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Gene Sequencing of Interferon-Gamma among Mice, Rabbits, and Men Infected with Leishmania Species

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Abstract: In this study, (15) whole blood samples of each mice and rabbit Pre-inoculated with leishmania amastigote were collected in the animal house of the College of Medicine, university of Diyala, and 15 whole blood were collected from 15 people infected with leishmaniasis, without mentioning the ages, and were taken for immunological and molecular analysis in Alkalis general hospital during the period from April to December 2024. The results showed that meddle levels of human Leishmania IgM antibody was (2.32±0.26) in comparison with the controls (0.08±0.05). Also, meddle levels of human Leishmania IgG antibody was (1.37±0.11) in comparison with controls (0.13±0.06) with mightily important variation (P≤0.0001). Furthermore, the meddile levels of Mice Leishmania IgM antibody was (2.06±0.23) in comparison to controls (0.08±0.04), and the meddle levels of Mice Leishmania IgG antibody was (1.23±0.11) in comparison to controls (0.08±0.04) with mightily important variation (P≤0.0001). Moreover, the meddle level of the Rabbit Leishmania IgM antibody was (1.67±0.15) in comparison to controls (0.08±0.04), and the meddle level of Rabbit Leishmania IgG antibody was (1.20±0.10) in comparison with controls (0.13±0.05) with mightily important variation (P≤0.0001). The meddle level of human Leishmania IgM antibody in males was (2.19±0.32), and the meddle level of human Leishmania IgM antibody in females was (2.63±0.43) with no important variations (P=0.4), also the meddle level of human Leishmania IgG antibody in males was (1.40±0.13), and the meddle level of human Leishmania IgG antibody in females was (1.44±0.17) with no important variations (P=0.87). In addition, the meddle level of Mice Leishmania IgM antibody in males was (2.45±0.32) and the meddle level of Mice Leishmania IgM antibody in females was (1.76±0.18) with no impotent variations (P=0.12). in addition, the meddle level of Mice Leishmania IgG antibody in males was (1.18±0.16) and the meddle level of Mice Leishmania IgG antibody in females was (1.37±0.16) with no significant variations (P=0.41). On the other hand, the meddle level of Rabbit Leishmania IgM antibody in males was (1.70±0.24) and the meddle level of Rabbit Leishmania IgM antibody in females was (1.72±1.72) with no significant variations (P=0.91) and the meddle level of Anti Rabbit Leishmania IgG antibody in males was (1.24±0.15) and the meddle level of Rabbit Leishmania IgG antibody in females was (1.23±0.14) with no important variations (P=0.92).

Keywords: Gene sequencing, Interferon-gamma, Mice, Rabbit, Man, Leishmania SPP.

INTRODUCTION

The infected sand fly (Phlebotomus Alexandria) is found in Iraq and is able to bite people and transmit Leishmania infantum to them to cause visceral leishmaniosis (VL) [Lakhal-Naouar, I. *et al*., 2021]. There is no vaccine or prophylactic medicine against the disease. North Americans usually possess no protective immunity. Many Iraqi people were exposed to the disease because of the deployment of more than one million US service members to Iraq [Lakhal-Naouar, I. *et al*., 2021]. The parasite has the ability for lifelong persistence inside the human macrophage. Activated visceral leishmaniosis presents with chronic sickness with weight loss, fever, cytopenia as well as hepatosplenomegalies. The host immune response (like tuberculosis) holds latent visceral leishmaniasis in check [Mody, R. M. *et al*., 2018]. Untreated Active untreated visceral leishmaniosis is related to >90% of mortalities but may be treated successfully with liposome amphotericin [Kaye, P. M. *et al*., 2023]. Because of the reactivation of AVL risks among soldiers who served in Iraq, we studied 200 highly risky military members approx [De Araujo, I. F. F. *et al*., 2023]. Outbreaks and new rural and urban recorded CL case foci were developed in Iraq, Syria, and Türkiye. The CL was spread in these areas due to wars in Syria and Iraq, immigration, poverty, and climate alteration [Rawlings, N. *et al*., 2024]. There are two types of cutaneous leishmaniasis, New World and Old-World CL, with the old CL causing the disease in the Arabian Peninsula, Middle East, Mediterranean littoral region, Near Asia, Africa, Indian Subcontinent as well as other parts in which L. major, L. tropica, L. aethiopica, and L. infantum exist [Mann, S. *et al*., 2021]. The two CL types include zoonotic cutaneous leishmaniosis (ZCL), which is caused by Leishmania major, and anthroponotic cutaneous leishmaniosis (ACL), which is caused by Leishmania tropica. The pre-clinical evaluation of the efficacy and safety of new models of animal components remains the main step in the new drug development of leishmaniasis [Sharifi, I. *et al*., 2023]. Nowadays, several various animal models are being used, and such in vivo models to discover new potential anti-leishmanial treatments vary widely in multiple features [Van den Ende, J. *et al*., 2023]. There is a huge difference in animals employed (strains, species, and sexes), Leishmanial species used for experimental infections, the route and dose of inoculation of challenge strain, response to disease in addition to symptom's clinical presentations [Smith, N. C. *et*

