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ASSOCIATION OF ANTI-BRUCELLA ANTIBODIES WITH CIRCULATORY IL-17, IL-18 AND INF-Γ GENES IN PATIENTS WITH BRUCELLOSIS IN DIYALA PROVINCE-IRAQ

Shahad Kh. Al-Qaisi[1], Hasan A. SH.[2], Maha F. Nazzal[3]
[1]*(MSc.Microbiol.) Middle Technical University-Baquba Technical Institute*
[2]*(Ph.D. Med. Microbiol.), University of Diyala-College of Medicine*
[3]*(Ph.D. Immunol.) University of Diyala, College of Education for pure science*

ABSTRACT

Background: Brucellosis is an infectious disease and one of the major public health problems worldwide. Among other areas, Brucellosis is endemic in the Middle East. Several studies have provided data that production of certain interleukins as well as polymorphisms in their genes may play a role in susceptibility or resistant of brucellosis.

Objective: Exploring the *Il-17*, *Il-18* and *INF-γ* gene detection rate and to figure out their association with anti-brucella IgM and IgG in patients clinically had brucellosis.

Patients and method: A total of 55 patients who were clinically suspected as having brucellosis and were tested positive for agglutination (Rose Bengal) test were included as patients group and 35 apparently healthy individuals were included as control group. In both patient and control groups, the highest age range was 20-29 years. 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. 20% of patients were males and 80% were females, while, 37.1 % of controls were males and 62.9% were females. The study groups were allocated from Baquba Teaching Hospital and some Healthcare Centers. A questionnaire form was preconstructed including socio-demographic and clinical information. Blood samples were collected from both study groups, sera were separated and submitted for slide agglutination (Rose Benal) test (Spin-React, Spain), Anti-Brucella IgM and IgG using ELISA technique (Demeditec - Germany). The IL-17, IL-18 and INF-γ genes was detected in the blood using RT-PCR (Promega, USA) after DNA extraction and quantification (Quantus Fluorometer). Human privacy was respected by obtaining verbal consent from all participants. Statistical analysis was done using SPSS version 25 and p values ≤ 0.05 were considered significant.

Results: The results found 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). on the other hand, 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). The results also revealed that the PCR 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%). None of the *IL-17*, *IL-18* and *INF- γ* genes detection rate had insignificant association with anti-brucella IgM although their detection rate among patients were higher compared to negativity rate. Similar insignificant association of the three genes with the anti-brucella IgG antibody.

Conclusion: The detection rate of IL-17, IL-18 and *INF- γ* were higher among patient with acute brucellosis, but they had insignificant association with anti-brucella IgM. However, the detection rate of these genes were lowered during chronic phase of the disease and insignificantly associated with anti-brucella IgG antibody.

Keywords: Brucellosis, Interleukin -17, Interleukin-18, Interferon- γ

I. INTRODUCTION:

Brucella are facultative intracellular, non-sporeforming, nonmotile, gram-negative bacteria causing brucellosis. Brucellosis is responsible for negatively affecting animal production and the health of humans in most developing countries (Elfaki *et al*., 2005). Transmission to humans through consumption of unpasteurized dairy products or through direct contact with infected animals, placentas or aborted fetuses (Ariza *et al*., 2007). Globally, there are 500 000 new cases of brucellosis each year, and the true incidence is always much higher than the reported number of cases (Pappas *et al*., 2006). The disease in areas like the Middle East, Eastern Europe, Africa, and Latin America is endemic (Lucero *et al*., 2008)..

Brucella has the propensity to localize inside macrophages in different body organs with protean clinical manifestations (Mantur *et al*., 2006). The intra- macrophages existence of brucellae represents one of the strategies for consequent alterations in both innate and adaptive immune responses and induction of chronic persistent infection (Elfaki *et al*., 2015; Jiao *et al*., 2021). It has been reported that cell mediated immunity is playing a crucial role in immunity response to brucellosis. $CD4^+$ and $CD8^+$ T lymphocytes had a key role in cellular immunity as they can release IFN-γ and activate the functions in macrophages (Golding *et al*., 2001; Ko and Splitter, 2003). Additionally, IL10 is a crucial cytokine contributing to resist inflammation, which makes various biological effects on multiple types of cell during infection with brucella, as IL-10 lead to the production drawdown of IFN-γ and inhibition of macrophages function (Saraiva and O'Garra, 2010).

