55 patients whose clinically suspected as having brucellosis were included as patients group and 35 apparently healthy individuals were included as control group. The age distribution of study groups was shown in table (4.1) and figure (4.1). In both patient and control groups, the highest age range was 20-29 years, 45.5% and 31.4% respectively. However, the difference between the two groups was statistically insignificant (P= 0.162). The mean ± SD and the age range of the patients and control groups were 29.8±10.1 (15-59) and 33.8±11.0 (18-58) years respectively, with insignificant difference statistically (P= 0.078).

**Table (4.1): Distribution of age categories in patients and control groups.**

| Age (Ys) | Patients | | Control | | P value |
|---|---|---|---|---|---|
| | No. | % | No. | % | |
| < 20 years | 6 | 10.9 | 2 | 5.7 | 0.162 * |
| 20---29 | 25 | 45.5 | 11 | 31.4 | |
| 30---39 | 17 | 30.9 | 10 | 28.6 | |
| 40---49 | 3 | 5.5 | 6 | 17.1 | |
| ≥ 50 years | 4 | 7.3 | 6 | 17.1 | |
| Total | 55 | 100% | 35 | 100% | |
| Mean ± SD (Range) | 29.8±10.1 (15-59) | | 33.8±11.0 (18-58) | | 0.078 ** |

*insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**insignificant difference between two independent means using Students-t-test at 0.05 level.

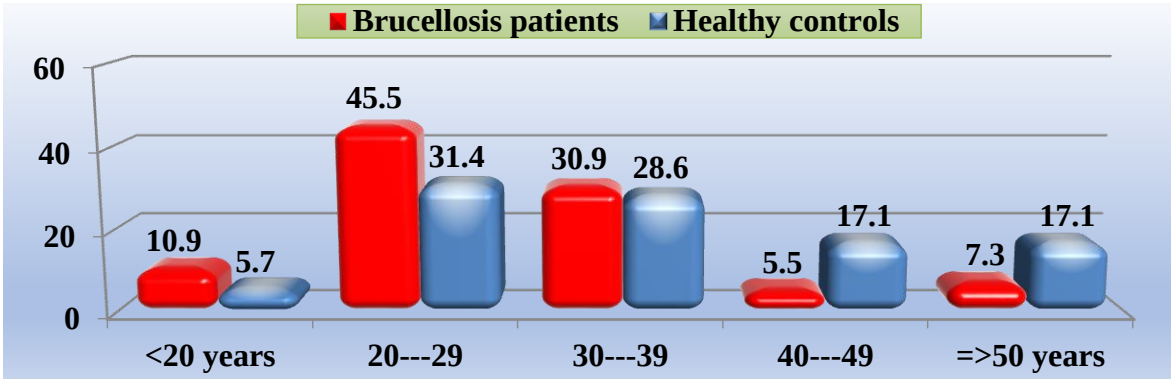



**Figure (4.1): Distribution of age categories of study groups.**

### 4.1.2. Gender:

Within the patients group, 44 (80%) were female and 11 (20%) were male. similarly among the control, 22 (62.9%) were female and 13 (37.1%) were male. The difference between the patient and control group was statistically insignificant (P= 0.073) as revealed in table (4.2) and figure (4.2).

**Table (4.2): Distribution of gender in patients and control groups.**

| Gender | Patients | | Control | | P value |
|---|---|---|---|---|---|
| | No. | % | No. | % | |
| Male | 11 | 20.0 | 13 | 37.1 | 0.073* |
| Female | 44 | 80.0 | 22 | 62.9 | |
| Total | 55 | 100 | 35 | 100 | |

*insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

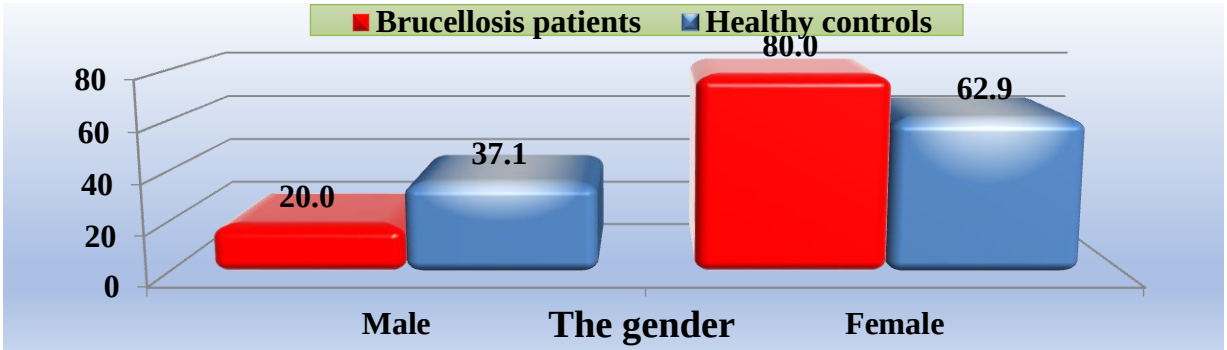



**Figure (4.2): Distribution of gender of study groups.**

### 4.1.3. Socio-Demographic Variables:

Table (4.3) and figures (4.3), (4.4) and (4.5) revealed the distribution of certain variables. So, regarding the residence in city or village, the results found that there was statistically insignificant difference between the patients and control groups (P= 0.809). The house breeding of productive animals showed that patients had higher rate of house breeding compared to control, but the difference was failed to reach the levels of statistical significance (P = 0.099). Concerning the consumption of dairy products and meat, the results found that there was a statistically significant difference between patients and control (P= 0.0001). The intra-familial transmission of brucellosis other than the index case showed that patient group had higher rate of intra-familial transmission compared to control (P= 0.045). About the disease history, the recent cases among patients was significantly higher than that of the control (P= 0.0001).

**Table (4.3): Distribution of Study groups according to soci-demographic variable.**

| Variables | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| Residence | City | 30 | 54.5 | 20 | 57.1 | 0.809** |
| | Village | 25 | 45.5 | 15 | 42.9 | |
| Home animals | Yes | 20 | 36.4 | 7 | 20.0 | 0.099** |
| | No | 35 | 63.6 | 28 | 80.0 | |
| Consumption of milk and meat, Dairy products | Yes | 50 | 90.9 | 21 | 60.0 | 0.0001* |
| | No | 5 | 9.1 | 14 | 40.0 | |
| Intra-familial brucellosis | Yes | 24 | 43.6 | 8 | 22.9 | 0.045* |
| | No | 31 | 56.4 | 27 | 77.1 | |
| Disease history | No infection | - | - | 34 | 97.1 | 0.0001* |
| | New infection | 44 | 80.0 | 1 | 2.9 | |
| | Old infection | 11 | 20.0 | - | - | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**insignificant between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

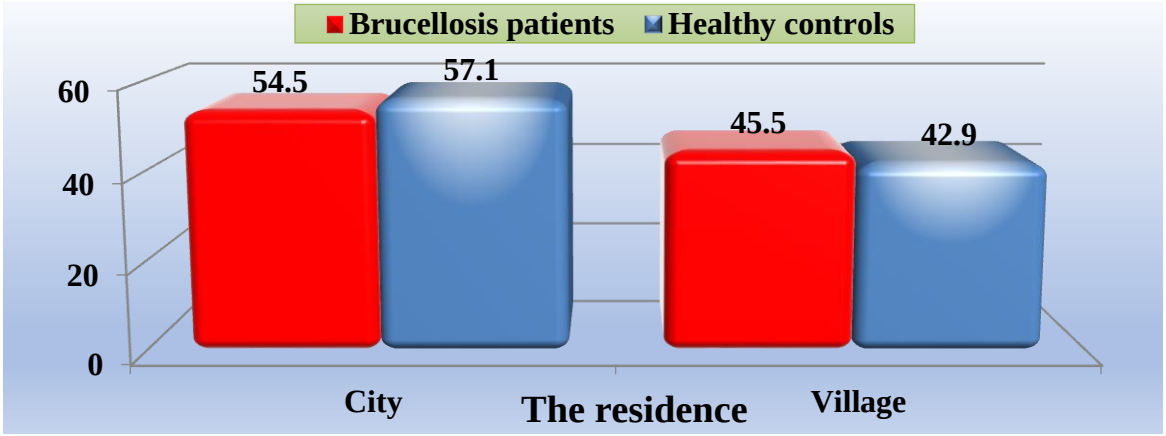



**Figure (4.3): Distribution of study groups according to residence.**

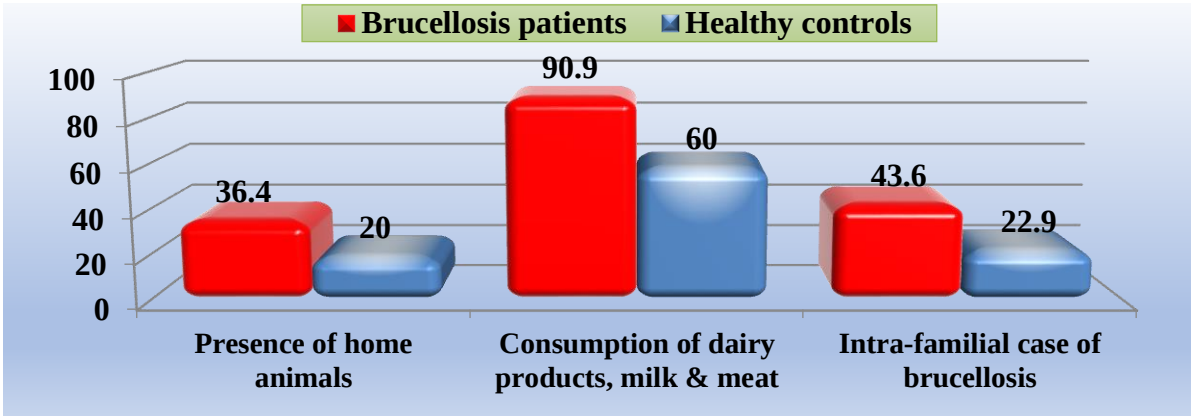



**Figure (4.4): Distribution of home animal, consumption of meat and dairy products and intra-familial cases of brucellosis.**

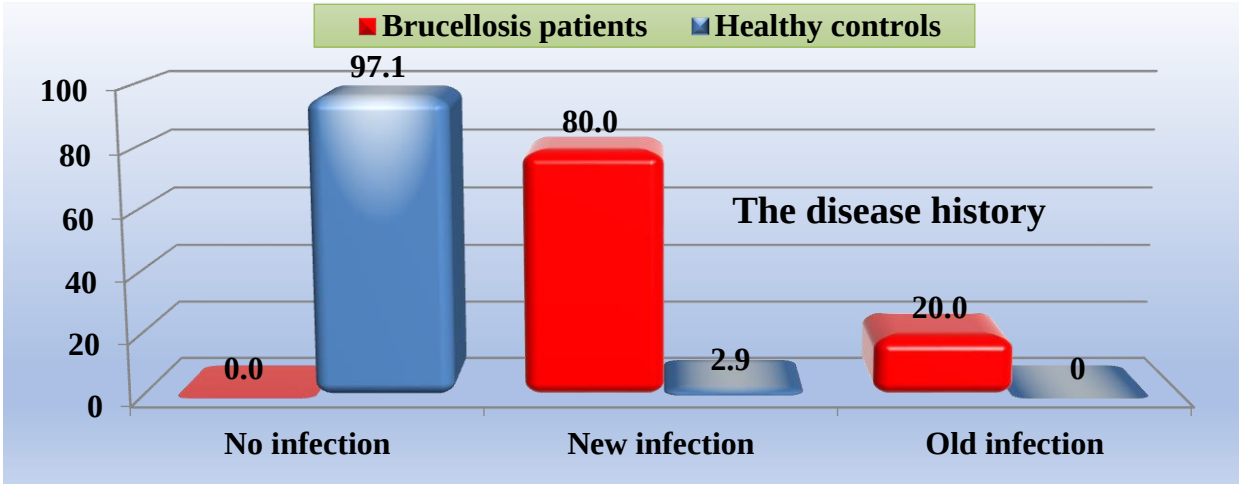



**Figure (4.5): Distribution of study groups according to disease history.**

### 4.1.4. Clinical Variables:

The results in table (4.4) and figure (4.6) showed that patients had significantly higher rate of fever compared to control (89.1% *Vs* 11.4), (P= 0.0001). Similarly, the night sweat, the patients had significantly higher rate of night sweat compared to the control group (80.0% *Vs* 5.7%), (P= 0.0001). Likewise, the patients had significantly higher rate of joint pain as compared to control (85.5% Vs 40%), (P= 0.0001).

**Table (4.4): Clinical variables among study groups.**

| Variables | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| Fever | Yes | 49 | 89.1 | 4 | 11.4 | 0.0001* |
| | No | 6 | 10.9 | 31 | 88.6 | |
| Night sweat | Yes | 44 | 80.0 | 2 | 5.7 | 0.0001* |
| | No | 11 | 20.0 | 33 | 94.3 | |
| Joint pain | Yes | 47 | 85.5 | 14 | 40.0 | 0.0001* |
| | No | 8 | 14.5 | 21 | 60.0 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

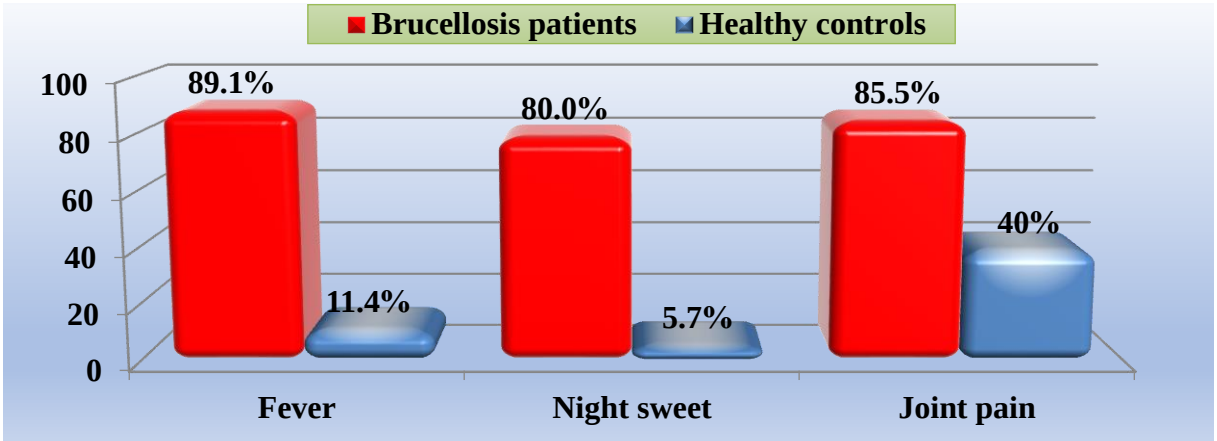



**Figure (4.6): Clinical variables among study groups.**

## 4.2. Rose Bengal Test:

Rose Bengal is a direct agglutination test was used as a screening test for brucellosis. The results showed that all patients were positive for Rose Bengal (100%), while, only 5.7% of the control were positive with a statistically significant difference (P= 0.0001).

**Table (4.5): Results of Rose Bengal test among study groups.**

| Test | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| Rose Bengal | Positive | 55 | 100.0 | 2 | 5.7 | 0.0001* |
| | Negative | - | - | 33 | 94.3 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

Undoubtedly the importance of the current study is attributed to many aspects; the most importantly, the high prevalence of brucellosis as an endemic zoonotic disease in both Iraqi population and domesticated reproductive animals (Shareef, 2006). In this regard it has been reported that human brucellosis is endemic in most of the Middle East countries with Syria, Iraq, Saudi Arabia, Turkey, and Iran having the world's highest incidence rates (Pappas *et al*., 2006; Dean *et al*., 2012). The annual incidence rate of human brucellosis in Iraq was 2.6/100000 population (Bagheri *et al*., 2020). On the animal side, It has been documented that the overall seroprevalence of brucellosis in food-producing animals over a period of 40 years was 14.14%, including 14.46% for sheep, 12.99% for goats, 11.69% for cattle, and 22.64% for buffalo with an increment rate of 9 times between 1979 and 2019 (Dahl, 2020).

It is worthy to mention the critical importance of small ruminants in the food supply with their considerable contribution to the provision of dairy products, meat, wool and hair for Iraqi's (Maxwell and Bill, 2008). For this reason, the people were routinely breed such food-producing ruminants in or near their houses to make benefit of their products. Furthermore, farmers had a custom to slaughter animals by themselves in their houses whenever they need meat or traditionally during religious or social occasions. Actually, this

explain the higher positivity rate of brucellosis among people who practice home breeding of such animals in rural and semi urban areas (Dahl, 2020). These customs which were away from veterinary supervision are responsible for the significantly higher positivity rate of brucellosis among those traditionally use raw milk and meat. In addition to consumption of raw milk and meat, in such home breeding, the farmer or probably members their family are aiding animals during delivery or abortion without safety precautions. Such high risk practices may responsible for the significantly higher positivity rate of brucellosis among family members or the clustering of brucellosis among Iraqi families (P= 0.045). Indeed, this fascinating result obtained in this study is largely attributed to the complete or partial absence of veterinary services, neglection of brucellosis as an important endemic zoonotic disease, beside the low health education and low living standard that promote perpetuation of the disease in Iraqi community (Shareef, 2006; Rossetti *et al*., 2017).

Brucella has the propensity to localize inside macrophages of the liver, spleen, bone marrow, uterus, heart, and brain with protean clinical manifestations (Mantur *et al*., 2006). The successful intracellular stealthy lifestyle has evolved through multiple strategies to evade immune response mechanisms, obligate the host cells to form a microenvironment conducive to its survival, reproduction and survive in the host cells for a long time, which eventually leads to the formation of chronic persistent infection (Ahmed *et al*., 2016; Jiao *et al*., 2021). The intra- macrophages existence of brucellae represents one of the strategies for consequent alterations in both innate and adaptive immune responses (Elfaki *et al*., 2015).

Compared to most other serological tests, RBT is rapid, easy to use, and relatively affordable as a screening test for Brucella species infection. Rose

Bengal test is so fast, but it has many false-negative results during the chronic brucellosis (Roushan *et al*., 2005).

In a study by Daood *et al.(* 2020) in Mosul city-Iraq, among a total of 385 sera of individuals suffering from clinical features suggestive of brucellosis, 115 (29.9%) of them showed positive reaction in RBT. In 2015, high rates of a positive result by RBT were reported among people in Al Sulaymaniyah and Baghdad regions (59.82% and 71.3% respectively) (AL-Bayaa, 2017; Mohammed, 2015). Brucellosis records in Iraq were higher than records from neighboring countries; For example, the prevalence of brucellosis in Turkey and Saudi Arabia was 6% and 12.5% respectively (Aloufi *et al.*,2016; Gul *et al.*,2014).

In the present study, the RBT positivity was used as an inclusion criteria, that is to say, all patients were already positive for RBT. However, among the control group who were apparently healthy individuals, the RBT positivity was 5.7%. Because the RBT detect total anti-brucella antibodies (IgM and IgG), so, it cannot differentiate between recent and past infections. If the anti-brucella IgM and IgG positivity of apparently healthy control by ELISA test were gathered, it appears that 6 out of 35 (17.1%) were positive which is higher than the RBT positivity. The difference in the positivity rate in this comparison is surly due to the low specificity and false positive of the RBT compared to ELISA technique (Diaz *et al*., 2011). Thus the current study is totally agree with others who stated that RBT can be useful as a screening test, but not for the final diagnosis of brucellosis especially in endemic areas (Ruiz-Mesa *et al*., 2005).

## 4.3. Anti-Brucella IgM:

### 4.3.1. Anti-IgM Positivity Rate:

Data presented in table (4.6) and figure (4.7) and figure (4.8) showed that 18 (32.7%) of the patients and 2 (5.7%) of the control were positive for anti-brucella IgM with statistically significant difference (P= 0.003).

**Table (4.6): Distribution of anti- IgM positivity rate among study groups.**

| Serological test | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| Anti-IgM | Positive | 18 | 32.7 | 2 | 5.7 | 0.003* |
| | Negative | 37 | 67.3 | 33 | 94.3 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

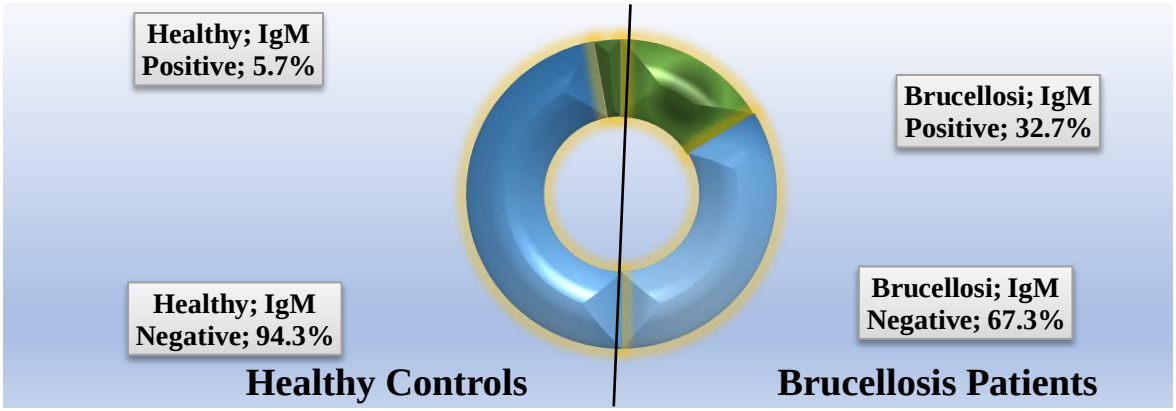
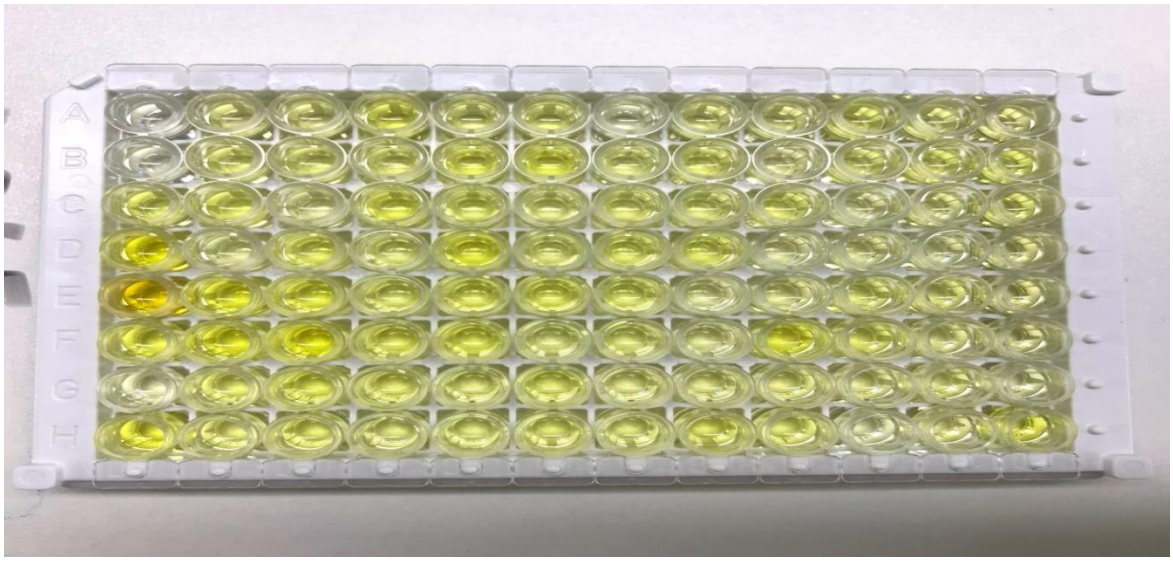



**Figure (4.7): Anti- IgM positivity rate among study groups.**



**Figure(4.8):Anti-IgM ELISA plate show anti-brucella IgM positivity rate.**

### 4.3.2. Anti-IgM Concentration:

Table (4.7) and figure (4.9) revealed that the anti-brucella IgM titer (IU/L) in patients with brucellosis is significantly higher than that of the control group (P= 0.011). Furthermore, the mean ± SD of anti-brucella IgM titer in the patient was significantly higher than that in the control group (6.382 ± 4.069 *Vs* 3.616 ± 2.141), (P= 0.0001).

**Table (4.7): Distribution of anti- IgM concentration among study groups.**

| **Serological test** | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| **Anti-IgM Concentration (IU/L)** | <1.0 IU/L | 2 | 3.6 | 4 | 11.4 | 0.011* |
| | 1.0--- | 2 | 3.6 | 5 | 14.3 | |
| | 2.0--- | 2 | 3.6 | 6 | 17.1 | |
| | 3.0--- | 11 | 20.0 | 8 | 22.9 | |
| | 4.0--- | 9 | 16.4 | 3 | 8.6 | |
| | 5.0--- | 9 | 16.4 | 3 | 8.6 | |
| | 6.0--- | 2 | 3.6 | 4 | 11.4 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 7.0--- | 4 | 7.3 | - | - | |
| | 8.0--- | 3 | 5.5 | 2 | 5.7 | |
| | 9.0--- | 1 | 1.8 | - | - | |
| | ≥10.0 IU/L | 10 | 18.2 | - | - | |
| Mean ± SD (Range) | | 6.382 ± 4.069 (0.06-18.791) | | 3.616 ± 2.141 (0.385-8.791) | | 0.0001** |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Significant difference between two independent means using Students-t-test at 0.05 level.

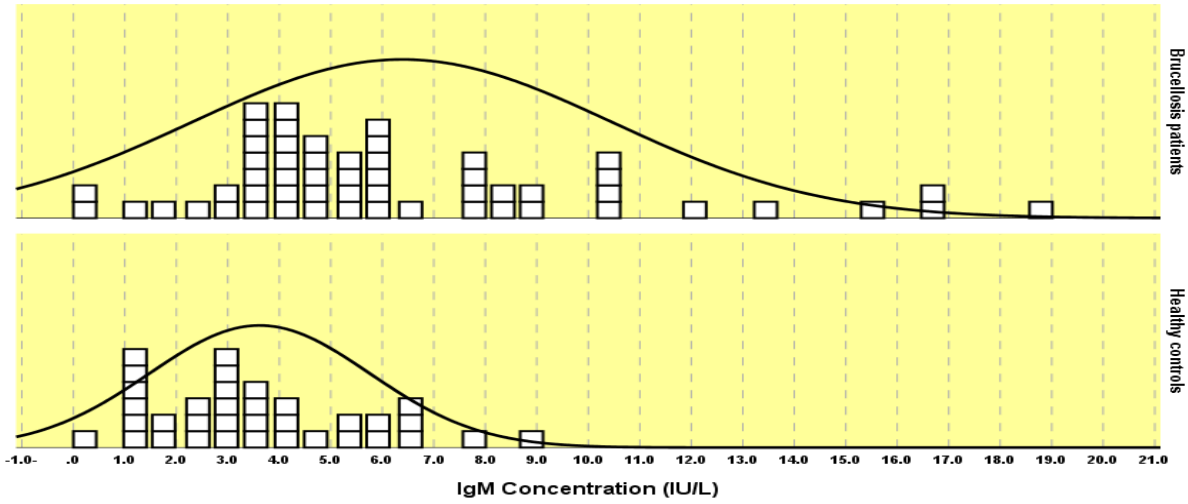



**Figure (4.9): anti- IgM concentration among study groups.**

## 4.4. Anti-Brucella IgG:

### 4.4.1. Anti-IgG Positivity Rate:

The results found that the anti-brucella IgG positivity rate among patients was 31(56.4%) while that of the control group was 4(11.4%). So the difference was statistically significant (P= 0.0001), as revealed in table (4.8) and figure (4.10).

**Table (4.8): Distribution of anti- IgG positivity rate among study groups.**

| Serological test | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| Anti-IgG | Positive | 31 | 56.4 | 4 | 11.4 | 0.0001* |
| | Negative | 24 | 43.6 | 31 | 88.6 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

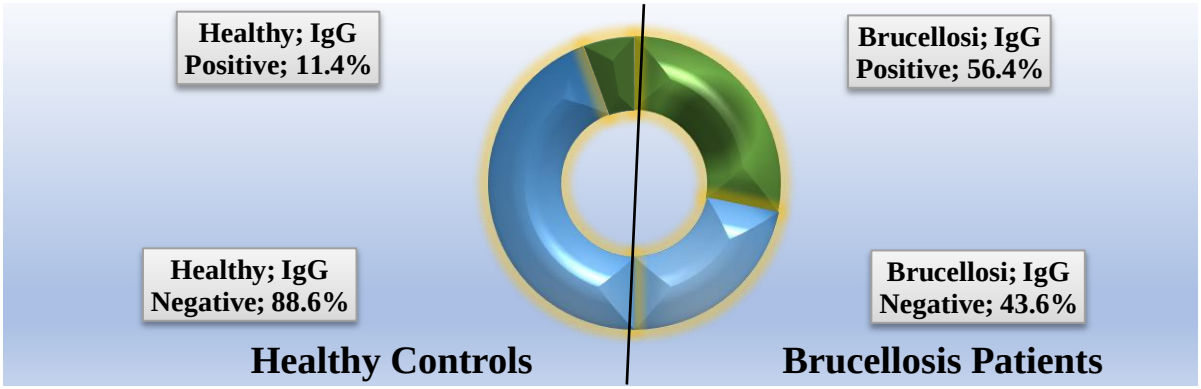



**Figure (4.10): Anti- IgG positivity rate among study groups.**

### 4.4.2. Anti-IgG Concentration:

The results also found that the titer of anti-brucella IgG (IU/L) in the patient group was significantly higher than its counterpart in control group (P= 0.0001). Additionally, the mean ± SD of anti-brucella IgG titer in the patient was significantly higher than that in the control group (9.702 ± 12.728 *Vs* 4.206 ± 1.993), (P= 0.013). Data were shown in table (4.9) and figure (4.11).

**Table (4.9): Distribution of anti- IgG concentration among study groups.**

| Serological test | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| **Anti-IgG Concentration (IU/L)** | <1.0 IU/L | - | - | - | - | 0.0001* |
| | 1.0--- | 1 | 1.8 | 1 | 2.9 | |
| | 2.0--- | - | - | 9 | 25.7 | |
| | 3.0--- | 5 | 9.1 | 11 | 31.4 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 4.0--- | 13 | 23.6 | 7 | 20.0 | |
| | 5.0--- | 3 | 5.5 | 3 | 8.6 | |
| | 6.0--- | 15 | 27.3 | - | - | |
| | 7.0--- | 5 | 9.1 | 2 | 5.7 | |
| | 8.0--- | 3 | 5.5 | 1 | 2.9 | |
| | 9.0--- | 2 | 3.6 | - | - | |
| | ≥10.0 IU/L | 8 | 14.5 | 1 | 2.9 | |
| Mean ± SD (Range) | | 9.702 ± 12.728 (1.954-69) | | 4.206 ± 1.993 (1.697-11.452) | | 0.013 ** |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Significant difference between two independent means using Students-t-test at 0.05 level.

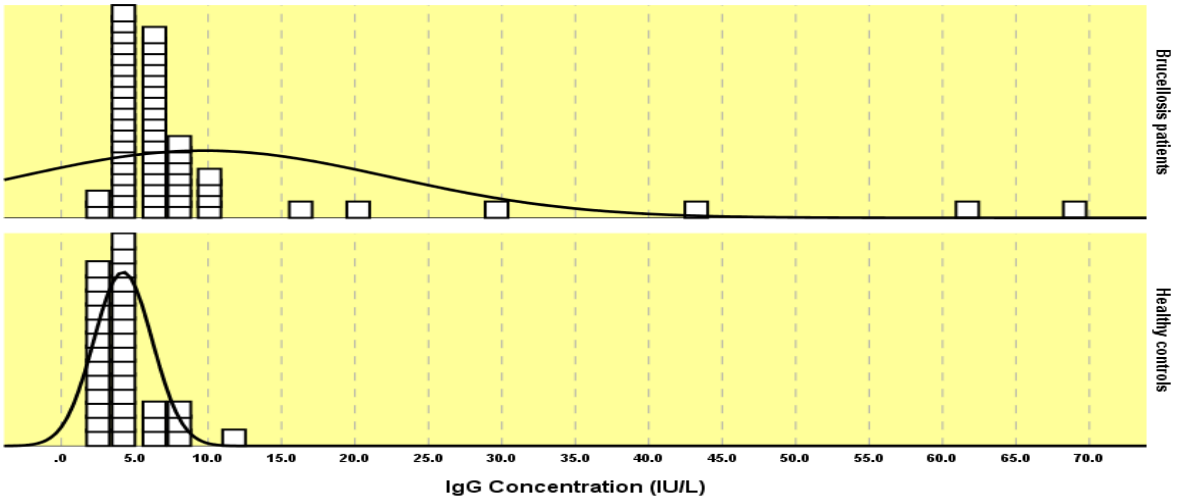



**Figure (4.11): Anti- IgG concentration among study groups.**

The anti-brucella IgM antibodies were the first to appear following infection and rise gradually during the course of acute infection. In contrast, the IgG antibodies appeared later after the onset of infection (Elfaki *et al*., 2015). The IgG antibodies may persist for many months after the successful antibiotic therapy. This explains the high seroprevalence of anti-brucella antibodies found in areas of endemicity and among individuals who had repeated flare up of brucellosis (Ariza *et al*., 1992).

The presence of IgM antibodies is indicative of acute infection but at the same time, IgM antibodies are well known for their cross-reactions with other bacterial species, like *Yersinia*, *Escherichia coli O157*, *Salmonella spp*. and *Francisella tularenis* (Aranis *et al*., 2008). Since infections have been identified as the cause of fever in most of our patients, cross-reactions are probably responsible for the detection of IgM anti-brucella antibodies in these patients. The result of IgM in this study is agree with AL-Jindan *et al.* (2019), who showed positivity rate of IgM was (22.1%) from all brucellosis patients.

However, the results of this study disagreed with Rahamathulla (2019) because their study showed IgM ELISA and IgG ELISA were positive for (86.9%) and (96.8%) cases respectively. Another study reports that ELISA had higher sensitivity than RBT, but, RBT yielded higher specificity than ELISA (Matope *et al*., 2011). Thus, this suggests that using both RBT and ELISA in the diagnosis of Brucellosis among patients, would yield better results.

The results found that the anti-brucella IgG positivity rate among patients was (56.4%) while that of the control group was (11.4%), as revealed in table (4.8) and figure (4.10). this result agree with Jasem *et al.*,( 2010) who recorded 60.5% was positive for IgG in Iraq patients. It has been reported that detection of IgG antibodies is more sensitive than detection of IgM antibodies for diagnosing cases of brucellosis but specificity is comparable (Gómez *et al.*, 2008). A possible explanation for the absence of IgM antibodies in these patients could be due to the fact that excess of IgG can lead to false-negative IgM in some immunoassays (Sharma *et al*., 2008). Furthermore, Al Dahouk *et al.*, (2011) reported that 11% of the patients with acute brucellosis were negative for IgM antibodies.

## 4.5. Results of CDs:

### 4.5.1. CD3 Positivity Rate:

Results in table (4.10) and figure (4.12) revealed that the CD3 positivity rate among brucella patients was 32(58.2%) versus 10 (28.6%) among the control group with a statistically significant difference (P= 0.006).

**Table (4.10): Distribution of $CD_3$ positivity rate among study groups.**

| CD Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| $CD_3$ | Positive | 32 | 58.2 | 10 | 28.6 | 0.006* |
| | Negative | 23 | 41.8 | 25 | 71.4 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

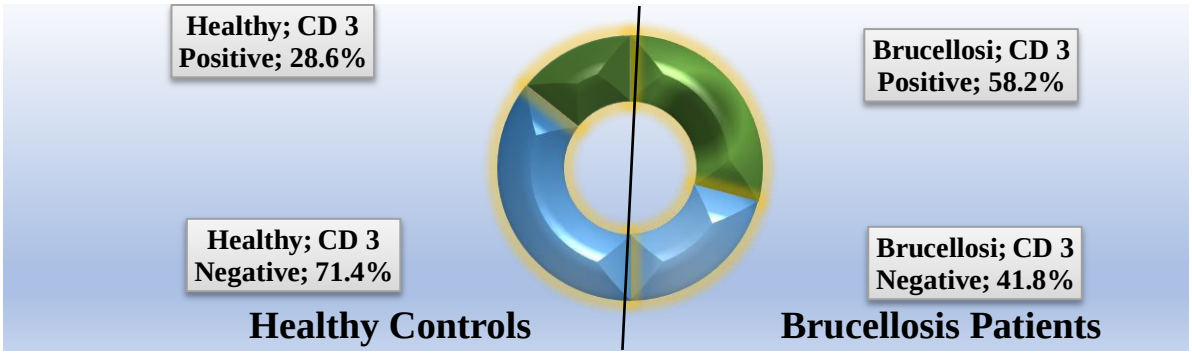



**Figure (4.12): $CD_3$ positivity rate among study groups.**

#### 4.5.1.1. $CD_3$ Concentration:

The results also found that although the CD3 concentration among the brucella patients was higher than that of control; however, the difference was failed to reach the levels of statistical significance (P= 0.080). Similarly, the Mean ± SD of CD3 concentration of patients was insignificantly higher than that of control (937.232 ± 700.197 *Vs* 699.968 ± 628.834), (P= 0.107). Data were illustrated in table (4.11) and figure (4.13).

**Table (4.11): Distribution of $CD_3$ concentration among study groups.**

| CD Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| **$CD_3$ Concentration (pg/ml)** | < 250 pg/ml | 1 | 1.8 | 2 | 5.7 | 0.080* |
| | 250--- | 11 | 20.0 | 12 | 34.3 | |
| | 500--- | 15 | 27.3 | 13 | 37.1 | |
| | 750--- | 12 | 21.8 | 4 | 11.4 | |
| | 1000--- | 8 | 14.5 | - | - | |
| | 1250--- | 3 | 5.5 | 3 | 8.6 | |
| | ≥ 1500 pg/ml | 5 | 9.1 | 1 | 2.9 | |
| Mean ± SD (Range) | | 937.232 ± 700.197 (210.657-4188.55) | | 699.968 ± 628.834 (169.755-3934.22) | | 0.107** |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Insignificant difference between two independent means using Students-t-test at 0.05 level.

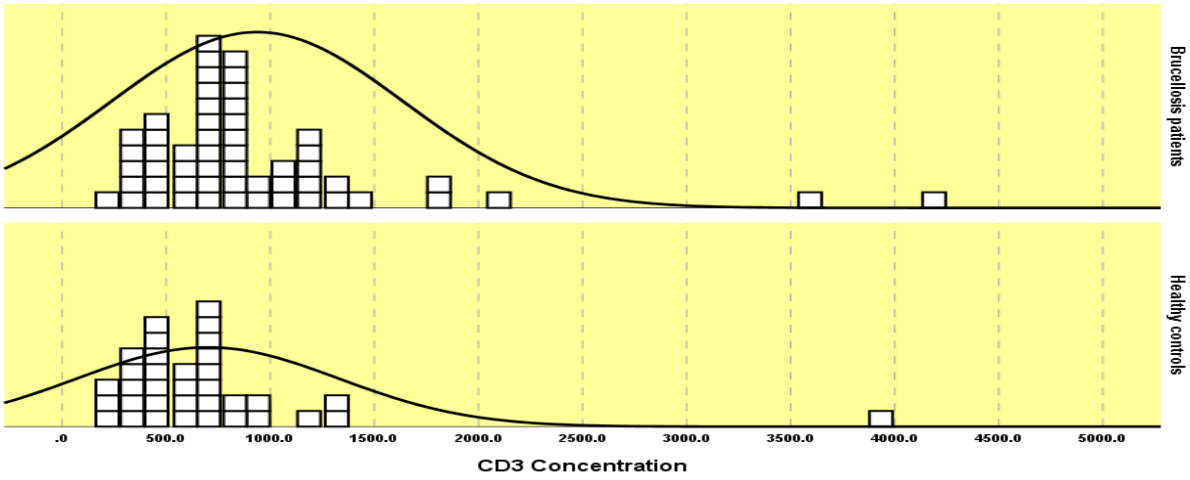



**Figure (4.13): $CD_3$ concentration among study groups.**

### 4.5.2. $CD_4$ Positivity Rate:

The results in table (4.12) and figure (4.14) revealed that the CD4 positivity rate among the patients was 25 (45.5%) while that of the controls was 15 (42.9%) with a statistically insignificant difference (P= 0.809).

**Table (4.12): Distribution of $CD_4$ positivity rate among study groups.**

| CD Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| $CD_4$ | Positive | 25 | 45.5 | 15 | 42.9 | 0.809* |
| | Negative | 30 | 54.5 | 20 | 57.1 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

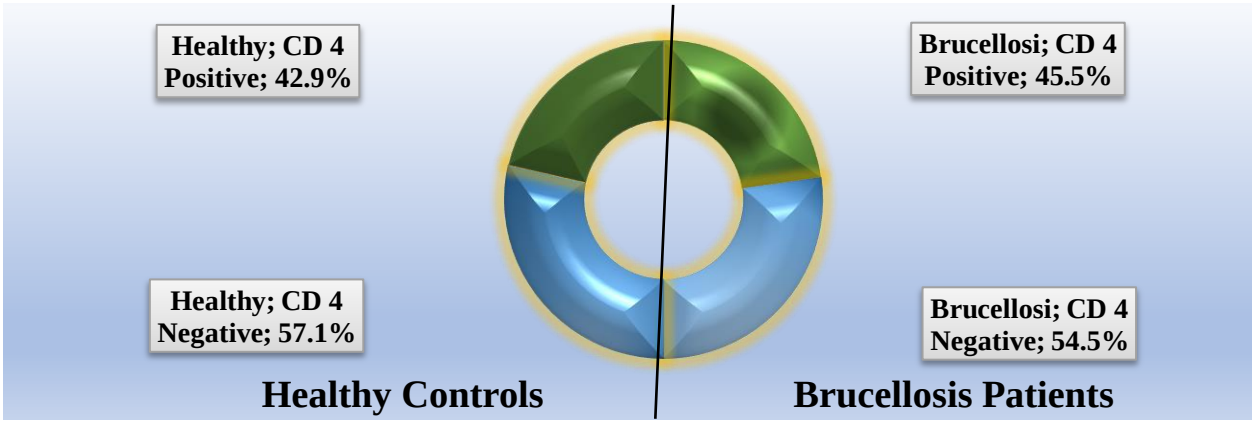



**Figure (4.14): $CD_4$ positivity rate among study groups.**

### 4.5.2.1. $CD_4$ Concentration:

The CD4 concentration among the patient group was insignificantly higher compared to that of the control group (P= 0.671). Likewise, the Mean ± SD of CD4 concentration of patients was insignificantly higher than that of control group (9.554 ± 4.129 *Vs* 8.945 ± 2.981), (P=0.452). Data were presented in table (413) and figure (4.15).

**Table (4.13): Distribution of $CD_4$ concentration among study groups.**

| CD Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| **$CD_4$ Concentration (pg/ml)** | < 4.0 pg/ml | 4 | 7.3 | 1 | 2.9 | 0.671* |
| | 4.0--- | 4 | 7.3 | 4 | 11.4 | |
| | 6.0--- | 8 | 14.5 | 9 | 25.7 | |
| | 8.0--- | 17 | 30.9 | 8 | 22.9 | |
| | 10.0--- | 13 | 23.6 | 8 | 22.9 | |
| | 12.0--- | 6 | 10.9 | 2 | 5.7 | |
| | ≥ 14.0 pg/ml | 3 | 5.5 | 3 | 8.6 | |
| Mean ± SD (Range) | | 9.554 ± 4.129 (2.577-26.659) | | 8.945± 2.981 (3.196-15.915) | | 0.452** |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

** Insignificant difference between two independent means using Students-t-test at 0.05 level.

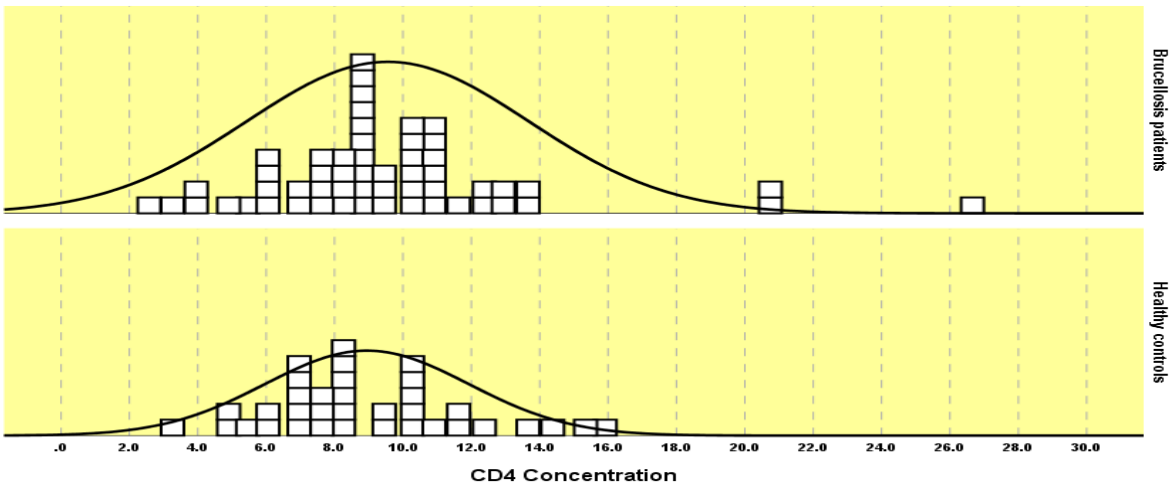



**Figure (4.15): $CD_4$ concentration among study groups.**

### 4.5.3. $CD_8$ Positivity Rate:

The positivity rate of CD8 among the patient group as revealed in table (4.14) and figure (4.16) was 53(96.4%) while that of the control group was 16(45.7%). The difference was statistically significant (P= 0.0001).