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al., 2022]. There is an essential role of interferon-gamma in the development and consequent controlling of Leishmaniasis. Interferon-gamma with other chemokines and cytokines are effective on each other to influence the infection course. Some factors are elaborated by Leishmanial parasites for activity modulation [Costa-da-Silva, A. C. *et al*., 2022].

MATERIAL AND METHODS

In the current study, (15) whole blood samples of each mice and rabbit Pre-inoculated with leishmania amastigote were collected in the animal house of the College of Medicine, university of Diyala, and 15 whole blood were collected from 15 people infected with leishmaniasis, without mentioning the ages, and were taken for immunological and molecular analysis in Alkalis general hospital during the period from April to December 2024. Mice and rabbits were intra-cardially inoculated with Leishmania species amastigote on day 0. All animals received 2×107 leishmanial parasites in 100 µl of RPMI media. On days 10, 20, 30, 45, 60, and 120, the animals were sacrificed after infections, and the weights of their spleen/liver were measured. Hepatic and splenic parasite load of the sacrificed animals was measured as: (No. of amastigotes/number of nucleated cells ×organ weight (mg)×2×105). Leshmania Spp. IgM and IgG were detected by Elisa assay, while IFN-ϒ gene detection by PCR and the sequences of IFN-ϒ was done by signer sequencer and the primer used:

rs2430561-F	TGTAAAACGACGGCCAGTCGTTGCTCACT GGGATTT
rs2430561-R	CAGGAAACAGCTATGACCATGTCTTCCTT GATGGTCTC

STATISTICAL ANALYSIS

An analysis of data was carried out using the available statistical package of IBM SPSS-22. The study parameters were analyzed by mean ±SD and Chi-square.

RESULTS

The results showed that the mean levels of human Leishmania IgM antibody was (2.32±0.26) in comparison with the controls (0.08±0.05). Also, meddle levels of human Leishmania IgG antibody was (1.37±0.11) in comparison with controls (0.13±0.06) with mightily important variation (P≤0.0001). Furthermore, meddle levels of Mice Leishmania IgM antibody was (2.06±0.23) in comparison to controls (0.08±0.04), and the medium levels of Mice Leishmania IgG antibody was (1.23±0.11) in comparison to controls (0.08±0.04) with higher important variation (P≤0.0001). Moreover, the medium level of Rabbit Leishmania IgM antibody was (1.67±0.15) in comparison to controls (0.08±0.04), and the middle level of Rabbit Leishmania IgG antibody was (1.20±0.10) in comparison with controls (0.13±0.05) with might important variation (P≤0.0001), as shown in Table (1).

Table (1): Mean levels of Leishmania infections between Mice, Rabbits, and men

Variables	Groups	Mean	SE	T-test	*P-value
Human Leishmania IgM	Case (n-31)	2.32	0.26	8.24	≤0.0001
	Control (n=14)	0.08	0.05		
Human Leishmania IgG	Case (n-31)	1.37	0.11	9.44	≤0.0001
	Control (n=14)	0.13	0.06		
Mice Leishmania IgM	Case (n-31)	2.06	0.23	8.36	≤0.0001
	Control (n=14)	0.08	0.04		
Mice Leishmania IgM	Case (n-31)	1.23	0.11	9.05	≤0.0001
	Control (n=14)	0.08	0.04		
Rabbit Leishmania IgM	Case (n-31)	1.67	0.15	8.71	≤0.0001
	Control (n=14)	0.18	0.06		
Rabbit Leishmania IgG	Case (n-31)	1.20	0.10	8.71	≤0.0001
	Control (n=14)	0.13	0.05		

