International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304, ISSN(Online): 2455-9563
Vol.9, No.9, pp 331-340, 2016

The association between periodontitis and polymorphism of tumor necrosis factor-α -308 in Iraqi population

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Abstract : A patients –healthy samples collection was conducted between February 2015- May 2015 and it included 100 Iraqi patients group with periodontitis and 30 number of healthy population group from Babylon province and it was conducted in Faculty of Dentistry**.** Two types of samples have been taken from each subjects in study , first :pocket samples for bacterial identification ,second: blood samples for DNA extraction from WBCs.

PCR-RFLP have been used to detect two SNPs and in TNFα at position -308, genotype and allele frequency have been calculated by using Hardy- Weinberg equation.

This study was showed that the number of aerobic bacteria in patients were $2.8 \times 10^5 \pm 187.93$ CFU/ml compare with healthy $3.1 \times 10^3 \pm 55.11$CFU/ml this difference was significant, while the number anaerobic in patients bacteria were $1.6 \times 10^4 \pm 43.45$ CFU/ml compare with healthy $2 \times 10^2 \pm 32.78$ CFU/ml.

The most bacteria isolated in this study were *P. gingivalis* 65 in patient and 5 in healthy control, *P. intermedia* 41 in patient and 3 in healthy control , *T. for sythensis* 33 in patient and 1 in healthy control, *Peptostreptococcus spp.* 100 in patient and 30 in healthy control , *A. actinomycetemcomitans*, 47 in patient and 9 in healthy control *Streptococcus spp.* 44 in patient and 22 in healthy control, *Staphylococcus aureus* 3 in patient and 52 in healthy control.

PCR-RFLP results of TNF-α -308 gene polymorphism showed the patients with genotype BB were more affected by periodontitis at 4.68 time than patients with AA and AB 0.2 and 0.67 respectively.

As the first finding ,we concluded that the polymorphism of TNF-α -308 associated with periodontitis in Iraqi population.

Key words PCR-RFLP , periodontitis , gene polymorphism , TNF-α -308, *P. gingivalis.*

Introduction

Periodontitis is considered as a chronic infectious disease ,which is featured by destruction of collagen fibers and other matrix constituents of periodontal ligaments and alveolar bone around the teeth in conjunction with initiation of periodontal pockets[1]. In critical cases, the disease could result to a complete loss of the teeth [2]. It has been established that the hardness of periodontitis is based on a dynamic balance of interactions between the microbial challenge and host immune inflammatory responses[3]. At certain point or period in host lifetime, these bacteria change in their numbers and relative proportions, and induce chronic periodontal inflammation, which may lead to teeth loss as a result of the damaging of the supporting alveolar bone[4]. Periodontitis is a highly prevalent disease, presenting substantial economical burden[5]. Furthermore, given a strong association of periodontal bacteria with other diseases, such as cardiovascular disease, rheumatoid arthritis or diabetes, it becomes evident that efficient periodontal treatment would be of great medical benefit to general health[6].

Scientists tried to make a classification of periodontal pathogens. The most popular classification has been divided into color coded clusters proposed by Socransky and his *et al* in 1998, and it was updated later[7] . This division identifies many complexes of bacteria and reflects their series of infection in the oral plaque and their association with the characters of the periodontitis. In this classification the bacteria forming dental plaque were classified into six clusters (red, orange, yellow, green, blue or *Actino* and purple), based on the structural features of the biofilm that extends away from the tooth surface. *Veillonella parvulla* and *Actinomyces odontolyticus* represented the 'purple' form. While Species of *Streptococci* including *S. sanguis*and*S. Oralis* represented the 'yellow' form.

It has been established that the hardness of periodontitis is based on a dynamic balance of interactions between the microbial challenge and host immune inflammatory responses (Goutoudi*et al*., 2004). Virulence factors of periodontopathogens, such as lipopolysaccharides, can induce expression of inflammatory cytokine[8] . Cytokines are small polypeptides with a huge range of inflammatory, immunomodulatory and metabolic properties, which are secreted by a various of cells, such as the macrophage/monocyte system, dendritic cells, lymphocytes, neutrophils, endothelial cells and fibroblasts, these cytokines such as IL-1β , IL-6 and TNF-α are released from previous cells in response to LPS of bacteria[9].

However, the current study was aimed to find the association between periodontitis and polymorphism TNF-α, to achieve that the following axes were applied : identified the types of bacteria associated with periodontitis specially anaerobic bacteria, detect the SNPs of TNF-α -308 by using RFLP-PCR technique and sequencing of mentioned gene for new SNPs if they found and for proving the type of SNPs.