**Table (4.14): Distribution of $CD_8$ positivity rate among study groups.**

| CD Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| $CD_8$ | Positive | 53 | 96.4 | 16 | 45.7 | 0.0001* |
| | Negative | 2 | 3.6 | 19 | 54.3 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

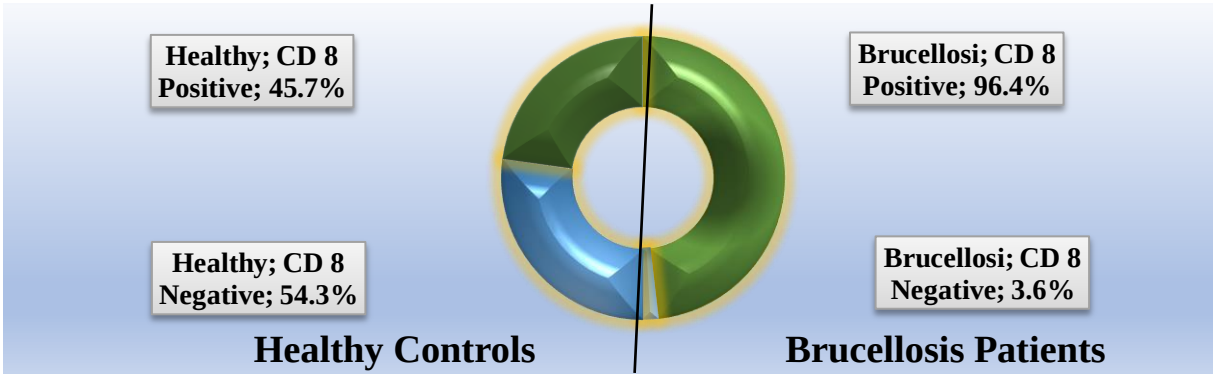



**Figure (4.16): $CD_8$ positivity rate among study groups.**

### 4.5.3.1.$CD_8$ Concentration:

Data concerning the CD8 concentration among study groups in table (4.15) and figure (4.17) showed that the concentration was significantly higher than that of the control (P= 0.0001). Likewise, the Mean ± SD of CD8 concentration of patients was significantly higher than that of control group (21.383 ± 7.489 *Vs* 12.093 ± 13.559), (P= 0.0001).

**Table (4.15): Distribution of $CD_8$ concentration among study groups.**

| CD Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| **$CD_8$ Concentration (pg/ml)** | <10 pg/ml | - | - | 18 | 51.4 | 0.0001* |
| | 10.0--- | 7 | 12.7 | 3 | 8.6 | |
| | 15.0--- | 19 | 34.5 | 3 | 8.6 | |
| | 20.0--- | 16 | 29.1 | 5 | 14.3 | |
| | 25.0--- | 9 | 16.4 | 3 | 8.6 | |
| | ≥ 30.0 pg/ml | 4 | 7.3 | 3 | 8.6 | |
| Mean ± SD (Range) | | 21.383 ± 7.489 (10.997-56.215) | | 12.093 ± 13.559 (0.040-48.335) | | 0.0001** |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Significant difference between two independent means using Students-t-test at 0.05 level.

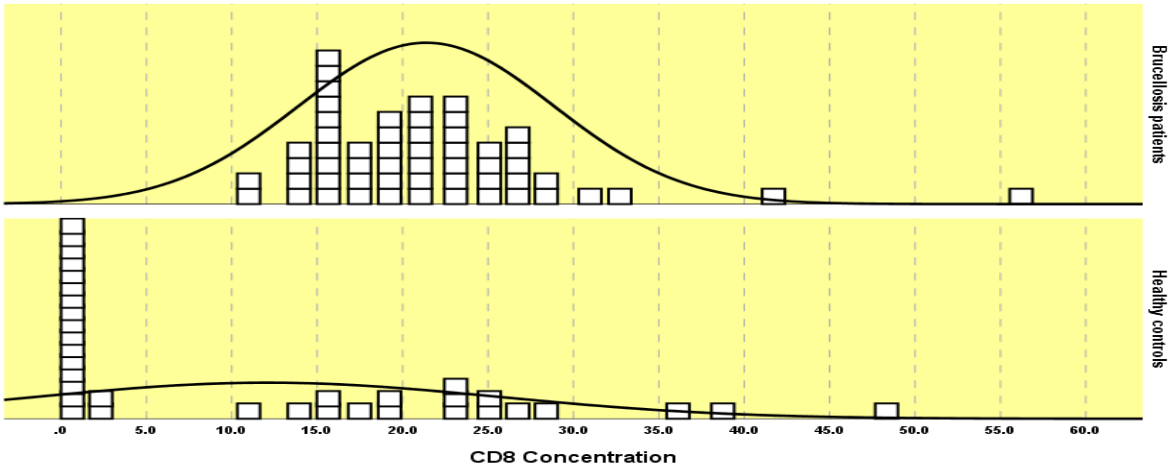



**Figure (4.17): $CD_8$ concentration among study groups.**

The CD3+ T cells represent mature lymphocytes, which are the main active cells in cellular immunity, and CD3+ T cells represent the overall level of cellular immunity. CD4+ T cell is an auxiliary T lymphocyte that assists other cells to participate in the immune response. The decrease of CD4+ T cells can lead to a series of dysfunction in TC, NK, macrophages, and B cells.

CD8+ T cells are the main effector cells that play specific cytotoxic effects, specifically killing target cells, mainly involved in the resistance to intracellular infection (Kato *et al.,* 2015), antitumor response (Nakajima *etal.*,2016) as well as participation in graft rejection (Cho *et al.*,2016). CD8+ T cells are not just a homogeneous cell group, some part of them are immunosuppressive cells that inhibit the function of other immune cells (Batliwalla *et al.,* 2000).

The results of CDs are agreed with Rodríguez-Zapata *et al.,* (2010) that found CD3, CD8 T lymphocytes that produced IL-2, IFN-γ, and TNF-α were significantly higher in untreated patients than in healthy controls. The percentages of CD3, CD4 and CD3, CD8 T cells expressing these cytokines in patients decreased after treatment but remained significantly higher than those found in healthy controls.

Two studies reported that the proportions of CD3+ T cells in human brucellosis patients were significantly increased compared to control individuals (Gao *et al.*, 2015; Celik and Akbulut.,2005) , while other 4 studies (Rodriguez-Zapata *et al.*,2010 ; Skendros *et al.,* 2007; Pourfathollah *et al.,* 1996; Akbulut *et al.,* 2005 ) showed that there was no significant difference between the two groups. Furthermore, another study showed significant decrease in the percentage of CD4+ was observed in chronic brucellosis (Skendros *et al.*,2008).

The analysis of the T-lymphocyte sub-groups showed a significant reduction in CD4+ T cell frequency in relapsed brucellosis patients compared with the acute brucellosis group ($p=0.028$), plus a significant increase in CD8+ T cell frequency was observed in acute and relapsed brucellosis compared with the healthy group ($p<0.05$). Furthermore, the CD4/CD8 ratio significantly increased toward to CD8+ T cells only in the relapsed brucellosis

group compared with the healthy individuals (p<0.001) (Kayhan *et al.*,2016), these result are consistent with the results of the current study.

There are several results of studies that disagree with the result of the current study, as these studies showed that proportions of $CD4^+$ T cells in human brucellosis patients were significantly decreased compared to control individuals (Akbulut *et al.*, 2005; Çelik and Akbulut, 2005; Rodríguez-Zapata *et al.*,2010; Gao *et al.*,2015; Gu *et al.*,2016 ).Additionally there were other two studies showed that there was insignificant difference between the two groups (Rodriguez-Zapata *et al.*,1996; Skendros *et al.*,2007).

## 4.6. Results of Interleukins:

### 4.6.1. Interleukin-17:

#### 4.6.1.1. Interleukin-17 Positivity Rate:

As shown in table (4.16) and figure (4.18), the IL-17 positivity rate among brucella patients was 32(58.2%) while that of the control group was 15 (42.9%). The difference was statistically insignificant (P= 0.156).

**Table (4.16): Distribution of Interleukin-17 positivity rate among study groups.**

| Interleukins Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| IL-17 | Positive | 32 | 58.2 | 15 | 42.9 | 0.156* |
| | Negative | 23 | 41.8 | 20 | 57.1 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

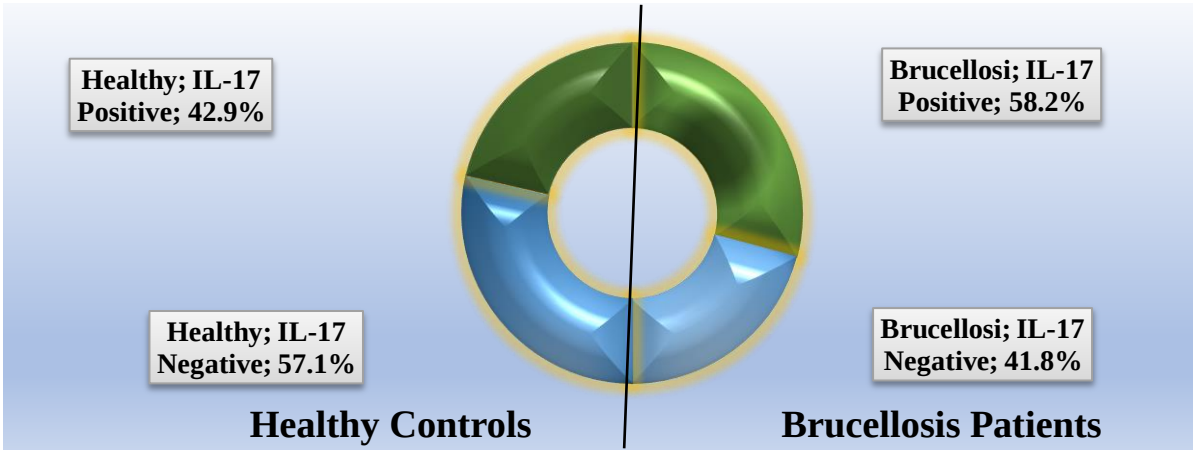



**Figure (4.18): Interleukin-17 positivity rate among study groups.**

### 4.6.1.2. Interleukin-17 Concentration:

Furthermore, the results found that the IL-17 concentration among the patient group was insignificantly higher than that of the control group (P= 0.346). Additionally, the Mean ± SD of IL-17 concentration among the patients (71.511 ± 175.567) was higher than that of controls (16.843 ± 20.061). However, the difference was failed to reach the levels of statistical significance (P= 0.071). Table (4.17) and figure (4.19).

**Table (4.17): Distribution of Interleukin-17 concentration among study groups.**

| Interleukins Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| IL-17 Concentration (pg/ml) | <10.0 pg/ml | 16 | 29.1 | 18 | 51.4 | 0.346* |
| | 10.0--- | 10 | 18.2 | 4 | 11.4 | |
| | 20.0--- | 9 | 16.4 | 6 | 17.1 | |
| | 30.0--- | 7 | 12.7 | 3 | 8.6 | |
| | 40.0--- | 5 | 9.1 | 2 | 5.7 | |
| | ≥ 50.0 pg/ml | 8 | 14.5 | 2 | 5.7 | |

| | | | |
|---|---|---|---|
| Mean ± SD (Range) | 71.511 ± 175.567 (0.375-875.792) | 16.843 ± 20.061 (0.375-79.542) | 0.071** |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Insignificant difference between two independent means using Students-t-test at 0.05 level.

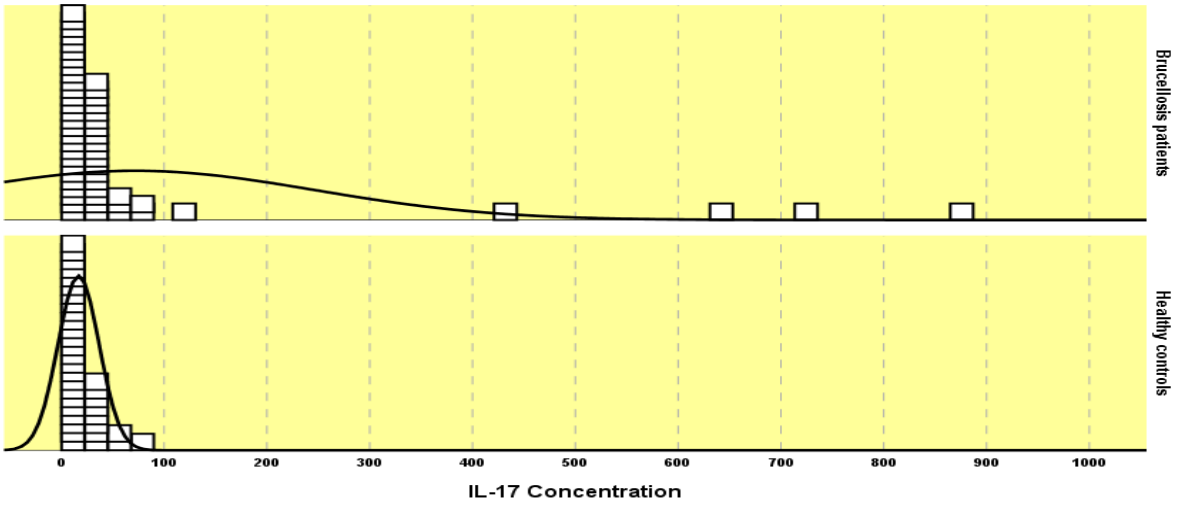



**Figure (4.19): Interleukin-17 concentration among study groups.**

The IL-17 cytokine played a principal role in both innate and adaptive immunity. So through its existence in the lamina propria and oral cavity mucosa, it play an essential role against infection through gastrointestinal tract (Marks and Craft, 2009). Therefore, IL-17 is a critical determinants of risk, severity or protection against brucellosis although its level decreased in patients at the end of treatment (Park *et al.*, 2005; Sofian *et al.*, 2016). The present result is consistent with that of Ghaznavi-Rad *et al.*, (2017) who reported insignificant increase in IL-17 suggesting that the cytokine production varies in the different stages of brucellosis.

Zheng *et al.* (2019) found that there were insignificant differences in the percentage of Th17 lymphocyte expression between convalescent patients and

healthy controls (P >0. 05). Also the percentage of CD4+ IL-17A (Th17) lymphocytes had insignificant differences between acute and chronic patients (P >0. 05), but the expression of CD4+ IL-17A was higher than that of healthy controls (P <0. 05). However, the current study inconsistent with that showed that the IL-17 level was significantly higher in the acute brucellosis group compared to controls (P < 0.01) and its value decreased in patients at the end of treatment without any significant difference (Sofian *et al.*,2016). Similarly, Kayhan *et al.* (2016) found that acute brucellosis induced a significant increase in IFN-γ and IL-17 levels compared with healthy individuals. Interestingly, IFN- γ and IL-17 levels in relapsed brucellosis patients were significantly lower than in acute brucellosis patients.

### 4.6.2. Interleukin-18:

#### 4.6.2.1. Interleukin-18 Positivity Rate:

Data presented in table (4.18) and figure (4.20) revealed that the IL-18 positivity rate among patients was 27(49.1%) which is higher than that of controls 11(31.4%). However, the difference was failed to reach the level of statistical significant (P= 0.098).

**Table (4.18): Distribution of Interleukin-18 positivity rate among study groups.**

| Interleukins Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| IL-18 | Positive | 27 | 49.1 | 11 | 31.4 | 0.098* |
| | Negative | 28 | 50.9 | 24 | 68.6 | |

*inSignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

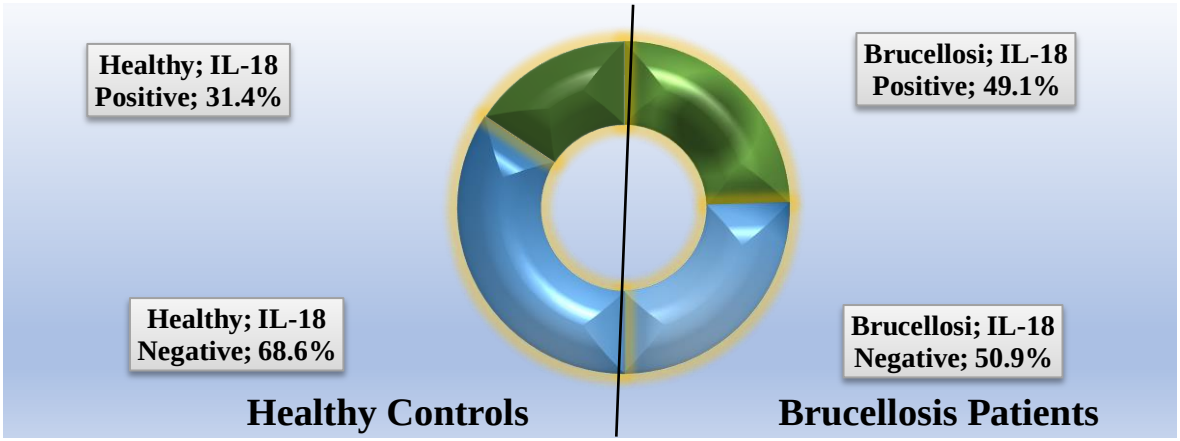



**Figure (4.20): Interleukin-18 positivity rate among study groups.**

### 4.6.2.2. Interleukin-18 Concentration:

Although the IL-18 concentration was higher than that of the control group; however, the difference was failed to reach the levels of statistical significant (P= 0.381). On the other hand, the mean ± SD of IL-18 concentration among the patients (0.841 ± 0.247) was higher than that of controls (0.778 ± 0.219 ). However the difference was failed to reach the levels of statistical significance (P= 0.221). Table (4.19) and figure (4.21).

**Table (4.19): Distribution of Interleukin-18 concentration among study groups.**

| Interleukins Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| IL-18 Concentration (pg/ml) | <0.5 pg/ml | 4 | 7.3 | - | - | 0.381* |
| | 0.5--- | 5 | 9.1 | 7 | 20.0 | |
| | 0.6--- | 7 | 12.7 | 7 | 20.0 | |
| | 0.7--- | 11 | 20.0 | 8 | 22.9 | |
| | 0.8--- | 7 | 12.7 | 4 | 11.4 | |
| | 0.9--- | 8 | 14.5 | 4 | 11.4 | |
| | ≥ 1.0 pg/ml | 13 | 23.6 | 5 | 14.3 | |
| Mean ± SD (Range) | | 0.841 ± 0.247 | | 0.778 ± 0.219 | | 0.221** |

| | | | |
|---|---|---|---|
| | (0.392-1.392) | (0.524-1.484) | |

*insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**insignificant difference between two independent means using Students-t-test at 0.05 level.

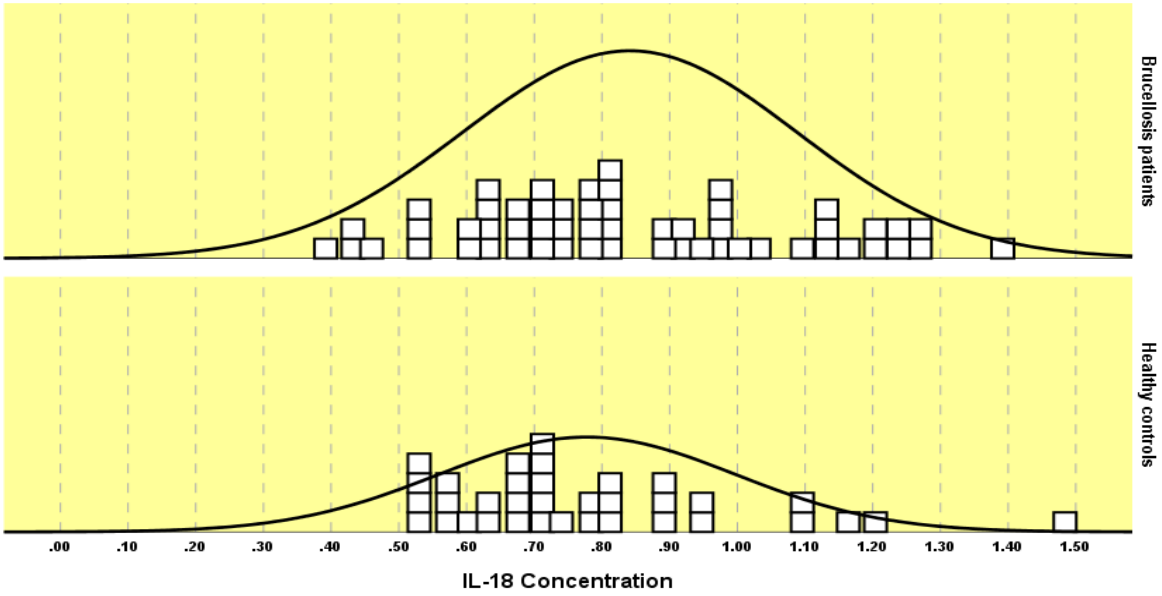



**Figure (4.21): Interleukin-18 concentration among study groups.**

Interleukine-18 plays a protective role against brucellosis and some other infectious diseases (Netea *et al.,* 2000; Pasquali *et al.*, 2001). The result of current study is inconcordant with Kayhan *et al* .(2016) who recorded that acute brucellosis induced significant elevation of IL-18 level in serum samples in comparison to healthy individuals. Moreover, the elevation reached to 3.2 times in acute brucellosis group in comparison to relapse group ($p<0.001$).

On the other hand, another results demonstrate that *B. abortus* infection induces a reduction of IL-18 secretion in mice at the macrophage level and once the infection was established, *B. abortus* selectively limits IL-18 secretion without affecting endogenous IFN- γ production (Fernandez-Lago *et al*., 2005). In another study, the IFN-γ, and IL-18 levels were investigated in

the serum samples of patient and healthy control, it appears that there was significantly increase in patients with acute brucellosis (Karaca *et al.*, 2019).

### 4.6.3. Interferon Gamma:

#### 4.6.3.1. Interferon Gamma Positivity Rate:

The positivity rate of **INF-γ** in patient group was 52(94.5%) which is highly significant compared to that of control group 12(34.3%), (P= 0.0001) as shown in table (4.20) and figure (4.22).

**Table (4.20): Distribution of INF-γ positivity rate among study groups.**

| Interleukins Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| INF-γ | Positive | 52 | 94.5 | 12 | 34.3 | 0.0001* |
| | Negative | 3 | 5.5 | 23 | 65.7 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

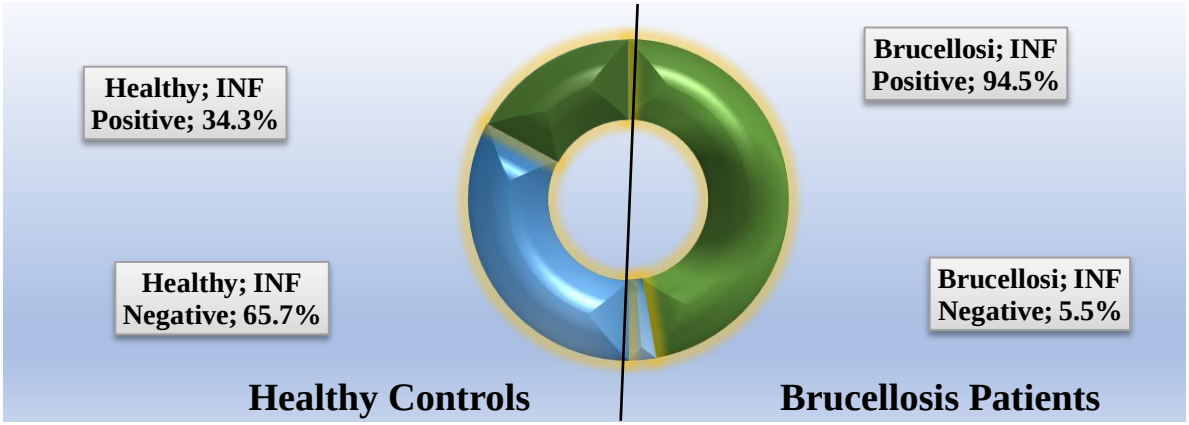



**Figure (4.22): INF-γ positivity rate among study groups.**

#### 4.6.3.2. Interferon Gamma Concentration:

Similarly, the INF-γ concentration was significantly higher among patient group compared to control group (P= 0.0001). Furthermore, the mean ± SD of INF-γ concentration among the patients (28.277 ± 8.660) was

significantly higher than that of controls (18.648 ± 9.886), (P=0.0001) as illustrated in table (4.21) and figure (4.23).

**Table (4.21): Distribution of INF-γ concentration among study groups.**

| Interleukins Marker | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| INF-γ (pg/ml) | <15.0 pg/ml | - | - | 10 | 28.6 | 0.0001* |
| | 15.0--- | 9 | 16.4 | 18 | 51.4 | |
| | 20.0--- | 12 | 21.8 | 6 | 17.1 | |
| | 25.0--- | 15 | 27.3 | - | - | |
| | 30.0--- | 7 | 12.7 | - | - | |
| | 35.0--- | 6 | 10.9 | - | - | |
| | ≥ 40.0 pg/ml | 6 | 10.9 | 1 | 2.9 | |
| Mean ± SD (Range) | | 28.277 ± 8.660 (17.457-64.373) | | 18.648 ± 9.886 (13.093-73.538) | | 0.0001** |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Significant difference between two independent means using Students-t-test at 0.05 level.

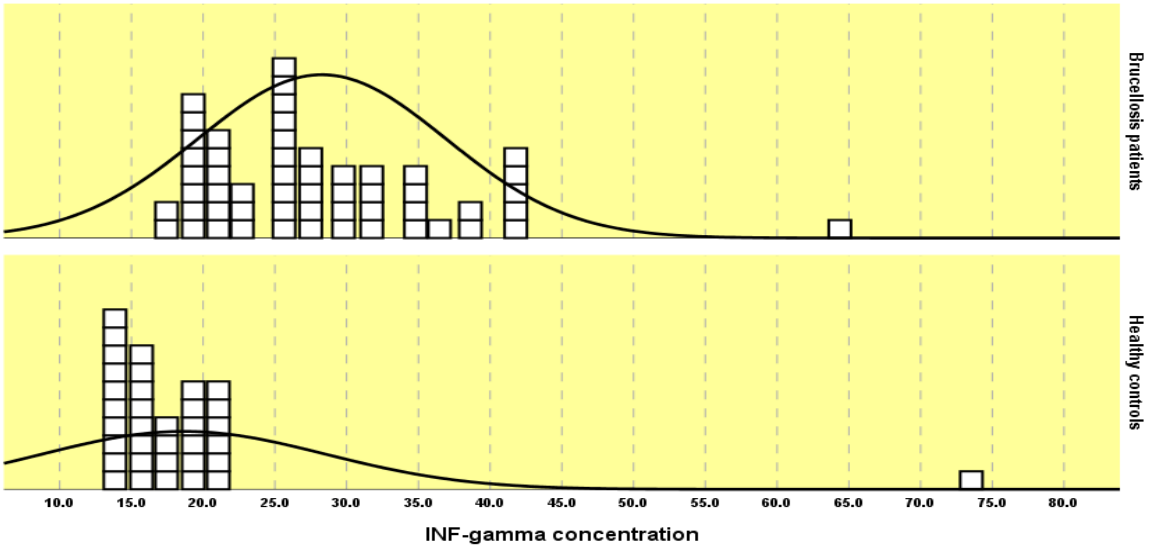



**Figure (4.23):** IFN-γ **concentration among study groups.**

The Interferon- γ is a critical cytokine for host control of brucella and a key mediator in conferring protection against brucella infection (Fernandez-Lago *et al.,* 2005). The current results regarding the IFN-γ are similar to two other studies; Giambartolomei *et al.* (2002) and Celli (2006) who found that IL-17, IFN-γ, and TNF-α levels were higher in patients with brucellosis than healthy controls, indicating a strong immune response was occurring. Indeed, previous studies have shown brucella is effectively cleared through the secretion of IFN-γ, IL-2, and TNF-α by T helper (Th) 1 cells ( Fernandez-Lago *et al*., 2005).

High levels of IFN-γ, have been reported in patients with acute or sub-acute brucellosis (Akbulut *et al.,* 2007). Rodríguez-Zapata *et al.* (2010), recorded a significantly higher levels of the IFN-γ, in serum and/or significantly higher percentages of samples with detectable levels than those for healthy controls, especially striking is the increased presence of high levels of IFN-γ in untreated patients. After treatment, significant reductions and normalization of the percentages of patients with detectable levels of IFN-γ in serum were found. These results are clearly consistent with the results of this study.

Additionally, the current results are agreeing with Han *et al.* (2017), who reported that there was a high levels of the cytokine interferon (IFN-γ) may be a characteristic of brucellosis chronicity. In another study observed that mean serum IFN-γ and TNF-α levels in acute brucellosis cases significantly decreased at the end of the treatment and that there was no significant difference between the post-treatment and the control group values (Demirdag *et al.,*2003).

In chronic brucellosis, detection of lower CD3+IFN-γ+ levels prior to antibiotic treatment, indicates a defective Th1 response in those patients. This

defect causes an imbalance between Th1 and T helper-2 (Th2) T cell responses. It is estimated that the diminished production of Th1 cytokines in these patients occurs because of the Th2 switch and/or the release of immune-regulatory cytokines, such as TGF-b1 (Skendros and Boura, 2013; Akbulut *et al.,* 2005; Rodríguez-Zapata *et al.*, 2010).

The IFN-γ as a key cytokine produced by Th1 cells induces immune response toward Th1 phenotype and this cytokine effectively increases the microbicidal activity of macrophages (Schordr *et al.*, 2004). Although, brucella evasion mechanisms have been implicated to interfere with protective effects of IFN-γ. Demirdag *et al*. (2003), and Akbulut *et al*. (2007), studies determined the serum levels of Th1 cytokines in acute and sub-acute brucellosis and healthy controls. They found that the serum IFN-γ levels in acute patients were significantly higher than sub-acute brucellosis and healthy subjects. Nevertheless, in this study, the anti-brucella IgM as a marker of acuteness as well as the anti-brucella IgG as a marker of chronicity, both are insignificantly associated with IFN-γ levels. In addition, they reported that the increased serum IFN-γ levels in acute patients were significantly decreased after effective treatment. Consistently, Rahmanpour *et al.* (2019), found that serum levels of IFN-γ were elevated in acute brucellosis as well as in the relapsed patients compared to under-treatment groups and healthy controls. Therefore, it seems that the levels of IFN-γ are varied according to the disease stages, previous treatment and probably the geographical areas.

Brucellosis as an intracellular latent infection, this study suggests a further studies on the usefulness of the interferon-gamma release assay in the differentiation of acute and latent brucellosis in Iraqi endemic community, as this technique approved efficacious in the diagnosis of latent tuberculosis (Tagmouti *et al*., 2014).

## 4.7. Gene Detection by PCR:

Results presented in table (4.22) and figure (4.24,4.25,26) and (4.27,4.28,4.29) revealed the PCR detection rate of the three genes included in this study, other figures (4.30, 4.31, 4.32, 4.33, 4.34, 4.35, 4.36) were presented in (Appendix II). The detection rate of IL-17 gene among the brucella patients was 33(60%) which is significantly higher compared to that of the control group 7(20%), (P= 0.0001). The detection rate of IL-18 gene among the patients was 40(72.7%) which is also highly significant compared to that of the controls 10(28.6%), (P= 0.0001). Additionally, the **INF-γ** gene detection rate among the patients was 39(70.9%) which is highly significant as compared to its counterpart in controls 11(31.4%).

**Table (4.22): Distribution of PCR detection rate among study groups.**

| **Molecular genes (PCR)** | | Patients | | Control | | P value |
|---|---|---|---|---|---|---|
| | | No. | % | No. | % | |
| *IL-17* | Gene detected | 33 | 60.0 | 7 | 20.0 | 0.0001* |
| | Gene not detected | 18 | 32.7 | 4 | 11.4 | |
| | Not done | 4 | 7.3 | 24 | 68.6 | |
| *IL-18* | Gene detected | 40 | 72.7 | 10 | 28.6 | 0.0001* |
| | Gene not detected | 11 | 20.0 | 2 | 5.7 | |
| | Not done | 4 | 7.3 | 23 | 65.7 | |
| *INF-γ* | Gene detected | 39 | 70.9 | 11 | 31.4 | 0.0001* |
| | Gene not detected | 12 | 21.8 | 2 | 5.7 | |
| | Not done | 4 | 7.3 | 22 | 62.9 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

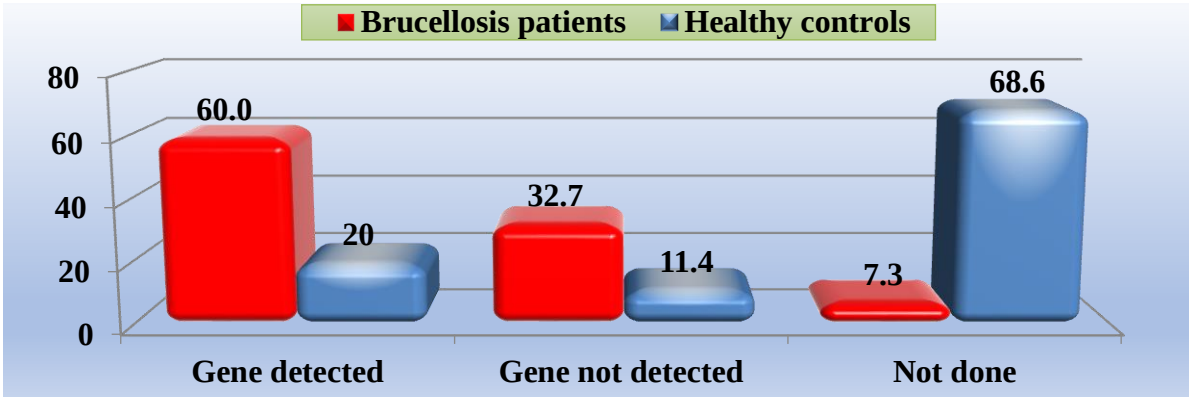



**Figure (4.24): Interleukin-17 detection rate among study groups.**

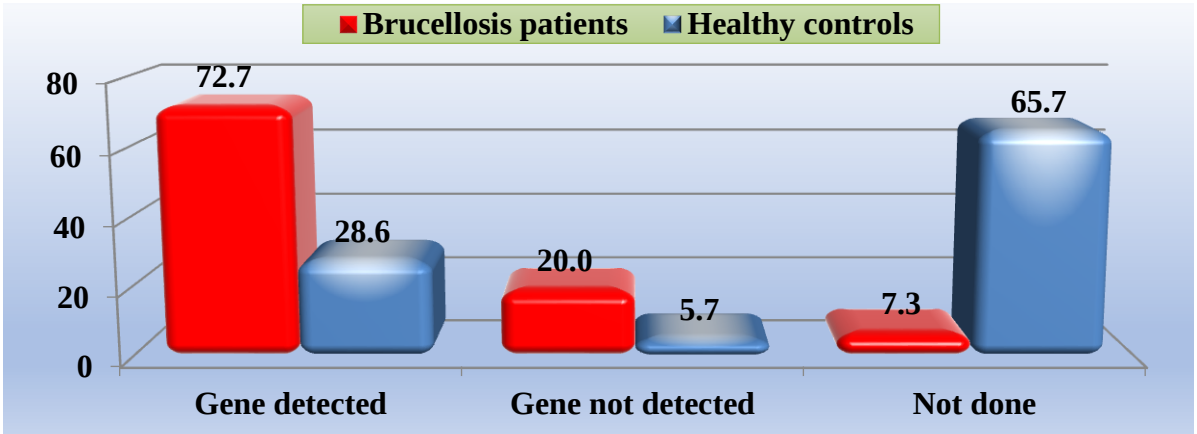



**Figure (4.25): Interleukin-18 detection rate among study groups.**

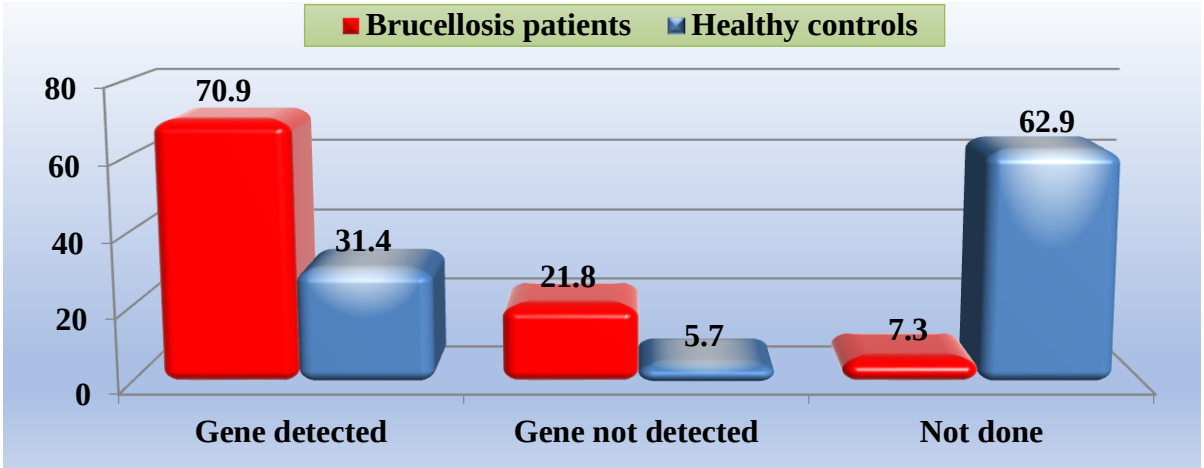



**Figure (4.26): INF-γ detection rate among study groups.**

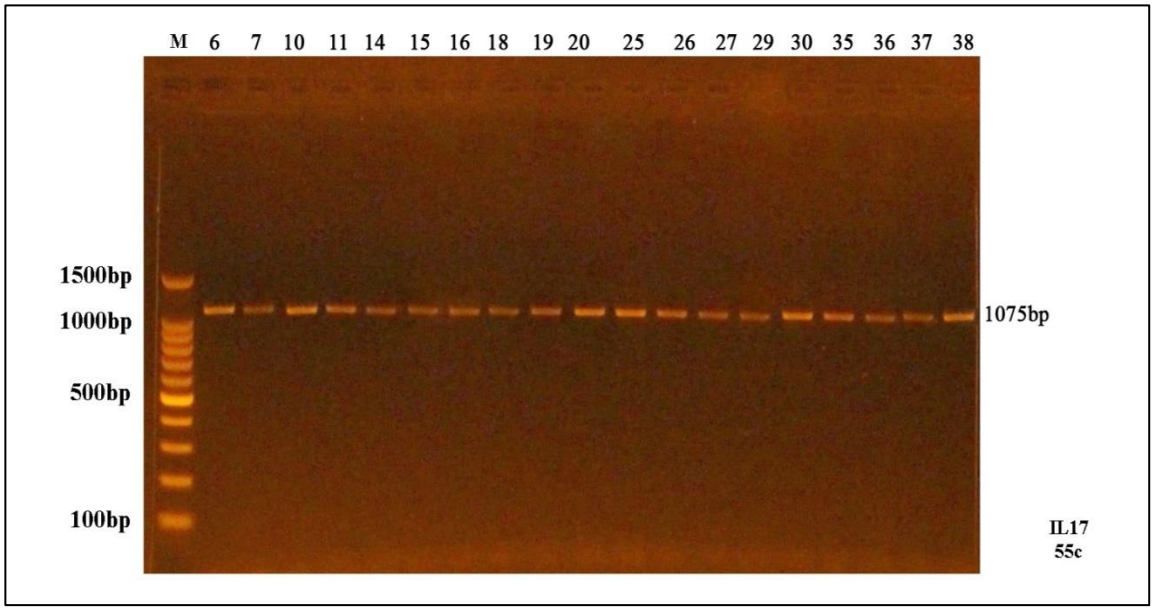
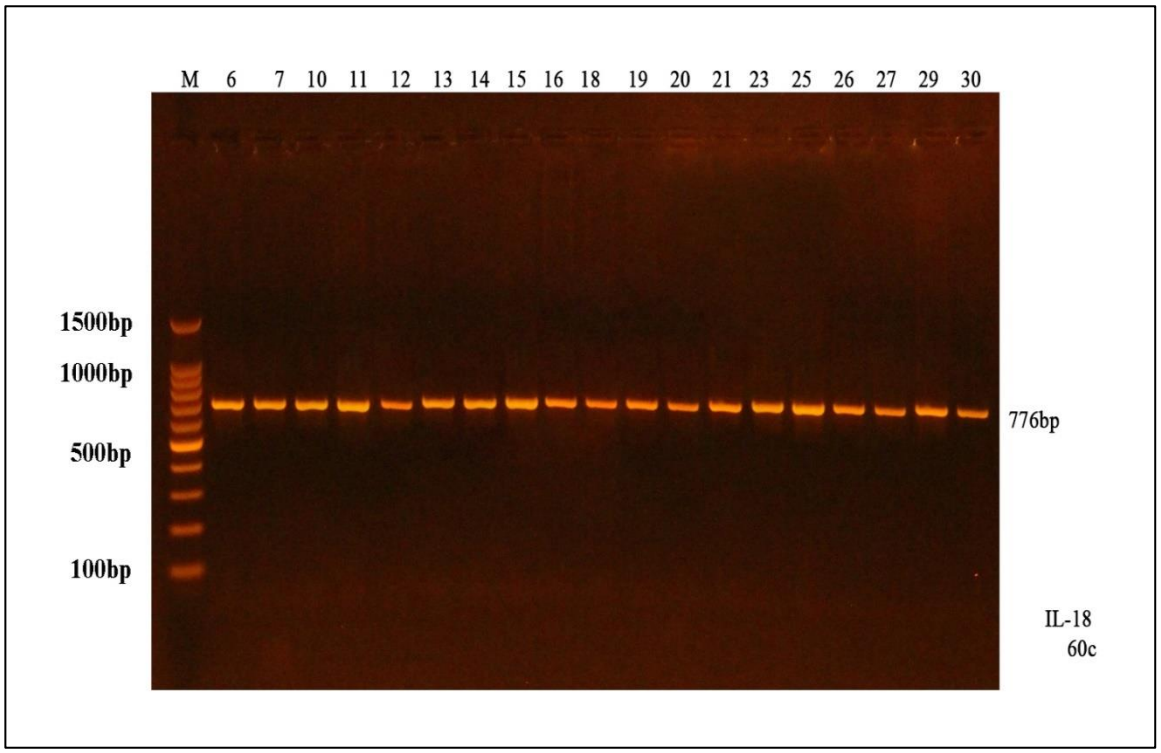
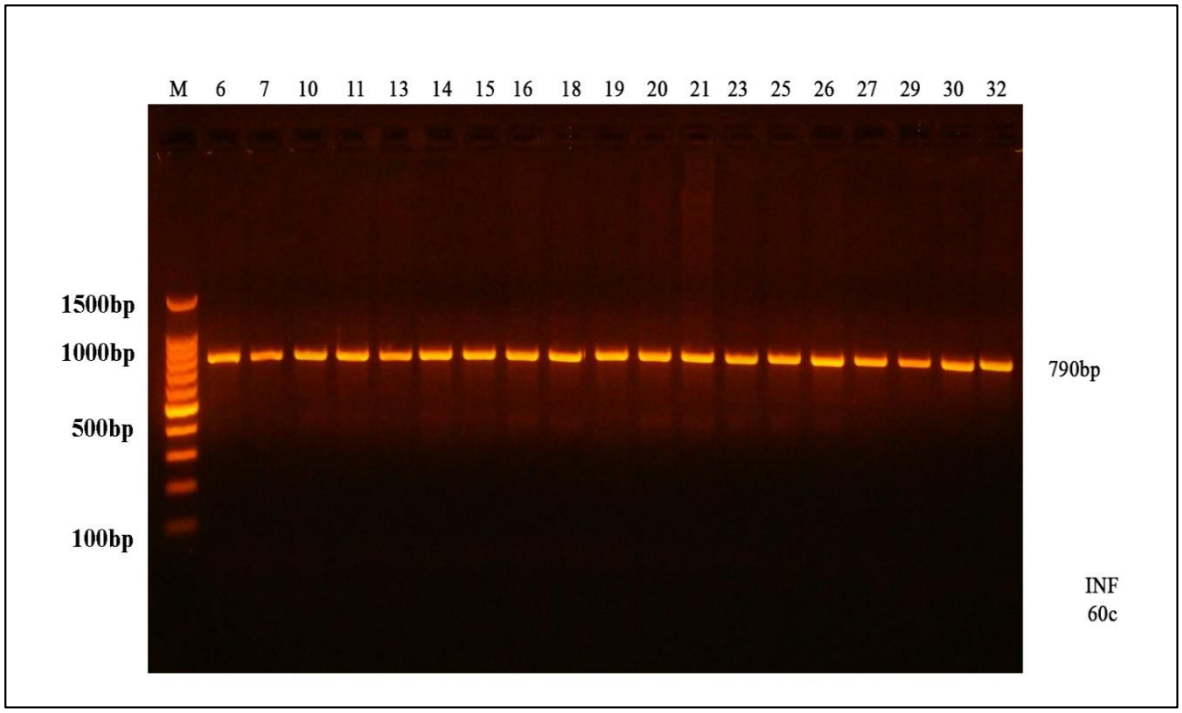



**Figure (4.27): Electrophoresis bands of IL-17 gene detection in this study.**




**Figure(4.28): Electrophoresis bands of IL-18 gene detection in this study.**




**Figure(4.29): Electrophoresis bands of INF-γ gene detection in this study.**

The study focused on specific sequences for each *IL-17*, *1L-18*, *INF-γ* genes. The *IL-17* coding gene is located on chromosome 6p12 and consists of three exons (Chen *et al.,* 2006). It was worthy to mention that the genetic variation in *IL-17* gene has been studied in various diseases (Zhang *et al*., 2008; Sadighi *et al*., 2014). Several researchers focused on studying the relation of the sequencing in the three genes and many illnesses such as brucellosis. IFN-γ gene detection targeted the +874 position which was previously employed in brucellosis (Rasouli and Kiany, 2007). The *IL-18* plays a protective role against brucellosis (Pasquali *et al.,* 2002).