***Highly significant**

Table (2) revealed that the meddle level of human Leishmania IgM antibody in males was (2.19±0.32) and the medium level of human Leishmania IgM antibody in females was (2.63±0.43) with no important variations (P=0.4), also the medium level of human Leishmania IgG antibody in males was (1.40±0.13) and the meddle level of human Leishmania IgG antibody in

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females was (1.44±0.17) with no important variations (P=0.87). In addition, the meddle level of Mice Leishmania IgM antibody in males was (2.45±0.32) and the meddle level of Mice Leishmania IgM antibody in females was (1.76±0.18) with no important variations (P=0.12). in addition, the medium level of Mice Leishmania IgG antibody in males was (1.18±0.16) and the mean level of Mice Leishmania IgG antibody in females was (1.37±0.16) with no significant variations (P=0.41). On the other hand, the meddle level of Rabbit Leishmania IgM antibody in males was (1.70±0.24), and the medium level of Rabbit Leishmania IgM antibody in females was (1.72±1.72) with no important variations (P=0.91), and the mean level of Rabbit Leishmania IgG antibody in males was (1.24±0.15), and the mean level of Anti goat Rabbit Leishmania IgG antibody in females was (1.23±0.14) with no important variations (P=0.92).

Table (2): Mean levels of Leishmania infections between Mice, Rabbits, and men according to the Sex

Variables	Gender	N	Mean	SE	T-test	P-value
Human Leishmania IgM	Male	16	2.19	0.32	0.82	0.4
	Female	14	2.63	0.43		
Human Leishmania IgG	Male	16	1.40	0.13	0.15	0.87
	Female	14	1.44	0.17		
Mice Leishmania IgM	Male	16	2.45	0.39	1.61	0.12
	Female	14	1.76	0.18		
Mice Leishmania IgM	Male	16	1.18	0.16	0.83	0.41
	Female	14	1.37	0.16		
Rabbit Leishmania IgM	Male	16	1.70	0.24	0.05	0.91
	Female	14	1.72	0.17		
Rabbit Leishmania IgG	Male	16	1.24	0.15	0.06	0.92
	Female	14	1.23	0.14		

Meddle levels of human Leishmania IgM antibody in the rural area is (2.44±0.38), and mean level of anti-human Leishmania IgM antibody in urban area was (2.35±0.37) with non-important variations P=0.87, but meddle levels of human Leishmania IgG antibody in rural area is (1.26±0.14), and medeium level of anti-human Leishmania IgG antibody in urban area was (1.57±0.15) with no significant variations P=0.14. Furthermore, medium levels of Mice Leishmania IgM antibody in rural areas is (2.09±0.27) and meddle level of Mice Leishmania IgM antibody in urban areas is (2.17±0.38) with non-important variations P=0.87. Mean level of Mice Leishmania IgG antibody in rural areas is (1.18±0.14), and the medium level of Mice Leishmania IgG antibody in an urban area is (1.36±0.17) with non-important variations P=0.45. Also, medium levels of Rabbit Leishmania IgM antibody in rural areas was (1.73±0.18), and the meddle level of Rabbit Leishmania IgM antibody in urban areas was (1.69±0.24) with no important variations P=0.89. Medium levels of Rabbit Leishmania IgG antibody in rural areas is (1.0507±0.13), and the meddle level of Rabbit IgG antibody in urban areas was (1.4340±0.151) with non-important variations P=0.70, as demonstrated in Table (3).

Table (3): Mean levels of Leshmania infections between Mice, Rabbits, and men according to the residency

Variables	Residency	N	Mean	SE	T-test	P-value
Human Leishmania IgM	Rural	15	2.44	0.38		
	Urban	15	2.35	0.37	0.15	0.87
Human Leishmania IgG	Rural	15	1.26	0.14		
	Urban	15	1.57	0.15	1.49	0.14
Mice Leishmania IgM	Rural	15	2.09	0.27		
	Urban	15	2.17	0.38	0.16	0.87
Mice Leishmania IgM	Rural	15	1.18	0.14		
	Urban	15	1.36	0.17	0.75	0.45
Rabbit Leishmania IgM	Rural	15	1.73	0.18		
	Urban	15	1.69	0.24	0.13	0.89
Rabbit Leishmania IgG	Rural	15	1.0507	0.13		0.70
	Urban	15	1.4340	0.151	1.88	

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Amplification of rs1800797 specific gene region of Human samples of IFN-ϒ gene resembles 969bp in agarose gel electrophoresis as arranged in figure (1).