Polymorphisms in the genes of cytokines can increase or decrease their expression and affect the determination of acute or chronic disease (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). In this regards, several studies have documented that IL-4, IL-6, IL-10, IL-17, IFN-γ, 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; Han *et al*., 2017; Lin *et al*., 2020). Kazemi *et al*. (2019), found that the Serum levels of IL-12 and TNF-α were significantly more frequent in the patients than in the control groups. The IL-13 gene polymorphism can be used as a biomarker for detecting susceptibility to Brucella disease. 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).

Several studies had demonstrating that hereditary factors play crucial parts in the development of Brucellosis (Bravo *et al*., 2003; Rezazadeh *et al*., 2006). It has been concluded that IL-10 - 819 loci polymorphism contributes no risk to Caucasian population but may be associated with decreased risk in Asian population, and IL-10 -1082 G/A, 592 loci and IL-6 -174 G/C polymorphism are not associated with brucellosis risk (Jin *et al*., 2020). Furthermore, the 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). No significant relationships were found between serum IL-17 titers and genotypes of the single-nucleotide polymorphisms (Keramat *et al*., 2019).

II. PATIENTS AND METHOD:

A total of 55 patients who were clinically suspected as having brucellosis and were tested positive for agglutination (Rose Bengal) test were included as patients group and 35 apparently healthy individuals were included as control group. In both patient and control groups, the highest age range was 20-29 years. 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. 20% of patients were males and 80% were females, while, 37.1 % of controls were males and 62.9% were females. The study groups were allocated from Baquba Teaching Hospital and some Healthcare Centers. A questionnaire form was preconstructed including socio-demographic and clinical information. Blood samples were collected from both study groups, sera were separated and submitted for slide agglutination (Rose Benal) test (Spin-React, Spain), Anti-Brucella IgM and IgG (Demeditec - Germany) as well as for detection of serum Interleukin-17, Interleukin-18, and Interferon –γ genes. Human privacy was respected by obtaining verbal consent from all participants. Statistical analysis was done using SPSS version 25 and p values ≤ 0.05 were considered significant.

Genomic DNA was extracted from blood sample according to the protocol ReliaPrep™ Blood gDNA Miniprep System, Promega (USA). For Quantitation of DNA, Quantus Fluorometer was used to determine the concentration of extracted DNA. 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 genetic variation in *IL-17* gene has been studied in various disease (Sadighi *et al*., 2014; Zhang *et al*., 2008). 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), IFN-γ gene detection targeted the +874 position which was previously empolyed in brucellosis (Rasouli and Kiany, 2007). The *IL-18* plays a protective role against brucellosis (Pasquali *et al.*, 2002). *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 (Folwaczny *et al.*,2005), such as the coding sequence, Exon–intron junctions, promoters and untranslated regions. Taking into account the involvement of these polymorphisms in altering IL-18 level.

Primers were used for molecular detection of *IL-17*, *IL-18* and *INF-γ* genes using RT-PCR from patients suspected as having brucellosis as well as from healthy controls. These primers were supplied by Macrogen Company in a lyophilized form.

Table (1): 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

III. RESULTS

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), as shown in table (2).

Table (2): 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	
20---29	25	45.5	11	31.4	
30---39	17	30.9	10	28.6	0.162 *
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 **

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

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

Table (3) showed that within the patients group, 44 (80%) were female and 11 (20%) were male. While in control, 22 (62.9%) were female and 13 (37.1%) were male. The difference between the two groups was statistically insignificant (P= 0.073).

Table (3): 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

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

Data presented in table (4) 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). on the other hand, 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),

Table (4): Distribution of anti- IgM and IgG positivity rate among study groups.

Serological Marker		Patients		Control		P value
		No.	%	No.	%	
Anti-IgM	Positive	18	32.7	2	5.7	0.003
	Negative	37	67.3	33	94.3	
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 (χ^2-test) at 0.05 level.

Results presented in table (5) revealed the PCR 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 (5): Distribution of PCR detection rate among study groups.

Molecular genes (PCR)		Patients		Control		P value
		No.	%	No.	%	
IL-17 gene	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	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	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 (χ^2-test) at 0.05 level.

Results in table (6) 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 an 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 (6): 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	

Significant difference between percentages using Pearson Chi-square test (χ^2-test) at 0.05 level.

The results in table (7) 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 significant (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 (7): 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	

Significant difference between percentages using Pearson Chi-square test (χ^2-test) at 0.05 level.