Materials and methods

The study included hundred consecutive patients with chronic periodontitis. All patients (25-65 years old) showed clinical evidence of alveolar loss and periodontal pockets. A 30 healthy subjects (20-60 years old)without clinical signs of periodontal disease were also selected. None of the periodontal patients or healthy subjects had received antibiotics for two months prior to specimen collection.

A questionnaire was applied to all individuals enrolled in this study, in order to obtain information

1. regarding dental history.
2. family history of periodontal disease.
3. smoking habit, as well as general health concerns.
4. Use of orthodontic appliances, chronic usage of anti-inflammatory drugs, history of diabetes, hepatitis or HIV infection, immunosuppressive chemotherapy.
5. bleeding disorders, severely compromised immune function, pregnancy or lactation were regarded as exclusion criteria. Except for the presence of periodontitis, the patients included in this study were systemically healthy. Since tobacco smoking is an important risk factor for periodontitis, we also analyzed our data taking the habit of smoking into consideration. Smokers were defined as current smokers (more than 10 cigarettes/day) and non-smokers included individuals that had never smoked. Individual consent form was obtained from all the patients and ethical committee approval was obtained before the beginning of collection of specimens.

Specimens collection

1- Pocket swap specimens

Specimen s were taken from subjects with complete aseptic precautions with the assistance of dentists. Initially the site of specimen collection was isolated with cotton rolls, carefully cleaned with sterile cotton. For single site, 2 sterile paper points (30-40#) were inserted to the periodontal pocket for at 30 second, the pocket depth is equal to or exceeding 3.5 -7 mm. and then transferred to Robertson's cooked meat medium. While the healthy control subjects specimen s were collected from sub gingival material with sterile paper points and transferred to Robertson's cooked meat media[10].The medium with specimen s directly transported to the laboratory in 1-2 houses .

2- **Blood specimens**

Five milliliters of venous blood were obtained from each subject, it was put into EDTA tubes. Blood in the EDTA tubes was stored in - 20˚C in order to be used later in genetic study,

Processing of specimens

1- Pocket specimens

One Inoculum from Robertson's cooked meat medium after shaking by Vortex for 1 mint was directly transported to three different media:

Frist: Brucella blood agar or blood agar (BBA) supplemented with hemin and vitamin K1(menadione) for isolation of strict aerobic bacteria. The medium incubated aerobically and anaerobically at 37C° for 5-7 days.

Second: Brucella blood agar or blood agar (BBAK) supplemented with hemin and vitamin K1(menadione) and Kanamycin for isolation facultative anaerobic bacteria, and the medium incubated aerobically and anaerobically at 37C° for 5-7 days .

Third: Brucella blood agar or blood agar(BBAKV) supplemented with hemin and vitamin K1(menadione) and kanamycin and vancomycin for isolation of strict anaerobic bacteria, and the medium incubated aerobically and anaerobically at 37C° for 7 days [11-12]. The plate incubated anaerobically in anaerobic jar were contained Gas bag to provide CO_2 necessary to growth of anaerobic bacteria.

The number of viable colonies were counted by using total viable plate count method[13].

C.F.U/mL original specimen = No. of colonies × Dilution factor / Inoculum size CFU/ml

When the period of incubation was completed. Colonies with differing characteristics were subjected to various test, and it were identified according to these references [14-15].

Diagnosis of some important bacteria genetically by multiplex PCR

The extracted DNA(Favoergen kit) from bacteria was amplified using specific primers that were listed in(table 3.5) and then subjected to multiplex PCR in two stages (PCR I and PCR II). The PCR conditions were listed in the(table 1) [16].

Table (1) :Multiplex PCR condition of oral bacteria genes

stages	Steps	Temperature C°	Time	Cycles
PCR I	Initial Denaturation	**95**	**5 mints**	**1**
	DNA denaturation	**95**	**30 sec**	**35**
	Primer annealing	**60**	**30 sec**	
	Extension	**72**	**1 mints**	
PCR II	Initial Denaturation	**95**	**5 mints**	**1**
	DNA denaturation	**95**	**30 sec**	**35**
	Primer annealing	**55**	**30 sec**	
	Extension	**72**	**1 mints**	

2- Blood specimens

Extraction of DNA from red blood cells (RBCs) for molecular study

Human genomic DNA from WBC_S for healthy control subjects and periodontitis subjects was extracted done using DNA extraction Kit (FAVORGEN).