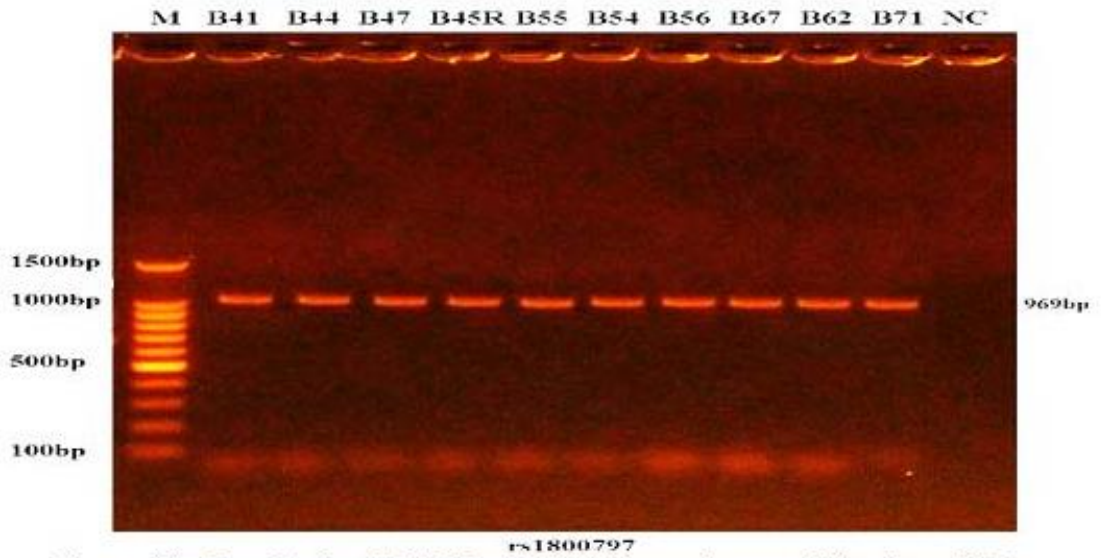

Figure (1): Result of *rs1800797* specific gene region amplification of Human samples were fractionated on 1.5% agarose gel electrophoresis stained withEth.Br. M: 100bp ladder markers. Lanes C1-A34 resembling 969bp PCR products

Amplification of rs1800797 specific gene region of cattle and goat samples of IFN-ϒ gene resemble 969bp in agarose gel electrophoresis as arranged in figure (2).