IV. DISCUSSION:

Human brucellosis is endemic in most of the East Mediterranean 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 (Shareef, 2006; Dahl, 2020). Certainly these documented evidences forming the central importance of such study in an endemic country like Iraq.

In this study, the Rose Bengal test was used as an inclusion criteria for enrollment of patients in this study. It is routinely used for the diagnosis of brucellosis in Iraq. It is well known that Rose Bengal test is so fast, but it has many false-negative results during the chronic brucellosis (Roushan et al., 2005). Serum agglutination test is the most common acceptable serological diagnostic test for human brucellosis. Certainly, in endemic areas the use of serum agglutination test titer ≥ 1:320 is more appropriate. It is necessary to explain that definitive treatment of patients has a correlation in declining serum agglutination test titers (Roushan et al., 2010).

The significantly higher positivity rate of anti-brucella IgM (P= 0.003) and anti-brucella IgG (P= 0.0001) 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. In contrast, IgG antibodies appeared later after the onset of infection (Elfaki et al., 2015). 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., 2007).

The successful intracellular stealthy lifestyle has evolved through multiple strategies to evade immune response, 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). Therefore, the cell mediated immunity play a crucial role in immune response to brucellosis infection. CD4+ and CD8+ T lymphocytes had the key role in cellular immunity as they can release IFN-γ and activate the functions in macrophages (Golding et al. 2001; Ko and Splitter, 2003). Among these effective immune responses are interleukins production. Furthermore, Polymorphisms in the genes of cytokines can increase or decrease their expression and affect the determination of acute or chronic disease (Hollegaard and Bidwell, 2006). Since genetic variation in various populations and the presence of specific polymorphisms in patients, understanding the cytokine pattern as an important factor in the clinical outcome of brucellosis infection can be effective in controlling the disease.

The results of molecular technique 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 higher among patient with acute brucellosis. However, it become lowered during the chronic brucellosis. Similar results were reported by other workers (Vitry et al., 2012;Corsetti et al., 2013). The 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, GM-CSF, MIP-2, monocyte chemoattractant protein-1 and TNF-α. The IL-17 family, is an important inflammatory cytokine, playing principal roles in both innate and adaptive immunities (Bedoya et al., 2013; Isailovic et al., 2015). The role of IL-17 was differentially relevant with respect to brucella species. IL-17-producing cells, found in the lamina propria, play an essential role against microorganisms infecting the gastrointestinal tract. Its production has also been observed in the lung and oral cavity mucosa. IL-17 is among critical determinants of risk, severity or protection of infectious diseases (Park et al., 2005). As the balance between Th1 and Th2 cytokines can cause resistance or susceptibility to infection with brucella species, Th1 cytokines inducing resistance, whereas Th2 cytokines cause predisposition to brucellosis (Gaffen, 2008). Furthermore, the role that IL-17 plays in the protection against brucellosis induced by vaccination in the intestinal mucosa (Pasquevich et al., 2011). However, no significant relationships were found between serum IL-17 titers and genotypes of the single-nucleotide polymorphisms (Keramat et al., 2019).

Regarding the IL-18 gene, the current results found that the detection rate was significantly higher in patients with brucellosis compared to control. Furthermore, it is higher in those patients with positive anti-brucella IgM and reduced thereafter during chronic phase of the disease. Increased levels of IL-18 expression was reported by (Akbulut, et al., 2007). Furthermore, IL-18 production was found to reduce the number of Brucella abortus mediated by an increased capability of spleen cells to produce INF-γ at the early phase of infection (Pasquali et al., 2002). A positive correlation of IL-18 with brucellosis, but, its association with single nucleotide polymorphisms (SNP) and brucellosis risk was not confirmed (Zafari et al., 2020).

The IL-18 is produced mainly by macrophages. It is a proinflammatory cytokine that facilitates type 1 responses. Together with IL-12, it induces cell-mediated immunity following infection. Together with IL12 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).

Similarly 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. Numerous studies had reported increased INF- γ expression during acute brucellosis (Ko and Splitter, 2003; Saraiva and O'Garra 2010). Moreover, INF-γ levels was found to be an independent predictive factors in the clinical diagnosis of brucellosis. (Lee et al., 2016; Han et al. 2017; Lin et al., 2020).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).

It can be concluded the determination of IL-17, IL-18 and INF-γ in patients with brucellosis may a prognostic predictors of disease severity in an endemic areas like Iraq.

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