Polymorphisms in the genes of cytokines can increase or decrease their expression and affect the determination of acute or chronic brucellosis (Hollegaard and Bidwell, 2006). It has been demonstrated that gene polymorphism can influence the expression of cytokine and play a crucial role in infectious diseases (Bidwell *et al.,* 2001; Sepanjnia *et al*., 2015). The results of molecular technique in the current study found that the *IL-17* gene detection

rate was significantly higher among patients with brucellosis compared to controls. The results also found that the *IL-17* gene detection rate was insignificantly associated with anti-brucella IgM and IgG antibodies among brucellosis patient, although, it become lowered during the chronic brucellosis. Similar results were reported by other workers (Vitry *et al*., 2012; Corestti *et al.*,2013).

The *IL-18* gene, the current results found that the detection rate was significantly higher in patients with brucellosis compared to control. Interleukine-18 gene is placed on the long arm of chromosome 11 and six single nucleotide polymorphisms (SNPs) have been recognized in the potentially functional region of its gene, such as the coding sequence, Exon–intron junctions, promoters and untranslated regions (UTRs). Taking into account the involvement of these polymorphisms in altering IL-18 level (Folwaczny *et al.*,2005).

The INF- γ gene detection rate was found to be significantly associated with patients with brucellosis and higher among those in the acute phase, but it re-decreases during the chronic phase. Pooled analysis suggested that the IFN-γ +874 AT vs AA as well as the TNF-α −308 AA vs GG + GA genotypes demonstrated a trend for the association with a higher risk of brucellosis (Eskandari- Nasab and Moghadampour, 2018).

### 4.8. Association of anti-Brucella IgM with Variables:

#### 4.8.1. Sociodemographic Variables:

data in table (4.23) showed that among 55 patients, the higher anti-brucella IgM positivity rate was among those less than 20 years old, but the difference was statistically insignificant (P= 0.278). Regarding the gender, the results showed that the anti-brucella IgM positivity rate was insignificantly higher in female compared to male (P= 0.666). Similarly, those reside in

villages had insignificantly higher rate of anti-brucella IgM compared to those reside in cities (P= 0.637). The anti-brucella IgM positivity rate was insignificantly higher among those breeds home animals compared to those did not (P= 0.786). Concerning the consumption of animal products (dairy or meat products), the results revealed that all participants 18(36%) with positive anti-brucella IgM were consumed animal products versus 32(64%) who were negative for anti-brucella IgM, the difference was statistically insignificant (P= 0.102). Regarding the presence of intrafamilial cases beside the index case, the results found that 20.8% of those with positive anti-brucella IgM had intrafamilial cases against 79.2% of those with intrafamilial cases but they are anti-brucella IgM negative. However, the difference was failed to reach the level of statistical insignificance (P= 0.098).

**Table (4.23): Association of anti-brucella IgM with sociodemographic variables.**

| sociodemographic variables | | Anti-brucella IgM | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 4 | 66.7 | 2 | 33.3 | 0.278* |
| | 20---29 | 8 | 32.0 | 17 | 68.0 | |
| | 30---39 | 4 | 23.5 | 13 | 76.5 | |
| | ≥ 40 years | 2 | 28.6 | 5 | 71.4 | |
| Sex | Male | 3 | 27.3 | 8 | 72.7 | 0.666* |
| | Female | 15 | 34.1 | 29 | 65.9 | |
| Residence | City | 9 | 30.0 | 21 | 70.0 | 0.637* |
| | Village | 9 | 36.0 | 16 | 64.0 | |
| Home animals | Yes | 7 | 35.0 | 13 | 65.0 | 0.786* |
| | No | 11 | 31.4 | 24 | 68.6 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Consumption of animal products** | Yes | 18 | 36.0 | 32 | 64.0 | 0.102* |
| | No | - | - | 5 | 100.0 | |
| **Intrafamilial cases** | Yes | 5 | 20.8 | 19 | 79.2 | 0.098* |
| | No | 13 | 41.9 | 18 | 58.1 | |

*insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The anti-brucella IgM antibody, as a marker of acuteness, showed the highest positivity rate among those less than 20 years old compared to other age groups. It is worth to mention that different studies have recorded different results, at least, in Middle East countries (Ruiz-Mesa et *al*., 2005; Bagheri *et al*., 2020). The most acceptable explanation is that this age group had incomplete protective antibodies probably because they had no previous exposure to *Brucellae* (Elfaki *et al*., 2015).

The results also showed that there are insignificant association of IgM marker with gender, residence, presence of home reproductive animals, consumption of dairy products as well as intrafamilial clustering of cases. Actually, these results required further studies to affirm or denied them.

### 4.8.2. Clinical Variables:

The results in table (4.24) showed that the anti-brucella IgM positivity rate was 15(34.1%) among those with new infection (first time diagnosed as brucellosis), while 29(65.9%) with new infection, but they had IgM negative. On the other hand, 3(27.3%) of those who had old infection (previously diagnosed as brucellosis) were IgM positive against 8(72.7%) who had old infection with negative anti-brucella IgM. The difference was statistically insignificant (P= 0.666). Regarding the presence of fever, 16(32.7%) of those with fever had anti-brucella IgM positive versus 2(33.3%) who were positive for IgM but without fever. On the other side, 33(67.3%) had fever although

they were negative for IgM against 4(66.7%) who were negative for IgM with no fever. The difference was statistically insignificant (P= 0.907).

The results also found that the night sweat was found in 15(34.1%) who were anti-brucella IgM positive versus 3(27.3%) who were positive for IgM but without night sweat. On the contrary, 29 (65.9%) had night sweat but they were negative for anti-brucella IgM. The difference was statistically insignificant (P= 0.666). Additionally, 14 (29.8%) of those positive for IgM had joint pain versus 33(70.2%) who had joint pain but negative to anti-brucella IgM. The difference was statistically insignificant (P= 0.260).

**Table (4.24): Association of anti-brucella IgM with clinical variables.**

| Clinical variables | | Anti-brucella IgM | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 15 | 34.1 | 29 | 65.9 | 0.666* |
| | Old infection | 3 | 27.3 | 8 | 72.7 | |
| Fever | Yes | 16 | 32.7 | 33 | 67.3 | 0.973* |
| | No | 2 | 33.3 | 4 | 66.7 | |
| Night sweat | Yes | 15 | 34.1 | 29 | 65.9 | 0.666* |
| | No | 3 | 27.3 | 8 | 72.7 | |
| Joint pain | Yes | 14 | 29.8 | 33 | 70.2 | 0.260* |
| | No | 4 | 50.0 | 4 | 50.0 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

These results indicate that the anti-brucella IgM was higher among those with new infection, presence of fever and night sweat, but these associations were failed to reach the levels of statistical significance in the present study. However, it is worthy to mention that arthralgia, fever, sweating, chills, headache, myalgia, malaise are among the main clinical

features of acute brucellosis according to case definition of brucellosis (Dean *et al*., 2012; Shi *et al*., 2018). Nevertheless, the osteoarthricular involvement is more prominent during chronic brucellosis (Tasova *et al*., 1999; Bodur *et al*., 2004). It seems that certain factors may affect the clinical presentation of brucellosis over different epidemiological areas (Hizel *et al*., 2007; Dean *et al*., 2012; Rubach *et al*., 2013). Furthermore, other study related factors including sample size, target population, the reliability of clinical data, manufacturer origin of laboratory kits may have a role in the correlation of serological test with clinical presentation (Al Dahouk *et al*.., 2003; Purwar *et al*., 2016). However, other workers denied correlation of brucella serological test with clinical outcomes nor with culture positivity at baseline. Suggesting considering the whole clinical picture of a brucellosis patient without relying solely on serological results during follow up and not replace culturing with serology testing alone at the time of diagnosis (Alsubaie *et al*., 2021). Keeping in the same connection anther idea was proposed by Al Jindan *et al*., (2019) who concluded that determination of single IgM or IgG anti-Brucella-antibodies by ELISA could both be considered as definite and should ideally be interpreted in the context of appropriate clinical scenario and confirmation by other laboratory assays.

It remains that 37 (67.2%) patients with brucellosis (29 new and 8 old infection) as indicated by strong clinical picture plus positive Rose Bengal test appeared as negative by anti-brucella IgM negative (table 4.24). The lack of seropositivity in patients with strongly suspected clinical picture may be attributed to the performance of tests early in the course of infection, the presence of blocking antibodies (non-agglutinating, incomplete) or the so-called "prezone" phenomenon. Enzyme immunoassays (ELISA) which measure specific IgM, IgG and IgA antibodies, allow for a better

interpretation of the clinical situation and overcome the false negativities/ positivities which may arise in Rose Bengal test or other rapid agglutination tests (Aliskan,2008).

Jindan *et al*. (2019), reported that all the double-positive (IgG- and IgM-positive) serology patterns were diagnosed as having brucellosis, suggesting that determination of single IgM or IgG anti-Brucella-antibodies by ELISA could both be considered as definite and should ideally be interpreted in the context of appropriate clinical scenario and confirmation by other laboratory assays. Several other studies had compared among serological cultural and molecular accuracy in the diagnosis of brucellosis in human and animals and had propose that the simultaneous use of serology and molecular techniques had the potential to overcome limitations of detection thereby enabling the selection of appropriate treatment for the patient (Ali *et al*., 2015; Mohseni *et al*., 2017; Dal *et al*., 2019).

### 4.8.3. Serological Variables:

Table (4.25) revealed that 10(32.3%) of those positive for anti-brucella IgM were also positive for anti-brucella IgG versus 8(33.3%) who were positive for IgM but negative for IgG. The difference was statistically insignificant (P= 0.933). Regarding the CD3, the results found that 14(43.8%) who were positive for IgM were also positive for CD3 versus 4(17.5%) who were positive for IgM but negative for CD3. The difference was statistically significant (P= 0.040). About the CD4, 9(36%) who were positive for anti-brucella IgM were also positive for CD4 while another 9(30.0%) were positive for IgM but negative for CD4, with statistically insignificant difference (P= 0.637). furthermore, the results showed that all 18(34.0%) who were positive for IgM were also positive for CD8 versus 35(66.0%) who were positive for

CD8 but negative for IgM. Again the difference was statistically insignificant (P= 0315).

Concerning the IL-17, the results found that 12(37.5%) who were anti-brucella IgM positive were also positive for IL-17 against 6(26.1%) who were positive for IgM but negative for IL-17 and on the contrary, 20(62.5%) were positive for IL-17 but negative for IgM. The difference was statistically insignificant (P= 0.374). Furthermore, 6(22.2%) who were positive for anti-brucella IgM were also positive for IL-18 versus 12 (42.9%) who were positive for IgM but negative for IL-18. On the contrary, 21(77.8%) who were negative for IgM but positive for IL-18. The difference was statistically insignificant (P= 0.103). Lastly, all 18(34.6%) of those positive for anti-brucella IgM were positive for INF-γ versus 34 (65.4%) who were positive for INF-γ but negative for IgM. The difference was statistically insignificant (P= 0.214).

**Table (4.25): Association of anti-brucella IgM with serological variables.**

| Serological variables | | Anti-brucella IgM | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgG | Positive | 10 | 32.3 | 21 | 67.7 | 0.933** |
| | Negative | 8 | 33.3 | 16 | 66.7 | |
| CD3 | Positive | 14 | 43.8 | 18 | 56.3 | 0.040* |
| | Negative | 4 | 17.4 | 19 | 82.6 | |
| CD4 | Positive | 9 | 36.0 | 16 | 64.0 | 0.637** |
| | Negative | 9 | 30.0 | 21 | 70.0 | |
| CD8 | Positive | 18 | 34.0 | 35 | 66.0 | 0.315** |
| | Negative | - | - | 2 | 100.0 | |
| IL-17 | Positive | 12 | 37.5 | 20 | 62.5 | 0.374** |
| | Negative | 6 | 26.1 | 17 | 73.9 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| IL-18 | Positive | 6 | 22.2 | 21 | 77.8 | 0.103** |
| | Negative | 12 | 42.9 | 16 | 57.1 | |
| INF-γ | Positive | 18 | 34.6 | 34 | 65.4 | 0.214** |
| | Negative | - | - | 3 | 100.0 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

** Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The current results found that the circulatory CD3 positivity rate and concentration was significantly higher among patients with brucellosis compared to control. Furthermore, the anti-brucella IgM positivity rate was significantly associated with CD3 but not IgG. It has been well documented that to establish themselves and cause chronic or latent disease in humans and animals, brucella species invade and proliferate within monocytic phagocytes (Dornand *et al*., 2002). Therefore, and subsequently, the acquired immunity against these intracellular bacteria is T cell dependent (Splitter *et al*., 1996). Consistent with present results using flowcytometry it has been found that the intracytoplasmic expression of IFN-γ, IL-2, and TNF-α, but not that of IL-4, the activated CD4+ CD3+ and CD8+ CD3+ T lymphocytes was significantly increased in untreated brucellosis patients that were partially normalized after therapy and that the rate of phagocytic cells, the mean phagocytic activity/cell, and the phagocytic indices for monocytes were defective (Rodríguez-Zapata *et al*., 2010). Similar results were also reported by (Durward *et al*., 2012).

### 4.8.4. Molecular variables:

Results in table (4.26) found that 12 (30.8%) of those with detected INF-γ gene were positive for anti-brucella IgM versus 6(50.0%) who were positive for IgM but negative for INF-γ gene, with insignificant difference (P= 0.223). Similarly, 12 (30.0%) of those with anti-brucella IgM positive had

detected IL-18 gene against 6(54.5%) who were IgM positive but they had no detectable IL-18 gene, with statistically insignificant difference (P= 0.131). finally, 11(33.3%) of those with positive anti-brucella IgM had detectable IL-17 gene versus 7(38.9%) who were positive for IgM but had no detectable IL-17 gene, with insignificant difference (P= 0.692).

**Table (4.26): Association of anti-brucella IgM with molecular variables.**

| **Molecular variables (PCR)** | | **Anti-brucella IgM** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| *INF-γ* gene | Gene detected | 12 | 30.8 | 27 | 69.2 | 0.223* |
| | Not detected | 6 | 50.0 | 6 | 50.0 | |
| *IL-18* gene | Gene detected | 12 | 30.0 | 28 | 70.0 | 0.131* |
| | Not detected | 6 | 54.5 | 5 | 45.5 | |
| *IL-17* gene | Gene detected | 11 | 33.3 | 22 | 66.7 | 0.692* |
| | Not detected | 7 | 38.9 | 11 | 61.1 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.9. Association of Anti-Brucella IgG with Variables:

### 4.9.1. Sociodemographic Variables:

The anti-brucella IgG positivity rate was highest among those with ≥ 40 years old with insignificant difference compared to other age groups (P= 0.586). Regarding the gender, females had slightly higher rate of IgG positivity rate compared to males (59.1% *Vs* 45.5%) with insignificant difference (P= 0.415). similarly, those reside in cities had higher but insignificant IgG positivity rate compared to those reside in villages (63.3% Vs 48.0%), (P= 0.254). Furthermore, the results also found that those breed domesticated animals at home has lower IgG positivity rate than their counterpart (60.0% *Vs* 50.0%), (P=0.472). On the other hand, those

participants consume dairy products had higher IgG positivity rate versus those who did not (60.0% Vs 20.0%), but the difference was failed to reach the level of statistical insignificant (P= 0.086). lastly, those participant with no intrafamilial cases has higher IgG positivity rate with insignificant difference (P= 0.166). these date were presented in table (4.27).

**Table (4.27): Association of anti-brucella IgG with sociodemographic variables.**

| sociodemographic variables | | Anti-brucella IgG | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 3 | 50.0 | 3 | 50.0 | 0.586* |
| | 20---29 | 12 | 48.0 | 13 | 52.0 | |
| | 30---39 | 11 | 64.7 | 6 | 35.3 | |
| | ≥ 40 years | 5 | 71.4 | 2 | 28.6 | |
| Sex | Male | 5 | 45.5 | 6 | 54.5 | 0.415* |
| | Female | 26 | 59.1 | 18 | 40.9 | |
| Residence | City | 19 | 63.3 | 11 | 36.7 | 0.254* |
| | Village | 12 | 48.0 | 13 | 52.0 | |
| Home animals | Yes | 10 | 50.0 | 10 | 50.0 | 0.472* |
| | No | 21 | 60.0 | 14 | 40.0 | |
| Consumption of animal products | Yes | 30 | 60.0 | 20 | 40.0 | 0.086* |
| | No | 1 | 20.0 | 4 | 80.0 | |
| Intrafamilial cases | Yes | 11 | 45.8 | 13 | 54.2 | 0.166* |
| | No | 20 | 64.5 | 11 | 35.5 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The results found that the highest anti-brucella IgG positivity rate was among those 40 years or more, while the highest anti-brucella IgM positivity rate was among those patients less than 20 years. This outcome seems logical

since the IgM antibodies against brucella LPS were the first to appear following infection and rise gradually during the course of acute infection. In contrast, IgG anti- brucella antibodies appeared later after the onset of infection and were likely provoked by brucella cytoplasmic proteins. On this basis, most of the available serological tests were based on LPS to differentiate between infected and uninfected hosts (Elfaki *et al*., 2015). Furthermore, since the brucellosis is a chronic latent disease, so the anti-brucella IgG remain in the body for long time and probably for the life after the disappearing of clinical signs and symptoms, and during the reactivation of latent brucellae, the IgM antibody rise shortly for transient period followed by four fold increase in IgG titer (Lopez-Santiago *et al*., 2019). Therefore the study assumes that the persistent of high titer of anti-brucella IgG was cumulative due to repeated reactivation infection of brucella, a feature that is very dominant among Iraqi patients.

### 4.9.2. Clinical Variables:

The results in table (4.28) found that 25(56.8%) of those with new brucellosis infection were positive for anti-brucella IgG, whereas 6(54.5%) of those with old brucellosis infection were positive for IgG and 19(43.2%) of those with new brucellosis infection were negative for IgG. The difference was insignificant (P=0.892). About the presence of fever, all 31(63.3%) of those with positive IgG had fever, while 18(36.7%) were negative for IgG but had fever. The difference was statistically significant (P=0.003). The night sweat was found in 25(56.8%) of those with IgG positive against 6(54.5%) who were positive for IgG but they had no night sweat. The difference was insignificant (P= 0.892). Regarding the joint pain, 24(51.1%) who had joint pain were positive for IgG against 7(87.5%) who were positive for IgG but had no joint pain. The difference was insignificant (P= 0.055)

**Table (4.28): Association of anti-brucella IgG with clinical variables.**

| Clinical variables | | Anti-brucella IgG | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 25 | 56.8 | 19 | 43.2 | 0.892** |
| | Old infection | 6 | 54.5 | 5 | 45.5 | |
| Fever | Yes | 31 | 63.3 | 18 | 36.7 | 0.003* |
| | No | - | - | 6 | 100.0 | |
| Night sweat | Yes | 25 | 56.8 | 19 | 43.2 | 0.892** |
| | No | 6 | 54.5 | 5 | 45.5 | |
| Joint pain | Yes | 24 | 51.1 | 23 | 48.9 | 0.055** |
| | No | 7 | 87.5 | 1 | 12.5 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

As shown in the previous table, there was a significant association of anti-brucella IgG and the presence of fever, and more than half of patients had night sweat as well as joint pain. Supposing that the IgG represented the chronic/latent stage, these results are logical, supporting by the finding that the IgG antibody was associated with more than half of patients with old infection. These signs and symptoms are among the main clinical features of acute brucellosis and reactivation brucellosis (Dean *et al.*, 2012; Shi *et al.*, 2018). Although the osteoarthricular involvement is more prominent during chronic brucellosis; however, spondylitis is the prominent one among Iraqi patients (Tasova *et al.*, 1999; Bodur *et al.*, 2004). This point probably not clearly specified in the questionnaire form of the patients. Generally, the

current results are consistent with the results of other workers in spite of different rates (Mermut *et al.*, 2012; Jia *et al.*,2017; Jiang *et al.*, 2019).

### 4.9.3. Serological Variables:

The association of anti-brucella IgG with serological markers were shown in table (4.29). 10(55.6%) of those with positive anti-brucella IgG were also positive for anti-brucella IgM against 8(44.4%) were negative for IgG but positive for IgM and 21(56.8%) were positive for IgG but negative for IgM. The difference was statistically insignificant (P= 0.933). About the CD3, 17(53.1%) were positive for both anti-brucella IgG and CD3, while 14(60.9%) were positive for IgG but negative for CD3. The difference was insignificant (P= 0.568). Similarly 17(68.0%) were positive for both anti-brucella IgG and CD4 versus 14(46.7%) who were positive for IgG but negative for CD4. The difference was again insignificant (P= 0.112). Regarding the CD8, 30(56.6%) were positive for IgG and CD8, while 23(43.4%) were positive for CD8 but negative for IgG with insignificant difference (P= 0.853).

In connection with IL-17, the results found that 21(65.6%) who were positive for IL-17 were also positive for IgG against 10(43.5%) who were positive for IgG bur negative for IL-17. The difference was insignificant (P= 0.102). The results also found that 12(44.4%) of those positive for IL-18 were also positive for IgG, while 19(67.9%) were positive for IgG but negative for IL-18. The negativity rate was higher but the difference was failed to reach the level of statistical significance (P=0.080). Regarding the INF-ɣ, the results found that 30 (57.7%) who were positive for INF-ɣ were positive to IgG, while 1(33.3%) who were positive for IgG were negative for INF-γ. The difference was insignificant (P= 0.408).

**Table (4.29): Association of anti-brucella IgG with serological variables.**

| Serological variables | | Anti-brucella IgG | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 10 | 55.6 | 8 | 44.4 | 0.933* |
| | Negative | 21 | 56.8 | 16 | 43.2 | |
| CD3 | Positive | 17 | 53.1 | 15 | 46.9 | 0.568* |
| | Negative | 14 | 60.9 | 9 | 39.1 | |
| CD4 | Positive | 17 | 68.0 | 8 | 32.0 | 0.112* |
| | Negative | 14 | 46.7 | 16 | 53.3 | |
| CD8 | Positive | 30 | 56.6 | 23 | 43.4 | 0.853* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |
| IL-17 | Positive | 21 | 65.6 | 11 | 34.4 | 0.102* |
| | Negative | 10 | 43.5 | 13 | 56.5 | |
| IL-18 | Positive | 12 | 44.4 | 15 | 55.6 | 0.080* |
| | Negative | 19 | 67.9 | 9 | 32.1 | |
| INF-γ | Positive | 30 | 57.7 | 22 | 42.3 | 0.408* |
| | Negative | 1 | 33.3 | 2 | 66.7 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.9.4. Molecular variables:

In connection with the detected genes, the results in table (4.30) revealed that 22(56.4%) of those with positive anti-brucella IgG had detectable INF- γ gene versus 17(43.6%) who had detectable INF-γ gene but were negative for IgG. The difference was statistically insignificant (P=0.249). Similarly, 23(57.5%) of those with positive IgG has detectable IL-18 gene versus 17(42.5%) who had detectable IL-18 gene but were IgG negative, with insignificant difference (P= 0.360). Lastly, the IL-17 was detected in 19(57.6%) of those with positive anti-brucella IgG while 14(42.4%) had

detectable IL-17 gene but negative foe IgG. The difference was statistically insignificant (P=0.525).

**Table (4.30): Association of anti-brucella IgG with molecular variables.**

| Molecular variables (PCR) | | Anti-brucella IgG | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| *INF-γ* gene | Gene detected | 22 | 56.4 | 17 | 43.6 | 0.249* |
| | Not detected | 9 | 75.0 | 3 | 25.0 | |
| *IL-18* gene | Gene detected | 23 | 57.5 | 17 | 42.5 | 0.360* |
| | Not detected | 8 | 72.7 | 3 | 27.3 | |
| *IL-17* gene | Gene detected | 19 | 57.6 | 14 | 42.4 | 0.525* |
| | Not detected | 12 | 66.7 | 6 | 33.3 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The IFN-*γ* levels secreted by Th1 cells remain significantly higher more than 12 months after treatment of brucellosis. The IFN-*γ*/TNF-*α* ratio may be a feasible parameter for assessing clinical severity of brucellosis (Xu *et al.*, 2019). IFN-γ gene detection targeted the +874 position which was previously employed in brucellosis (Rasouli and Kiany, 2007). Pooled analysis suggested that the IFN-γ +874 AT vs AA as well as the TNF-α −308 AA vs GG + GA genotypes demonstrated a trend for the association with a higher risk of brucellosis (Eskandari-Nasab and Moghadampour, 2018).

### 4.10. Association of CD3 with Variables:

#### 4.10.1. Sociodemographic Variables:

Results in table (4.31) showed that the CD3 positivity rate was highest among those with 30-39 years old compared to other age groups, but the difference was insignificant (P= 0.508). Additionally, the IgG positivity rate

was slightly higher in females than that of males (59.1% *Vs* 54.5%) with insignificant difference (P= 0.785). The IgG positivity rate was found to be higher but in significant among cities residents compared to villages residents (66.7% *Vs* 48.0%), (P= 0.162). The results also revealed that those participants who didn't breeds home animals had higher but insignificant IgG positivity rate compared to their counterpart (65.7% *Vs* 45.0%), (P= 0.134). The IgG positivity rate was higher among those consume dairy products compared to their counterpart (62.0% *Vs* 20.0%); however, the difference was failed to reach the levels of statistical significant (P= 0.069). finally, those participants with intrafamilial cases of brucellosis had higher IgG positivity rate, but with insignificant difference (P= 0.262).

**Table (4.31): Association of CD3 with sociodemographic variables.**

| sociodemographic variables | | CD3 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 4 | 66.7 | 2 | 33.3 | 0.508* |
| | 20---29 | 13 | 52.0 | 12 | 48.0 | |
| | 30---39 | 12 | 70.6 | 5 | 29.4 | |
| | ≥ 40 years | 3 | 42.9 | 4 | 57.1 | |
| Sex | Male | 6 | 54.5 | 5 | 45.5 | 0.785* |
| | Female | 26 | 59.1 | 18 | 40.9 | |
| Residence | City | 20 | 66.7 | 10 | 33.3 | 0.162* |
| | Village | 12 | 48.0 | 13 | 52.0 | |
| Home animals | Yes | 9 | 45.0 | 11 | 55.0 | 0.134* |
| | No | 23 | 65.7 | 12 | 34.3 | |
| Consumption of animal products | Yes | 31 | 62.0 | 19 | 38.0 | 0.069* |
| | No | 1 | 20.0 | 4 | 80.0 | |
| Intrafamilial cases | Yes | 16 | 66.7 | 8 | 33.3 | 0.262* |
| | No | 16 | 51.6 | 15 | 48.4 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.10.2. Clinical Variables:

Results presented in table (4.32) revealed the association of CD3 positivity and the clinical variables. Regarding the patient history, 26(59.1%) who were positive for CD3 had new brucellosis infection, while 6(54.5%) who were positive for CD3 had old brucellosis infection. The difference was insignificant (P= 0.785). Fever was found to be present in 29(59.2%) of those positive with CD3 but it was absent in 3(50.0%) of those with positive CD3. The difference was statistically insignificant (P= 0.667). The night sweat was found in 27(61.4%) of those with positive CD3, but it was absent in 5(45.5%) of those with positive CD3. The difference was insignificant (P= 0.339). The results also found that 26(55.3%) who were positive for CD3 had joint pain against 6(75.0%) who were positive for CD3 but had no joint pain. The difference was insignificant (P= 0.297).

**Table (4.32): Association of CD3 with clinical variables.**

| Clinical variables | | CD3 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 26 | 59.1 | 18 | 40.9 | 0.785* |
| | Old infection | 6 | 54.5 | 5 | 45.5 | |
| Fever | Yes | 29 | 59.2 | 20 | 40.8 | 0.667* |
| | No | 3 | 50.0 | 3 | 50.0 | |
| Night sweat | Yes | 27 | 61.4 | 17 | 38.6 | 0.339* |
| | No | 5 | 45.5 | 6 | 54.5 | |
| Joint pain | Yes | 26 | 55.3 | 21 | 44.7 | 0.297* |
| | No | 6 | 75.0 | 2 | 25.0 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.10.3. Serological Variables:

Results in table (4.33) showed that 14(77.8%) who were positive for CD3 were also positive for anti-brucella IgM, while 18(48.6%) were positive for CD3 but negative for IgM. The difference was statistically significant (P= 0.040). Regarding the INF-γ, the results showed that all 32(61.5%) who were positive for CD3 were also positive for INF-γ, whereas, 20(38.5%) of those negative for CD3 were positive for INF-γ. The difference was significant (P=0.036). Regarding the IgG, the results found that 17(54.8%) were positive for CD3 and IgG against 15(62.5%) who were positive for CD3 but negative for IgG. Thus the difference was insignificant (P=0.568). Likewise, the CD3 was insignificantly associated with CD4 and CD8 (P= 0.178 and P=0. 811) respectively.

In connection with IL-17, 22(68.8%) were positive for CD4 and IL-17 which was higher than those positive for CD3 but negative for IL-17(43.5%). However, the difference was failed to reach the level of statistical significance (P= 0.061). the association with IL-18 was also insignificant (P= 0.139).

**Table (4.33): Association of CD3 with serological variables.**

| Serological variables | | CD3 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 14 | 77.8 | 4 | 22.2 | 0.040* |
| | Negative | 18 | 48.6 | 19 | 51.4 | |
| IgG | Positive | 17 | 54.8 | 14 | 45.2 | 0.568** |
| | Negative | 15 | 62.5 | 9 | 37.5 | |
| CD4 | Positive | 17 | 68.0 | 8 | 32.0 | 0.178** |
| | Negative | 15 | 50.0 | 15 | 50.0 | |
| CD8 | Positive | 31 | 58.5 | 22 | 41.5 | 0.811** |
| | Negative | 1 | 50.0 | 1 | 50.0 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| IL-17 | Positive | 22 | 68.8 | 10 | 31.3 | 0.061** |
| | Negative | 10 | 43.5 | 13 | 56.5 | |
| IL-18 | Positive | 13 | 48.1 | 14 | 51.9 | 0.139** |
| | Negative | 19 | 67.9 | 9 | 32.1 | |
| INF-γ | Positive | 32 | 61.5 | 20 | 38.5 | 0.036* |
| | Negative | - | - | 3 | 100.0 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The significantly higher positivity rate of anti-brucella IgM among patients compared to controls are not unusual since the patients were enrolled in this study based on their Rose-Bengal test positivity. These results are consistent with other local or abroad (Ruiz-mesa *et al.,*2005; Hasanjani and Ebrahimpour, 2015). Indeed, IgM antibodies were the first to appear following infection and rise gradually during the course of acute infection.

Two studies reported that the proportions of CD3+ T cells in human brucellosis patients were significantly increased compared to control individuals (Gao *etal*., 2015; Celik and Akbulut.,2005). The CD3 positivity rate was significantly associated with anti-brucella IgM but not IgG. It has been well documented that to establish themselves and cause chronic or latent disease in humans and animals, *brucella species* invade and proliferate within monocytic phagocytes (Dornand *et al.*, 2002). Therefore and subsequently, the acquired immunity against these intracellular bacteria is T cell dependent (Splitter *et al*., 1996).

Rodríguez-Zapata *et al.* (2010), recorded significantly higher levels of the IFN-γ, in serum and/or significantly higher percentages of samples with detectable levels than those for healthy controls. Especially striking is the

increased presence of high levels of IFN-γ in untreated patients. After treatment, significant reductions and normalization of the percentages of patients with detectable levels of IFN-γ in serum were found. Consistently, Rahmanpour *et al.* (2019), they found that serum levels of IFN-γ were elevated in acute brucellosis as well as in the relapsed patients compared to under-treatment groups and healthy controls.

### 4.10.4. Molecular Variables:

In connection with CD3 positivity and gene detection, the results showed that 21(53.8%) of those positive for CD3 had detectable *INF-γ* gene, while 9(75.0%) were positive for CD3 but had no detectable *INF-γ* gene. The difference was insignificant (P= 0.193). On the other hand, 22(55.0%) of those who were positive for CD3 had detectable *IL-18* gene against 8(72.7%) who were positive for CD3 but had no detectable *IL-18* gene. The difference was insignificant (P= 0.290). The *IL-17* gene was detected in 20(60.6%) of those with positive for CD3, but it was undetected in 10(55.6%) of those with positive CD3. The difference was insignificant (P= 0.726).

**Table (4.34): Association of CD3 with molecular variables.**

| Molecular variables (PCR) | | CD3 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| *INF-γ* gene | Gene detected | 21 | 53.8 | 18 | 46.2 | 0.193* |
| | Not detected | 9 | 75.0 | 3 | 25.0 | |
| *IL-18* gene | Gene detected | 22 | 55.0 | 18 | 45.0 | 0.290* |
| | Not detected | 8 | 72.7 | 3 | 27.3 | |
| *IL-17* gene | Gene detected | 20 | 60.6 | 13 | 39.4 | 0.726* |
| | Not detected | 10 | 55.6 | 8 | 44.4 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.11. Association of CD4 with Variables:

### 4.11.1. Sociodemographic Variables:

Results in table (4.35) revealed that the highest CD4 positivity rate was among those with 20-29 years old (56.0%) compared to other age groups; however the difference was insignificant (P= 0.531). The results also found that the CD4 positivity rate was significantly higher among those reside in cities compared to those reside in villages (63.3% *Vs* 24.0%), (P= 0.004). Whereas, the CD4 positivity rate was negatively associated with the presence of home animal (25.0% *Vs* 57.1%), (P= 0.021). Similarly, the CD4 positivity rate was high among those without intrafamilial cases compared to their counterpart (48.4% *Vs* 41.7%), (P=0. 620). Finally, the CD4 positivity rate was insignificantly higher among those consume dairy products compared to those did not (48.0% Vs 20.0%), (P= 0.231).

**Table (4.35): Association of CD4 with sociodemographic variables.**

| sociodemographic variables | | CD4 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 2 | 33.3 | 4 | 66.7 | 0.531 |
| | 20---29 | 14 | 56.0 | 11 | 44.0 | |
| | 30---39 | 6 | 35.3 | 11 | 64.7 | |
| | ≥ 40 years | 3 | 42.9 | 4 | 57.1 | |
| Sex | Male | 5 | 45.5 | 6 | 54.5 | |
| | Female | 20 | 45.5 | 24 | 54.5 | |
| Residence | City | 19 | 63.3 | 11 | 36.7 | 0.004* |
| | Village | 6 | 24.0 | 19 | 76.0 | |
| Home animals | Yes | 5 | 25.0 | 15 | 75.0 | 0.021* |
| | No | 20 | 57.1 | 15 | 42.9 | |
| Consumption of | Yes | 24 | 48.0 | 26 | 52.0 | 0.231 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **animal products** | No | 1 | 20.0 | 4 | 80.0 | |
| **Intrafamilial cases** | Yes | 10 | 41.7 | 14 | 58.3 | 0.620 |
| | No | 15 | 48.4 | 16 | 51.6 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

A meta-analysis showed a significantly decreased proportion of CD4+ T cells in human brucellosis patients compared to healthy individuals, with an immunologic dysfunction of T lymphocyte, suggesting that CD4+ and CD8+ T cells might be the important factors affecting the progress of brucellosis (Zheng *et al.,* 2018). Another study showed significant decrease in the percentage of CD4+ was observed in chronic brucellosis (Skendros *et al.,*2008).

### 4.11.2. Clinical Variables:

The association of CD4 positivity rate and clinical variables were shown in table (4.36). Regarding the patient history, the CD4 positivity rate was insignificantly higher among those with old infection versus those with new infection (63.6% *Vs* 40.9%), (P= 0.176). Similarly the CD4 positivity rate was insignificantly associated with neigh sweat (63.6% *Vs* 40.9%), joint pain (62.5% *Vs* 42.6%) with (P= 0.176) and (P= 0.295) respectively. The fever was negatively associated with the CD4 positivity rate (50.0% *Vs* 44.9%), (P= 0.813).

**Table (4.36): Association of CD4 with clinical variables.**

| **Clinical variables** | | **CD4** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| Patients history | New infection | 18 | 40.9 | 26 | 59.1 | 0.176* |
| | Old infection | 7 | 63.6 | 4 | 36.4 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Fever | Yes | 22 | 44.9 | 27 | 55.1 | 0.813* |
| | No | 3 | 50.0 | 3 | 50.0 | |
| Night sweat | Yes | 18 | 40.9 | 26 | 59.1 | 0.176* |
| | No | 7 | 63.6 | 4 | 36.4 | |
| Joint pain | Yes | 20 | 42.6 | 27 | 57.4 | 0.295* |
| | No | 5 | 62.5 | 3 | 37.5 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.11.3. Serological Variables:

The results in table (4.37) revealed that the CD4 positivity rate was significantly higher among those with IL-17 positivity rate (62.5% vs 21.7%), (P= 0.003). Similarly, the CD4 positivity rate was higher but insignificant among those with IgM positive (50.0% Vs 43.2%), (P= 0. 637) and IgG positive (54.8% Vs 33.3%), (P= 0.112). Furthermore, the CD4 positivity rate was insignificantly higher with those with CD3 (P- 0.178) and negatively associated with CD8 positivity (P= 0.895). Additionally, the association of CD4 positivity with IL-18 and INF-γ positivity were also insignificant (P= 0.218) and (P= 0.665) respectively.

**Table (4.37): Association of CD4 with serological variables.**

| **Serological variables** | | **CD4** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| IgM | Positive | 9 | 50.0 | 9 | 50.0 | 0.637** |
| | Negative | 16 | 43.2 | 21 | 56.8 | |
| IgG | Positive | 17 | 54.8 | 14 | 45.2 | 0.112** |
| | Negative | 8 | 33.3 | 16 | 66.7 | |
| CD3 | Positive | 17 | 53.1 | 15 | 46.9 | 0.178** |
| | Negative | 8 | 34.8 | 15 | 65.2 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| CD8 | Positive | 24 | 45.3 | 29 | 54.7 | 0.895** |
| | Negative | 1 | 50.0 | 1 | 50.0 | |
| IL-17 | Positive | 20 | 62.5 | 12 | 37.5 | 0.003* |
| | Negative | 5 | 21.7 | 18 | 78.3 | |
| IL-18 | Positive | 10 | 37.0 | 17 | 63.0 | 0.218** |
| | Negative | 15 | 53.6 | 13 | 46.4 | |
| INF-γ | Positive | 24 | 46.2 | 28 | 53.8 | 0.665** |
| | Negative | 1 | 33.3 | 2 | 66.7 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

**Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.11.4. Molecular Variables:

The association of CD4 positivity rate with the detection rate of *INF-γ, IL-18* and *IL-17* genes was statistically insignificant (P= 0.815, P= 0.904 and P= 0.138) respectively. Data were shown in table (4.38).

**Table (4.38): Association of CD4 with molecular variables.**

| **Molecular variables (PCR)** | | **CD4** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| *INF-γ* gene | Gene detected | 18 | 46.2 | 21 | 53.8 | 0.815* |
| | Not detected | 6 | 50.0 | 6 | 50.0 | |
| *IL-18* gene | Gene detected | 19 | 47.5 | 21 | 52.5 | 0.904* |
| | Not detected | 5 | 45.5 | 6 | 54.5 | |
| *IL-17* gene | Gene detected | 13 | 39.4 | 20 | 60.6 | 0.138* |
| | Not detected | 11 | 61.1 | 7 | 38.9 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.12. Association of CD8 with Variables:

### 4.12.1. Sociodemographic Variables:

Results in table (4.39) showed that participants who were consume dairy products had higher CD8 positivity rate than their counterpart (98.0% *Vs* 80.0%); However, the difference was failed to reach the levels of statistical significance (P= 0.040). On the other hand, the CD8 positivity rate was insignificantly associated with other variables.

**Table (4.39): Association of CD8 positivity rate with sociodemographic variables.**

| sociodemographic variables | | CD8 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 6 | 100.0 | - | - | 0.370* |
| | 20---29 | 24 | 96.0 | 1 | 4.0 | |
| | 30---39 | 17 | 100.0 | - | - | |
| | ≥ 40 years | 6 | 85.7 | 1 | 14.3 | |
| Sex | Male | 10 | 90.9 | 1 | 9.1 | 0.280* |
| | Female | 43 | 97.7 | 1 | 2.3 | |
| Residence | City | 28 | 93.3 | 2 | 6.7 | 0.188* |
| | Village | 25 | 100.0 | - | - | |
| Home animals | Yes | 20 | 100.0 | - | - | 0.276* |
| | No | 33 | 94.3 | 2 | 5.7 | |
| Consumption of animal products | Yes | 49 | 98.0 | 1 | 2.0 | 0.040* |
| | No | 4 | 80.0 | 1 | 20.0 | |
| Intrafamilial cases | Yes | 23 | 95.8 | 1 | 4.2 | 0.853* |
| | No | 30 | 96.8 | 1 | 3.2 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.12.2. Clinical Variables:

In connection with the clinical signs, the results found that patients with old brucellosis had insignificantly higher rate of CD8 compared to those with new infection (100.0% Vs 95.5%), (P= 0.471). Similarly, the CD8 was negatively associated with the presence of fever, night sweat and joint pain (P= 0.614), (P= 0.471) and (P= 0.552) respectively. Data were shown in table (4.40).

**Table (4.40): Association of CD8 positivity rate with clinical variables.**

| Clinical variables | | CD8 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 42 | 95.5 | 2 | 4.5 | 0.471* |
| | Old infection | 11 | 100.0 | - | - | |
| Fever | Yes | 47 | 95.9 | 2 | 4.1 | 0.614* |
| | No | 6 | 100.0 | - | - | |
| Night sweat | Yes | 42 | 95.5 | 2 | 4.5 | 0.471* |
| | No | 11 | 100.0 | - | - | |
| Joint pain | Yes | 45 | 95.7 | 2 | 4.3 | 0.552* |
| | No | 8 | 100.0 | - | - | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.12.3. Serological Variables:

The results in table (4.41) found that the INF-γ positivity rate was significantly associated with the CD8 positivity rate (98.1% Vs 66.7%), (P= 0.005). The results also found that the IL-17 positivity rate was 32(100%) against (91.3%) among those with CD8 positive. However, the difference was failed to reach the level of statistical significant (P= 0.089). While the IL-18 was negatively associated with the CD4 positivity rate (100.0% Vs 92.6%),

(P= 0.142). Further results found that although the anti-brucella IgM positivity rate was 18(100%) among those positive for CD8; however, the difference was statistically insignificant (P= 0. 315).

**Table (4.41): Association of CD8 positivity rate with serological variables.**

| Serological variables | | CD8 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 18 | 100.0 | - | - | 0.315 |
| | Negative | 35 | 94.6 | 2 | 5.4 | |
| IgG | Positive | 30 | 96.8 | 1 | 3.2 | 0.853 |
| | Negative | 23 | 95.8 | 1 | 4.2 | |
| CD3 | Positive | 31 | 96.9 | 1 | 3.1 | 0.811 |
| | Negative | 22 | 95.7 | 1 | 4.3 | |
| CD4 | Positive | 24 | 96.0 | 1 | 4.0 | 0.895 |
| | Negative | 29 | 96.7 | 1 | 3.3 | |
| IL-17 | Positive | 32 | 100.0 | - | - | 0.089 |
| | Negative | 21 | 91.3 | 2 | 8.7 | |
| IL-18 | Positive | 25 | 92.6 | 2 | 7.4 | 0.142 |
| | Negative | 28 | 100.0 | - | - | |
| INF-γ | Positive | 51 | 98.1 | 1 | 1.9 | 0.005* |
| | Negative | 2 | 66.7 | 1 | 33.3 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

There are three mechanisms of acquired immunity in brucella infection: (1) the antimicrobial effect of macrophage induced by interferon-γ secreted by CD4+, CD8+, γ, and β T lymphocyte (Durward *et al*., 2012); (2) the hosts eliminate the macrophages infected by *brucella* through the cytotoxic effect of CD8+, γ, and β T lymphocytes (Durward-Diioia *et al*., 2015); and (3) the modulating effect of the antibody can enhance the phagocytosis of

macrophages (Baldwin and Goenka, 2006). Activated CD8+ T lymphocytes and CD4+ Th1 type immune responses play an important role in the scavenging of intracellular pathogens (Wang *et al*., 2014). CD8+ T cell exhaustion, suppressed INF- γ production, and delayed memory response induced by chronic *B. melitensis* infection (Durward-Diioia *et al*., 2015). Additionally, Cytotoxic CD4+ T cells which appears at early *B.abortus* infection concomitantly with high levels of IFN-γ and granzyme B expression. CD4+ CTLs represent a so far uncharacterized immune cell sub-type triggered by early immune responses (Martirosyan *et al.*, 2013).