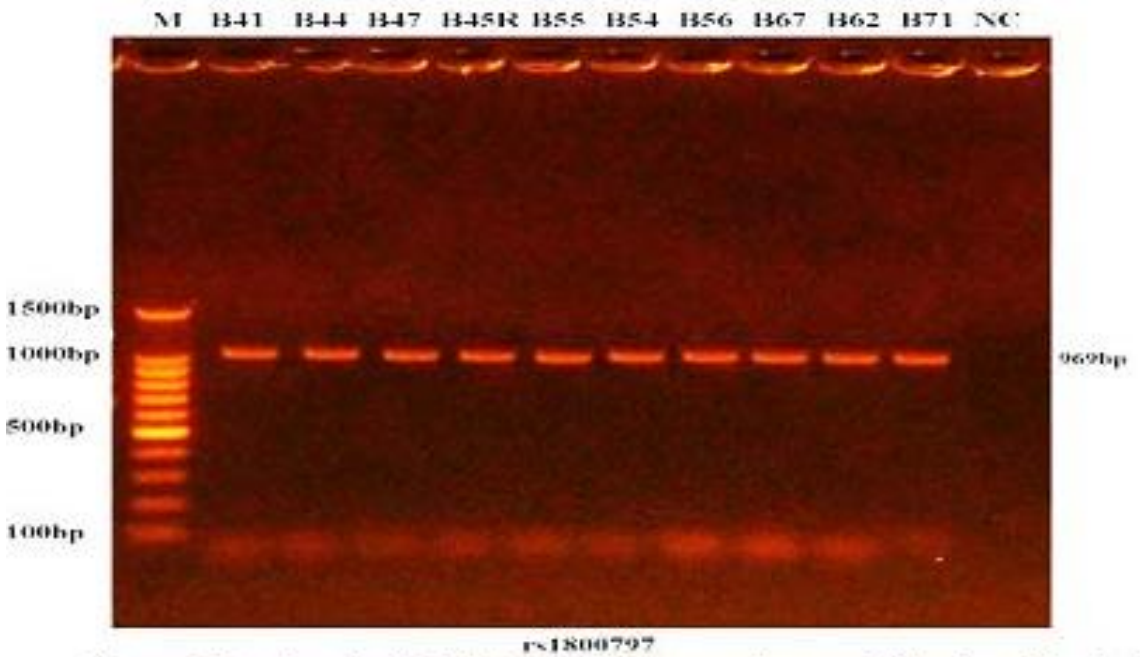

Figure 2:Results of *rs1800797* specific gene region amplification of Animals samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder markers. Lanes B41-B45 for Mice and B55-B71 for Rabbit resemble 969bp PCR products

Table (5) and figure (3) showed a mutation occurrence on IFNG GENE ID 3569; the wild AA was altered to the A>G compared to the control group in cattle, goats, and man infected with CCFHV.

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Table (5): Variation of wild SNPs of IFN-ϒ gene

IL6 GENE ID 3569	
SNPs	rs1800797
Wild	AA
Variation	A>G
Samples	
A3	AG
A8	AG
A11	GG
A13	GG
A18	GG
A28	AG
A36	AG
A41	GG
B41	GG
B54	AG
B55	GG
B62	GG
C1	AG
C2	AG
C3	AG
C4	GG
C5	AG

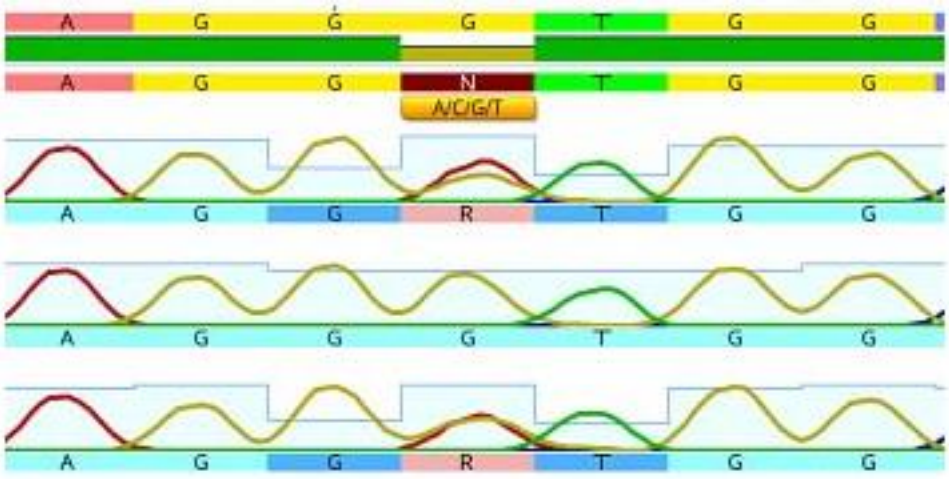

Figure (3): Analysis of rs1800797 SNP of TNF-? gene using Sanger sequencing. Single "G" peak indicating a G homozygous allele. Single "A" peak indicating an A homozygous allele. Presence of "G" and "A" peak indicating G/A heterozygous allele

DISCUSSION

According to the results, De Lima, *et al*., (2021) reported that patients in stages IV and V of the disease formed higher IgG & IgG1 antibody levels in comparison with patients in stages I and II. There was a significant decrease in IgG and IgG2 antibody levels in the majority of patients who showed good response to the treatments [De Lima, C. M. F. *et al*., 2021] and who found that serum Leishmania-specific IgG, IgG1, and IgG2 antibody levels are investigated by ELISA prior and following treatments with antimony or antimony + pentoxifyllin. Our finding demonstrated a relationship between IgG titer and mucosal disease severity. The remarkable decrease in antibody formation following successful treatments in the majority of patients primarily suggests that such examinations may be useful to help in the therapeutic response evaluation [De Lima, C. M. F. *et al*., 2021]. Muñoz-Durango (2022) proved