Another results using flowcytometry it has been found that the intracytoplasmic expression of IFN-γ, IL-2, and TNF-α, but not that of IL-4, the activated CD4+ CD3+ and CD8+ CD3+ T lymphocytes was significantly increased in untreated brucellosis patients that were partially normalized after therapy and that the rate of phagocytic cells, the mean phagocytic activity/cell, and the phagocytic indices for monocytes were defective (Rodríguez-Zapata *et al.*, 2010). Similar results were also reported by (Durward *et al*., 2012).

The current results are also consistent with (Zheng *et al*., 2018) who stated that the frequency of CD8+ T cells was significantly higher in human brucellosis patients compared to healthy. Actually, in humans, IFN-γ producing CD4+ T cells, CD8+ T cells and γδ T cells have been implicated in the control of brucellosis (Cannella *et al*., 2012). Therefore, CD8+ T cell exhaustion, suppressed INF- γ production, and delayed memory response promote chronic *Brucella melitensis* infection (Durward-Diioia *et al*., 2015). Therefore systemic and mucosal immune protection via CD8+ T cell engagement is an attractive means for B. *melitensis* vaccination (Clapp *et al*., 2016).

### 4.12.4. Molecular Variables:

The association of CD8 positivity rate with *INF-γ, IL-18* and *IL-17* gene detection rates was shown in table (4.42). The *INF-γ* gene was detected in (97.4% against 91.7%) of those with CD4 positive. The difference was insignificant (P= 0.368). Similarly, the *IL-18* gene was detected in (97.5% *Vs* 90.9%) of those with CD8 positive and the *IL-17* gene was detected in (97.0% *Vs* 94.4%) of those with CD8 positive with (P= 0. 319) and (P= 0.657) respectively.

**Table (4.42): Association of CD8 positivity rate with molecular variables.**

| Molecular variables (PCR) | | CD8 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| *INF-γ* gene | Gene detected | 38 | 97.4 | 1 | 2.6 | 0.368* |
| | Not detected | 11 | 91.7 | 1 | 8.3 | |
| *IL-18* gene | Gene detected | 39 | 97.5 | 1 | 2.5 | 0.319* |
| | Not detected | 10 | 90.9 | 1 | 9.1 | |
| *IL-17* gene | Gene detected | 32 | 97.0 | 1 | 3.0 | 0.657* |
| | Not detected | 17 | 94.4 | 1 | 5.6 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.13. Association of IL-17 with Variables:

### 4.13.1. Sociodemographic Variables:

Regarding the age group, the IL-17 positivity rate was insignificantly higher among those ≥ 40 years old (P=0.281). The IL-17 positivity rate was insignificantly higher among males versus female (63.6% Vs 56.8%), (P= 0.682). The IL-17 positivity rate was insignificantly higher in those reside in cities against those reside in villages (63.3% VS 52.0%), (P= 0.682). Furthermore, other variables including the presence of animals in home,

consumption of dairy products and intrafamilial cases, all were insignificantly associated with the IL-17 positivity (P= 0.718), (P=0.387) and (p= 0.984) respectively.

**Table (4.43): Association of IL-17 positivity rate with sociodemographic variables.**

| sociodemographic variables | | IL-17 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 4 | 66.7 | 2 | 33.3 | 0.281* |
| | 20---29 | 11 | 44.0 | 14 | 56.0 | |
| | 30---39 | 12 | 70.6 | 5 | 29.4 | |
| | ≥ 40 years | 5 | 71.4 | 2 | 28.6 | |
| Sex | Male | 7 | 63.6 | 4 | 36.4 | 0.682* |
| | Female | 25 | 56.8 | 19 | 43.2 | |
| Residence | City | 19 | 63.3 | 11 | 36.7 | 0.396* |
| | Village | 13 | 52.0 | 12 | 48.0 | |
| Home animals | Yes | 11 | 55.0 | 9 | 45.0 | 0.718* |
| | No | 21 | 60.0 | 14 | 40.0 | |
| Consumption of animal products | Yes | 30 | 60.0 | 20 | 40.0 | 0.387* |
| | No | 2 | 40.0 | 3 | 60.0 | |
| Intrafamilial cases | Yes | 14 | 58.3 | 10 | 41.7 | 0.984* |
| | No | 18 | 58.1 | 13 | 41.9 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.13.2. Clinical Variables:

Results presented in table (4.44) found that the IL-17 positivity rate was higher among those with old brucella infection compared to those with recent cases of brucellosis (81.8% *Vs* 52.3%), (P= 0.076). Other variables including

Fever, night sweat, joint pain were negatively associated with the Il-17 positivity rate (P= 0.655), (P=, 0.682) and (P= 0.279) respectively.

**Table (4.44): Association of IL-17 positivity rate with clinical variables.**

| Clinical variables | | IL-17 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 23 | 52.3 | 21 | 47.7 | 0.076* |
| | Old infection | 9 | 81.8 | 2 | 18.2 | |
| Fever | Yes | 28 | 57.1 | 21 | 42.9 | 0.655* |
| | No | 4 | 66.7 | 2 | 33.3 | |
| Night sweat | Yes | 25 | 56.8 | 19 | 43.2 | 0.682* |
| | No | 7 | 63.6 | 4 | 36.4 | |
| Joint pain | Yes | 26 | 55.3 | 21 | 44.7 | 0.297* |
| | No | 6 | 75.0 | 2 | 25.0 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

Protective immunity to this pathogen is mainly dependent to cell-mediated immune responses so that, the contribution of antigen-presenting cells (macrophages and dendritic cells) and consequently induction of $CD4^+$ and $CD8^+$ T-lymphocytes play crucial roles in these responses (Gorvel,2008). Two subsets of $CD4^+$ T cells, Th1 and Th17, are central players of the immune responses to intracellular bacteria (Peck and Mellins,2010).

CD3+ T cells represent mature lymphocytes, which are the main active cells in cellular immunity, and CD3+ T cells represent the overall level of cellular immunity. Kayhan *et al*. (2016), observed that Patients with acute brucellosis had a significantly higher percentage of CD3+ T lymphocytes compared with healthy individuals and relapse patients.

### 4.13.3. Serological Variables:

The IL-17 positivity rate was significantly higher among those participants with CD4 positive compared to those who were CD4 negative (80.0% *Vs* 40.0%), (P= 0.003). Similarly, the IL-17 positivity rate among those with CD4 positive was higher than those who were CD3 negative (68.8% *Vs* 43.5%); However, the difference was failed to reach the levels of statistical significant (P= 0.061). Likewise, the IL-17 positivity rate was higher among those with CD8 positive, but again the difference was failed to reach the levels of statistical significant (P= 0.089). On the other hand, the association of IL-17 with anti-brucella IgG and IgM was higher in both antibodies but statistically insignificant (P= 0.374) and (P= 0.102) respectively. Lately, the association of IL-17 with IL-18 and INF-γ were also insignificant (P= 0. 139) and (P= 0.370).

**Table (4.45): Association of IL-17 positivity rate with serological variables.**

| Serologica variables | | IL-17 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 12 | 66.7 | 6 | 33.3 | 0.374 |
| | Negative | 20 | 54.1 | 17 | 45.9 | |
| IgG | Positive | 21 | 67.7 | 10 | 32.3 | 0.102 |
| | Negative | 11 | 45.8 | 13 | 54.2 | |
| CD3 | Positive | 22 | 68.8 | 10 | 31.3 | 0.061 |
| | Negative | 10 | 43.5 | 13 | 56.5 | |
| CD4 | Positive | 20 | 80.0 | 5 | 20.0 | 0.003* |
| | Negative | 12 | 40.0 | 18 | 60.0 | |
| CD8 | Positive | 32 | 60.4 | 21 | 39.6 | 0.089 |
| | Negative | - | - | 2 | 100.0 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| IL-18 | Positive | 13 | 48.1 | 14 | 51.9 | 0.139 |
| | Negative | 19 | 67.9 | 9 | 32.1 | |
| INF-γ | Positive | 31 | 59.6 | 21 | 40.4 | 0.370 |
| | Negative | 1 | 33.3 | 2 | 66.7 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The present results found that there was a significant association of IL-17 with CD4 and CD8 T lymphocytes among Iraqi patients with brucellosis. The present study is totally agree with the fact that Activated CD8+ T lymphocytes and CD4+ Th1 type immune responses play an important role in the scavenging of intracellular pathogens (Wang *et al*., 2014). The current results are also consistent with Vitry *et al*. (2014), who stated that both brucella-specific humoral response and $CD4^+$ Th1 cells must act together to confer protective immunity to *B. melitensis* infection.

The IL-17-producing cells, found in the lamina propria, play an essential role against microorganisms infecting the gastrointestinal tract. IL-17 production has also been observed in the lung and oral cavity mucosa. The cells producing the cytokine migrate toward these sites due to CCR6 expression (Marks and Craft, 2009). In oral vaccination of BALB/c mice with *B. melitensis* strain it has been found that the IL-17 and IL-22 compensate for the absence of IFNγ and that the Th1, Th17, and CD8+ T lymphocytes was increased in the gut mucosa and system, leading to a high production of IFN-γ and IL-17 (Ibanez *et al*., 2015). Additionally, a cooperative role for Th1 and Th17 cells in immunity to brucellosis is more evident during acute and relapse phases of brucellosis (Rahmanpour *et al*., 2019).

The natural invasion of the host by Brucellae induces a specific immune response mediated by Th1 lymphocytes which are characterized by the

production of proinflammatory cytokines as IL-1β, IL-6, TNF-α, and more importantly, IFNγ (Byndloss and Tsolis, 2016). These proinflammatory cytokines play a protective role since they activate macrophages to increase their bactericide capacity and CTL that kill infected cells (Brandao *et al*., 2012). It has been observed that IFN-γ and IL-12 production by macrophages is induced by IL-17 (Onishi and Gaffen, 2010). Also, the differentiation to Th17 lymphocytes is induced by a combination of IL-1, IL-6, and the TGF-β. The *in vivo* participation of IL-23 in this differentiation process is necessary to achieve a stable differentiation and proliferation of Th17 cells when activated (Korn *et al*., 2009). Therefore, IL-17 is a critical determinants of risk, severity or protection against brucellosis although it level decreased in patients at the end of treatment (Park *et al*., 2005; Sofian *et al.*, 2016).

The current results are also consistent with (Zheng *et al*., 2018) who stated that the frequency of CD8+ T cells was significantly higher in human brucellosis patients compared to healthy. Actually, in humans, IFN-γ producing CD4+ T cells, CD8+ T cells and γδ T cells have been implicated in the control of brucellosis (Cannella *et al*., 2012). Therefore, CD8+ T cell exhaustion, suppressed INF- γ production, and delayed memory response promote chronic *Brucella melitensis* infection (Durward-Diioia *et al*., 2015). Therefore systemic and mucosal immune protection via CD8+ T cell engagement is an attractive means for B. *melitensis* vaccination (Clapp *et al*., 2016).

Collectively, based on these observations, the results of the current study are concordant with that of Xavier *et al*., (2013) who stated that both $CD4^+$ and $CD8^+$ T cells as well as IL-17 were implicated in primary protection against brucellosis. While $CD4^+$ T cells exert their effect via cytokine secretion, cytotoxic $CD8^+$ T cells eliminate infection by lysing autologous

Macrophage infected with brucella species. Furthermore, cytotoxic CD4+ T cells which appears at early *Brucella abortus* infection concomitantly with high levels of IFN-γ and granzyme B expression, it represent a so far uncharacterized immune cell sub-type triggered by early immune responses (Martirosyan *et al*., 2013).

### 4.13.4. Molecular Variables:

The association of IL-17 positivity rate with molecular markers was shown in table (4.46). The IL-17 positivity rate was insignificantly higher among those who had no *INF-γ* gene detected compared to those who has detected *INF-γ* gene (75.0% *Vs* 59.0%), (P=.0 315). Similarly the IL-17 gene and IL-18 gene were inversely associated with IL-17 positivity rate (57.6% *Vs* 72.7%), (P= 0.301) and (60.0% *Vs* 72.7%), (P= 0.439) respectively.

**Table (4.46): Association of IL-17 positivity rate with molecular variables.**

| **Molecular variables (PCR)** | | **IL-17** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| *INF-γ* gene | Gene detected | 23 | 59.0 | 16 | 41.0 | 0.315* |
| | Not detected | 9 | 75.0 | 3 | 25.0 | |
| *IL-18* gene | Gene detected | 24 | 60.0 | 16 | 40.0 | 0.439* |
| | Not detected | 8 | 72.7 | 3 | 27.3 | |
| *IL-17* gene | Gene detected | 19 | 57.6 | 14 | 42.4 | 0.301* |
| | Not detected | 13 | 72.2 | 5 | 27.8 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.14. Association of IL-18 with Variables:

### 4.14.1. Sociodemographic Variables:

Results in table (4.47) found that the higher IL-18 positivity rate was among those ≥ 40 years old compared to other age groups (P=0.507). Regarding the gender, males were found to have higher rate of IL-18 compared to female (63.6% *Vs* 45.5%). With insignificant difference (P= 0.281). Furthermore, the association of IL-18 positivity rate with residence, presence of home animals, consumption of dairy products and intrafamilial cases, all were insignificantly associated (P= 0.349). (P=0.221), (P= 0.609) respectively.

**Table (4.47): Association of IL-18 positivity rate with sociodemographic variables.**

| sociodemographic variables | | IL-18 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 3 | 50.0 | 3 | 50.0 | 0.507* |
| | 20---29 | 10 | 40.0 | 15 | 60.0 | |
| | 30---39 | 9 | 52.9 | 8 | 47.1 | |
| | ≥ 40 years | 5 | 71.4 | 2 | 28.6 | |
| Sex | Male | 7 | 63.6 | 4 | 36.4 | 0.281* |
| | Female | 20 | 45.5 | 24 | 54.5 | |
| Residence | City | 13 | 43.3 | 17 | 56.7 | 0.349* |
| | Village | 14 | 56.0 | 11 | 44.0 | |
| Home animals | Yes | 12 | 60.0 | 8 | 40.0 | 0.221* |
| | No | 15 | 42.9 | 20 | 57.1 | |
| Consumption of animal products | Yes | 24 | 48.0 | 26 | 52.0 | 0.609* |
| | No | 3 | 60.0 | 2 | 40.0 | |
| Intrafamilial cases | Yes | 12 | 50.0 | 12 | 50.0 | 0.906* |
| | No | 15 | 48.4 | 16 | 51.6 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.14.2. Clinical Variables:

The IL-18 positivity rate was higher among those participants complaining joint pain compared to their counterpart (55.3% *Vs* 12.5%) with statistically significant difference (P= 0.025). However, other variables namely patient history, fever and night sweat were insignificantly associated with the IL-18 (P= 0. 345), (P= 0.362) , (P= 0.686) and (0.906) respectively. Results were presented in table (4.48).

**Table (4.48): Association of IL-18 positivity rate with clinical variables.**

| Clinical variables | | IL-18 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 23 | 52.3 | 21 | 47.7 | 0.345** |
| | Old infection | 4 | 36.4 | 7 | 63.6 | |
| Fever | Yes | 23 | 46.9 | 26 | 53.1 | 0.362** |
| | No | 4 | 66.7 | 2 | 33.3 | |
| Night sweat | Yes | 21 | 47.7 | 23 | 52.3 | 0.686** |
| | No | 6 | 54.5 | 5 | 45.5 | |
| Joint pain | Yes | 26 | 55.3 | 21 | 44.7 | 0.025* |
| | No | 1 | 12.5 | 7 | 87.5 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

** Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The significant association of IL-18 with joint pain as one main sign of recent as well as chronic/latent brucellosis. The IL-18 is produced mainly by macrophages. It is a proinflammatory cytokine that facilitates type 1 responses. Together with IL-12 acts on CD4, CD8 T cells and NK cells to induce IFN-γ production (Yasuda *et al.,* 2019). Experimentally, it has been found that combined inoculation of IL-12 and IL-18 reduced the number of *B.*

*abortus* in the spleen and that the effect of the treatment was mediated by an increased capability of spleen cells to produce INF- γ at the early phase of infection (Pasquali *et al.*, 2002). Conversely, once the infection was established, *B. abortus* selectively limits IL-18 secretion without affecting endogenous IFN- γ production (Fernandez-Lago *et al.*, 2005). Similar elevated levels of IL-18 was reported in acute brucellosis (Kayhan *et al*., 2016).

It has been reported that the serum IL-18 and IFN-γ levels were significantly increased in patients with acute brucellosis in a Caspase-1-related inflammasomes mediated (Karaca *et al*., 2018). However, generally the cytokine levels in acute or chronic brucellosis is a marker of immune response and an indicative marker of severity of brucellosis (Lin *et al.*, 2020).

### 4.14.3. Serological Variables:

The IL-18 positivity rate was higher among those participants with negative anti-brucella IgG compared to those with positive IgG (62.5% Vs 38.7%); However, the difference was insignificant (P= 0.080). Similarly the anti-brucella IgM was inversely associated with IL-18 positivity (33.3% Vs 56.8%) with insignificant difference (P= 0.103). Furthermore, all other variables including CD3, CD4, CD8, IL-17 and INF-γ were inversely associated with the IL-18 positivity. All results were shown in table (4.49).

**Table (4.49): Association of IL-18 positivity rate with serological variables.**

| Serological variables | | IL-18 | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 6 | 33.3 | 12 | 66.7 | 0.103* |
| | Negative | 21 | 56.8 | 16 | 43.2 | |
| IgG | Positive | 12 | 38.7 | 19 | 61.3 | 0.080* |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Negative | 15 | 62.5 | 9 | 37.5 | |
| CD3 | Positive | 13 | 40.6 | 19 | 59.4 | 0.139* |
| | Negative | 14 | 60.9 | 9 | 39.1 | |
| CD4 | Positive | 10 | 40.0 | 15 | 60.0 | 0.218* |
| | Negative | 17 | 56.7 | 13 | 43.3 | |
| CD8 | Positive | 25 | 47.2 | 28 | 52.8 | 0.142* |
| | Negative | 2 | 100.0 | - | - | |
| IL-17 | Positive | 13 | 40.6 | 19 | 59.4 | 0.139* |
| | Negative | 14 | 60.9 | 9 | 39.1 | |
| INF-γ | Positive | 25 | 48.1 | 27 | 51.9 | 0.531* |
| | Negative | 2 | 66.7 | 1 | 33.3 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.14.4. Molecular Variables:

The IL-18 positivity rate was lower among those who had detected *IL-17* gene compared to those who had no detectable *IL-17* gene (39.4% *Vs* 66.7%); However, the difference was statistically insignificant (P= 0.063). Other two genes namely the *INF-γ* and *IL-18* genes were also inversely associated with the IL-18 positivity rate with insignificant difference (P= 0.162) and (P= 0.274). Data were shown in table (4.50).

**Table (4.50): Association of IL-18 positivity rate with molecular variables.**

| **Molecular variables (PCR)** | | **IL-18** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| *INF*-γ gene | Gene detected | 17 | 43.6 | 22 | 56.4 | 0.162* |
| | Not detected | 8 | 66.7 | 4 | 33.3 | |
| *IL-18* gene | Gene detected | 18 | 45.0 | 22 | 55.0 | 0.274* |
| | Not detected | 7 | 63.6 | 4 | 36.4 | |
| *IL-17* gene | Gene detected | 13 | 39.4 | 20 | 60.6 | 0.063* |
| | Not detected | 12 | 66.7 | 6 | 33.3 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.15. Association of INF-γ with Variables:

### 4.15.1. Sociodemographic Variables:

Results in table (4.51) showed that the INF-γ positivity rate was significantly associated with those who consume dairy products compared to their counterpart (98.0% Vs 60.0%), (P= 0.0001). The intrafamilial brucellosis was insignificantly associated with the positivity rate of INF-γ (95.8% Vs 93.5%), (P=0. 711). The highest INF-γ positivity rate was among those with < 20 years old compared to other age groups (100.0%), but the difference was insignificant (P= 679). Furthermore, the INF-γ positivity rate was insignificantly higher in female compared to male (P= 0.553).

**Table (4.51): Association of INF-γ positivity rate with sociodemographic variables.**

| sociodemographic variables | | INF-γ | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 6 | 100.0 | - | - | 0.679** |
| | 20---29 | 24 | 96.0 | 1 | 4.0 | |
| | 30---39 | 16 | 94.1 | 1 | 5.9 | |
| | ≥ 40 years | 6 | 85.7 | 1 | 14.3 | |
| Sex | Male | 10 | 90.9 | 1 | 9.1 | 0.553** |
| | Female | 42 | 95.5 | 2 | 4.5 | |
| Residence | City | 28 | 93.3 | 2 | 6.7 | 0.665** |
| | Village | 24 | 96.0 | 1 | 4.0 | |
| Home animals | Yes | 19 | 95.0 | 1 | 5.0 | 0.911** |
| | No | 33 | 94.3 | 2 | 5.7 | |
| Consumption of animal products | Yes | 49 | 98.0 | 1 | 2.0 | 0.0001* |
| | No | 3 | 60.0 | 2 | 40.0 | |
| Intrafamilial cases | Yes | 23 | 95.8 | 1 | 4.2 | 0.711** |
| | No | 29 | 93.5 | 2 | 6.5 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The results in previous table showed that all (100%) of brucellosis patients with less than 20 years had positive INF-γ positive. This is most probably due to the fact that these patients may exposed to brucella infection for the first time. Several studies had documented that high levels of the IFN-γ may be a characteristic of acute, relapsed and chronic brucellosis (Han *et al*., 2017; Rahmanpour *et al.,* 2019). Furthermore, Rodríguez-Zapata *et al.* (2010), recorded significantly higher levels of the IFN-γ, in serum especially striking is the increased presence of high levels of IFN-γ in untreated patients, with significant reductions and normalization after treatment (Ghaznavi *et al*., 2017).

Beside the IFN-γ other cytokines; IL-4, IL-6, IL-10, IL-17 and TNF-α levels were found to be higher in patients with brucellosis, suggesting that assessing cytokine levels in patients with acute and chronic brucellosis is not only useful for detecting the immune response, but can also be indicative of the severity of brucellosis particularly, the IL-6 and INF-γ levels may be useful independent predictive factors in the clinical diagnosis of brucellosis. (Lee *et a*l., 2016; Lopez-Santiago *et al*., 2019; Lin *et al*., 2020).On the other hand, it has been found that the IFN-*γ* levels secreted by Th1 cells remain significantly higher more than 12 months after treatment of brucellosis. The IFN-*γ*/TNF-*α* ratio may be a feasible parameter for assessing clinical severity of brucellosis (Xu *et al*., 2019). Therefore, the current result is consistent with all these studies affirming the elevation of IFN-γ levels in the sera of patients with brucellosis.

The results of consumption of diary product are in agreement with other study that performed in Iran, palstain, Lebanon and Saudi-Arabia (Golshani and Buozari,2017; Dadar *et al.*,2019; Rajab *et al.*,2020) that find the major risk factor of brucellosis arise when consumption un pasteurization diary product . The milk of infected sheep and goats may contain large numbers of viable organisms, which become concentrated in products such as soft cheese (Ongor *et al.,* 2006).

### 4.15.2. Clinical Variables:

The results in table (4.52) revealed that the INF-γ positivity rate was higher among those with old brucellosis compared to those with recent cases (100.0% *Vs* 93.2%); However, the difference was insignificant (P= 0. 373). Similarly, the INF-γ positivity was higher among those without fever compared to those with fever (100.0% *Vs* 93.9%) as well as among those without joint pain compared to those with joint pain (100.0% *Vs* 93.6%) with insignificant difference (P= 0.533) and (P= 0.462). Additionally, the INF-γ positivity was insignificantly higher among those with night sweat compared to their counterpart (95.5% Vs 90.9%) with insignificant difference (P= 0.553).

**Table (4.52): Association of INF-γ positivity rate with clinical variables.**

| Clinical variables | | INF-γ | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 41 | 93.2 | 3 | 6.8 | 0.373* |
| | Old infection | 11 | 100.0 | - | - | |
| Fever | Yes | 46 | 93.9 | 3 | 6.1 | 0.533* |
| | No | 6 | 100.0 | - | - | |
| Night sweat | Yes | 42 | 95.5 | 2 | 4.5 | 0.553* |

| | | | | | | |
|---|---|---|---|---|---|---|
| | No | 10 | 90.9 | 1 | 9.1 | |
| Joint pain | Yes | 44 | 93.6 | 3 | 6.4 | 0.462* |
| | No | 8 | 100.0 | - | - | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.15.3. Serological Variables:

Data presented in table (4.53) found that the INF-γ positivity rate was significantly higher among those participants with CD3 positive compared to their counterpart (100.0% *Vs* 87.0%), (P= 0.036). Furthermore, the INF-γ positivity was significantly higher among those with positive CD8 compared to those with negative CD8 (96.2% Vs 50.0%) with significant difference (P= 0.005). The anti-brucella IgM and IgG were higher among those with positive INF-γ compared to their counterparts (100.0% *Vs* 91.9%) and (96.8% *Vs* 91.7%) respectively with insignificant difference (P= 0. 214) and (P= 0.408). respectively. The IL-17 was insignificantly associated and the IL-18 was inversely associated with INF-γ positivity (P= 0.370) and (P= 0.531) respectively.

**Table (4.53): Association of INF-γ positivity rate with serological variables.**

| **Serological variables** | | **INF-γ** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| IgM | Positive | 18 | 100.0 | - | - | 0.214 |
| | Negative | 34 | 91.9 | 3 | 8.1 | |
| IgG | Positive | 30 | 96.8 | 1 | 3.2 | 0.408 |
| | Negative | 22 | 91.7 | 2 | 8.3 | |
| CD3 | Positive | 32 | 100.0 | - | - | 0.036* |
| | Negative | 20 | 87.0 | 3 | 13.0 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| CD4 | Positive | 24 | 96.0 | 1 | 4.0 | 0.665 |
| | Negative | 28 | 93.3 | 2 | 6.7 | |
| CD8 | Positive | 51 | 96.2 | 2 | 3.8 | 0.005* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |
| IL-17 | Positive | 31 | 96.9 | 1 | 3.1 | 0.370 |
| | Negative | 21 | 91.3 | 2 | 8.7 | |
| IL-18 | Positive | 25 | 92.6 | 2 | 7.4 | 0.531 |
| | Negative | 27 | 96.4 | 1 | 3.6 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

The key function played by IFN-γ-producing Th1 CD4+ T cells in the control of *B. melitensis* infection, whereas IFN-γ-producing CD8+ T cells or B cell-mediated humoral immunity plays only a modest role in the clearance of bacteria during primary infection(Vitry *et al*.,2012), on the other hand , it has been demonstrate that IL-10 modulates the proinflammatory immune response to *B. abortus* infection leads to bacterial clearance which was preceded by an enhanced IFN-γ, TNF-α and IL-17 responses in both the serum and the spleen. Additionally, dendritic cells from produced elevated levels of IL-12 and TNF-α (Corsetti *et al*., 2013).

### 4.15.4. Molecular Variables:

The INF-γ positivity rate was insignificantly higher among those with detectable *INF-γ* gene, *IL-18* gene and *IL-17* gene compared to their counterparts (97.4% *Vs* 91.7%), (97.5% *Vs* 90.9%) and (97.0% *Vs* 94.4%) respectively with insignificant difference (P=0.368), (P= 0.319) and (P= 0. 657). Results were presented in table (4.54).

**Table (4.54): Association of INF-γ positivity rate with molecular variables.**

| Molecular variables (PCR) | | INF-γ | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| *INF-γ* gene | Gene detected | 38 | 97.4 | 1 | 2.6 | 0.368* |
| | Not detected | 11 | 91.7 | 1 | 8.3 | |
| *IL-18* gene | Gene detected | 39 | 97.5 | 1 | 2.5 | 0.319* |
| | Not detected | 10 | 90.9 | 1 | 9.1 | |
| *IL-17* gene | Gene detected | 32 | 97.0 | 1 | 3.0 | 0.657* |
| | Not detected | 17 | 94.4 | 1 | 5.6 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.16. Association of *INF-γ* Gene with Variables:

### 4.16.1. Sociodemographic Variables:

Table (4.55) showed that the highest *INF-γ* gene detection rate was among those with 20-29 years old compared to other age groups but the difference was insignificant (P= 0.270). Furthermore, female had insignificantly higher *INF-γ* gene detection rate compared to males (78.6% *Vs* 66.7%), (P= 0.445). It was also found that those who breed home animals had higher *INF-γ* gene detection versus their counterpart (83.3% *Vs* 72.7%), (P= 0.393). The *INF-γ* gene detection was appeared to be higher in those with family cluster cases of brucellosis compared to their counterpart (77.3% *Vs* 75.9%) with insignificant difference (P= 0.906).

**Table (4.55): Association of *INF-γ* gene detection rate with sociodemographic variables.**

| sociodemographic variables | | *INF-γ* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 3 | 50.0 | 3 | 50.0 | 0.270* |
| | 20---29 | 20 | 87.0 | 3 | 13.0 | |
| | 30---39 | 11 | 73.3 | 4 | 26.7 | |
| | ≥ 40 years | 5 | 71.4 | 2 | 28.6 | |
| Sex | Male | 6 | 66.7 | 3 | 33.3 | 0.445* |
| | Female | 33 | 78.6 | 9 | 21.4 | |
| Residence | City | 20 | 71.4 | 8 | 28.6 | 0.349* |
| | Village | 19 | 82.6 | 4 | 17.4 | |
| Home animals | Yes | 15 | 83.3 | 3 | 16.7 | 0.393* |
| | No | 24 | 72.7 | 9 | 27.3 | |
| Consumption of animal products | Yes | 35 | 74.5 | 12 | 25.5 | 0.248* |
| | No | 4 | 100.0 | - | - | |
| Intrafamilial cases | Yes | 17 | 77.3 | 5 | 22.7 | 0.906* |
| | No | 22 | 75.9 | 7 | 24.1 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.16.2. Clinical Variables:

The results found that the *INF-γ* gene detection rate was insignificantly higher among those with old brucella infection compared to those with new infection (80.0% Vs 75.6%), (P= 0.769). Furthermore, *INF-γ* gene was also insignificantly higher among those with fever compared to their counterpart (77.8% Vs 66.7%), (P= 0.547). The *INF-γ* gene detection rate was also insignificantly higher among those with night sweat versus their counterpart (78.0% *Vs* 70.0%). On the contrary, the *INF-γ* gene detection rate was

inversely associated with joint pain (74.4% *Vs* 87.5%), (P= 0.423). Data were shown in table (4.56).

**Table (4.56): Association of *INF-γ* gene detection rate with clinical variables.**

| Clinical variables | | *INF-γ* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 31 | 75.6 | 10 | 24.4 | 0.769* |
| | Old infection | 8 | 80.0 | 2 | 20.0 | |
| Fever | Yes | 35 | 77.8 | 10 | 22.2 | 0.547* |
| | No | 4 | 66.7 | 2 | 33.3 | |
| Night sweat | Yes | 32 | 78.0 | 9 | 22.0 | 0.591* |
| | No | 7 | 70.0 | 3 | 30.0 | |
| Joint pain | Yes | 32 | 74.4 | 11 | 25.6 | 0.423* |
| | No | 7 | 87.5 | 1 | 12.5 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.16.3. Serological Variables:

The *INF-γ* gene detection rate was inversely associated with anti-brucella IgM and IgG (66.7% Vs 81.8%) and (71.0% Vs 85.0%) with insignificant difference (P= 0.223) and (P= 0.249). Similarly, the CD3 and CD4 were also inversely associated with the *INF-γ* gene detection rate (70.0% *Vs* 85.7%) and (75.0% *Vs* 77.8%) respectively with insignificant difference (P= 0.193) and (p= 0. 815). Likewise, the IL-17 and IL-18 were inversely associated with the *INF-γ* gene detection rate (71.9% *Vs* 84.2%) and (68.0% vs 84.6%) with insignificant difference (P= 0.315) and (P= 0. 162) respectively.

**Table (4.57): Association of *INF-γ* gene detection rate with serological variables.**

| Clinical variables | | *INF-γ* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 12 | 66.7 | 6 | 33.3 | 0.223* |
| | Negative | 27 | 81.8 | 6 | 18.2 | |
| IgG | Positive | 22 | 71.0 | 9 | 29.0 | 0.249* |
| | Negative | 17 | 85.0 | 3 | 15.0 | |
| CD3 | Positive | 21 | 70.0 | 9 | 30.0 | 0.193* |
| | Negative | 18 | 85.7 | 3 | 14.3 | |
| CD4 | Positive | 18 | 75.0 | 6 | 25.0 | 0.815* |
| | Negative | 21 | 77.8 | 6 | 22.2 | |
| CD8 | Positive | 38 | 77.6 | 11 | 22.4 | 0.368* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |
| IL-17 | Positive | 23 | 71.9 | 9 | 28.1 | 0.315* |
| | Negative | 16 | 84.2 | 3 | 15.8 | |
| IL-18 | Positive | 17 | 68.0 | 8 | 32.0 | 0.162* |
| | Negative | 22 | 84.6 | 4 | 15.4 | |
| INF-γ | Positive | 38 | 77.6 | 11 | 22.4 | 0.368* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.16.4. Molecular Variables:

The association of *INF-γ* gene detection rate with other gene were shown in table (4.58). It was found that the *INF-γ* gene was detected in 39(97.5%) of those who had detectable *IL-18* gene versus 1(2.5%) who were negative for *INF-γ* gene but positive for *IL-18* gene. The difference was statistically significant (P= 0.0001). Similarly, the *INF-γ* gene was detected in

all (100.0%) of those participants who had detectable *IL-17* gene, while only 6(33.3%) were positive for *INF-γ* gene but negative for *IL-17* gene. The difference was significant (P= 0.0001).

**Table (4.58): Association of *INF-γ* gene detection rate with molecular variables.**

| Molecular variables (PCR) | | *INF-γ* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| *IL-18* gene | Gene detected | 39 | 97.5 | 1 | 2.5 | 0.0001* |
| | Not detected | - | - | 11 | 100.0 | |
| *IL-17* gene | Gene detected | 33 | 100.0 | - | - | 0.0001* |
| | Not detected | 6 | 33.3 | 12 | 66.7 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.17. Association of *IL-18* Gene with Variables:

#### 4.17.1. Sociodemographic Variables:

The *IL-18* gene detection rate was highest (91.3%) among those with 20-29 years old compared to other age groups (P= 0.135). Furthermore, females had higher *IL-18* gene detection rate compared to males (81.0% Vs 66.7%), (P= 0.344). The *IL-18* gene detection rate was insignificantly higher among those who had home animals compared to their counterpart (P= 0.530). On the other hand, The *IL-18* gene detection rate was inversely associated consumption of dairy products and the presence of intrafamilial cluster cases with insignificant difference (P= 0. 275) and (P= 0.861). All results were shown in table (4.59).

**Table (4.59): Association of *IL-18* gene detection rate with sociodemographic variables.**

| sociodemographic variables | | *IL-18* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 3 | 50.0 | 3 | 50.0 | 0.135* |
| | 20---29 | 21 | 91.3 | 2 | 8.7 | |
| | 30---39 | 11 | 73.3 | 4 | 26.7 | |
| | ≥ 40 years | 5 | 71.4 | 2 | 28.6 | |
| Sex | Male | 6 | 66.7 | 3 | 33.3 | 0.344* |
| | Female | 34 | 81.0 | 8 | 19.0 | |
| Residence | City | 21 | 75.0 | 7 | 25.0 | 0.511* |
| | Village | 19 | 82.6 | 4 | 17.4 | |
| Home animals | Yes | 15 | 83.3 | 3 | 16.7 | 0.530* |
| | No | 25 | 75.8 | 8 | 24.2 | |
| Consumption of animal products | Yes | 36 | 76.6 | 11 | 23.4 | 0.275* |
| | No | 4 | 100.0 | - | - | |
| Intrafamilial cases | Yes | 17 | 77.3 | 5 | 22.7 | 0.861* |
| | No | 23 | 79.3 | 6 | 20.7 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.17.2. Clinical Variables:

The *IL-18* gene detection rate was insignificantly higher among those with old brucellosis infection compared to those with new cases (80.0% *Vs* 78.0%), (P= 0.893). The results also found that the *IL-18* gene detection rate was insignificantly higher among those with fever compared to their counterpart (80.0% *Vs* 66.8%), (P= 0. 456). On the contrary, the *IL-18* gene detection rate was inversely associated with night sweat and joint pain (78.0%

Vs 80.0%) and (76.7% *Vs* 87.5%) respectively wit insignificant difference (P= 0.893) and (P= 0.497) respectively. Data were shown in table (4.60).

**Table (4.60): Association of *IL-18 gene* detection rate with clinical variables.**

| Clinical variables | | *IL-18* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Patients history | New infection | 32 | 78.0 | 9 | 22.0 | 0.893* |
| | Old infection | 8 | 80.0 | 2 | 20.0 | |
| Fever | Yes | 36 | 80.0 | 9 | 20.0 | 0.456* |
| | No | 4 | 66.7 | 2 | 33.3 | |
| Night sweat | Yes | 32 | 78.0 | 9 | 22.0 | 0.893* |
| | No | 8 | 80.0 | 2 | 20.0 | |
| Joint pain | Yes | 33 | 76.7 | 10 | 23.3 | 0.497* |
| | No | 7 | 87.5 | 1 | 12.5 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.17.3. Serological Variables:

The results in table (4.61) found that association of IL-18 gene with anti-brucella IgM and IgG (66% vs 84.8%) and (74.2% vs 85%) with insignificant difference (P =0.131 and 0.360). similarly, the CD3 and CD4 were also insignificant association (P= 0.290 and 0.904). furthermore , all result to other variables including CD8, IL-17, IL-18, INF-γ were shown in table (4.61).

**Table (4.61): Association of *IL-18* gene detection rate with serological variables.**

| Serological variable | | *IL-18* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 12 | 66.7 | 6 | 33.3 | 0.131* |
| | Negative | 28 | 84.8 | 5 | 15.2 | |
| IgG | Positive | 23 | 74.2 | 8 | 25.8 | 0.360* |
| | Negative | 17 | 85.0 | 3 | 15.0 | |
| CD3 | Positive | 22 | 73.3 | 8 | 26.7 | 0.290* |
| | Negative | 18 | 85.7 | 3 | 14.3 | |
| CD4 | Positive | 19 | 79.2 | 5 | 20.8 | 0.904* |
| | Negative | 21 | 77.8 | 6 | 22.2 | |
| CD8 | Positive | 39 | 79.6 | 10 | 20.4 | 0.319* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |
| IL-17 | Positive | 24 | 75.0 | 8 | 25.0 | 0.439* |
| | Negative | 16 | 84.2 | 3 | 15.8 | |
| IL-18 | Positive | 18 | 72.0 | 7 | 28.0 | 0.274* |
| | Negative | 22 | 84.6 | 4 | 15.4 | |
| INF-γ | Positive | 39 | 79.6 | 10 | 20.4 | 0.319* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.17.4. Molecular Variables:

The results in table (4.62) found that the *IL-18* gene was detected in all those who had detectable INF-γ gene against 1(8.3%) who had detectable *IL-18* gene but had none detectable *INF*-γ gene. The difference was statistically significant (P= 0.0001). Likewise, the *IL-18* gene was detected in all those

who had detectable *IL-17* gene versus 7(38.9%). The difference was also significant (P= 0.0001).

**Table (4.62): Association of *IL-18* gene detection rate with molecular variables.**

| Molecular variables (PCR) | | *IL-18* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| *INF*-γ gene | Gene detected | 39 | 100.0 | - | - | 0.0001* |
| | Not detected | 1 | 8.3 | 11 | 91.7 | |
| *IL-17*gene | Gene detected | 33 | 100.0 | - | - | 0.0001* |
| | Not detected | 7 | 38.9 | 11 | 61.1 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.18. Association of *IL-17* Gene with Variables:

### 4.18.1. Sociodemographic Variables:

The *IL-17* gene detection rate was highest among those with 30-39 years old compared to other age groups (73.3%), but the difference was insignificant (P= 0.719). Males had insignificantly higher rate of *IL-17* gene detection compared to females (P= 0.892). The consumption of dairy products has insignificant association with *IL-17* gene detection rate (63.8% *Vs* 75.0%), (P= 0.654).

**Table (4.63): Association of *IL-17* gene detection rate with sociodemographic variables.**

| sociodemographic variables | | *IL-17* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| Age | < 20 years | 3 | 50.0 | 3 | 50.0 | 0.719* |
| | 20---29 | 14 | 60.9 | 9 | 39.1 | |
| | 30---39 | 11 | 73.3 | 4 | 26.7 | |
| | ≥ 40 years | 5 | 71.4 | 2 | 28.6 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Sex** | Male | 6 | 66.7 | 3 | 33.3 | 0.892* |
| | Female | 27 | 64.3 | 15 | 35.7 | |
| **Residence** | City | 18 | 64.3 | 10 | 35.7 | 0.945* |
| | Village | 15 | 65.2 | 8 | 34.8 | |
| **Home animals** | Yes | 12 | 66.7 | 6 | 33.3 | 0.829* |
| | No | 21 | 63.6 | 12 | 36.4 | |
| **Consumption of animal products** | Yes | 30 | 63.8 | 17 | 36.2 | 0.654* |
| | No | 3 | 75.0 | 1 | 25.0 | |
| **Intrafamilial cases** | Yes | 14 | 63.6 | 8 | 36.4 | 0.889* |
| | No | 19 | 65.5 | 10 | 34.5 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.18.2. Clinical Variables:

The *IL-17* gene detection rate was higher among those with old brucellosis infection compared to those with new brucellosis cases (70.0% *Vs* 63.4%). The difference was insignificant (P= 0. 696). Similarly, the *IL-17* gene detection was insignificantly higher among those with fever compared to those without fever (68.9% *Vs* 33.3%); However, the difference was failed to reach the level of significance (P= 0.087). Additionally, the *IL-17* gene detection rate was insignificantly associated with both night sweat and joint pain (P= 0. 278) and (P= 0.507) respectively.

**Table (4.64): Association of *IL-17 gene* detection rate with clinical variables.**

| **Clinical variables** | | ***IL-17* gene** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| Patients history | New infection | 26 | 63.4 | 15 | 36.6 | 0.696* |
| | Old infection | 7 | 70.0 | 3 | 30.0 | |
| Fever | Yes | 31 | 68.9 | 14 | 31.1 | 0.087* |
| | No | 2 | 33.3 | 4 | 66.7 | |
| Night sweat | Yes | 28 | 68.3 | 13 | 31.7 | 0.278* |
| | No | 5 | 50.0 | 5 | 50.0 | |
| Joint pain | Yes | 27 | 62.8 | 16 | 37.2 | 0.507* |
| | No | 6 | 75.0 | 2 | 25.0 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.18.3. Serological Variables:

The IL-17 gene detection rate was insignificantly higher in those with anti-brucella IgM negative compared to those with IgM positive (66.7% *Vs* 61.1%), (P= 0.692). Similarly, the *IL-17* gene detection rate was inversely associated with anti-brucella IgG with insignificant difference (70.0% *Vs* 61.3), (P=0. 525). The CD3 and CD8 were also insignificantly associated with the *IL-17* gene detection rate (P= 0.726) and (P= 0.657). Additionally, the *IL-17* gene detection rate was higher among those with negative IL-18 compared to those with positive counterpart (76.9% *Vs* 52.0%), but the difference was failed to reach the level of statistical significance (P= 0.063). Data were presented in table (4.65).

**Table (4.65): Association of *IL-17* gene detection rate with serological variables.**

| Serological variables | | *IL-17* gene | | | | P value |
|---|---|---|---|---|---|---|
| | | Positive | | Negative | | |
| | | No | % | No | % | |
| IgM | Positive | 11 | 61.1 | 7 | 38.9 | 0.692* |
| | Negative | 22 | 66.7 | 11 | 33.3 | |
| IgG | Positive | 19 | 61.3 | 12 | 38.7 | 0.525* |
| | Negative | 14 | 70.0 | 6 | 30.0 | |
| CD3 | Positive | 20 | 66.7 | 10 | 33.3 | 0.726* |
| | Negative | 13 | 61.9 | 8 | 38.1 | |
| CD4 | Positive | 13 | 54.2 | 11 | 45.8 | 0.138* |
| | Negative | 20 | 74.1 | 7 | 25.9 | |
| CD8 | Positive | 32 | 65.3 | 17 | 34.7 | 0.657* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| IL-17 | Positive | 19 | 59.4 | 13 | 40.6 | 0.301* |
| | Negative | 14 | 73.7 | 5 | 26.3 | |
| IL-18 | Positive | 13 | 52.0 | 12 | 48.0 | 0.063* |
| | Negative | 20 | 76.9 | 6 | 23.1 | |
| INF-γ | Positive | 32 | 65.3 | 17 | 34.7 | 0.657* |
| | Negative | 1 | 50.0 | 1 | 50.0 | |

*Insignificant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

### 4.18.4. Molecular Variables:

The results in table (4.66) revealed that the *Il-17* gene detection rate was higher among those who has detectable *INF*-γ gene compared to those who are *Il-17* gene negative (84.6% *Vs* 15.4%). The difference was statistically significant (P= 0.0001). Similarly, the *IL-17* gene detection rate was higher among those who had detectable *IL-18* gene against those who were negative for *IL-17* gene detection (82.5% *Vs* 17.5%) with significant difference (P= 0.0001).

**Table (4.66): Association of *IL-17* gene detection rate with molecular variables.**

| **Molecular variables (PCR)** | | ***IL-17* gene** | | | | P value |
|---|---|---|---|---|---|---|
| | | **Positive** | | **Negative** | | |
| | | **No** | **%** | **No** | **%** | |
| *INF*-γ gene | Gene detected | 33 | 84.6 | 6 | 15.4 | 0.0001* |
| | Not detected | - | - | 12 | 100.0 | |
| *IL-18gene* | Gene detected | 33 | 82.5 | 7 | 17.5 | 0.0001* |
| | Not detected | - | - | 11 | 100.0 | |

*Significant difference between percentages using Pearson Chi-square test ($\chi^2$-test) at 0.05 level.

## 4.19.Sequencing Results:

### 4.19.1. Sequencing of the 1075 bp Region within the IL-17A Gene:

In the present study, the *IL-17A* genetic sequences in chromosome no. 6 was analyzed in 51 samples. This gene encodes for IL-17A protein, which is involved in several activities related to connecting T cell-mediated adaptive immunity and acute inflammatory response to destroy extracellular bacteria and fungi (https://www.uniprot.org/uniprot/Q16552). The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Concerning the 1075 bp amplicons, the NCBI BLASTn engine shown about 99 % sequence similarities between the sequenced samples and the intended reference target sequences, which partially cover the 5-(untranslated region) UTR, the entire exon 1, and the upstream portion of intron 1 of this gene. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NG_033021.1), the accurate positions and other details of the retrieved PCR fragments were identified Figure (4.37).

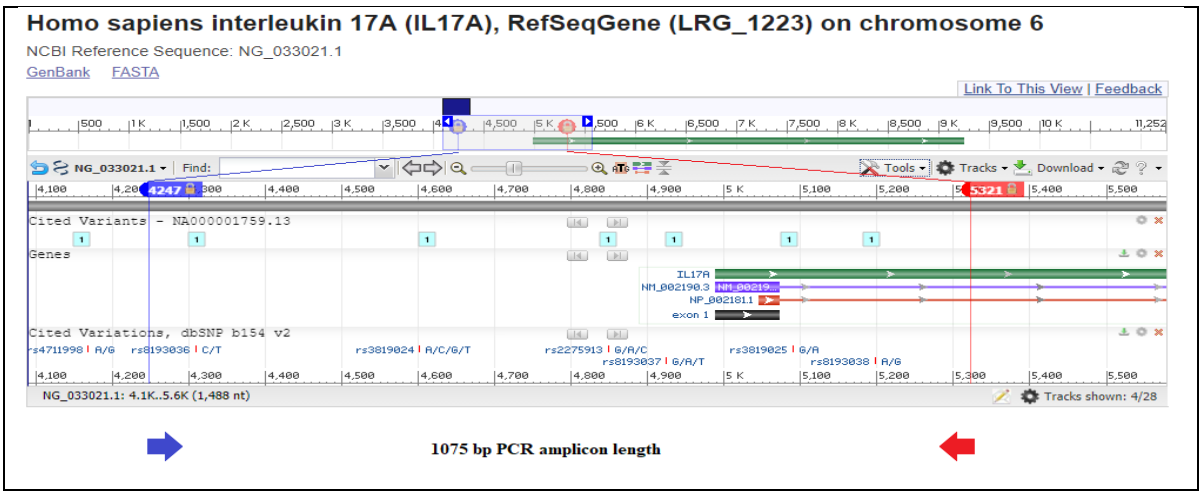



**Figure (4.37): The exact position of the retrieved 1075 bp amplicons that covered a portion of the 5′-UTR, exon 1, and a portion of intron 1 of the *ILA17* gene within chromosome no. 6 (GenBank acc. no. NG_033021.1). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.**

After positioning the 1075 bp amplicons' sequences within chromosome no. 6, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 1075 bp amplified amplicons, Table (4.67).

**Table (4.67): The position and length of the 1075 bp PCR amplicons used to amplify a portion of the 5′-UTR, exon 1, and a portion of intron 1 of the *ILA17* gene located within chromosome no. 6 (GenBank acc. no. NG_033021.1). The gray-colored sequences referred to the position of the reverse and forward primers, respectively.**

| Amplicon | Reference locus sequences (5′ - 3′) | length |
|---|---|---|
| DNA sequences within the *IL-17* gene | *GTCTCCTCTCCTTTCTAGTTCTCATCACTCTCTACTCCCCCCTGCCCCCCTTTTCTC<br>CATCTCCATCACCTTTGTCCAGTCTCTATCCCCATTTTCAATTCCTTCCTCAAAACAC<br>CAAGTTGCTTGGTAGCATGCAGGGTTGGAACATGCCTTTAACAGAAAATCTCGTGTCT<br>CTTGAACCTAGTTATTTATTCCTTGAGCAGAGTAGATATTCAACAAAAGAATTGTTAA<br>ATTCAATTAAATAGGATATATCTTATTATTAAATATTTTTTTCATTTTTTGTTTACTT<br>ATATGATGGGAACTTGAGTAGTTTCCGGAATTGTCTCCACAACACCTGGCCAAGGAAT<br>CTGTGAGGAAAAGAAAGATCAAATGGAAAATCAAGGTACATGACACCAGAAGACCTAC<br>ATGTTACTTCAAACTTTTTCTTCCTCATGAACCATTAAAATAGAGCATAACTCTTCTG<br>GCAGCTGTACATATGTTCATAAATACATGATATTGACCCATAGCATAGCAGCTCTGCT<br>CAGCTTCTAACAAGTAAGAATGAAAAGAGGACATGGTCTTTAGGAACATGAATTTCTG<br>CCCTTCCCATTTTCCTTCAGAAGGAGAGATTCTTCTATGACCTCATTGGGGGCGGAAA<br>TTTTAACCAAAATGGTGTCACCCCTGAACCCACTGCGACACGCCACGTAAGTGACCAC<br>AGAAGGAGAAAAGCCCTATAAAAAGAGAGACGATAGCGCTACATTTTGTCCATCTCAT<br>AGCAGGCACAAACTCATCCATCCCCAGTTGATTGGAAGAAACAACGATGACTCCTGGG<br>AAGACCTCATTGGTGGTGAGTCCTGCACTAACGTGCGATGCTCTTGCTGATTTGGACC<br>AGATAGTATTTCTGGACCGTGGGCATGAAACGCTGGGTTCTGACTATGGAGATCCAGG<br>AATACTGTATATGTAGGATAGGAAATGAAAGCTTTGGTAGGTATTTAAGTCATTGTGC<br>AGCATTTTCAAGAACTGATACACAGCAGTTTGAAAGATAAGATTAAAACTGAAAGATA<br>GCTATATTGGGGCTAAACCACACAAGAAGTGT** | 1075 bp |

* refers to the forward primer sequences (placed in a forward complement direction)

** refers to the reverse primer sequences (placed in a reverse complement direction)

The alignment results of the 1075 bp samples revealed the presence of seven variations in some of the analyzed samples in comparison with the referring reference DNA sequences, figure (4.38) was shown in appendix III.

The sequencing chromatogram of the identified variation regions, as well as their detailed annotations, were documented, and the chromatograms of these sequences were shown according to their positions in the PCR amplicons, figure (4.39).

Highly interesting nucleic acid polymorphisms (SNPs) were detected in this study in the investigated samples, in which seven were replaced with other nucleotides at positions 215, 219, 341, 356, 537, 603, and 679. Two of these SNPs were detected in three polymorphic patterns. In the case of A356G, a homozygous A/A status was observed in C10, C11, C25, C32, S4, S7, S10, S11, S18, S19, S20, S26, S27, S29, S30, S35, S37, S38, S40, S41, S43, S48, S53, and S55, heterozygous A/G pattern in C5, C15, S5, S14, S36, S38, S44, S47, and S49, and homozygous G/G pattern in C20, S3, S6, S15, S16, S25, and S45.

Likewise, three polymorphic patterns were also observed in the case of A603G; a homozygous A/A status in C20, S3, S6, S16, and S25, a heterozygous A/G status in C5, C15, S5, S10, S14, S15, S19, S36, S38, S41, S44, S45, S47, and S49, and a homozygous G/G status in C10, C11, C24, C32, S4, S7, S11, S18, S20, S26, S27, S29, S30, S35, S37, S40, S43, S46, S48, S53, and S55. Meanwhile, the other five observed nucleic acid substitutions exhibited only two polymorphic forms; a wild type homozygous form and a mutant heterozygous form. These mutations were A215T, A219C, A341G, A537C, and G679A.

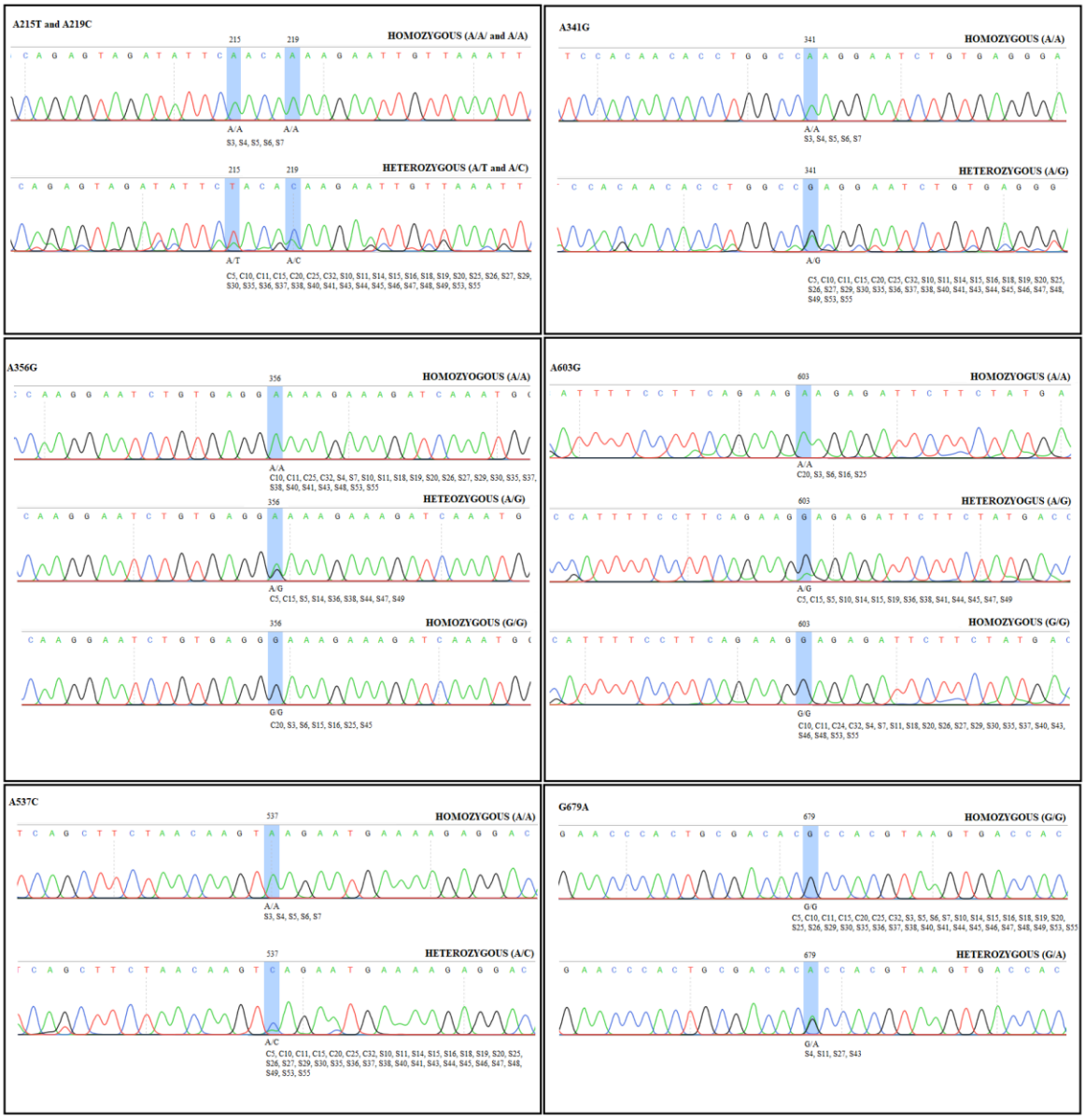



**Figure (4.39): The pattern of the detected single nucleotide polymorphisms (SNPs) within the DNA chromatogram of the targeted 1075 bp amplicons of the *ILA17* gene. The identified SNPs were highlighted according to their position in the PCR amplicons. The letters S# and C# refer to the sample and control codes respectively.**

The reason behind the absence of the mutant homozygous form may be associated with the low number of the investigated samples. Alternatively, the absence of this homozygous form may also be attributed to the low frequency of the observed SNPs in the investigated samples. Thus, it is mandatory to

explore further details of these SNPs in their original positions in the genomic DNA sequences of the IL-17A gene deposited in the dbSNP database.

To elucidate the positions of the targeted SNPs concerning their deposited SNP database of the sequenced 1075 bp fragments, the corresponding positions of the *IL-17A* gene were retrieved from the dbSNP server (https://www.ncbi.nlm.nih.gov/projects/SNP/) . To check the possible novelty of each observed SNP, graphical representations were performed concerning the *IL-17A* dbSNP database within chromosome no. 6 using the GenBank Acc. No. NC_000006.12.

By reviewing the dbSNP engine, it was found that four of the detected SNPs were previously known as they were deposited as rs1582236522, rs3819024, rs2275913, and rs8193037 for A341G, A356G, A603G, and G679A respectively (Figure 4.39). The observed rs1582236522 SNP is highly recognized in the targeted *IL-17A* gene due to its high frequency (0.5) recorded (https://www.ncbi.nlm.nih.gov/snp/rs1582236522) . However, this SNP was not reported in any publications indexed in the dbSNP. Likewise, rs3819024 SNP was also deposited in high frequency (0.33) in the dbSNP (https://www.ncbi.nlm.nih.gov/snp/rs3819024) . As well, it was also reported in the literature (https://www.ncbi.nlm.nih.gov/snp/rs3819024#publications). In addition to both rs1582236522 and rs3819024, the rs2275913 SNP was also deposited in considerable frequency in the dbSNP, which ranged from 0.2 to 0.34 (https://www.ncbi.nlm.nih.gov/snp/rs2275913). Compared with rs3819024, the rs2275913 SNP is more intensively reported in the literature, which entailed more potential importance for its observation in the *IL-17A* gene than the other observed polymorphisms (https://www.ncbi.nlm.nih.gov/snp/rs2275913#publications) . In contrast to the previous three observed SNPs, the rs8193037 SNP was deposited in very

low frequency in the dbSNP, which ranged between 0.015 to 0.020 (https://www.ncbi.nlm.nih.gov/snp/rs8193037) . Despite its low frequency, this SNP was also reported in the literature (https://www.ncbi.nlm.nih.gov/snp/rs8193037#publications) .

By reviewing the same dbSNP records, it was found that three of our observed SNPs (A215T, A219C, and A537C) were not deposited previously in the dbSNP, so their novel nucleic acid substitutions were confirmed and validated.

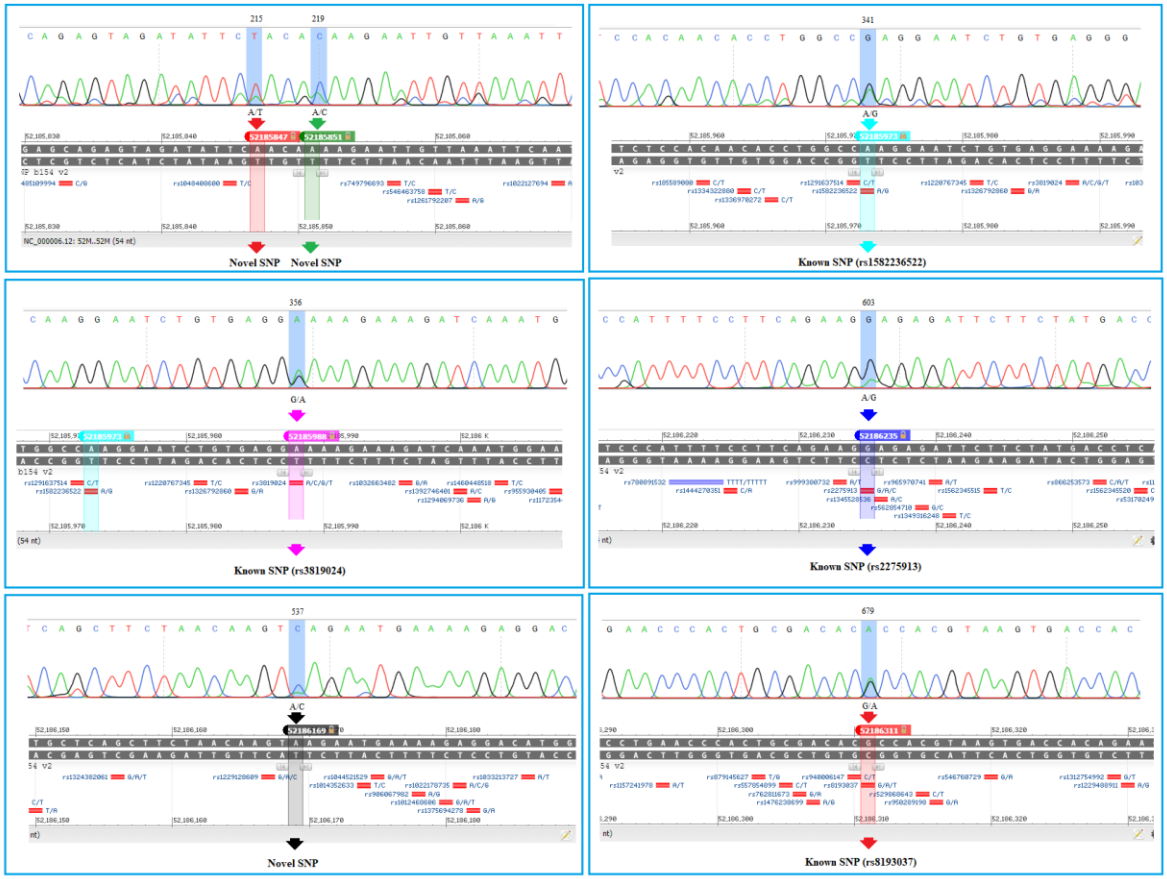



**Figure (4.40): The SNP's novelty checking of *IL-17A* genetic single nucleotides polymorphisms using the dbSNP server. The identified SNPs were marked with different colors. The GenBank acc. no. NC_000006.12 was used in the positioning of the highlighted substitution SNPs. The positions of the targeted sequences were found in the positive strand.**

To summarize the results obtained from the sequenced 1075 bp fragments, the exact positions of the observed variations were described in the NCBI reference sequences Table (4.68).

**Table (4.68): The patterns of the observed SNPs in the 1075 bp amplicons that are designed to amplify a portion of the 5′-UTR, the entire exon 1, and the upstream portion of intron 1 within the *ILA17* gene in comparison with the NCBI referring sequences (GenBank acc. no. NC_000006.12). The symbol "C" refers to the control samples, and the symbol "S" refers to the sample number.**

| Sample No. | Zygosity status | Position in the PCR fragment | Position in the reference genome | SNP type | SNP summary |
|---|---|---|---|---|---|
| **S3, S4, S5, S6, S7** | Homozygous (A/A) | 215 | 52185847 | Upstream variant (5′-UTR) | Wild type |
| **C5, C10, C11, C15, C20, C25, C32, S10, S11, S14, S15, S16, S18, S19, S20, S25, S26, S27, S29, S30, S35, S36, S37, S38, S40, S41, S43, S44, S45, S46, S47, S48, S49, S53, S55** | Heterozygous (A/T) | 215 | 52185847 | Upstream variant (5′-UTR) | Novel SNP (A215T) |
| **S3, S4, S5, S6, S7** | Homozygous (A/A) | 219 | 52185851 | Upstream variant (5′-UTR) | Wild type |
| **C5, C10, C11, C15, C20, C25, C32, S10, S11, S14, S15, S16, S18, S19, S20, S25, S26, S27, S29, S30, S35, S36, S37, S38, S40, S41, S43, S44, S45, S46, S47, S48, S49, S53, S55** | Heterozygous (A/C) | 219 | 52185851 | Upstream variant (5′-UTR) | Novel SNP (A219C) |
| **S3, S4, S5, S6, S7** | Homozygous (A/A) | 341 | 52185973 | Upstream variant (5′-UTR) | Wild type |
| **C5, C10, C11, C15, C20, C25, C32, S10, S11, S14, S15, S16, S18, S19, S20, S25, S26, S27, S29, S30, S35, S36, S37, S38, S40, S41, S43, S44, S45, S46, S47, S48, S49, S53, S55** | Heterozygous (A/G) | 341 | 52185973 | Upstream variant (5′-UTR) | rs1582236522 |
| **C10, C11, C25, C32, S4, S7, S10, S11, S18, S19, S20, S26, S27, S29, S30, S35, S37, S38, S40, S41, S43, S48, S53, S55** | Homozygous (A/A) | 356 | 52185988 | Upstream variant (5′-UTR) | Wild type |
| **C5, C15, S5, S14, S36, S38, S44, S47, S49** | Heterozygous (A/G) | 356 | 52185988 | Upstream variant (5′-UTR) | rs3819024 |
| **C20, S3, S6, S15, S16, S25, S45** | Homozygous (G/G) | 356 | 52185988 | Upstream variant (5′-UTR) | rs3819024 |

| | | | | | |
|---|---|---|---|---|---|
| **S3, S4, S5, S6, S7** | Homozygous (A/A) | 537 | 52186169 | Upstream variant (5′-UTR) | Wild type |
| **C5, C10, C11, C15, C20, C25, C32, S10, S11, S14, S15, S16, S18, S19, S20, S25, S26, S27, S29, S30, S35, S36, S37, S38, S40, S41, S43, S44, S45, S46, S47, S48, S49, S53, S55** | Heterozygous (A/C) | 537 | 52186169 | Upstream variant (5′-UTR) | Novel SNP (A537C) |
| **C20, S3, S6, S16, S25** | Homozygous (A/A) | 603 | 52186235 | Upstream variant (5′-UTR) | Wild type |
| **C5, C15, S5, S10, S14, S15, S19, S36, S38, S41, S44, S45, S47, S49** | Heterozygous (A/G) | 603 | 52186235 | Upstream variant (5′-UTR) | rs2275913 |
| **C10, C11, C24, C32, S4, S7, S11, S18, S20, S26, S27, S29, S30, S35, S37, S40, S43, S46, S48, S53, S55** | Homozygous (G/G) | 603 | 52186235 | Upstream variant (5′-UTR) | rs2275913 |
| **C5, C10, C11, C15, C20, C25, C32, S3, S5, S6, S7, S10, S14, S15, S16, S18, S19, S20, S25, S26, S29, S30, S35, S36, S37, S38, S40, S41, S44, S45, S46, S47, S48, S49, S53, S55** | Homozygous (G/G) | 679 | 52186311 | Upstream variant (5′-UTR) | Wild type |
| **S4, S11, S27, S43** | Heterozygous (G/A) | 679 | 52186311 | Upstream variant (5′-UTR) | rs8193037 |

In the studied 1075 bp *IL-17A* amplicons, it was found that the total number of retrieved variants was seven, namely A215T, A219C, A341G (rs1582236522), A356G (rs3819024), A537C, A603G (rs2275913), and G679A (rs8193037). Thus, it is mandatory to explore the potential for their co-inheritance among them in all investigated samples to unravel their possible contribution in the analyzed *IL17A* fragments. After SNPs association data were retrieved, they were plotted collectively. To investigate the average linkage disequilibrium (LD) panel, significant LD values were plotted against the genetic distance between markers. The calculations of the pairwise LD values for these tag-SNPs are shown in Figure (4.41).

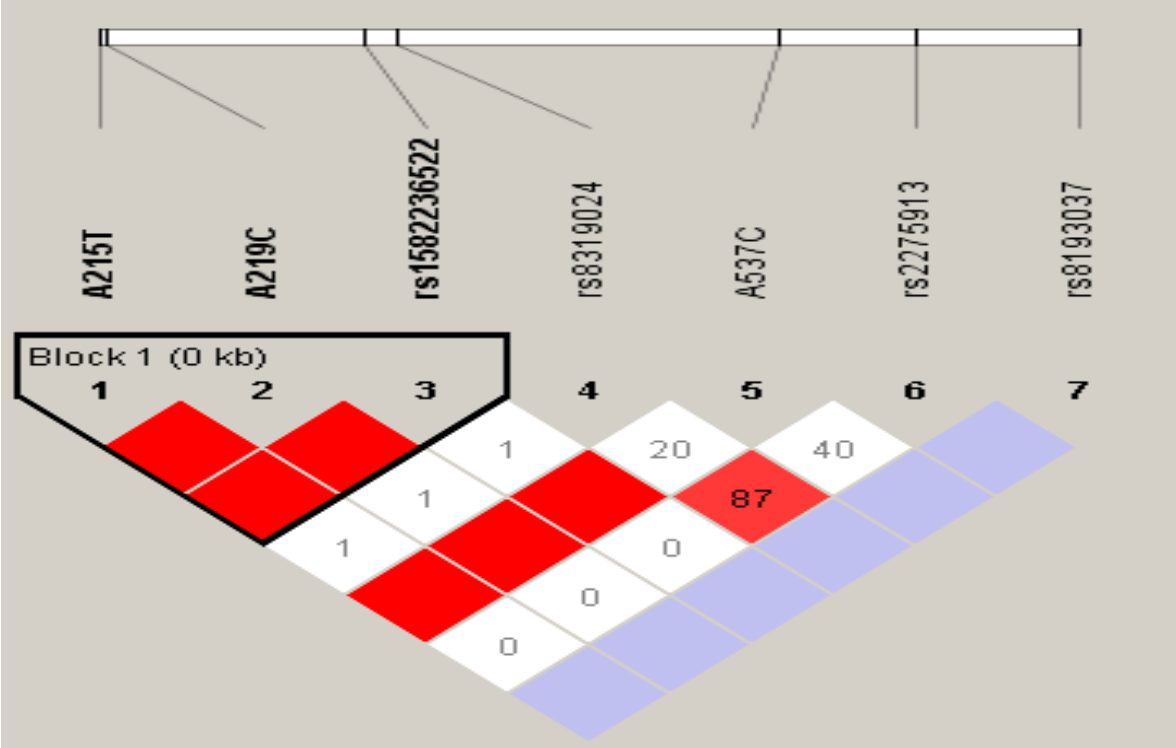



**Figure (4.41): Linkage disequilibrium graphical representation among all possible pairs of seven variants observed in the amplified 1075 bp fragment of the *IL-17A* gene using standard D'/LOD Color Scheme. LD values are expressed as differences in the shade of each color. The red color refers to high LD. The numbers given at the top of the figure indicate the coordinate of sites in the 1075 bp amplicons**.

In the center of the plot, the identity of each SNP was mentioned, and all LD values that are contained within both upstream and downstream of the observed SNPs were shown. LD values between any two variants in these plots are graphically displayed using standard colored triangles varying from white (low LD values) to red (high LD values). The graphical plotting of the uploaded SNPs indicated a tight connection of the first three observed SNPs with each other, namely A215T, A219C, and rs1582236522. Whilst, the other observed four SNPs were not connected at all or not connected to that extent. This observation of high LD values among the first three observed SNPs could be explained by the ability of these SNPs to be transferred as one block with each other and the effect on the development and progression of brucellosis.

This observation was also confirmed by statistically significant increase in! L-17 detection rate among brucellosis patients compared to healthy control. These results indicated a significant association between these SNPs and the development and intensity of brucellosis among Iraqi population.

### 4.19.2. Sequencing of the 776 bp Region within the *IL-18* Gene:

In the present study, the *IL18* genetic sequences in chromosome no. 11. This gene encodes for interleukin 18 protein, which is involved in several activities related to polarized T-helper 1 (Th1) cell and natural killer (NK) cell immune responses (https://www.uniprot.org/uniprot/Q14116) . The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) . Concerning the 776 bp amplicons, the NCBI BLASTn engine shown about 99 % sequence similarities between the sequenced samples and the intended reference target sequences, which partially cover the intron 1 and a portion of exon 2 of this gene. By comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences (GenBank acc. NG_028143.1), the accurate positions and other details of the retrieved PCR fragments were identified figure (4.42).

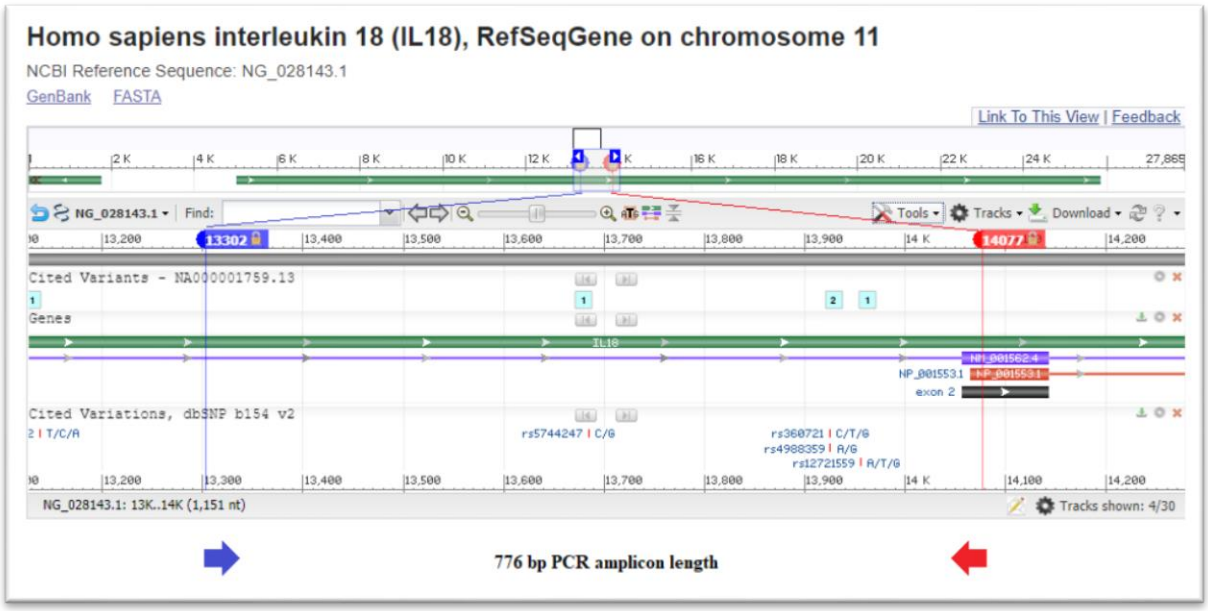



**Figure (4.42): The exact position of the retrieved 776 bp amplicon that partially cover the intron 1 and a portion of exon 2 of the *IL-18* gene within chromosome no. 11 (GenBank acc. no. NG_028143.1). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint**

After positioning the 776 bp amplicons' sequences within chromosome no. 11, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 776 bp amplified amplicons, table (4.69).

**Table (4.69): The position and length of the 776 bp PCR amplicons used to amplify the downstream portion of the intron 1 and the upstream portion of exon 2 of the *IL-18* gene located within chromosome no. 11 (GenBank acc. no. NG_028143.1). The gray-colored sequences referred to the position of the reverse and forward primers, respectively**.

| Amplicon | Reference locus sequences (5′ - 3′) | Length |
|---|---|---|
| DNA sequences within the *IL-18* gene | *CTCACACTGAGAACTCCTATTCCATAACAAAATTCTGGAAGCCTGCACACC<br>GTATTGGAAGAGGGGCAGAAAGGAAAAGCAAATGGAAGGATTTAAATTTTTT<br>TCAAATCCTGTATCCCTTGATTTTACAGCAAGATTGTATTTATGTATTACTT<br>GTGTTAAAAATATAGTATAATCGAGACTCCAGATCAAAAATCACCGCAGCTC<br>AGGGAGAAAGAGGGCCACCAAATGCCAGAGCCCTTCAGCCTTCTCCCACCCT<br>GCCTGTACCCTCAGATGGAAGCACTTTTTTATCATTGTTTCACCTTTAGCAT<br>TTTGACAATGAAGTCACAAACCTTCAGCCTCTCACCCATAGGAACCCACTGG<br>TTGTAAGAGAAGGATGAAGCCAGTCCTTCCTAAAGGGCACGATTAGATGTGT<br>TTATGGCATCCTCAGGTGAAACTATATTTATATTGACAATATATTTATATTT<br>CTCAAGGAATACTAGAATAATGATTCAGTTCAGTACTAGGCCATTTATCTAC<br>CCTTTATAATATTGTTTAATGAGAAAATGCTTTCTATCTTCCAAATATCTGA<br>TGATTTGTAAGAGAACACTTAAACATGGGTATTCATAAGCTGAAACTTCTGG<br>CATTTATTGAATGTCAAGATTGTTCATCAGTATACTAGGTGATTAACTGACC<br>ACTGAACTTGAAGGTAGTATAAAGTAGTAGTAAAAGGTACAATCATTGTCTC<br>TTAACAGATGGCTCTTTGCTTTCATTAGGAATAAAGATGGCTGCTGAAC<br>** | 776 bp |

* refers to the forward primer sequences (placed in a forward complement direction)

** refers to the reverse primer (sequences (placed in a reverse complement direction)

The alignment results of the 776 bp samples revealed the presence of three variations in some of the analyzed samples in comparison with the referring reference DNA sequences, figure (4.43) was shown in **appendix III**.

The sequencing chromatogram of the identified variation regions, as well as their detailed annotations, were documented, and the chromatograms of these sequences were shown according to their positions in the PCR amplicons, figure (4.44).

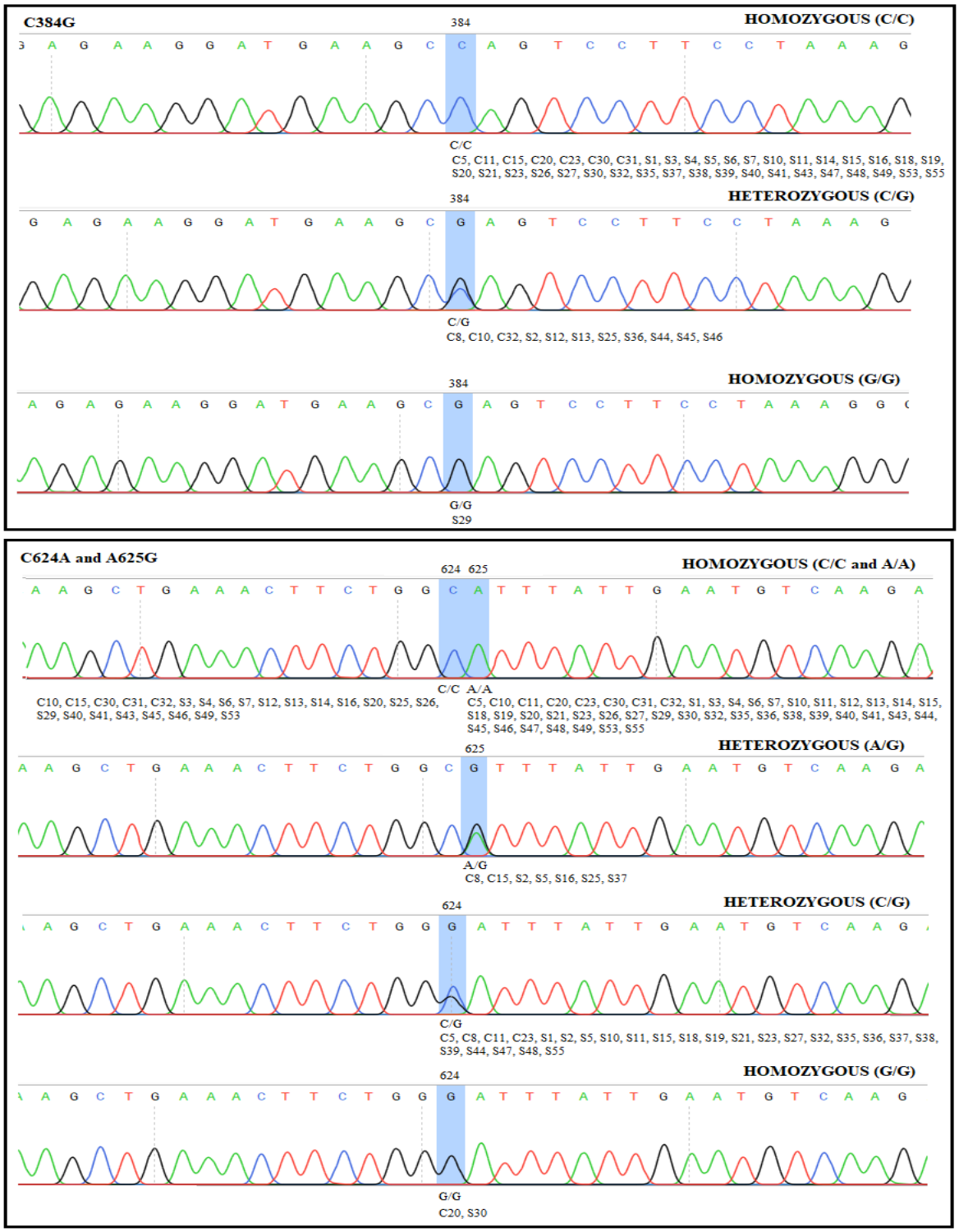



**Figure (4.44): The pattern of the detected C384G, C624A, and A625G SNPs within the DNA chromatogram of the targeted 776 bp amplicons of the downstream portion of the intron 1 and the upstream portion of exon 2 the *IL-18* gene. The identified SNPs were highlighted according to their position in the PCR amplicons. The letter S# refers to the sample code, while the letter "C" refers to the controls.**

Highly interesting SNPs were detected in this study in the investigated samples, in which cytosine was replaced with guanine at position 384 (C384G), cytosine was replaced with adenine at 624 (C624A), and adenine was replaced with guanine at position 625 (A625G). Concerning C384G, three patterns of polymorphisms were detected in this locus, homozygous C/C pattern in C5, C11, C15, C20, C23, C30, C31, S1, S3, S4, S5, S6, S7, S10, S11, S14, S15, S16, S18, S19, S20, S21, S23, S26, S27, S30, S32, S35, S37, S38, S39, S40, S41, S43, S47, S48, S49, S53, and S55, heterozygous C/G pattern in C8, C10, C32, S2, S12, S13, S25, S36, S44, S45, and S46, and homozygous G/G pattern in S29. Likewise, C624A has also exhibited three polymorphic patterns in this locus, homozygous C/C pattern in C10, C15, C30, C31, C32, S3, S4, S6, S7, S12, S13, S14, S16, S20, S25, S26, S29, S40, S41, S43, S45, S46, S49, and S53, heterozygous C/A pattern in C5, C8, C11, C23, S1, S2, S5, S10, S11, S15, S18, S19, S21, S23, S27, S32, S35, S36, S37, S38, S39, S44, S47, S48, and S55, and homozygous A/A pattern in C20 and S30. In contrast to both C384G and C624A, only two polymorphic forms were observed in the case of A625G SNP as it was observed in C5, C10, C11, C20, C23, C30, C31, C32, S1, S3, S4, S6, S7, S10, S11, S12, S13, S14, S15, S18, S19, S20, S21, S23, S26, S27, S29, S30, S32, S35, S36, S38, S39, S40, S41, S43, S44, S45, S46, S47, S48, S49, S53, and S55, a wild type homozygous (A/A) form, and a mutant heterozygous (A/G) form as it was observed in C8, C15, S2, S5, S16, S25, and S37.

To elucidate the positions of the targeted SNPs concerning their deposited SNP database of the sequenced 776 bp fragments, the corresponding positions of the *IL18* gene were retrieved from the dbSNP server. To check the possible novelty of each observed SNP, graphical representations were performed concerning the *IL18* dbSNP database within chromosome no. 11

using the GenBank Acc. No. NC_000011.10. By reviewing the dbSNP engine, it was found that the three detected SNPs (C384G, C624G, and A625G) were previously known as it was deposited as rs5744247, rs360721, and rs4988359 respectively, figure (4.45).

Concerning the rs5744247 SNP, relatively low frequencies that were ranged from 0.09 to 0.1 were reported in the dbSNP (https://www.ncbi.nlm.nih.gov/snp/rs5744247) . However, this SNP was described in several publications that concerned with *IL-18*-associated metabolic issues (https://www.ncbi.nlm.nih.gov/snp/rs5744247#publications) .

In comparison to rs5744247, a higher frequency (0.22 to 0.27) was reported for the rs360721 SNP in the dbSNP database. Accordingly, it was not unusual to observe this SNP in three polymorphic forms in the currently investigated samples. As in the case of the rs5744247 SNP, this SNP was also reported in the literature (https://www.ncbi.nlm.nih.gov/snp/rs360721#publications) . In contrast to the adjacent rs5744247 SNP, lower frequencies that ranged from 0.07 to 0.16 of the rs4988359 SNP were reported in the dbSNP (https://www.ncbi.nlm.nih.gov/snp/rs4988359). For this reason, only two polymorphic patterns were reported for this SNP in the current study. However, this SNP was also described in a few publications in the literature (https://www.ncbi.nlm.nih.gov/snp/rs4988359#publications).

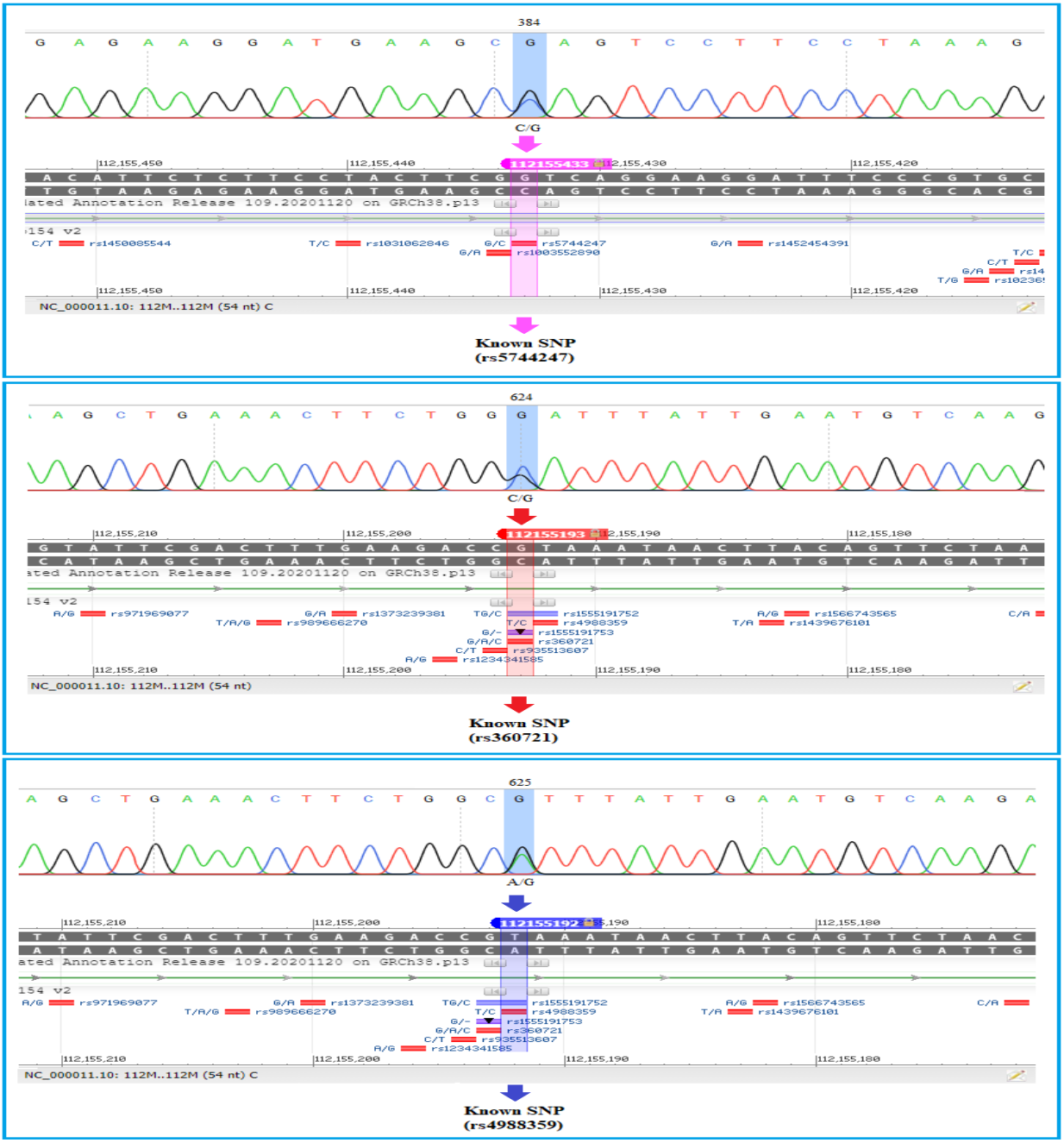



**Figure (4.45): The SNP's novelty checking of *IL-18* genetic single nucleotides polymorphisms using the dbSNP server. A) The identified SNPs were marked with different colors. The GenBank acc. no. NC_000011.10 was used in the positioning of the highlighted substitution SNPs. The positions of the targeted sequences were found in the negative strand.**

To summarize the results obtained from the sequenced 776 bp fragments, the exact positions of the observed variations were described in the NCBI reference sequences Table (4.70).