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that the adaptive humoral response is described by early IgG1 production followed by the production of IgG2a type of L(V) p-specific antibodies. The ratio of IFNγ/IL-4 & IgG2a/IgG1 suggested that the response of primary non-protective Th-2 is redirected towards the response of protective Th-1. In situ, investigations reported an abundant recruitment of myeloid cells and IFN-γ and IL-4-producing T-cells to the infection site and a typical histopathologic alteration prompted by dermotropic Leishmanial spp. It has been evidenced that such a model is appropriate for pharmacological and immunomodulatory intervention investigation and antigen discovery. All of these findings confirm the utility and validity of this novel mouse model for studying the immunity, pathogenesis as well as therapeutics of L(V)p infection [Muñoz-Durango, N, 2022]. About infected rabbits, the results indicated that the maturation affinity of the Anti-Leishmanial IgG antibody avidity was promoted in the early stage, affecting the suitable interactions between antigens and influencing the progression of the disease. Such facts can be related to a monovalent immune complex, as shown in experimental and human visceral leishmaniasis. Such a scenario could be associated with the activation of the independent processes of immune cells by the parasites but lacking in the preparation of antigens used as an immunogen (De Carvalho, 2020). The role of IFN-ϒ, Gurjar, *et al*., (2022) demonstrated that IFNγ, which is a cytokine and type-2 interferon, is important for both adaptive and innate immunities. IFNγ is bound to the IFN-γRs on the macrophage's cell membranes, and it signals through the pathway of JAK1-STAT-1 and induces the genes of IFN-γ-stimulated. Since Leishmanial amastigotes replicate and reside in the macrophage, the activation of IFN-γ mediated macrophage eventually occurs in Leishmanial eliminations.

Genetic sequences of IFN-ϒ was a mutation occurrence on IFNG GENE ID 3569, the wild AA altered to the A>G compared to the control group; these results match with (Chong *et al*., 20), who found that the allele of IFN-γ +874A was related to susceptibilities to SARS in a dose-dependent manner. People with IFN-γ +874 AA and AT genotypes showed 5.19-fold (95% confidence intervals [CI], 2.78-9.68) & 2.57-fold (95% CI, 1.35-4.88) elevated risks to develop SARS. The IL-10 and TNF-α polymorphisms are not related to the susceptibility of SARS [Habeeb, M. R. *et al*., 2021]. Also (Wei, *et al*., 2017) reported that Interferon gamma (IFNG) is an essential cytokine that contributed to provide resistance to mycobacterium diseases. Common IFNG variants, e.g., IFNG +874 T/A(rs2430561), could be associated with the susceptibility of TB, although such a relationship hisas not consistently noticed. Our updated meta-analysis was conducted to assess the relationship between the polymorphism of IFNG+874 T/A (rs2430561) and the susceptibility of tuberculosis. The databases of Sino Med and PubMed were examined until October 2016, and odd ratios (ORs) with 95% confidence interval (CI) were applied for assessing the relationship strength, according to the criteria of searching for manuscripts that report the susceptibility of tuberculosis and its association to the polymorphism of IFNG T/A. Forty-two case-control studies from 39 various articles have been retrieved. A significant positive decrease and protective relationships have been detected between the polymorphism of IFNG T/A and the risk of tuberculosis in 5 genetic models. Furthermore, in analyzing the stratified subgroups, a protective association was found in 4 various sources and ethnicities of the control group. In addition, the polymorphism of IFNG T/A played a key role to protect people from TB. The meta-analysis study indicated that the polymorphism of IFNG T/A is highly related to the susceptibility of tuberculosis and can be applied as a predictive biomarker. Additional research with greater specimen size and considerations of interactions of gene-environment must be carried out for elucidation of the IFNG T/A polymorphism role in the susceptibility of tuberculosis. Furthermore, Habeeb, *et al*., (2021) concluded the polymorphisms in cytokine genes, including interferon-gamma (IFN-γ), have been implicated in numerous autoimmune disorders and chronic inflammatory diseases. Certain polymorphisms described in coding and regulatory regions of IFN-γ genes are C/G, G/T, A/T, A/G, G/A, and G/A loci 20. The roles of the polymorphism of interferon-gamma gene in 3 regions: 9A/G, A/T, and G/T in various outcomes of HCV infection, whether chronicity among Iraqi patients.

CONCLUSION

There was a mutation occurrence on IFNG GENE ID 3569; the wild AA altered to the A>G compared to the control group in cattle, goats, and man infected with CCFHV.

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