**Table (4.70): The patterns of the observed SNPs in the 776 bp amplicons that are designed to amplify the downstream portion of the intron 1 and the upstream portion of exon 2 within the *IL-18* gene in comparison with the NCBI referring sequences (GenBank acc. no. NC_000011.10). The symbol "C" and "S" refer to the samples and controls respectively.**

| Sample No. | Zygosity status | Position in the PCR fragment | Position in the reference genome | SNP type | SNP summary |
|---|---|---|---|---|---|
| **C5, C11, C15, C20, C23, C30, C31, S1, S3, S4, S5, S6, S7, S10, S11, S14, S15, S16, S18, S19, S20, S21, S23, S26, S27, S30, S32, S35, S37, S38, S39, S40, S41, S43, S47, S48, S49, S53, S55** | Homozygous (C/C) | 384 | 112155433 | Intronic variant | Wild type |
| **C8, C10, C32, S2, S12, S13, S25, S36, S44, S45, S46** | Heterozygous (C/G) | 384 | 112155433 | Intronic variant | rs5744247 |
| **S29** | Homozygous (G/G) | 384 | 112155433 | Intronic variant | rs5744247 |
| **C10, C15, C30, C31, C32, S3, S4, S6, S7, S12, S13, S14, S16, S20, S25, S26, S29, S40, S41, S43, S45, S46, S49, S53** | Homozygous (C/C) | 624 | 112155193 | Intronic variant | Wild type |
| **C5, C8, C11, C23, S1, S2, S5, S10, S11, S15, S18, S19, S21, S23, S27, S32, S35, S36, S37, S38, S39, S44, S47, S48, S55** | Heterozygous (C/G) | 624 | 112155193 | Intronic variant | rs360721 |
| **C20, S30** | Homozygous (G/G) | 624 | 112155193 | Intronic variant | rs360721 |
| **C5, C10, C11, C20, C23, C30, C31, C32, S1, S3, S4, S6, S7, S10, S11, S12, S13, S14, S15, S18, S19, S20, S21, S23, S26, S27, S29, S30, S32, S35, S36, S38, S39, S40, S41, S43, S44,** | Homozygous (A/A) | 625 | 112155192 | Intronic variant | Wild type |

| | | | | | |
|---|---|---|---|---|---|
| **S45, S46, S47, S48, S49, S53, S55** | | | | | |
| **C8, C15, S2, S5, S16, S25, S37** | Heterozygous (A/G) | 625 | 112155192 | Intronic variant | rs4988359 |

In the studied 776 bp *IL-18* amplicons, it was found that the total number of retrieved variants were only three, namely rs5744247, rs360721, and rs4988359. After SNPs association data were retrieved, they were plotted collectively. To investigate the average LD panel, significant LD values were plotted against the genetic distance between markers. The calculations of the pairwise LD values for these tag-SNPs are shown in Figure (4.46).

In the center of the plot, the identity of each SNP was mentioned, and all LD values that are contained both upstream and downstream of the observed SNPs were shown. LD values between any two variants in these plots are graphically displayed using standard colored triangles varying from white (low LD values) to red (high LD values). The graphical plotting of the observed SNPs indicated no tight connection among all the observed SNPs with each other. In the plotted graph, a neutral distribution was observed for rs5744247 and rs360721 SNPs. whereas both rs360721 and rs4988359 SNPs showed entirely independent inheritance for each investigated locus. This observation of low LD values could be explained by the inability of these SNPs to be transferred with each other. However, statistical results indicated a significant (or non-significant) association between these SNPs and the development of brucellosis associated with the currently investigated patients.

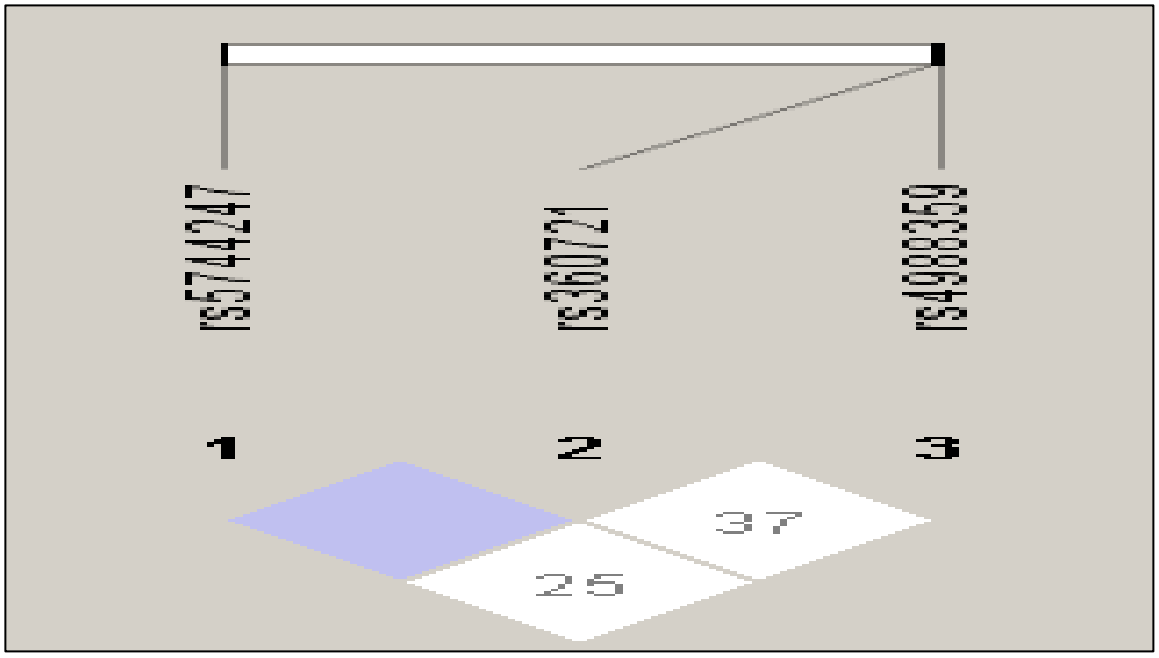



**Figure (4.46): Linkage disequilibrium graphical representation among all possible pairs of three variants observed in the amplified 776 bp fragment of the *IL-18* gene using standard D'/LOD Color Scheme. LD values are expressed as differences in the shade of each color. The blue color refers to the neutral LD, while the white color refers to the low LD. The numbers given at the top of the figure indicate the coordinate of sites in the 776 bp amplicons.**

### 4.19.3. Sequencing of the 790 bp Region within the INF-γ Gene:

In the present study, the *INF*-γ genetic sequences in chromosome no. 12. This gene is responsible for encoding *INF*-γ protein, which was involved in several activities related to immunoregulatory functions in the body (https://www.uniprot.org/uniprot/P01579) . The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI blastn. Concerning the 790 bp amplicons, the NCBI BLASTn engine shown about 99 % sequence similarities between the sequenced samples and the intended reference target sequences, which partially covered the intron 2 of this gene. By comparing the observed DNA sequences of these investigated

samples with the retrieved DNA sequences (GenBank acc. NG_015840.1), the accurate positions and other details of the retrieved PCR fragments were identified Figure (4.47).

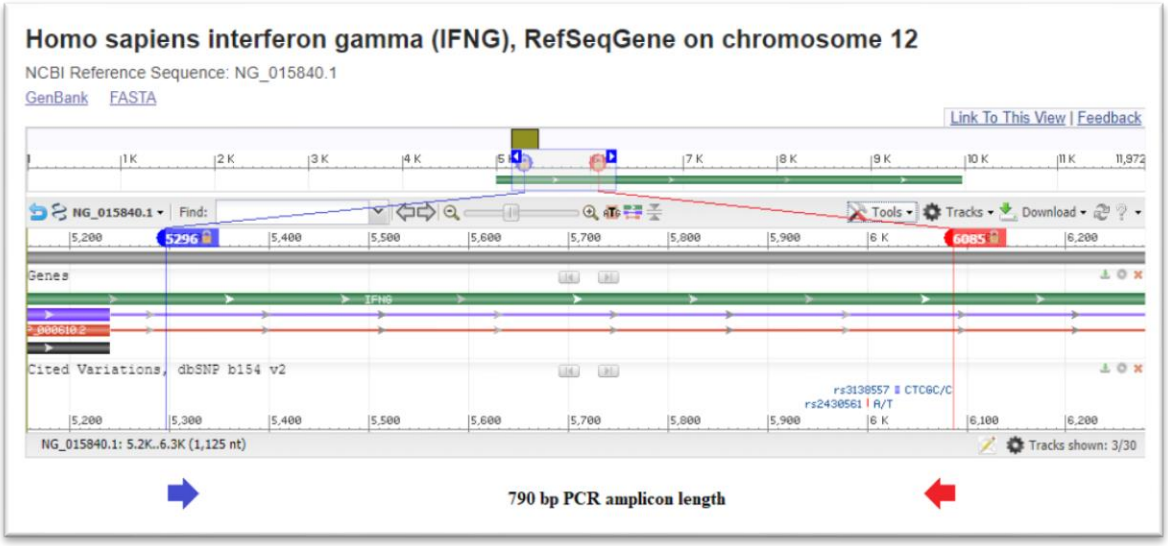



**Figure (4.47): The exact position of the retrieved 790 bp amplicon that partially covered a portion of the intron 2 of the *INF-γ* gene within chromosome no. 12 (GenBank acc. no. NG_015840.1). The cyan arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.**

After positioning the 790 bp amplicons' sequences within chromosome no. 12, the details of its sequences were highlighted, in terms of the positioning of both forward and reverse primers of the 790 bp amplified amplicons Table (4.71).

**Table (4.71): The position and length of the 790 bp PCR amplicons used to amplify a portion of the intron 2 of the *INF-γ* gene located within chromosome no. 12 (GenBank acc. no. NG_015840.1). The gray-colored sequences referred to the position of the reverse and forward primers, respectively.**

| Amplicon | Reference locus sequences (5′ - 3′) | Length |
|---|---|---|
| DNA sequences within the *IFNG* gene | *CTTGCTGTAGCATCTCTGATAGGCTGTCATCTCTTGTAGGCAGTCATTTTG<br>AGATTTGGTGTTATTTTGTTAATTATTGACTAGATGAGTTCCTTGACTAAAT<br>AATCTAGATATTGTTTTAACCTTCTGCTCAGTTTGTATAGAGACTTAAAAGG<br>GATTTATGAATTTTCCAAAAGATGGGCATAATATGGGTATGAAGCATAATGA<br>TGTTAATAATTTTGTGGTGGGAACTCATTCAGTTGTGATAGTCAAGGAGTAT<br>GCAGATTGAAAAAAATGATTGGTTATTAGTTTTTGACTTCTCAGACTCTAAG<br>GTCAAGATTAGCATTAAAAAGGTAATAGGAAATGTTTACAAATTAAAGTCAA<br>AAAGGTCCTTAAAGCTTTGGCTTAAAAAAATAACTGATAGGTGATTTTCTCC<br>AAAAAGTGATTTCAACATTCTGCTTCTCTATCTATATTACTTGTGAAGTATT<br>CCGGAACTTCGTTGCTCACTGGGATTTTGGAAGAATTATGATTCTGGCTAAG<br>GAATGTTTAAAAATTTTAAGTGAATTTTTTGAGTTTCTTTTAAAATTTTATT<br>GATGGTTAATGAAAAGTTTTTACATTTTAAATATTTCATTATTTGTTTAAAA<br>CTTAGCTGTTATAATTATAGCTGTCATAATAATATTCAGACATTCACAATTG<br>ATTTTATTCTTACAACACAAAATCAAATCACACACACACACACACACACACA<br>CACACTCGCACATGTTTGGAACTATCTTTTAAAGCTCGTATAATAATACCCT<br>ACAGGAAGGCA** | 790 bp |

* refers to the forward primer sequences (placed in a forward complement direction)

** refers to the reverse primer (sequences (placed in a reverse complement direction)

The alignment results of the 790 bp samples revealed the presence of only one variation in some of the analyzed samples in comparison with the referring reference DNA sequences, as shown in figure (4.48) in the **appendix III.**

The sequencing chromatogram of the identified variation region, as well as its detailed annotations, were documented, and the chromatograms of these sequences were shown according to their positions in the PCR amplicons, figure (4.49).

Highly interesting SNP was detected in this study in the investigated samples, in which adenine was replaced with thymine at position 705 in the analyzed locus. This SNP was detected in three patterns, homozygous A/A pattern in C31 and S3, heterozygous A/T pattern in C5, C8, C10, C11, C15, C32, C24, C30, C32, S2, S4, S5, S6, S7, S11, S19, S21, S25, S27, S29, S30, S32, S35, S37, S41, S43, S44, S46, S48, S49, and S55, and homozygous T/T pattern in C20, S1, S10, S13, S14, S15, S16, S18, S20, S23, S26, S36, S38, S39, S40, S45, S47, and S53.

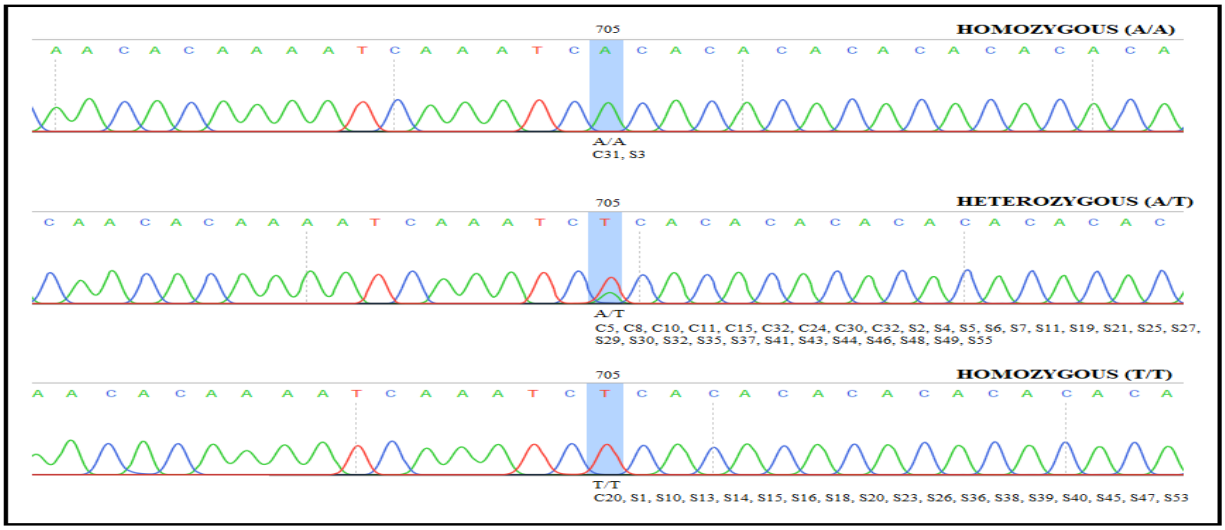



**Figure (4.49): The pattern of the detected A705T within the DNA chromatogram of the targeted 790 bp amplicons of the intron 2 of the *INF-γ* gene. The identified SNP was highlighted according to its position in the PCR amplicons. The letter S# refers to the sample code, while the letter "C" refers to the control samples.**

To elucidate the positions of the observed SNP concerning its deposited SNP database of the sequenced 790 bp fragments, the corresponding position of the *INF-γ* gene was retrieved from the dbSNP server

(https://www.ncbi.nlm.nih.gov/projects/SNP/) . To check the possible novelty of this observed SNP, a graphical representation was performed concerning the *INF-γ* gene dbSNP database within chromosome no. 12 using the GenBank Acc. No. NC_000012.12. By reviewing the dbSNP engine, it was found that the detected A705T SNP was previously deposited intronic variant as it was deposited as rs2430561, figure (4.50). However, this SNP is highly recognized in the targeted *INF-γ* gene gene due to its high frequency that ranged between 0.25 to 0.35 (https://www.ncbi.nlm.nih.gov/snp/rs2430561) . Furthermore, this SNP was intensively reported in many publications that described its association with many INF-γ related dysfunctions (https://www.ncbi.nlm.nih.gov/snp/rs2430561#publications) .

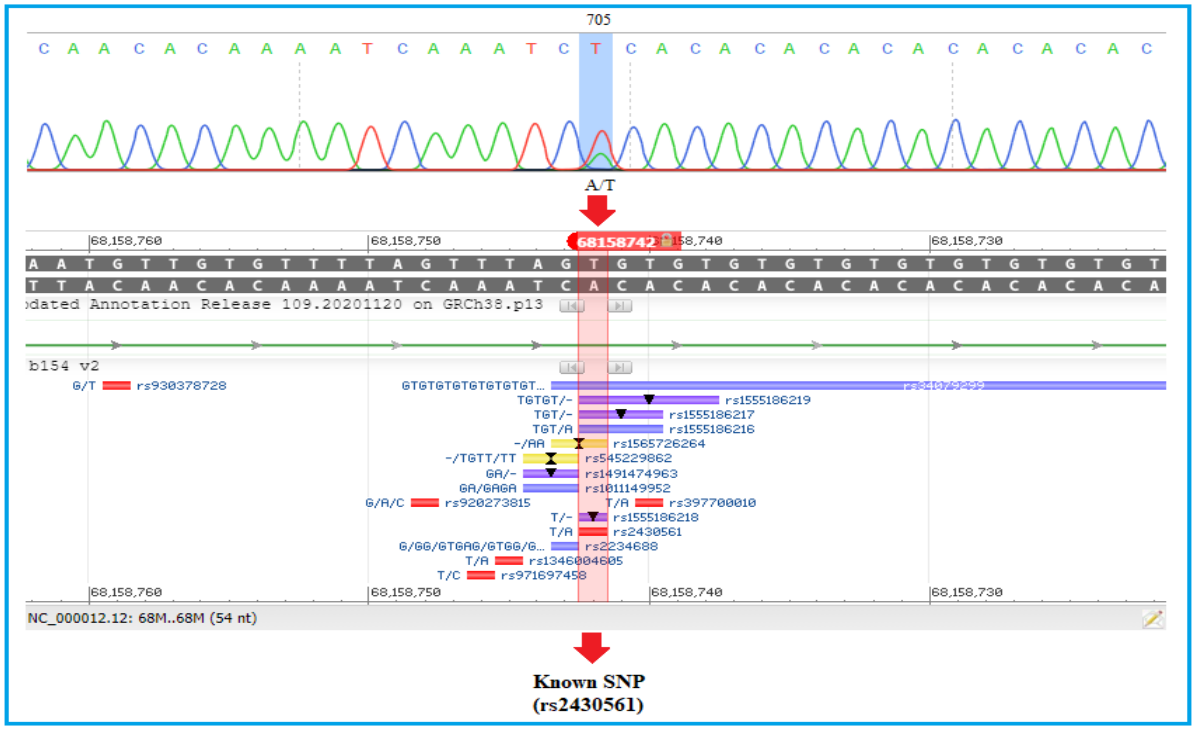



**Figure (4.50): The SNP's novelty checking of *INF-γ* genetic single nucleotides polymorphisms using the dbSNP server. A) The identified known SNP (rs2430561) was marked with a red color. The GenBank acc. no. NC_000012.12 was used in the positioning of the highlighted substitution SNP. The position of the targeted sequence was found in the negative strand.**

The GenBank acc. no. for all samples in the current study in **appendix III.**

To summarize the results obtained from the sequenced 790 bp fragments, the exact position of the observed variation was described in the NCBI reference sequences, table (4.72).

**Table (4.72): The patterns of the observed SNP in the 790 bp amplicons that designed to amplify a portion of the intron 2 within the *INF-γ* gene in comparison with the NCBI referring sequences (GenBank acc. no. NC_000012.12). The letters "S" and "C" refer to the sample numbers and control numbers respectively.**

| Sample No. | Zygosity status | Position in the PCR fragment | Position in the reference genome | SNP type | SNP summary |
|---|---|---|---|---|---|
| **C31, S3** | Homozygous (A/A) | 705 | 68158742 | Intronic variant | Wild type |
| **C5, C8, C10, C11, C15, C32, C24, C30, C32, S2, S4, S5, S6, S7, S11, S19, S21, S25, S27, S29, S30, S32, S35, S37, S41, S43, S44, S46, S48, S49, S55** | Heterozygous (A/T) | 705 | 68158742 | Intronic variant | rs2430561 |
| **C20, S1, S10, S13, S14, S15, S16, S18, S20, S23, S26, S36, S38, S39, S40, S45, S47, S53** | Homozygous (T/T) | 705 | 68158742 | Intronic variant | rs2430561 |

Results in table (4.58, 4.62 and 4.66) found that there were cross association among the three genes detected in this study; namely *IL-17*, *IL-18* and *IFN- γ* genes. These three genes were originally (table 4.22) had significantly higher detection rate in patients with brucellosis compared to health control. Furthermore, these gene expression namely; the IL-17, IL-18 and INF- γ cytokine positivity rate and their concentration were higher in sera of patients with brucellosis (table 4.16, 4.18 and 4.19).

More importantly, a genetic analysis of 1075 bp *IL-17A* amplicons revealed the presence of 7 pairs of SNAPs in the amplified 1075 bp fragment of the *IL-17A* gene. All these 7 SNAPs are with high LD values. The high LD values among the first three observed SNPs could be explained by the ability of these SNPs to be associated with development and progression of the Brucellosis. This observation was also confirmed by the statistically significant detection of *IL-17* gene in Iraqi patients with brucellosis compared to healthy population (Table 4.22). These results indicated a significant association between these SNPs and the development and intensity of brucellosis among Iraqi population. Based on these observation, the current study may affirm the role of *IL-17 A* gene polymorphism in susceptibility and progression of brucellosis.

In the studied 776 bp *IL-18* amplicons, it was found that the total number of retrieved SNPs were only three, namely rs5744247, rs360721, and rs4988359. One of these with neutral LD value, while the others were with low LD values. These observations of low LD value SNPs can be explained as inability of these SNPs to be transferred with each other. Thus the genetic analysis suggest that the statistical results can solve whether yes or no association between these SNPs and the development of brucellosis associated with the currently investigated patients. Since the statistical analysis found that the IL-18 gene detection was significantly higher among brucella patients compared to healthy control (table 4.22). Thus the current study claimed that there was an association between the *IL-18* gene polymorphism and brucellosis, leaving the scientific door opened for further studies.

Concerning the *INF-γ* genetic analysis, the alignment results of the 790 bp amplicon revealed the presence of only one SNPs in some samples in

comparison with the referring reference DNA sequences. this SNP is highly recognized in the targeted *INF*-γ gene due to its high frequency and was intensively reported in many publications that described its association with many INF-γ related dysfunctions.It is important to remind that the *INF*-γ gene detection rate was significantly higher in brucellosis patients compared to healthy control (table 4.22). Again the study claim an association between *INF*-γ gene polymorphism with brucellosis among Iraqi population.

Collectively these immunological, molecular and genetic analysis results are generally making the current study in agreement with all publication affirming the role of *IL-17*, *IL-18* and *INF*-γ gene polymorphism in susceptibility and progression of brucellosis.

It is extensively documented the role of cytokines in protection and amelioration of the clinical course of brucellosis (Pasquali *et al*., 2002; Elfaki *et al*., 2015; Kayhan *et al*., 2016; Lopez-Santiago *et al*., 2019). Some of these cytokines were remain in the body for longer time after treatment (Rasouli *et al.*,2013; Xu *et al*., 2019) . Therefore, it has been suggested that assessing cytokine levels in patients with acute and chronic brucellosis is not only useful for detecting the immune response, but can also be indicative of the severity of brucellosis particularly, the IL-6 and INF-γ levels (Lee *et a*l., 2016; Han *et al*., 2017; Lin *et al*., 2020).

Several studies had documented that polymorphisms in the regulatory regions of cytokine genes may not only increase susceptibility to some infectious diseases but also affect the course and prognosis of the disease (Bidwell *et al.,* 2001). The connection between cytokine gene polymorphisms and brucellosis disease status was studied in various populations with variable results (Bravo *et al*.,2003; Karaoglan *et al*., 2009; Eskandari-Nasab *et al*., 2013). A few studies report an association between cytokine gene

polymorphism and development of brucellosis (Rasouli *et al*., 2007, Bravo *et al*., 2003, Rezazadeh *et al*., 2006, Caballero *et al*., 2000). Keeping in the same line, it has been reported that The *IL-13* gene polymorphism can be used as a biomarker for detecting susceptibility to Brucella (Kazemi *et al*., 2019).

Moreover, it has been found that polymorphisms of IL-10 (-819) and TGFβ1 codons 10 and 25 were associated with susceptibility or resistance to brucellosis. The IL-6 (-174) GC genotype may be a risk factor for the development of focal complications of brucellosis, whereas the GG genotype may be a protective factor against brucellosis (Karaoglan *et al*., 2009). Additionally, several potentially functional single nucleotide polymorphisms (SNPs) have been identified in the human IFN-γ R1 gene promoter and the protective role of TNF-α-857C/T and TNF-α-308A polymorphism against human brucellosis (Ismael *et al*., 2016). A total of 17 studies were included in pooled analysis demonstrated that the IFN-γ +874 AT vs AA genotype in a codominant model may confer an increased risk of brucellosis in the overall population,while TNF-α −308 G/A, revealed that the AA vs GG + GA (recessive) genotype increased the risk of brucellosis (Eskandari-Nasab and Moghadampour, 2018). In another meta-analysis including 25 eligible studies concluded that IL-4 rs2243250 and IL-18 rs1946519 have a positive correlation with brucellosis whereas the IFN-γ UTR5644, TGF-β rs1800470 and rs1800471, TNF-α rs1800629, and IL-10 rs1800872 showed a negative association with this disease ( Zafari, *et al*., 2020). Likewise, Fu *et al*. (2019) found that IL-6 (-174 G/C), IL-10 (-1082 A/G, -819C/T), and TGF-β1 (codon 10C/T) polymorphisms is not a risk factor for brucellosis infection. TGF-β1 codon 25 GG genotype may be considered as a risk factor for brucellosis.

The presence of C allele in position -611 in IFNγ R1 gene promoter was related to a higher risk of disease and susceptibility to brucellosis. Moreover, the presence of T allele in position -611 in IFN-γ R1 gene promoter was related to a lower risk of disease (Naseri *et al*., 2019). On the contrary, Ismael *et al*. (2018) found that polymorphisms in IFN-γ R1-56 and IFN-γ R1-611 polymorphism had no role in genetic susceptibility to human brucellosis.

Keramat *et al*. (2019), reported that the serum IL-17 titers were significantly higher in patients with brucellosis than in controls as measured by ELISA. The AA genotype at positions rs3748067, rs3819025 and rs4711998 and GG genotype at position rs3819024 are likely protective factors against brucellosis, whereas the GG genotype at positions rs3748067, rs3819025 and rs4711998 and AA genotype at position rs3819024 may be risk factors against the disease. Indicating that there was no significant relationships between serum IL-17 titers and genotypes of the single-nucleotide polymorphisms. On the other hand, it has been stated that IL-17 rs4711998, rs8193038, rs3748067 AA genotypes and AAGAA haplotype could be considered as susceptibility factors for brucellosis while the inheritance of IL-17 rs3819024GG and rs3819025AA genotypes might be resistance factors against brucellosis (Rasouli *et al* 2013).

It is well known that Th17 cells, a distinct subset of CD4+ T cells, produce various cytokines, namely IL-17, IL-6, IL-9, IL-21, IL-22, IL-23, IL-26, GMCS factor, MIP-2, MCP-1and TNF-α. The IL-17 family has six members, namely IL-17A to IL-17F. IL-17A is an important inflammatory cytokine, playing principal roles in both innate and adaptive immunities (Bedoya *et al*., 2013; Isailovic *et al*., 2015). The IL-17 coding gene is located on chromosome 6p12 and consists of three exons. Genetic variation in IL-17 has been studied in various diseases and cancers (Zhang *et al*., 2008; Ren *et*

*al*., 2017). In this regard, it has been found that there was a cooperative role of Th1 and Th17 cells in immunity to brucellosis which is more evident during acute and relapse phases of brucellosis (Rahmanpour *et al*., 2019).

# Conclusions and Recommendations

## 5.1. Conclusions:

- About one third of clinically suspected brucellosis patients whose Rose Bengal test positive had anti-brucella IgM positive as a marker of recent infection.
- About 5% of anti-brucella IgM positive were recorded among apparently healthy individuals In Diyala community.
- More than one half of clinically suspected brucellosis patients who were Rose Bengal test positive had anti-brucella IgG.
- About 10% of apparently healthy individuals had anti-brucella IgG antibody as a marker of past infection in Diyala community.
- The CD3 positivity rate was significantly higher among brucellosis patients compared to healthy control.
- The CD4 positivity rate was insignificantly higher among brucellosis patients compared to healthy control.
- Patients with brucellosis had a highly significant increase in CD8 positivity rate compared to healthy control as a marker of cytotoxic T cells against intracellular brucellae.
- The IL-17 positivity rate was insignificantly higher in patients with brucellosis compared to health control.
- The IL-18 positivity rate among brucellosis patients was higher compared to healthy control, but the difference was failed to reach the level of statistical significance.
- The INF-γ positivity rate significantly higher among patients with brucellosis compared to health control as it is a crucial mediator of cellular immunity.

- The PCR technique found that the *IL-17*, *IL-18* and *INF-γ* genes detection rates were significantly higher among patients with brucellosis competed to health control.
- The anti-brucella IgM antibody positivity rate was highest among those <20 years old and significantly associated with CD3 positivity rate.
- The anti-brucella IgG positivity rate was highest among those ≥ 49 years old and it is also significantly associated with those consume dairy products.
- The anti-brucella IgG positivity rate was significantly associated with fever and joint pain. As well as IL-18 positivity rate.
- The CD3 positivity rate was significantly associated with anti-brucella IgM antibody, IL-17 and INF-γ positivity.
- The CD4 positivity rate was significantly associated with IL-17 positivity rate.
- The CD8 positivity rate was positively associated with IL-17 and negatively associated with IL-18 positivity rate.
- The IL-17 positivity rate was significantly associated with CD3 and CD4 positivity and insignificantly associated with CD8 positivity.
- The IL-18 positivity rate was significantly associated with joint pain and anti-brucella IgG antibody plus insignificantly associated with *IL-17* gene detection rate.
- The INF-γ positivity rate was significantly associated with consumption of dairy products CD3 and CD8 positivity rates.
- The *INF-γ* gene detection rate was significantly associated with *IL-18* gene detection rate.
- The *IL-18* gene detection rate was significantly associated with IL-17 and *INF-γ* gene detection rates.

- The *IL-17* gene detection rate was significantly associated with IL-18 and *INF-γ* gene detection rates.
- The genetic and sequencing analysis of *IL-17* 1075 bp amplicons assure the presence of seven sequence nucleotide polymorphism (SNPs).These SNPs with high LD values may explain the ability of these SNPs for the development and progression of brucellosis.
- The genetic and sequencing analysis of *IL-18* 776 bp amplicons affirm the presence of three SNPs. These SNPs with low LD values may explain the insignificant association of these SNPs and the development of brucellosis.
- The genetic and sequencing analysis of *INF-γ* gene targeting the 790 bp amplicons affirm the presence of one SNP. This SNP has high frequency and intensively reported in many publications including its association with brucellosis.

## 5.2. Recommendation:

A summary of the results of this study should be informed to Diyala Directory of Health to make benefits from its contents.

- As Iraq is still one of few prominent endemic countries, motivation and strengthen the brucella institutes and communicable disease prevention and control for training and research as a reference centers are highly recommended.
- Expansion and follow up of periodically screening of Iraqi community for brucellosis as well as the reproductive animals with the collaboration of veterinary services are highly recommended.

- Authorities of public health and communicable diseases have to adopt a real and scientific programs about the protection of the community and prevention of the disease are highly recommended.
- Since, Brucella is a fastidious intracellular bacteria, further molecular studies on the detection of other immune parameters particularly those which had a role in promoting and regulating cellular immune response are recommended.
- Further studies on the role of cytokines in susceptibility and progression of brucellosis particularly *INF*-α, IL-10 IL-12, IL-6 are recommended.

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# Appendix I

## Questionnaire sheet:

Name:

Age:

Sex:

Residence:

Presence of home animals:

Consumption of dairy products& milk and meat:

Intrafamilial case of brucellosis:

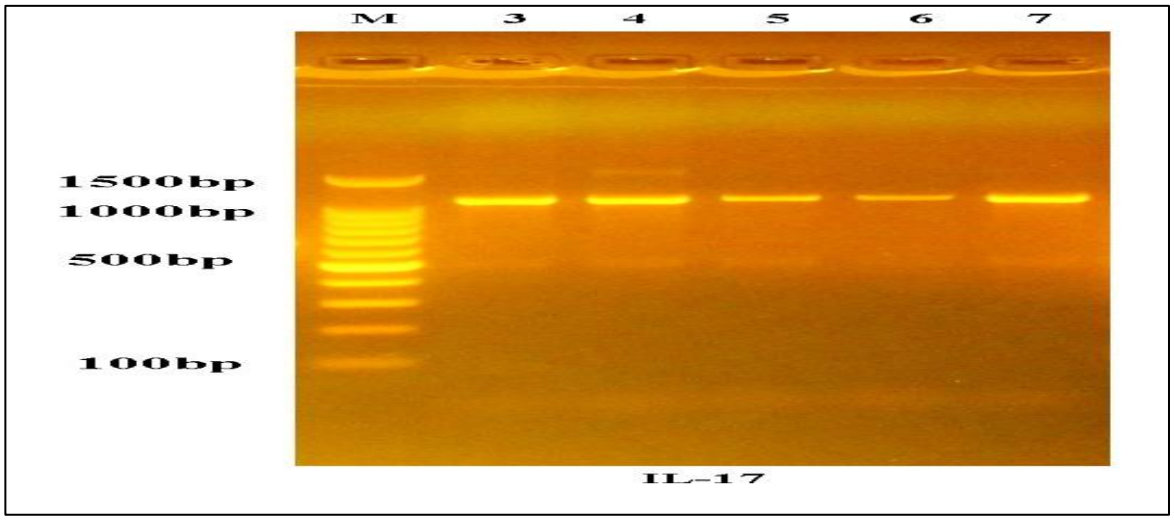
Patients history:

Sign: A. Fever.

B. Night sweet.

C. Joint pain.

# Appendix II




**Figure(4.30): Electrophoresis bands of IL-17 gene detection in this study**.

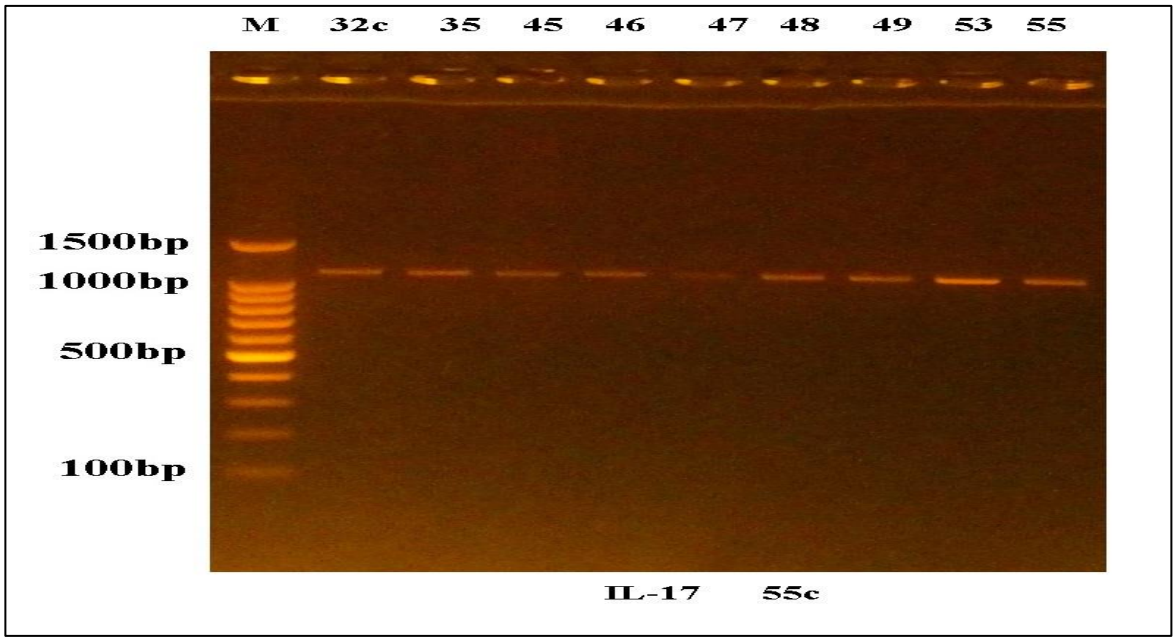
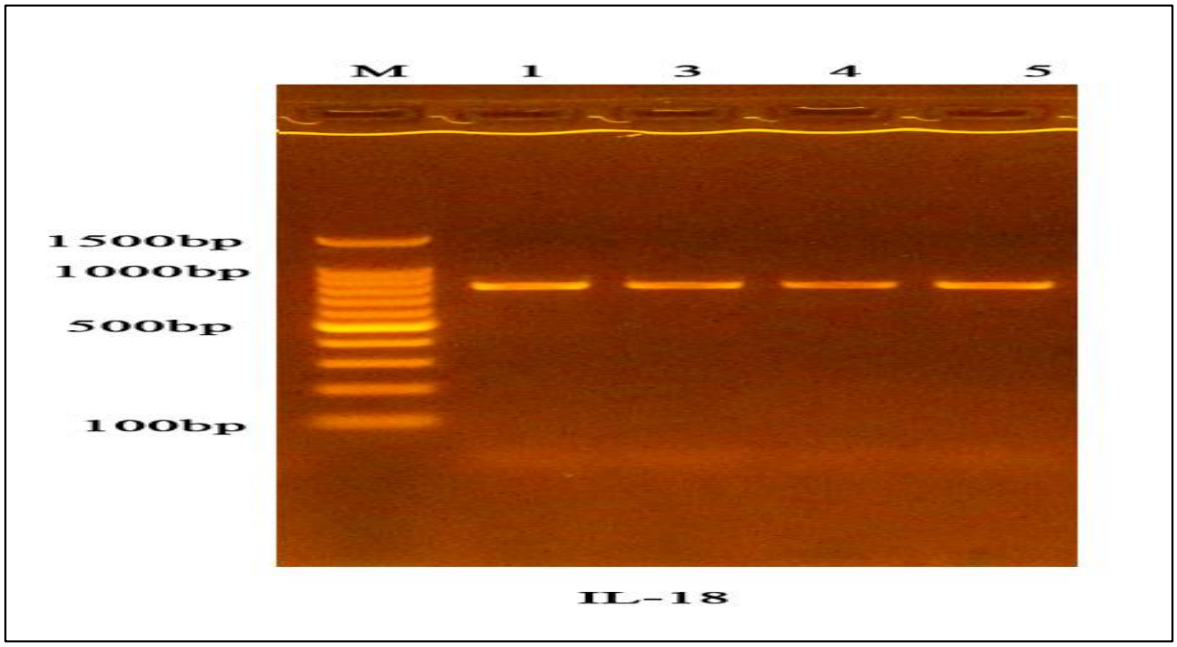
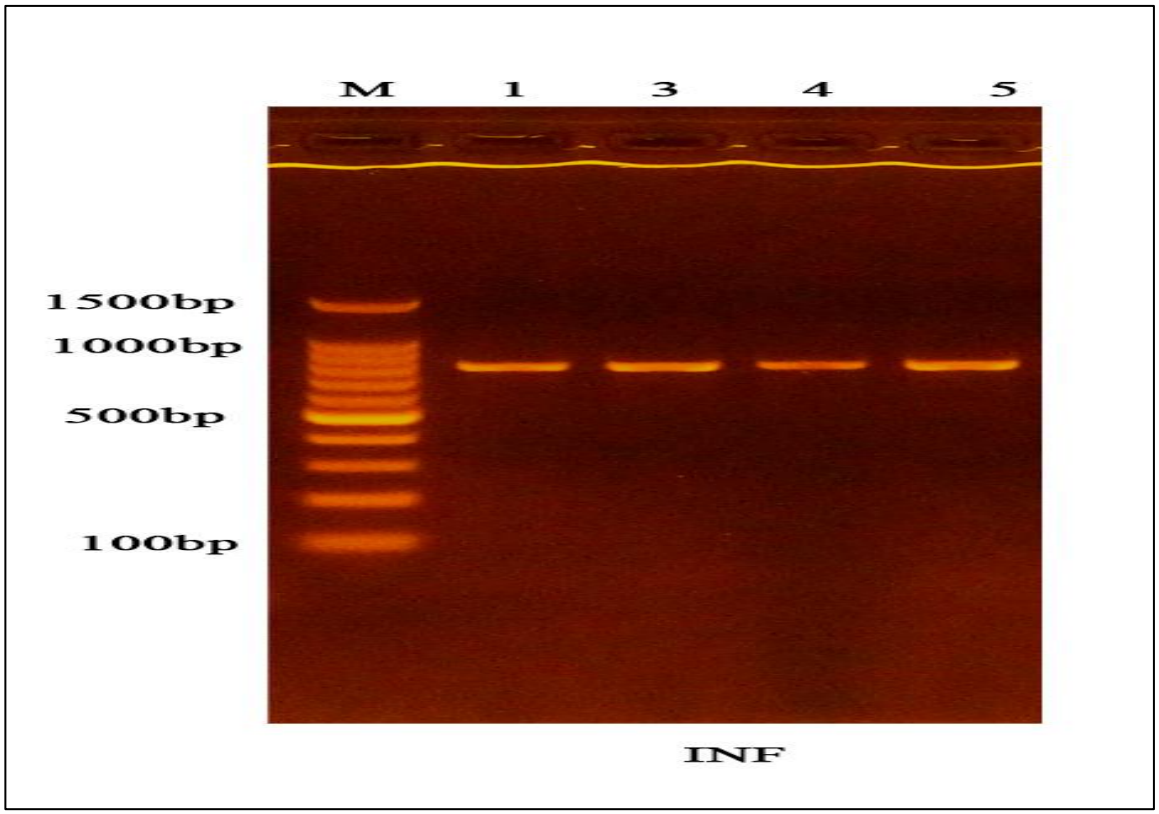


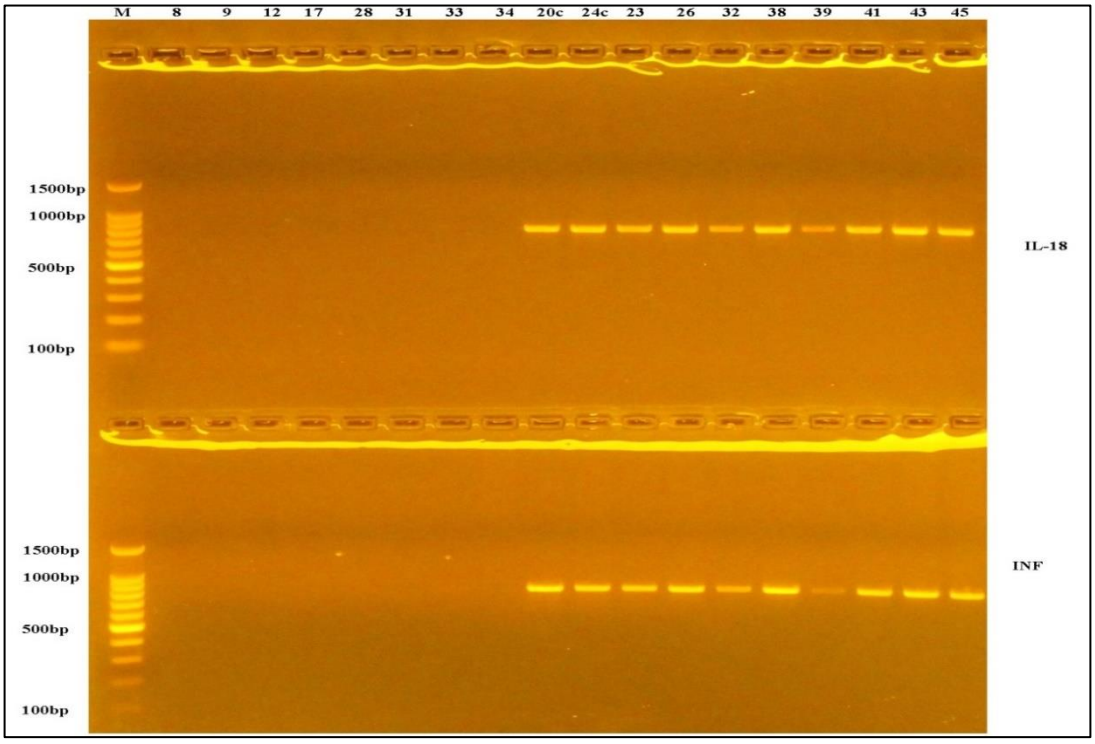

**Figure (4.31):Electrophoresis bands of IL-17 gene detection in this study**.




**Figure(4.32):Electrophoresis bands of IL-18 gene detection in this study**.




**Figure(4.33):Electrophoresis bands of IL-INF-γ gene detection in this study**.




**Figure(4.34):Electrophoresis bands of IL-18 and INF-γ gene detection in this study.**

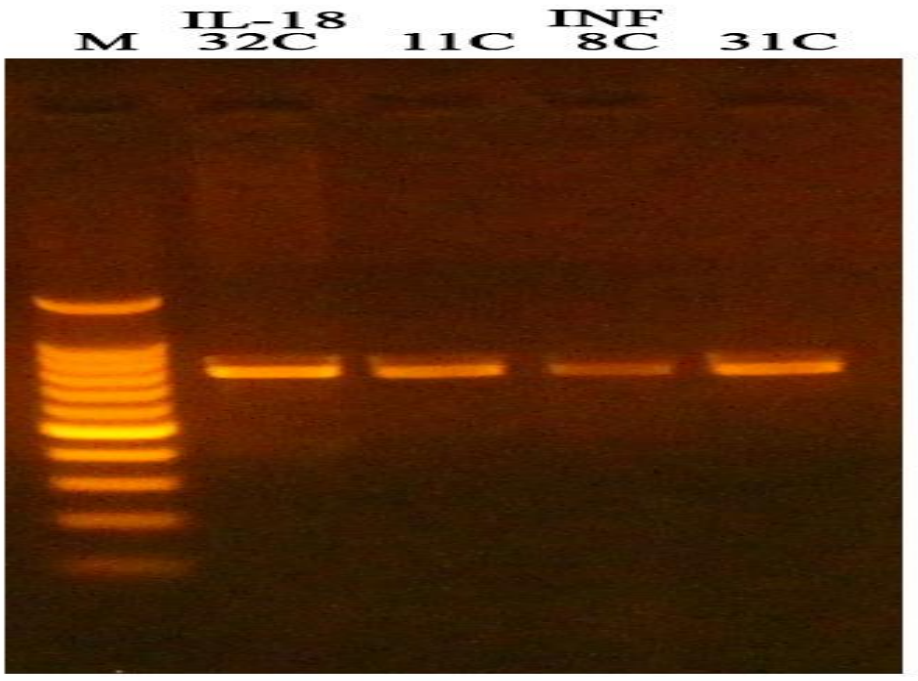



**Figure (4.35): Electrophoresis bands of IL-18 and INF-γ gene detection in this study.**

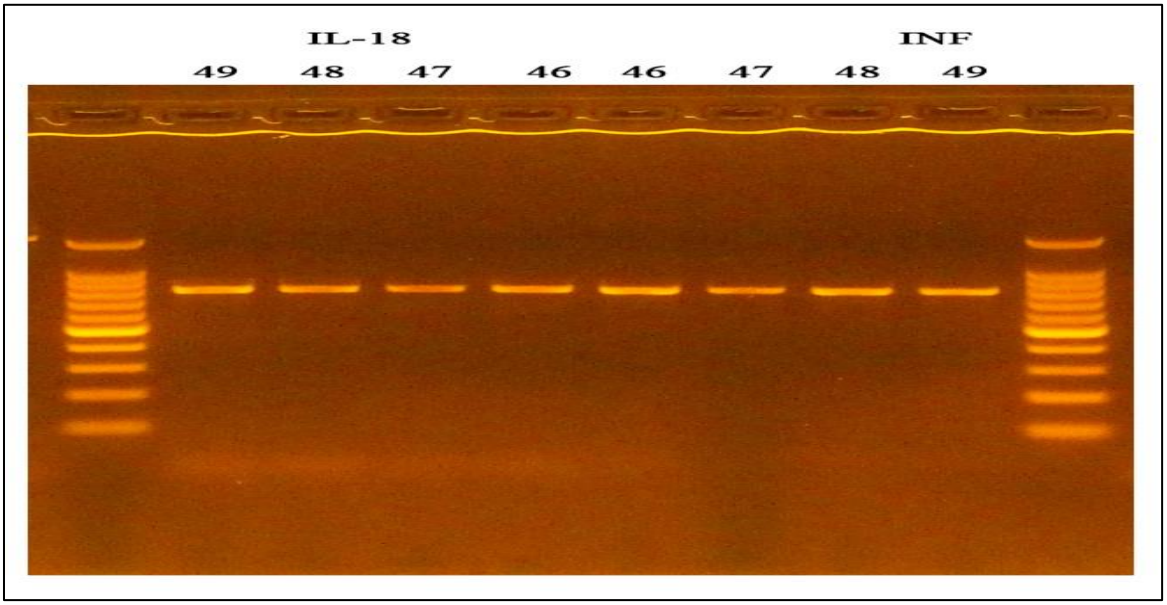
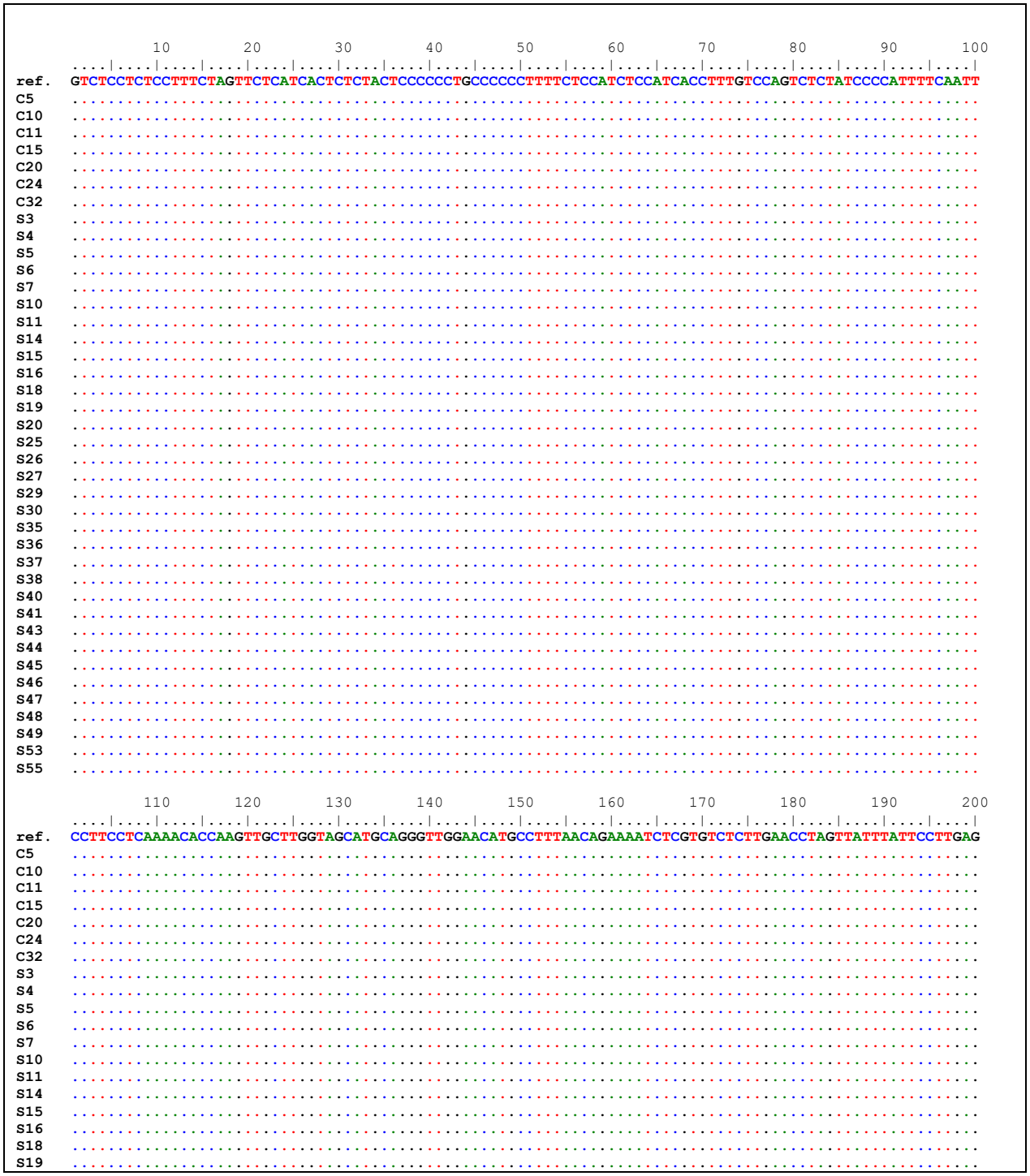
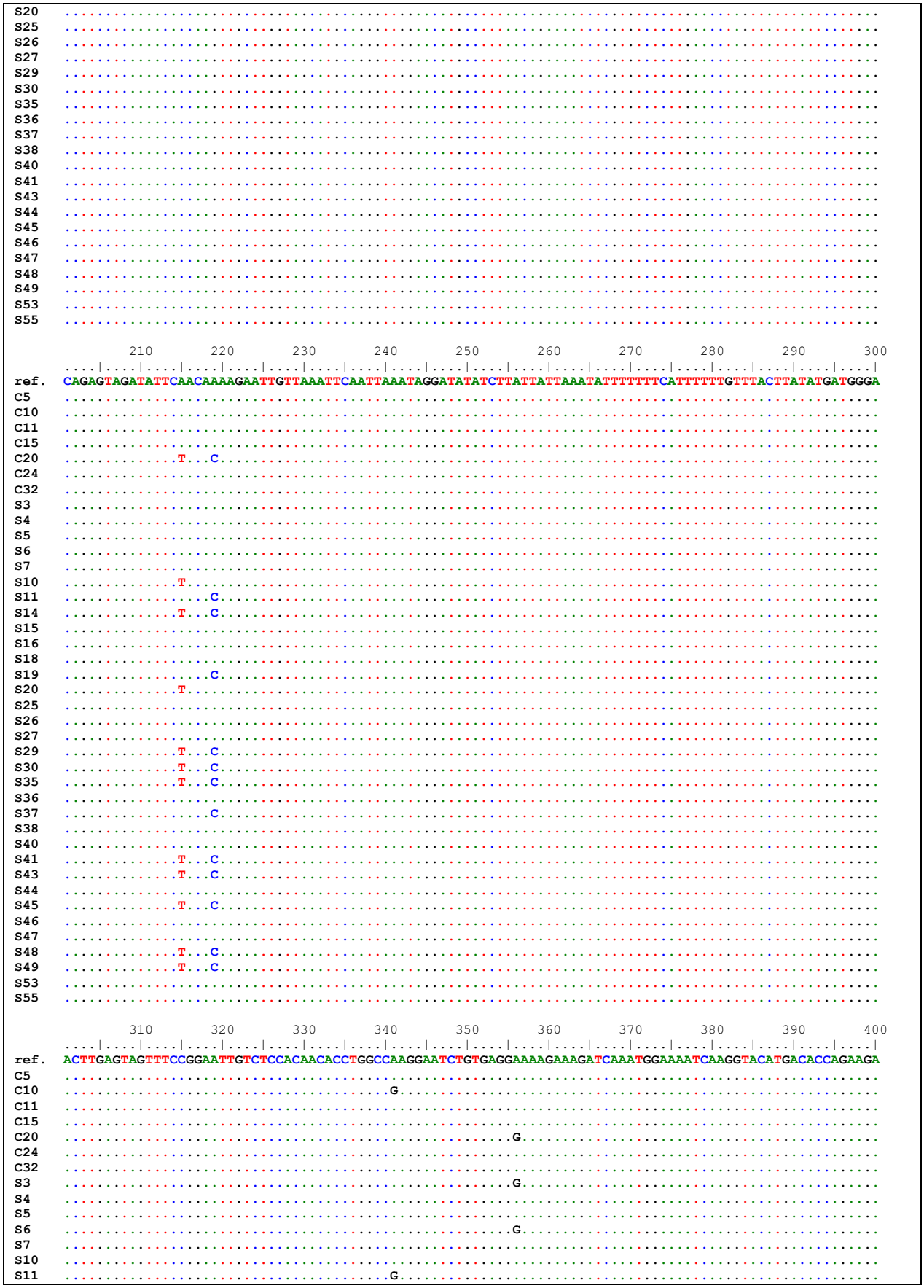


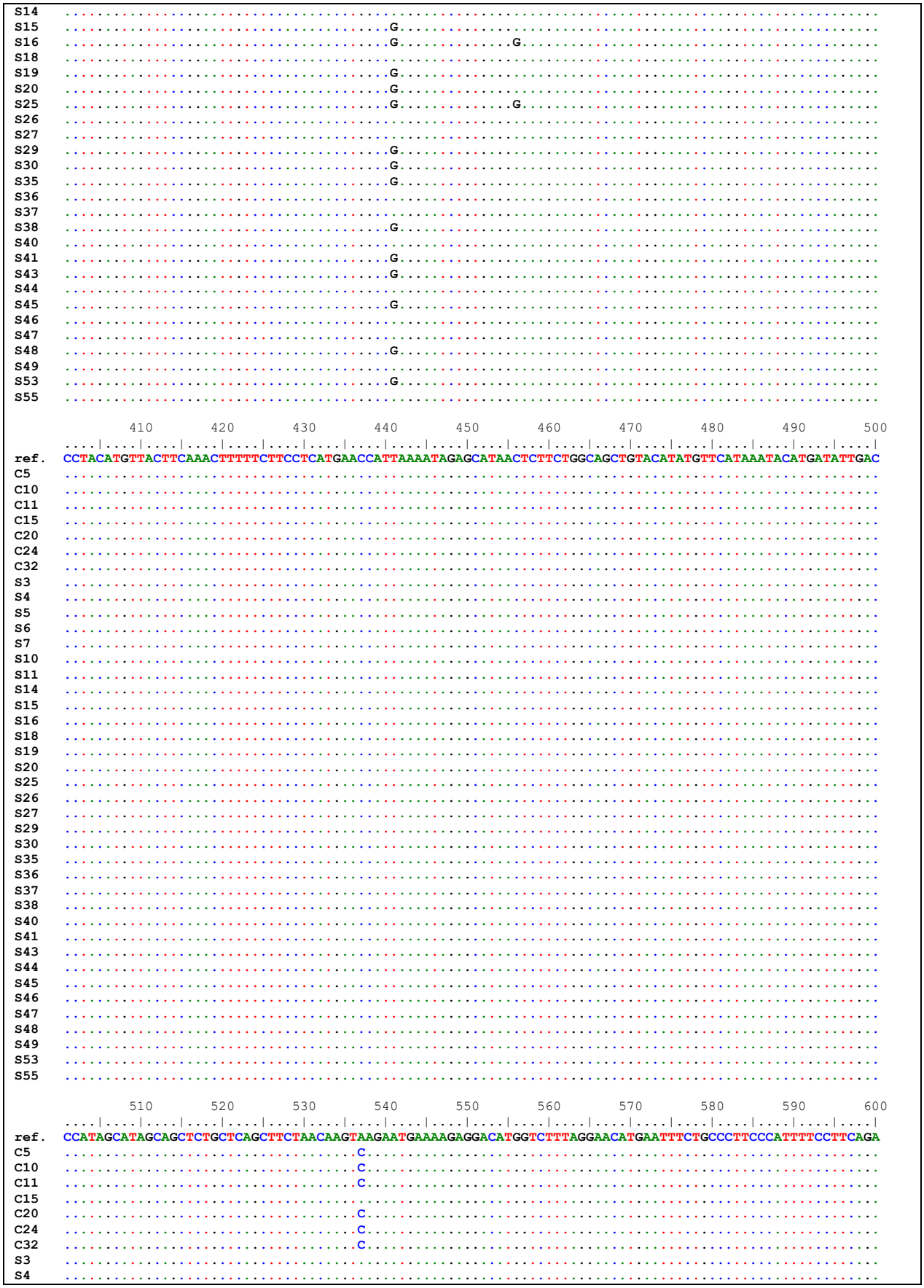

**Figure (4.36): Electrophoresis bands of IL-18 and INF-γ gene detection in this study.**

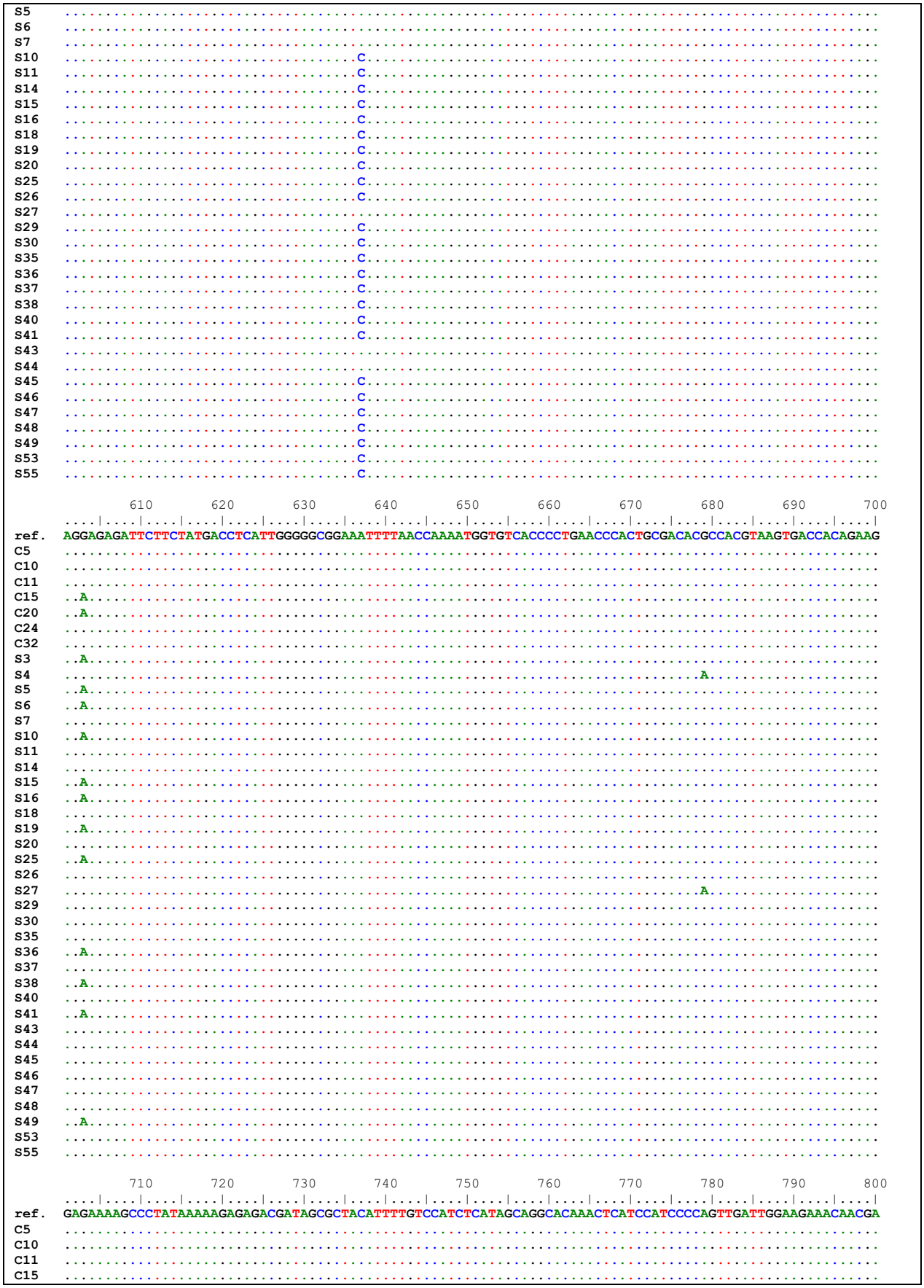
# Appendix III

The alignment results of the 1075 bp samples revealed the presence of seven variations in some of the analyzed samples in comparison with the referring reference DNA sequences **Figure (4.38).**




S20 S25 S26 S27 S29 S30 S35 S36 S37 S38 S40 S41 S43 S44 S45 S46 S47 S48 S49 S53 S55
210 220 230 240 250 260 270 280 290 300
ref. CAGAGTAGATATTCAACAAAAGAATTGTTAAATTCAATTAAATAGGATATATCTTATTATTAAATATTTTTTTCATTTTTTGTTTACTTATATGATGGGA
C5 C10 C11 C15 C20 C24 C32 S3 S4 S5 S6 S7 S10 S11 S14 S15 S16 S18 S19 S20 S25 S26 S27 S29 S30 S35 S36 S37 S38 S40 S41 S43 S44 S45 S46 S47 S48 S49 S53 S55
310 320 330 340 350 360 370 380 390 400
ref. ACTTGAGTAGTTTCCGGAATTGTCTCCACAACACCTGGCCAAGGAATCTGTGAGGAAAAGAAAGATCAAATGGAAAATCAAGGTACATGACACCAGAAGA
C5 C10 C11 C15 C20 C24 C32 S3 S4 S5 S6 S7 S10 S11


S14
S15
S16
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S19
S20
S25
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S53
S55
410 420 430 440 450 460 470 480 490 500
ref. CCTACATGTTACTTCAAACTTTTTCTTCCTCATGAACCATTAAAATAGAGCATAACTCTTCTGGCAGCTGTACATATGTTCATAAATACATGATATTGAC
C5
C10
C11
C15
C20
C24
C32
S3
S4
S5
S6
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S11
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S47
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S49
S53
S55
510 520 530 540 550 560 570 580 590 600
ref. CCATAGCATAGCAGCTCTGCTCAGCTTCTAACAAGTAAGAATGAAAAGAGGACATGGTCTTTAGGAACATGAATTTCTGCCCTTCCCATTTTCCTTCAGA
C5
C10
C11
C15
C20
C24
C32
S3
S4


S5
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S55
610 620 630 640 650 660 670 680 690 700
ref. AGGAGAGATTCTTCTATGACCTCATTGGGGGCGGAAATTTTAACCAAAATGGTGTCACCCCTGAACCCACTGCGACACGCCACGTAAGTGACCACAGAAG
C5
C10
C11
C15
C20
C24
C32
S3
S4
S5
S6
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S53
S55
710 720 730 740 750 760 770 780 790 800
ref. GAGAAAAGCCCTATAAAAAGAGAGACGATAGCGCTACATTTTGTCCATCTCATAGCAGGCACAAACTCATCCATCCCCAGTTGATTGGAAGAAACAACGA
C5
C10
C11
C15


C20
C24
C32
S3
S4
S5
S6
S7
S10
S11
S14
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S45
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S49
S53
S55
810 820 830 840 850 860 870 880 890 900
ref. TGACTCCTGGGAAGACCTCATTGGTGGTGAGTCCTGCACTAACGTGCGATGCTCTTGCTGATTTGGACCAGATAGTATTTCTGGACCGTGGGCATGAAAC
C5
C10
C11
C15
C20
C24
C32
S3
S4
S5
S6
S7
S10
S11
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S40
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S43
S44
S45
S46
S47
S48
S49
S53
S55
910 920 930 940 950 960 970 980 990 1000


ref. GCTGGGTTCTGACTATGGAGATCCAGGAATACTGTATATGTAGGATAGGAAATGAAAGCTTTGGTAGGTATTTAAGTCATTGTGCAGCATTTTCAAGAAC
C5 .....
C10 .....
C11 .....
C15 .....
C20 .....
C24 .....
C32 .....
S3 .....
S4 .....
S5 .....
S6 .....
S7 .....
S10 .....
S11 .....
S14 .....
S15 .....
S16 .....
S18 .....
S19 .....
S20 .....
S25 .....
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S36 .....
S37 .....
S38 .....
S40 .....
S41 .....
S43 .....
S44 .....
S45 .....
S46 .....
S47 .....
S48 .....
S49 .....
S53 .....
S55 .....
1010 1020 1030 1040 1050 1060 1070
ref. TGATACACAGCAGTTTGAAAGATAAGATTAAAACTGAAAGATAGCTATATTGGGGCTAAACCACACAAGAAGTGT
C5 .....
C10 .....
C11 .....
C15 .....
C20 .....
C24 .....
C32 .....
S3 .....
S4 .....
S5 .....
S6 .....
S7 .....
S10 .....
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S46 .....
S47 .....
S48 .....
S49 .....

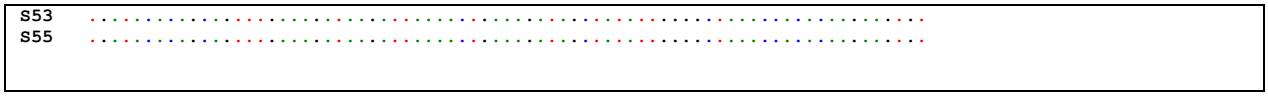
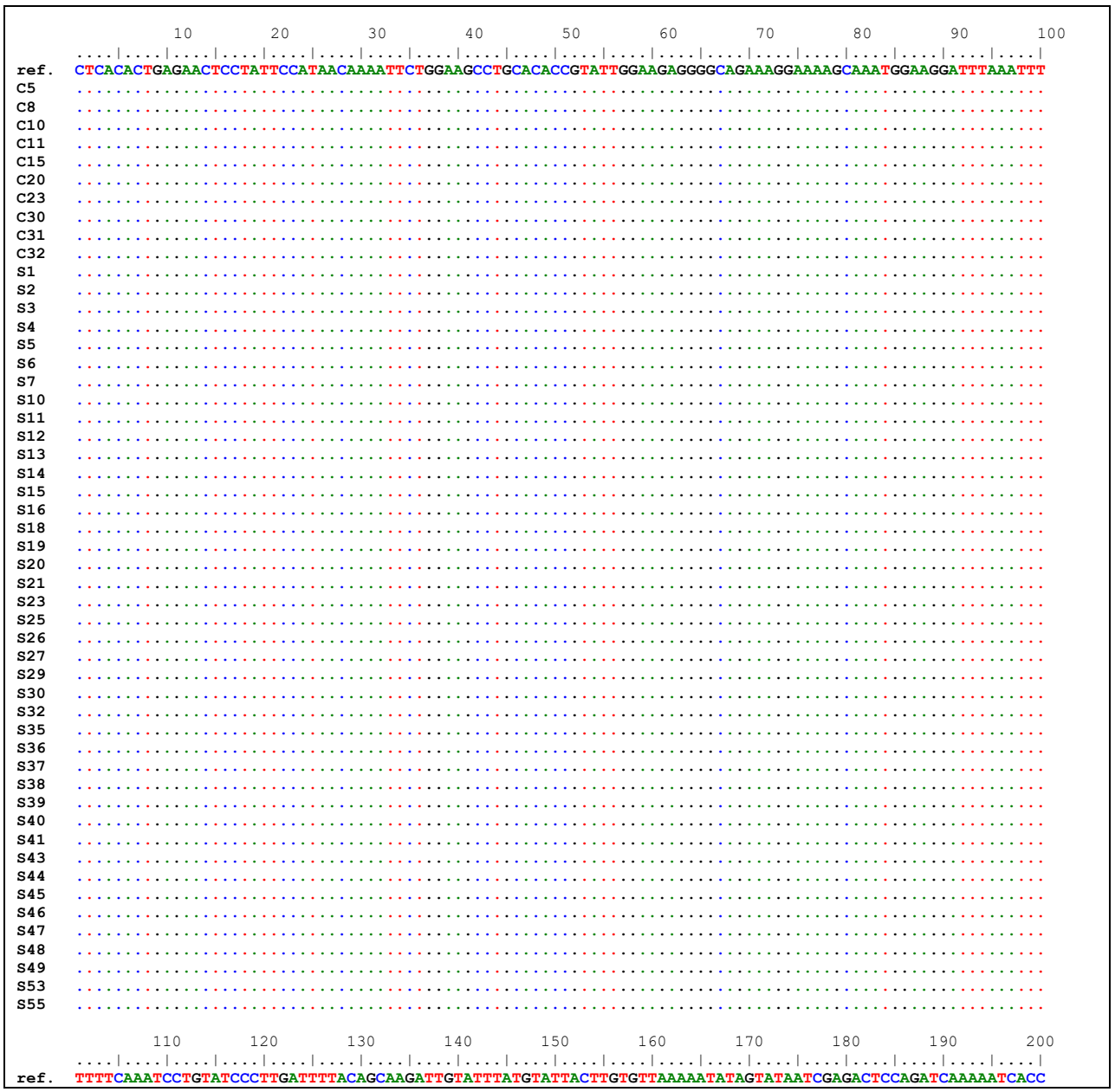
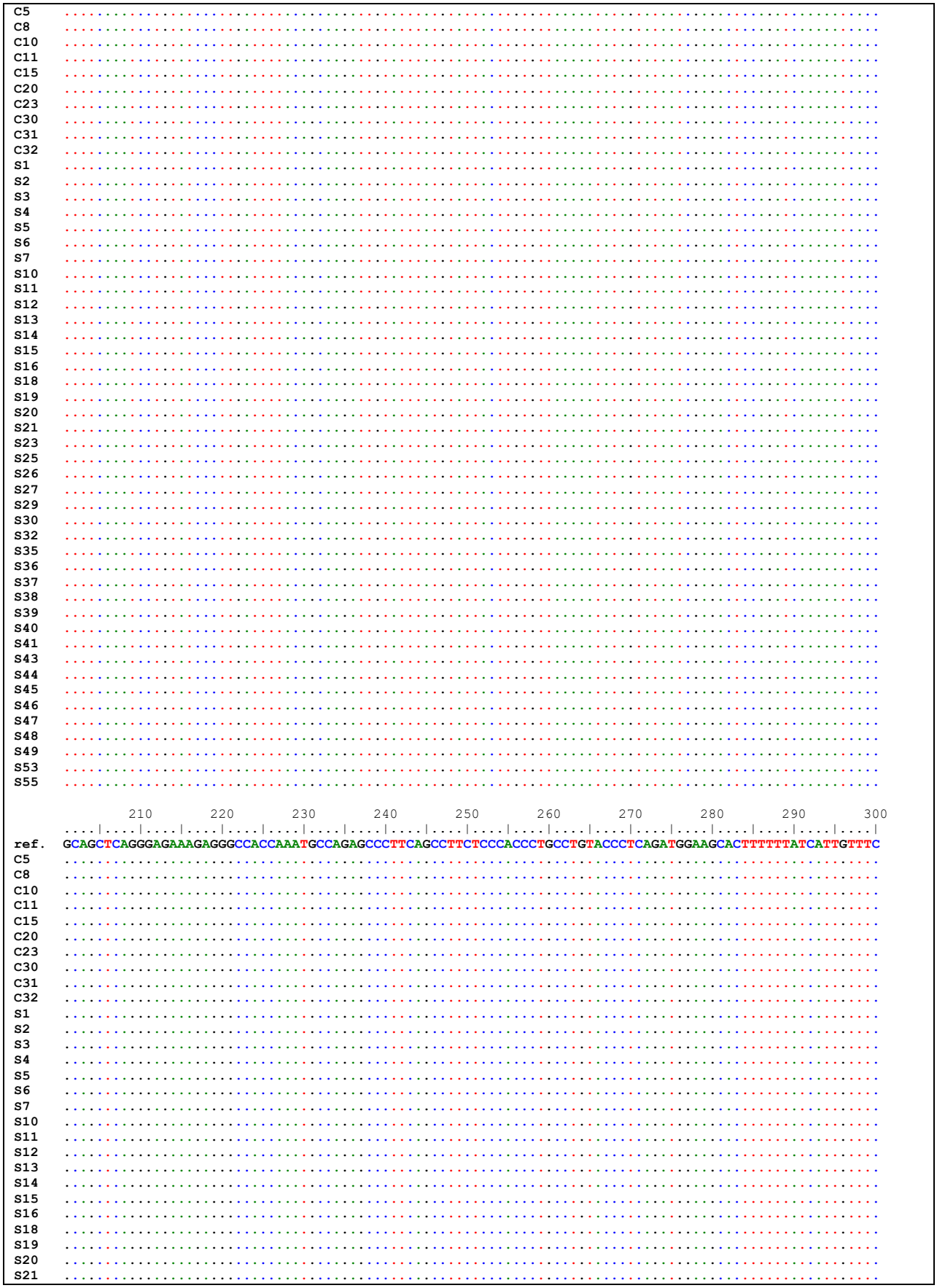
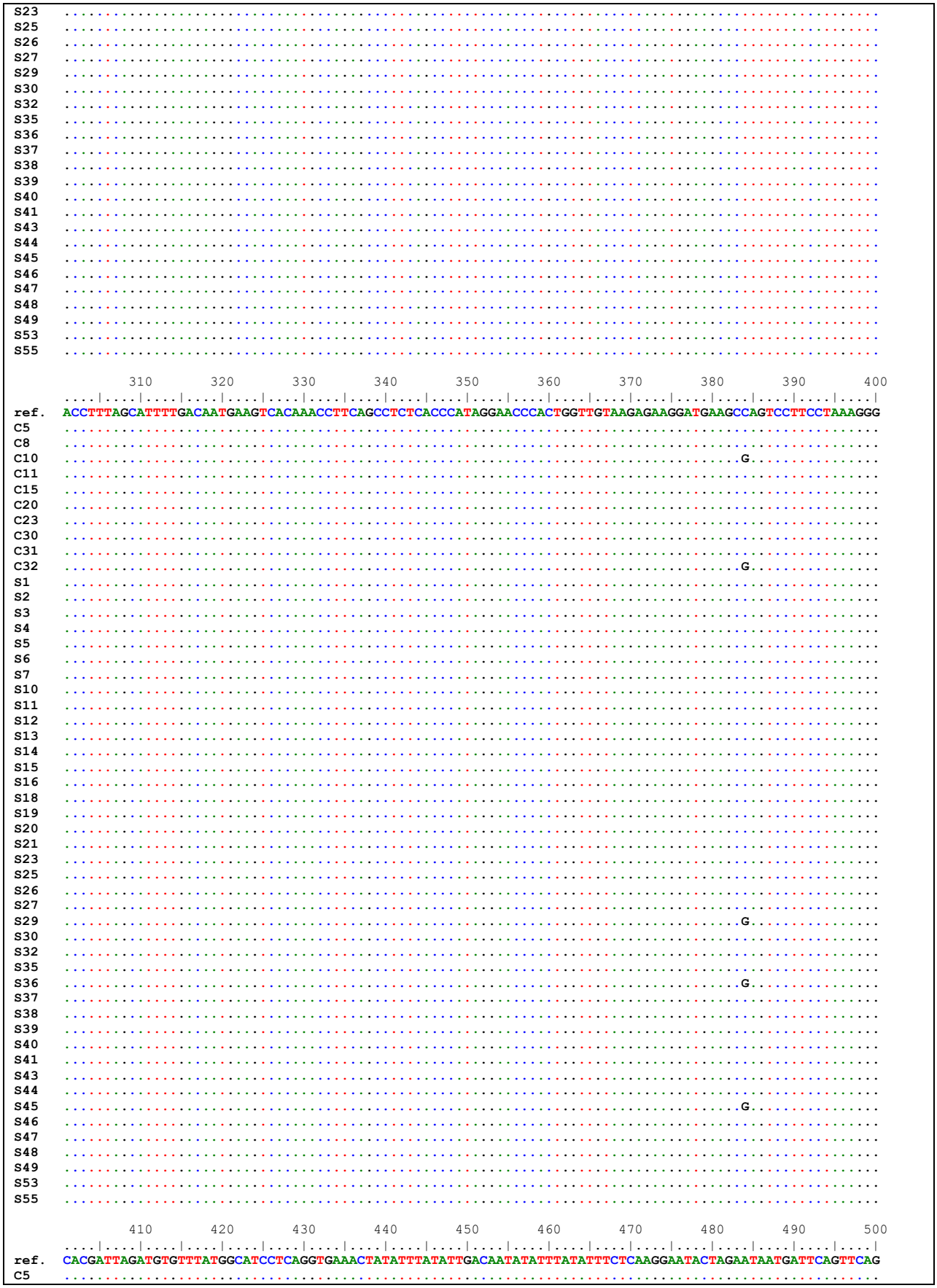


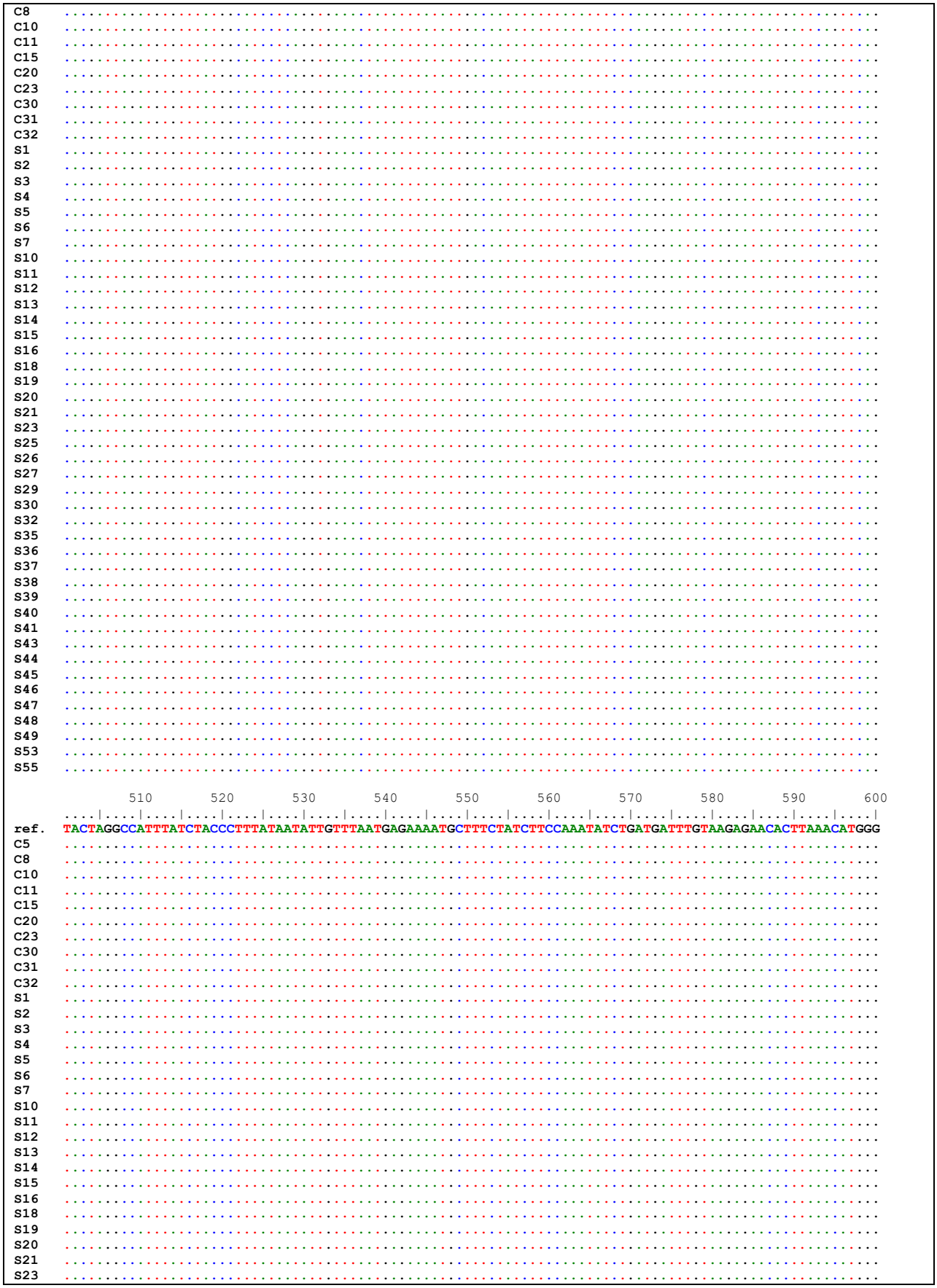

**Figure (4.38): DNA sequences alignment of fourty samples with their corresponding reference sequences of the 1075 bp amplicons of the *ILA17* gene. The symbols "ref." refers to the NCBI referring sequence, "S#" refers to the sample code, and "C" refers to controls.**

The alignment results of the 776 bp samples revealed the presence of three variations in some of the analyzed samples in comparison with the referring reference DNA sequences ,**Figure (4.43).**




C5
C8
C10
C11
C15
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C23
C30
C31
C32
S1
S2
S3
S4
S5
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S7
S10
S11
S12
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S43
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S49
S53
S55
210 220 230 240 250 260 270 280 290 300
ref. GCAGCTCAGGGAGAAAGAGGGCCACCAAATGCCAGAGCCCTTCAGCCTTCTCCCACCCTGCCTGTACCCTCAGATGGAAGCACTTTTTTATCATTGTTTC


S23
S25
S26
S27
S29
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S36
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S38
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S45
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S49
S53
S55
310 320 330 340 350 360 370 380 390 400
ref. ACCTTTAGCATTTTGACAATGAAGTCACAAACCTTCAGCCTCTCACCCATAGGAACCCACTGGTTGTAAGAGAAGGATGAAGCCAGTCCTTCCTAAAGGG
C5
C8
C10
C11
C15
C20
C23
C30
C31
C32
S1
S2
S3
S4
S5
S6
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S46
S47
S48
S49
S53
S55
410 420 430 440 450 460 470 480 490 500
ref. CACGATTAGATGTGTTTATGGCATCCTCAGGTGAAACTATATTTATATTGACAATATATTTATATTTCTCAAGGAATACTAGAATAATGATTCAGTTCAG
C5


C8
C10
C11
C15
C20
C23
C30
C31
C32
S1
S2
S3
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S5
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S48
S49
S53
S55
510
520
530
540
550
560
570
580
590
600
ref. TACTAGGCCATTTATCTACCCTTTATAATATTGTTTAATGAGAAAATGCTTTCTATCTTCCAAATATCTGATGATTTGTAAGAGAACACTTAAACATGGG
C5
C8
C10
C11
C15
C20
C23
C30
C31
C32
S1
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S3
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S23

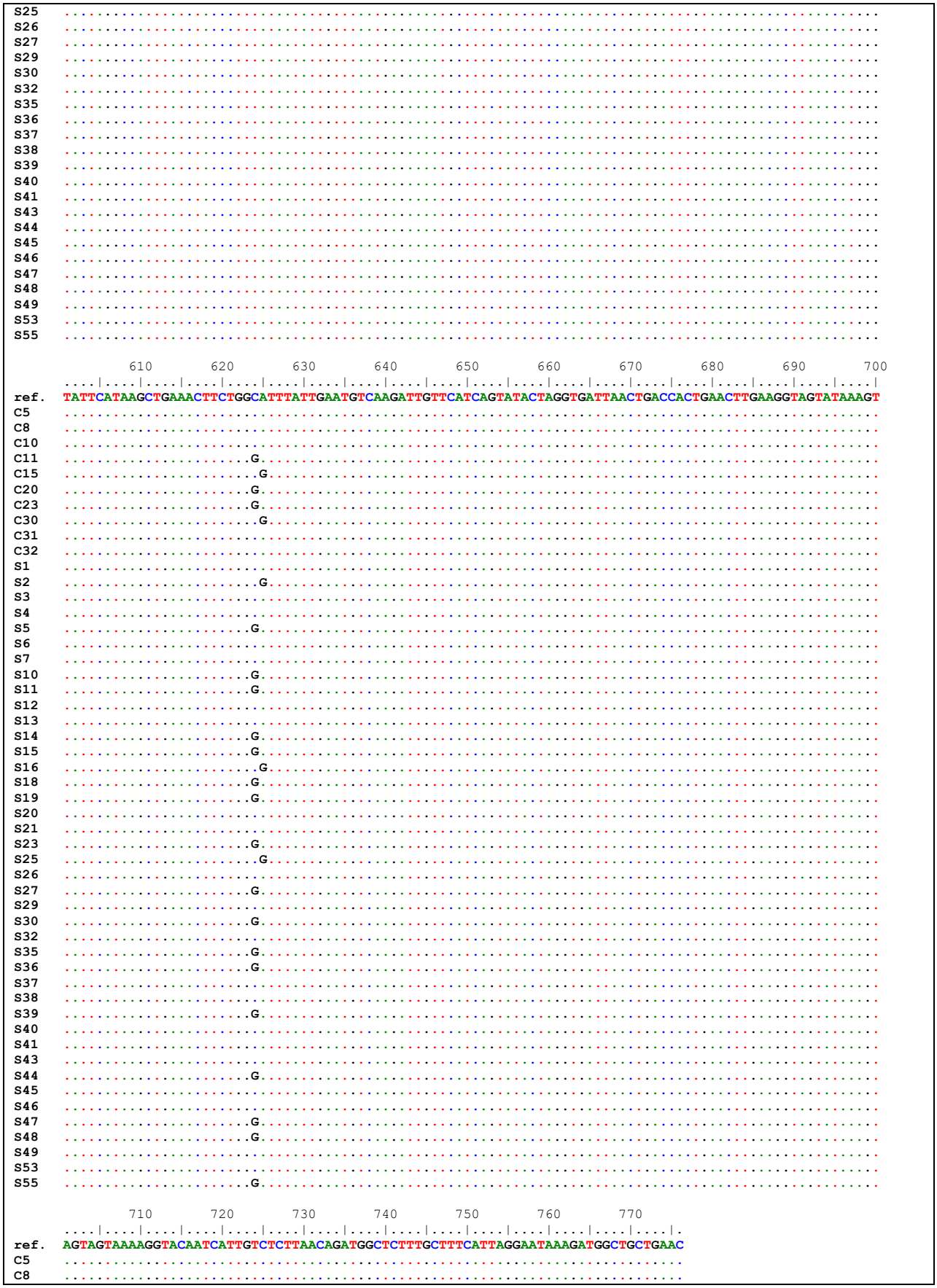
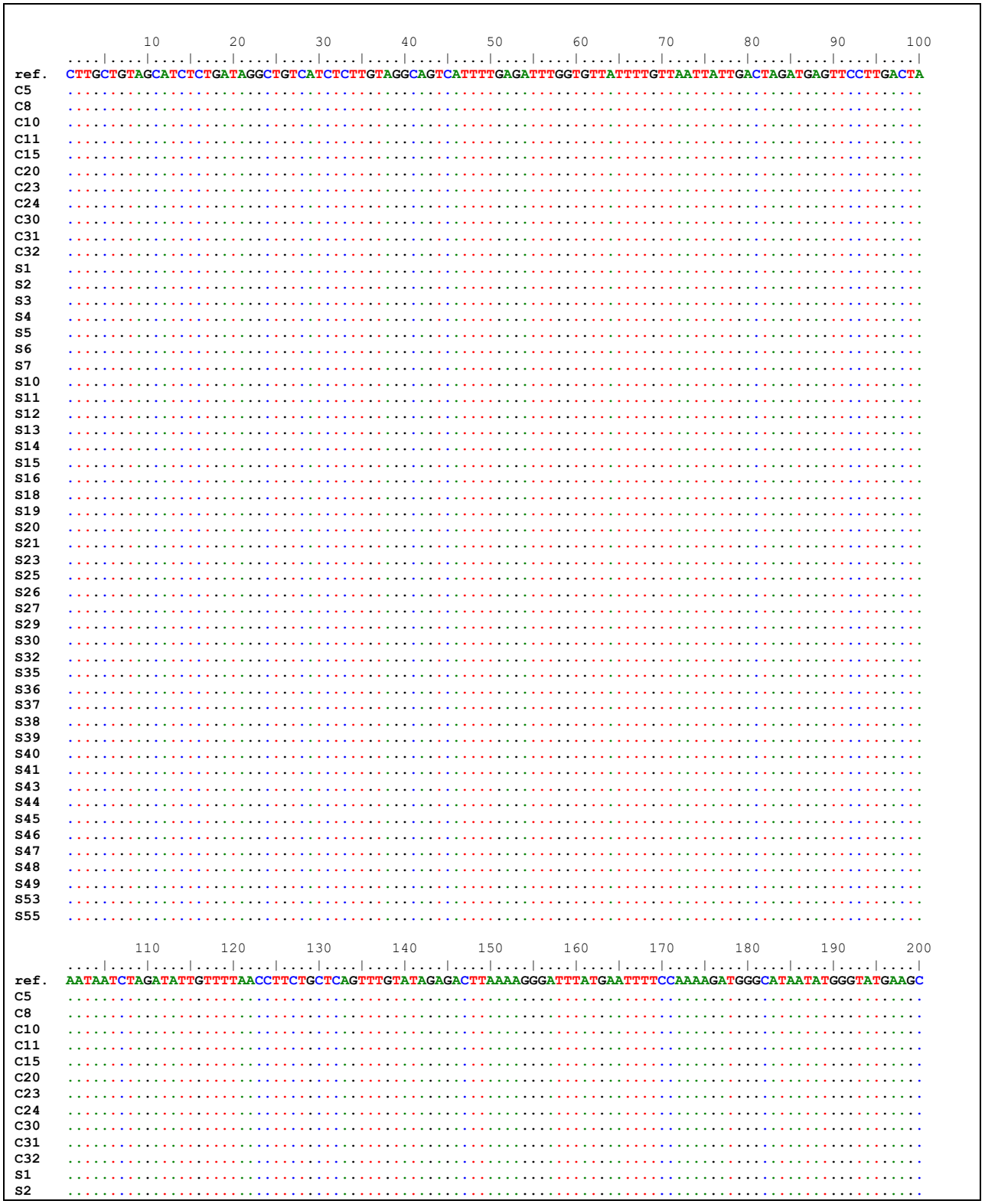
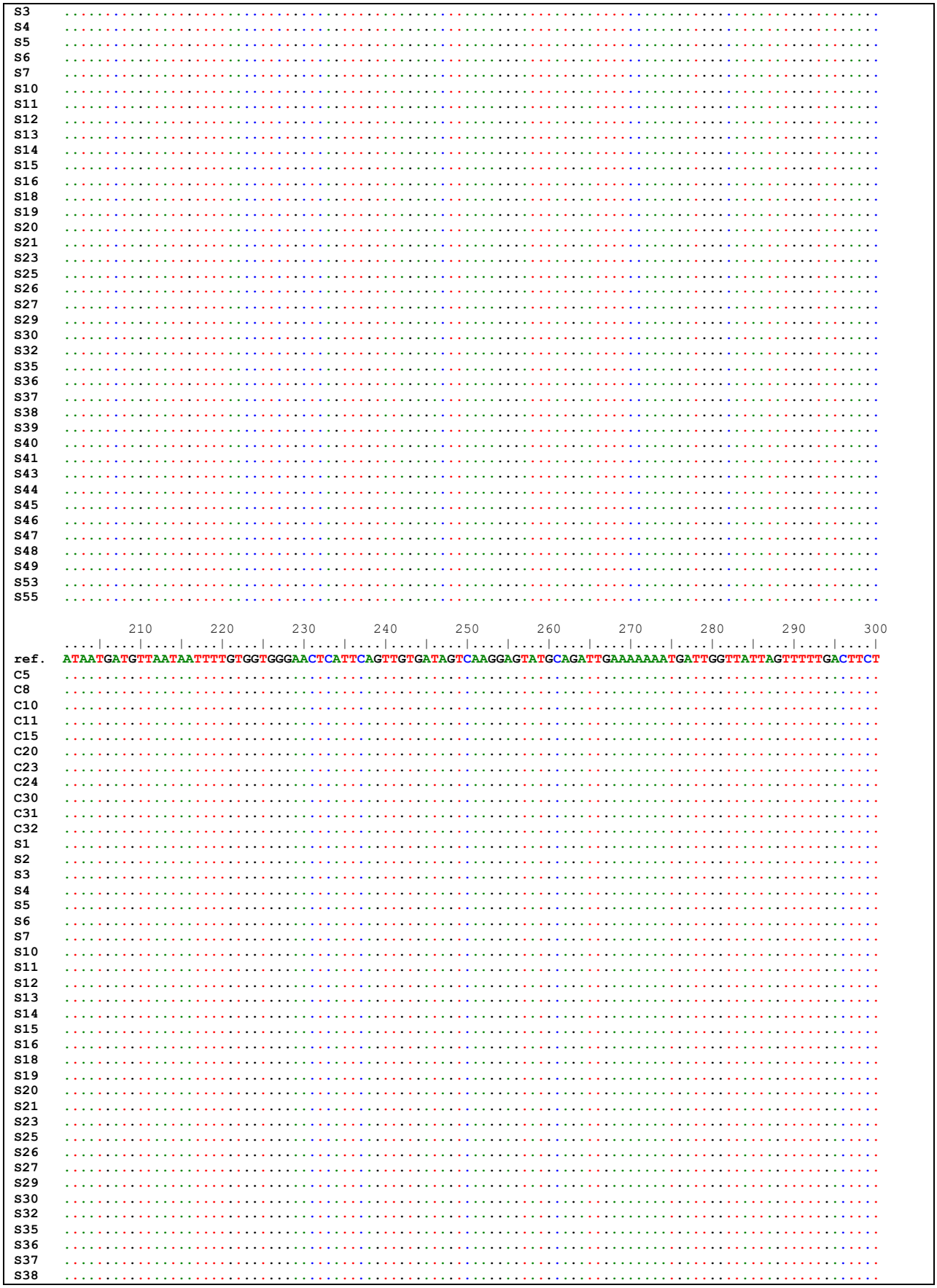

S25 S26 S27 S29 S30 S32 S35 S36 S37 S38 S39 S40 S41 S43 S44 S45 S46 S47 S48 S49 S53 S55
610 620 630 640 650 660 670 680 690 700
ref. TATTCATAAGCTGAAACTTCTGGCATTTATTGAATGTCAAGATTGTTCATCAGTATACTAGGTGATTAACTGACCACTGAACTTGAAGGTAGTATAAAGT
C5 C8 C10 C11 C15 C20 C23 C30 C31 C32 S1 S2 S3 S4 S5 S6 S7 S10 S11 S12 S13 S14 S15 S16 S18 S19 S20 S21 S23 S25 S26 S27 S29 S30 S32 S35 S36 S37 S38 S39 S40 S41 S43 S44 S45 S46 S47 S48 S49 S53 S55
710 720 730 740 750 760 770
ref. AGTAGTAAAAGGTACAATCATTGTCTCTTAACAGATGGCTCTTTGCTTTCATTAGGAATAAAGATGGCTGCTGAAC
C5 C8

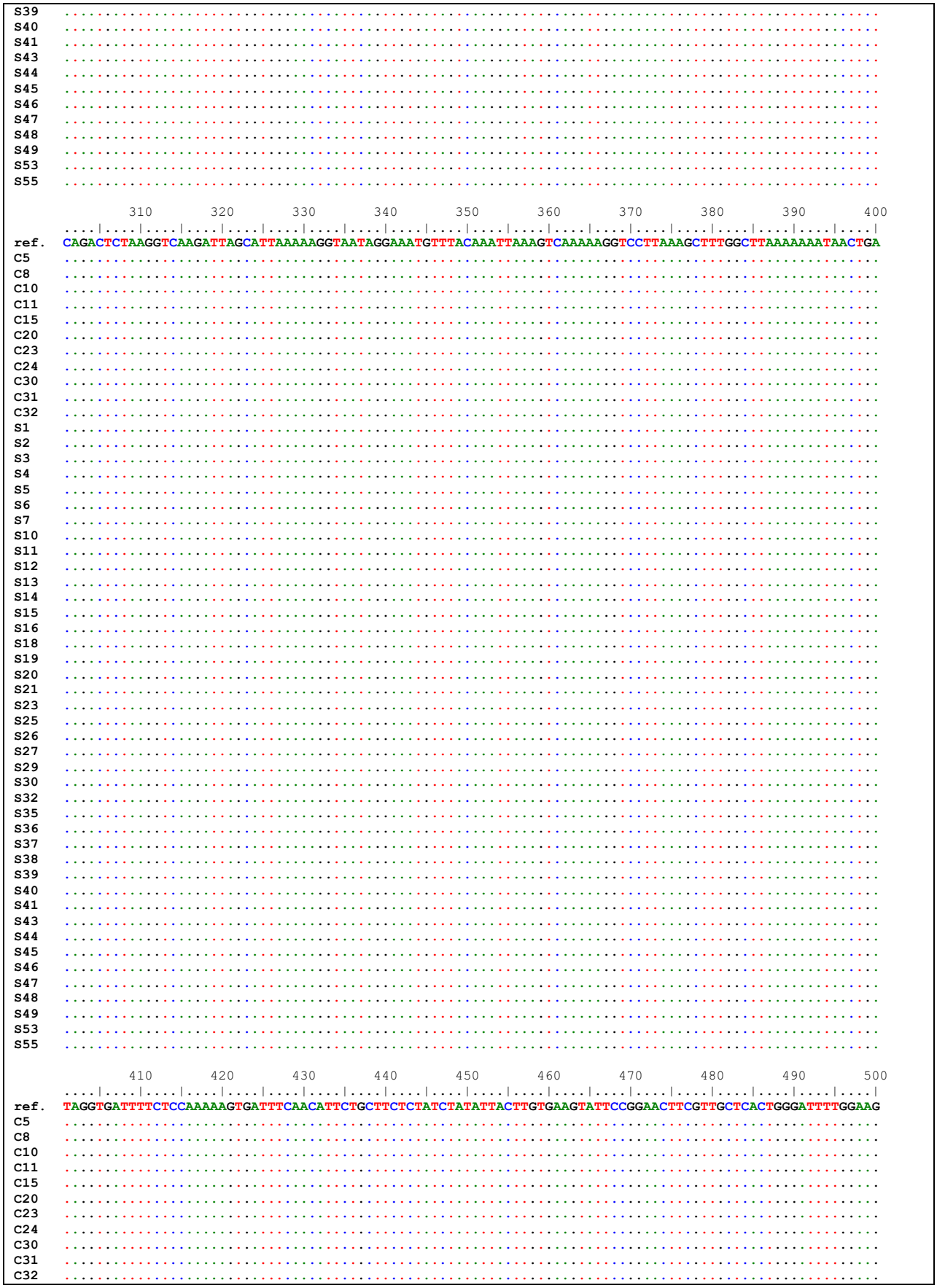


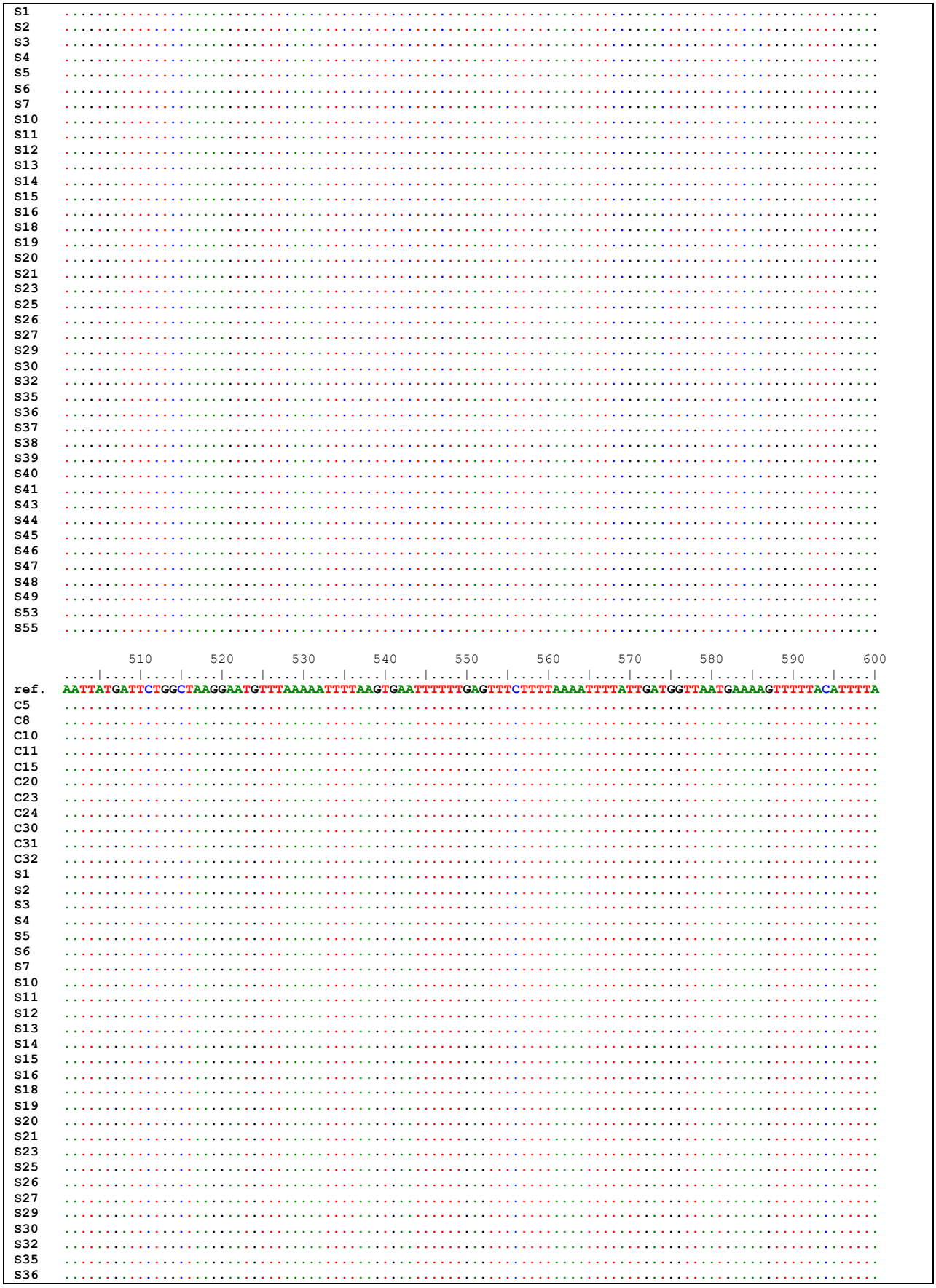

**Figure (4.43):** **DNA sequences alignment of fifty-one samples with their corresponding reference sequences of the 776 bp amplicons the downstream portion of the intron 1 and the upstream portion of exon 2 the *IL18* gene. The symbol "ref." refers to the NCBI referring sequence, "S#" refers to the sample code, and "C" refers to controls.**

The alignment results of the 790 bp samples revealed the presence of only one variation in some of the analyzed samples in comparison with the referring reference DNA sequences, **Figure (4.48).**




S3
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S18
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S40
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S43
S44
S45
S46
S47
S48
S49
S53
S55
210 220 230 240 250 260 270 280 290 300
ref. ATAATGATGTTAATAATTTTGTGGTGGGAACTCATTCAGTTGTGATAGTCAAGGAGTATGCAGATTGAAAAAAATGATTGGTTATTAGTTTTTGACTTCT
C5
C8
C10
C11
C15
C20
C23
C24
C30
C31
C32
S1
S2
S3
S4
S5
S6
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S38


S39
S40
S41
S43
S44
S45
S46
S47
S48
S49
S53
S55
310 320 330 340 350 360 370 380 390 400
ref. CAGACTCTAAGGTCAAGATTAGCATTAAAAAGGTAATAGGAAATGTTTACAAATTAAAGTCAAAAAGGTCCTTAAAGCTTTGGCTTAAAAAAATAACTGA
C5
C8
C10
C11
C15
C20
C23
C24
C30
C31
C32
S1
S2
S3
S4
S5
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S48
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S53
S55
410 420 430 440 450 460 470 480 490 500
ref. TAGGTGATTTTCTCCAAAAAGTGATTTCAACATTCTGCTTCTCTATCTATATTACTTGTGAAGTATTCCGGAACTTCGTTGCTCACTGGGATTTTGGAAG
C5
C8
C10
C11
C15
C20
C23
C24
C30
C31
C32


S1
S2
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S5
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S55
510 520 530 540 550 560 570 580 590 600
ref. AATTATGATTCTGGCTAAGGAATGTTTAAAAATTTTAAGTGAATTTTTTGAGTTTCTTTTAAAATTTTATTGATGGTTAATGAAAAGTTTTTACATTTTA
C5
C8
C10
C11
C15
C20
C23
C24
C30
C31
C32
S1
S2
S3
S4
S5
S6
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S11
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S36

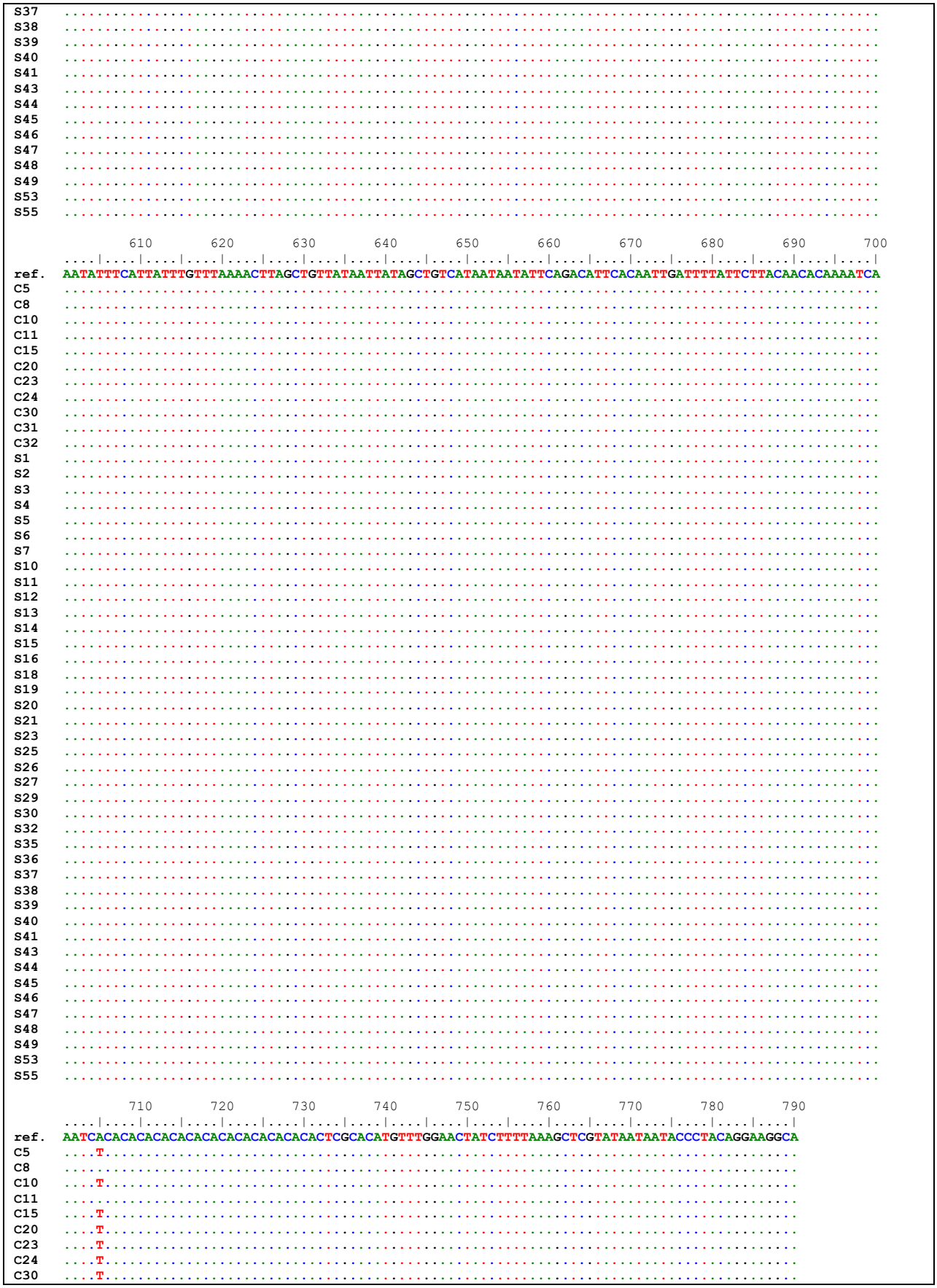

S37
S38
S39
S40
S41
S43
S44
S45
S46
S47
S48
S49
S53
S55
610 620 630 640 650 660 670 680 690 700
ref. AATATTTCATTATTTGTTTAAAACTTAGCTGTTATAATTATAGCTGTCATAATAATATTCAGACATTCACAATTGATTTTATTCTTACAACACAAAATCA
C5
C8
C10
C11
C15
C20
C23
C24
C30
C31
C32
S1
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S47
S48
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S53
S55
710 720 730 740 750 760 770 780 790
ref. AATCACACACACACACACACACACACACACACTCGCACATGTTTGGAACTATCTTTTAAAGCTCGTATAATAATACCCTACAGGAAGGCA
C5 ....T
C8
C10 ....T
C11
C15 ....T
C20 ....T
C23 ....T
C24 ....T
C30 ....T




**Figure (4.48): DNA sequences alignment of fifty-one samples with their corresponding reference sequences of the 790 bp amplicons of the intron 2 of the *INFG* gene. The letters "ref." refers to the NCBI referring sequence, "S" and "C" refer to the sample and control numbers respectively.**

- **GenBank accession numbers for all samples in the current study:**

BankIt2421762 seq1 MW526081

BankIt2421762 seq2 MW526082

BankIt2421762 seq3 MW526083

BankIt2421762 seq4 MW526084

BankIt2421762 seq5 MW526085

BankIt2421762 seq6 MW526086

| | |
|---|---|
| BankIt2421762 seq7 | MW526087 |
| BankIt2421762 seq8 | MW526088 |
| BankIt2421762 seq9 | MW526089 |
| BankIt2421762 seq10 | MW526090 |
| BankIt2421762 seq11 | MW526091 |
| BankIt2421762 seq12 | MW526092 |
| BankIt2421762 seq13 | MW526093 |
| BankIt2421762 seq14 | MW526094 |
| BankIt2421762 seq15 | MW526095 |
| BankIt2421762 seq16 | MW526096 |
| BankIt2421762 seq17 | MW526097 |
| BankIt2421762 seq18 | MW526098 |
| BankIt2421762 seq19 | MW526099 |
| BankIt2421762 seq20 | MW526100 |
| BankIt2421762 seq21 | MW526101 |
| BankIt2421762 seq22 | MW526102 |
| BankIt2421762 seq23 | MW526103 |
| BankIt2421762 seq24 | MW526104 |
| BankIt2421762 seq25 | MW526105 |
| BankIt2421762 seq26 | MW526106 |
| BankIt2421762 seq27 | MW526107 |
| BankIt2421762 seq28 | MW526108 |
| BankIt2421762 seq29 | MW526109 |
| BankIt2421762 seq30 | MW526110 |
| BankIt2421762 seq31 | MW526111 |
| BankIt2421762 seq32 | MW526112 |

| | |
|---|---|
| BankIt2421762 seq33 | MW526113 |
| BankIt2421762 seq34 | MW526114 |
| BankIt2421762 seq35 | MW526115 |
| BankIt2421762 seq36 | MW526116 |
| BankIt2421762 seq37 | MW526117 |
| BankIt2421762 seq38 | MW526118 |
| BankIt2421762 seq39 | MW526119 |
| BankIt2421762 seq40 | MW526120 |
| BankIt2421771 seq1 | MW526121 |
| BankIt2421771 seq2 | MW526122 |
| BankIt2421771 seq3 | MW526123 |
| BankIt2421771 seq4 | MW526124 |
| BankIt2421771 seq5 | MW526125 |
| BankIt2421771 seq6 | MW526126 |
| BankIt2421771 seq7 | MW526127 |
| BankIt2421771 seq8 | MW526128 |
| BankIt2421771 seq9 | MW526129 |
| BankIt2421771 seq10 | MW526130 |
| BankIt2421771 seq11 | MW526131 |
| BankIt2421771 seq12 | MW526132 |
| BankIt2421771 seq13 | MW526133 |
| BankIt2421771 seq14 | MW526134 |
| BankIt2421771 seq15 | MW526135 |
| BankIt2421771 seq16 | MW526136 |
| BankIt2421771 seq17 | MW526137 |
| BankIt2421771 seq18 | MW526138 |

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| BankIt2421771 seq19 | MW526139 |
| BankIt2421771 seq20 | MW526140 |
| BankIt2421771 seq21 | MW526141 |
| BankIt2421771 seq22 | MW526142 |
| BankIt2421771 seq23 | MW526143 |
| BankIt2421771 seq24 | MW526144 |
| BankIt2421771 seq25 | MW526145 |
| BankIt2421771 seq26 | MW526146 |
| BankIt2421771 seq27 | MW526147 |
| BankIt2421771 seq28 | MW526148 |
| BankIt2421771 seq29 | MW526149 |
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| BankIt2421771 seq37 | MW526157 |
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| BankIt2421771 seq45 | MW526165 |
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| BankIt2421771 seq48 | MW526168 |
| BankIt2421771 seq49 | MW526169 |
| BankIt2421771 seq50 | MW526170 |
| BankIt2421771 seq51 | MW526171 |
| BankIt2421774 seq1 | MW526172 |
| BankIt2421774 seq2 | MW526173 |
| BankIt2421774 seq3 | MW526174 |
| BankIt2421774 seq4 | MW526175 |
| BankIt2421774 seq5 | MW526176 |
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| BankIt2421774 seq46 | MW526217 |
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| BankIt2421774 seq48 | MW526219 |
| BankIt2421774 seq49 | MW526220 |
| BankIt2421774 seq50 | MW526221 |
| BankIt2421774 seq51 | MW526222 |
| BankIt2421774 seq52 | MW526223 |

## الخلاصة

يحدث داء البروسيلات بسبب بكتريا من جنس البروسيلا *Brucella* والتي هي بكتريا صغيرة سالبة لصبغة جرام غير متحركة وغير مكونة للابواغ وهي عصيات مكورة هوائية اختيارية توجد داخل الخلايا قادرة على غزو الخلايا الظهارية والبانيات التغذوية المشيمية والخلايا التغصنية اضافة الى البلاعم الكبيرة. توجد أربعة أنواع منها تسبب الاصابة للبشر وهي: *B.abortus* و *B.canis* و*B.mellitunsis* و *B.suis*. تشمل الأعراض الحمى المتموجة والتعرق الغزير وآلام المفاصل والعضلات. يعد داء البروسيلات أحد أكثر الأمراض حيوانية المصدر شيوعًا في جميع أنحاء العالم مع وجوده كمرض متوطن في بعض المناطق في الشرق الأوسط وآسيا الوسطى وأفريقيا وأمريكا الوسطى والجنوبية. تحدث الاصابة في الإنسان بشكل اساسي من ملامسة الماشية المصابة أو استهلاك منتجات حيوانية ملوثة و تعتبر الماشية والحيوانات المجترة الصغيرة مضائف خازنة رئيسية للمرض.

استهدفت الدراسة الحالية تحديد أبرز المظاهر المناعية الخلوية والخلطية لداء البروسيلا الحاد والمزمن وهي ( CD3، CD4، CD8، IL-17، IL-18، IFN-γ) واستكشاف تعدد اشكال جينية معينة للمضيف والتي قد تؤثر على حساسية وضراوة داء البروسيلات.

أجريت هذه الدراسة المقطعية العرضية في مدينة بعقوبة – محافظة ديالى للفترة من شهر شباط 2020 إلى شهر حزيران 2021 وشملت 55 مريضاً يشتبه سريرياً بإصابتهم بداء البروسيلات حيث كانت نتائجهم إيجابية اساسا لاختبار تراص روز بنغال. تراوحت أعمار المرضى بين (12 و 65) عامًا (11 من الذكور و 44 من الاناث) تم اختيارهم من مستشفى بعقوبة التعليمي ومستشفى البتول التعليمي للولادة والأطفال بالإضافة الى 35 شخصا من الاصحاء كمجموعة سيطرة (16 من الذكور و 19 من الاناث) تراوحت أعمارهم بين (14و65) سنة. تمت الموافقة على اجراء هذه الدراسة

من قبل اللجنة العلمية في مديرية صحة ديالى وكذلك من قبل اللجنة العلمية والأخلاقية في كلية التربية للعلوم الصرفة–جامعة ديالى. جمعت المعلومات الاجتماعية–الديموغرافية والسريرية من كل شخص من خلال استبيان خاص معد مسبقًا لهذا الغرض. تم احترام خصوصية المرضى من خلال موافقتهم الشفهية. اجري التحليل الإحصائي باستخدام برنامج الحزمة الإحصائية للعلوم الاجتماعية (SPSS) الإصدار 27. واعتبرت القيمة $P < 0.05$ ذات دلالة معنوية إحصائيا. اخضعت مجموعتي الدراسة أولاً للكشف عن الأجسام المضادة للبروسيلا (IgG و IgM) باستخدام تقنية مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA) ثم تحديد مستوى كل من الانترلوكينات IL–17 و IL–18 و IFN–γباستخدام عدد منفصلة من تقنيات ELISA. علاوة على ذلك تم تقييم مستويات CD3 و CD4 و CD8 باستخدام تقنية ELISA وأخيرًا تم الكشف عن جينات كل من IL–17 و IL–18 و IFN–γ باستخدام تقنية تفاعل البلمرة المتسلسل Conventional–PCR.

أظهرت النتائج أن جميع المرضى (100%) كانوا إيجابيين لاختبار تراص روز بنغال Rose–Bengal مقابل 5.7 ٪ من مجموعة السيطرة مع وجود فروق ذات دلالة معنوية عند (P=0.0001). كان معدل الإيجابية للضد IgM بين المرضى 32.7٪ بينما كانت ايجابية بنسبة 5.7٪ عند مجموعة السيطرة مع فرق ذو دلالة معنوية إحصائياً عند (P=0.003). علاوة على ذلك، كان العيار الحجمي لاضداد IgM المضادة للبروسيلا أعلى بكثير من العيار الحجمي لمجموعة السيطرة عند (P=0.011). وجد أن 43.8٪ من الذين كانوا إيجابيين للضد IgM كانوا إيجابيين أيضًا لخلايا CD3 مقابل 17.5٪ كانوا إيجابيين للضد IgM لكن سلبيين لخلايا CD3 وبفروقات ذات دلالة معنوية احصائيا عند (P=0.040). بلغت نسبة الإيجابية لضد البروسيلا IgG بين المرضى 56.4٪ والتي كانت ذات دلالة معنوية إحصائيا مقارنة بمجموعة السيطرة عند (P=0.0001). وجدت النتائج

أيضًا أن العيار الحجمي لضد البروسيلا IgG في المرضى كان أعلى بكثير من نظيره في مجموعة السيطرة عند (P=0.0001). كانت الحمى موجودة في 63.3٪ من المرضى المصابين الايجابيين للضد IgG والتي كانت ذات دلالة معنوية إحصائيا مقارنة بالذين لا يعانون من الحمى عند (P=0.0001). بالاضافة لذلك، أظهرت النتائج امكانية اكتشاف جينات الانترفيرون غاما IFN-γلدى 56.4٪ من المصابين بالبروسيلات والايجابيين للضد IgG مع وجود فروقات ليست لها دلالة معنوية احصائيا عند (P=0.249).

كانت نسبة إيجابية خلايا CD3 بين المرضى 58.2٪ مقابل 28.6٪ لدى مجموعة السيطرة مع وجود فرق ذو دلالة معنوية إحصائياً عند (P=0.006). وأظهرت النتائج أن 77.8٪ من المرضى الايجابيين لخلايا CD3 كانوا ايجابيين أيضا لضد البروسيلا IgM مع وجود فرق ذو دلالة معنوية إحصائية مقارنة بنظيره عند (P=0.040). وجد أن 61.5٪ ممن كانوا إيجابيين لخلايا CD3 كانوا إيجابيين أيضًا للانترفيرون غاما INF-γ مع فروقات كبيرة مقارنة بنظيره عند (P=0.036)، وان نسبة إيجابية خلايا CD4 بين المرضى كانت 45.5٪ بينما كانت النسبة لدى مجاميع السيطرة 42.9٪ مع وجود فروقات ليس لها دلالة معنوية إحصائياً عند (P=0.809). بالاضافة لذلك، كانت نسبة الإيجابية لخلايا CD4 أعلى بشكل ملحوظ بين الاشخاص المقيمين في المدن مقارنة بهؤلاء المقيمين في القرى (63.3٪ مقابل 24.0٪) عند (P=0.004). كانت نسبة إيجابية خلايا CD8 بين المرضى 96.4٪ بينما بلغت تلك النسبة 45.7٪ مع وجود فرق ذو دلالة معنوية إحصائياً عند (P=0.0001). وجد أن نسبة إيجابية الانترفيرونIFN-γ- مرتبطة بشكل ملحوظ مع نسبة إيجابية خلايا CD8 (98.1٪ مقابل 66.7٪) عند (P=0.005).

أظهرت نتائج الإنترلوكينات أن نسبة إيجابية الانترلوكين السابع عشر IL−17 بين مرضى البروسيلات كانت 58.2٪ بينما بلغت تلك النسبة في مجموعة السيطرة 42.9٪ مع وجود فروقات ليست لها دلالة معنوية إحصائياً (P=0.156) و كانت نسبة إيجابية الانترلوكين السابع عشر IL−17 أعلى قليلا بين الذكور مقارنة بالإناث (63.6٪ مقابل 56.8٪) عند (P=0.682). كانت نسبة إيجابية الانترلوكين السابع عشر IL−17 أعلى بشكل ملحوظ بين الاشخاص المشاركين الايجابيين لخلايا CD4 مقارنة بأولئك الذين كانوا سلبيين لخلايا CD4 (80.0%) مقابل (40.0%) عند (P=0.003). بلغت نسبة إيجابية الانترلوكين الثامن عشر IL−18 بين المرضى 27 (49.1٪) وهي أعلى من تلك التي سجلت لدى الاشخاص في مجموعة السيطرة 11 (31.4٪). ومع ذلك لم يتمكن الفارق من الوصول إلى مستوى الدلالة الإحصائية عند (P=0.098). كانت نسبة إيجابية الانترلوكين الثامن عشر IL−18 أعلى بين المشاركين الذين يشكون من آلام المفاصل مقارنة بنظرائهم من الاصحاء (55.3٪ مقابل 12.5٪) مع وجود فرق ذو دلالة معنوية إحصائيًا عند (P=0.025). بلغت نسبة إيجابية الانترفيرون غاما IFN−γ لدى المرضى 94.5٪ وهي ذات دلالة معنوية عالية احصائيا مقارنة مع مجموعة السيطرة (34.3٪) عند (P=0.0001). أظهرت النتائج أيضًا أن نسبة إيجابية الانترفيرون غاما INF−γ كان مرتبطًا بشكل كبير مع الاشخاص الذين يستهلكون منتجات الألبان مقارنة بنظرائهم (98.0٪ مقابل 60.0٪) عند (P=0.0001). كانت نسبة إيجابية الانترفيرون غاما IFN−γ أعلى بشكل ملحوظ بين المشاركين الذين كانوا ايجابيين لخلايا CD3 مقارنة بنظرائهم (100.0٪ مقابل 87.0٪) عند (P=0.036). بالاضافة لذلك، كانت نسبة إيجابية الانترفيرون غاما INF−γ أعلى بشكل ملحوظ بين أولئك الايجابيين لخلايا CD8 مقارنة بأولئك السلبيين لخلايا CD8 (96.2)٪ مقابل (50.0٪) مع وجود فرق ذو دلالة معنوية عند (P=0.005).

اظهرت نتائج الكشف بتقنية PCR عن الجينات الثلاثة ان نسبة الجين للانترلوكين السابع عشر IL−17 كانت 60٪ بين مرضى البروسيلات وهو أعلى بكثير مقارنة بمجموعة السيطرة 20٪ عند (P=0.0001). وكانت نسبة الكشف عن جين الانترلوكين الثامن عشر IL−18 بين المرضى 72.7٪ وهو أيضًا ذو دلالة معنوية عالية مقارنة بمجموعة السيطرة 28.6٪ عند (P=0.0001). بالإضافة إلى ذلك، كانت نسبة الكشف عن جين الانترفيرون غاما IFN−γ بين المرضى 70.9٪ وهي نسبة ذات أهمية معنوية عالية مقارنة بنظيرها في مجموعة السيطرة 31.4٪.

تم تحليل التسلسلات الجينية للانترلوكين IL−17A في الكروموسوم 6 في 51 عينة. كشفت نتائج المحاذاة لعينات 1075bp عن وجود سبعة اختلافات (تعدد أشكال النوكليوتيدات المفردة (SNPs) في العينات التي تم تحليلها مقارنة بتسلسل الحامض النووي المرجعي. تم استبدال هذه النيوكلوتايدات السبعة بالنيوكليوتيدات الأخرى في المواقع 215 و 219 و 341 و 356 و 537 و 603 و 679. تم الكشف عن اثنين من هذه الأشكال المتعددة في ثلاثة أنماط متعددة الأشكال وهي النمط A356G متماثل الجينات A/A والنمط s55 متغاير الجينات A/G وكذلك النمط S49 متماثل الجينات G/G. أظهرت بدائل الأحماض النووية الخمسة الأخرى التي تمت ملاحظتها شكلين فقط متعددة الأشكال وهي الشكل متماثل الجينات من النمط البري وشكل متغاير الجين الطافر او المتحور. كانت هذه الطفرات عبارة عن A215T و A219C و A341G و A537C و G679A. بالإضافة لهذا، وجد ان اربعة من الاشكال الجينية المتعددة (SNP) كانت تعرف سابقا كما كانت مودعة بالاسماء rs8193037 and rs2275913, rs3819024, rs1582236522 بدلا من A341G, A356G, A603G, and G679A على التوالي. يتم التعرف على الشكل الجيني rs1582236522 بشكل ملحوظ في جين الانترلوكين IL−17A المستهدف بسبب تردده العالي

(0.5). ومع ذلك، لم يتم الإبلاغ عن هذا الشكل الجيني المفرد SNP في أي منشورات مفهرسة في قاعدة بيانات dbSNP. وبالمثل تم إيداع الشكل rs3819024أيضًا وبتردد عالٍ هو (0.33) في قاعدة بيانات dbSNP. ومن خلال مراجعة نفس سجلات البيانات dbSNP، وجد أن ثلاثة من الاشكال الجينية المتعددة المفردة من (SNP) التي تمت ملاحظتها وهي (A215T و A219C و A537C) لم يتم إيداعها مسبقًا في قاعدة بيانات dbSNP، لذلك تم تأكيد بدائل الحامض النووي الجديدة والتحقق من صحتها. يمكن لارتفاع قيم اختلال توازن الارتباط LD المرتفعة لأول ثلاثة أشكال من النيوكليوتيدات المتعددة المفردة SNPs ان يفسر قدرة هذه النيوكلوتايدات على تسريع تطور وتقدم داء البروسيلات.

يقوم التسلسل الجيني للانترلوكين الثامن عشر IL-18 الموجود في الكروموسوم 12 بالتشفير لتصنيع بروتين الإنترلوكين الثامن عشر 18. كشفت نتائج المحاذاة لعينات 776bp وجود ثلاثة تعددات للأشكال في العينات التي تم تحليلها مقارنة بتسلسل الحامض النووي المرجعي. في هذه النيوكلوتايد التي تم الكشف عنها، تم استبدال الحامض الاميني سايتوسين cytosine بالجوانين guanin في الموقع 384 (C384G) وتم استبدال السيتوزينcytosine بالأدينين adenine عند الموقع 624 (C624A) ، وكذلك تم استبدال الأدينين adenine بالجوانين guanin في الموقع 625 (A625G). اما فيما يتعلق بالموقع C384G، تم الكشف عن ثلاثة أنماط من الأشكال المتعددة في هذا المكان وهي النمط متماثل الجينات C/C والنمط متغاير الجينات C/G والنمط متماثل الجينات G/G. على عكس كل من الموقعين C384G و C624A، لوحظ وجود شكلين فقط من الاشكال المتعددة في حالة الموقع A625G SNP. وبالتالي فإن مضخما 776 bp للانترلوكين

الثامن عشر18 احتوت فقط على ثلاثة أشكال مفردة SNPs مسترجعة . تراوحت قيم اختلال توازن الارتباط LD لهذه النيوكلوتايدات بين قيم منخفضة و مرتفعة.

تقوم التسلسلات الجينية للانترفيرون غاما IFN–γ الموجودة في كروموسوم 12 بالتشفير لتصنيع بروتين الانترفيرون غاما IFN–γ . كشفت نتائج المحاذاة لعينات 790bp وجود شكل مفرد واحد فقط (SNP) في العينات التي تم تحليلها مقارنة بتسلسل الحامض النووي المرجعي. في هذا الشكل المفرد SNP، تم استبدال الحامض الاميني أدينينadenine بالثايمين thymine في الموقع 705 في الموقع الذي تم تحليله. تم الكشف عن هذا الشكل المفرد SNP في ثلاثة أنماط وهي النمط A/A متماثل الجينات والنمط متغاير الجينات A/T وأنماط T/T متماثلة الجينات. من خلال مراجعة محرك dbSNP، وجد أن الموقع A705T SNP الذي تم الكشف عنه قد تم إيداعه مسبقًا كمتغير انتروني حيث تم إيداعه كـ rs2430561. ومع ذلك، تم التعرف عليه بشكل كبير في جين الانترفيرون غاما IFN–γالمستهدف نظرًا لزيادة تردده الذي يتراوح من 0.25 إلى 0.35 (https://www.ncbi.nlm.nih.gov/snp/rs2430561).

يمكن الاستنتاج بان الدراسة الحالية وجدت أن داء البروسيلات يثير الاستجابات المناعية الخلوية والخلطية المعقدة وأن تعدد أشكال النوكليوتيدات المفردة في بعض الجينات تشارك في تنظيم المناعة الوقائية، وخاصة جين الانترلوكين السابع عشر IL–17 الذي قد يكون له دور في تطور داء البروسيلات وشدته.

وزارة التعليم العالي والبحث العلمي

جامعة ديالى كلية التربية للعلوم الصرفة

قسم علوم الحياة

# دراسة وراثية مناعية عن حمى مالطا الحاد والمزمن في محافظة ديالى

أطروحة مقدمة

إلى مجلس كلية التربية للعلوم الصرفة بجامعة ديالى كجزء من متطلبات نيل درجة دكتوراه فلسفة في علوم الحياة

من قبل

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