Amplification of TNF-α -308 gene

TNF-α -308 gene was amplified using specific primer:

forward 5'-GAGGCAATAGGTTTTGAGGGCCAT-3'

reverse 5'-GGGACACACAAGCATCAAG-3'

The conditions of the PCR and annealing temperature was determined as reported in [17]. Conditions of PCR were listed in the table (2) . Amplified DNA fragments were put in 1% agaros , concentration of TBE buffer was 0.5x , electrophoresis time was 45 mints at 75v and the bands of TNF-α was visualized under UV light illuminator (France) when ethidium bromide had been added, A 100 base- pair ladder was used as a DNA marker (Eurex) for estimation of fragment sizes.

Table(2) PCR condition of Tumor Necrosis Factor-α -308

Cycles	**Time (mints))**	**Temp.(C°)**	**Steps**	**Stage**
1	4	94	Initial Denaturation	1
35	30 sec	94	DNA denaturation	2
	30sec	63	Primer Annealing	
	30sec	72	Extension	
1		4	Hold	3

Restriction Fragment Length Polymorphism (RFLP)

TNF-α has a single nucleotide polymorphism at nucleotide position -308 in the promoter reign that results by altering the nucleotide at position -308 from guanine to adenine. A recognition site for the restriction enzyme ***NcoI***(Promega) was detected by using specific primer .However, in this study the ***Nco I*** had been used as a restriction enzyme for this SNP , the enzyme recognized the sequence:

Recognition sequence : C▼CATG G

G GTAC▲C

Source : An *E. coli* strain that carries the *Nco*I gene from *Nocardiacorallina* (ATCC 19070).

Then the PCR product with enzyme was electrophoresis on 9% PAGE for three hours.

To compare frequency of genotypes and alleles in normal and patient groups, $\chi 2$ (SPSS-19) was used, and $p<0.05$ was considered as statistically significant. Logistic regression test was used to estimate the odds ratio (OR) and confidence interval 95% (CI).

Results and discussion

The results of the present study included microbiological study and molecular study. In molecular study was presented the total viable count of bacteria associated with periodontitis , and it was determined by selecting plates that have 30 to 300 colonies , and the number of bacteria was calculated by using the following formula:

Total viable count =No. of colonies × Dilution factor / Inoculum sizeCFU/ml

Table (3) showed total viable count of bacteria that collect from patients with chronic periodontitis and healthy control . There was significant association between study groups in the aerobic bacteria , the number of bacteria in healthy groups was $3.1\times10^3\pm55.11$ CFU/ml, and in patient was $2.8\times10^5\pm87.93$ CFU/ml $P=0.001$

and the anaerobic bacteria in healthy and patient was $2\times10^{2}\pm32.78$ CFU/ml and $1.6\times10^{4}\pm43.45$ CFU/ml respectively ($P\leq 0.05$).

Table (3) Viable cells count of bacteria associated with chronic periodontitis

Culture type	Mean ±SD CFU/ml		*P* value
	Healthy N=30	**Patients** N=100	
Aerobic	$3.1\times10^{3}\pm55.11$	$2.8\times10^{5}\pm187.93$	0.001
Anaerobic	$2\times10^{2}\pm32.78$	$1.6\times10^{4}\pm43.45$	0.001
Total	$3.3\times10^{2}\pm37.1$	$29.6\times10^{4}\pm63.61$	0.001

Bacteria were isolated in this study and their results were mentioned in table (4) , The most bacteria isolated in this study were *P. gingivalis* 65 in patients and 5 in healthy control, *P. intermedia* 41 in patients and 3 in healthy control , *T. forsythensis* 33 in patients and 1 in healthy control, *Peptostreptococcus spp.* 100 in patients and 30 in healthy control , *A. actinomycetemcomitans*, 47 in patients and 9 in healthy control *Streptococcus spp.* 44 in patients and 22 in healthy control and *S. aureus* 52 in patients and 3 in control.

Table (4) Number of bacteria in chronic periodontitis patients and healthy control isolated from oral

Microorganisms	Subjects N =130	
	Healthy N=30	**Patients N=100**
1. Anaerobic	**Culture**	**Culture**
P. gingivalis	5	65
P. intermedia	3	41
T. forsythensis	1	33
Peptostreptococcus spp.	30	100
2. Aerobic and facultative anaerobic		
A. actinomycetemcomitans	9	47
Streptococcus spp.	22	44
S. aureus	3	52
A. Other	yes	yes

Identification of some oral bacteria by multiplex PCR

Bacteria which identified by multiplex PCR included *P. gingivalis*, *P. intermedia*, *T. forsythensis*, and *A .actinomycetemcomitans* , the bands of pervious bacteria were 197bp, 575bp, 641bp and 593 respectively, figure (1).

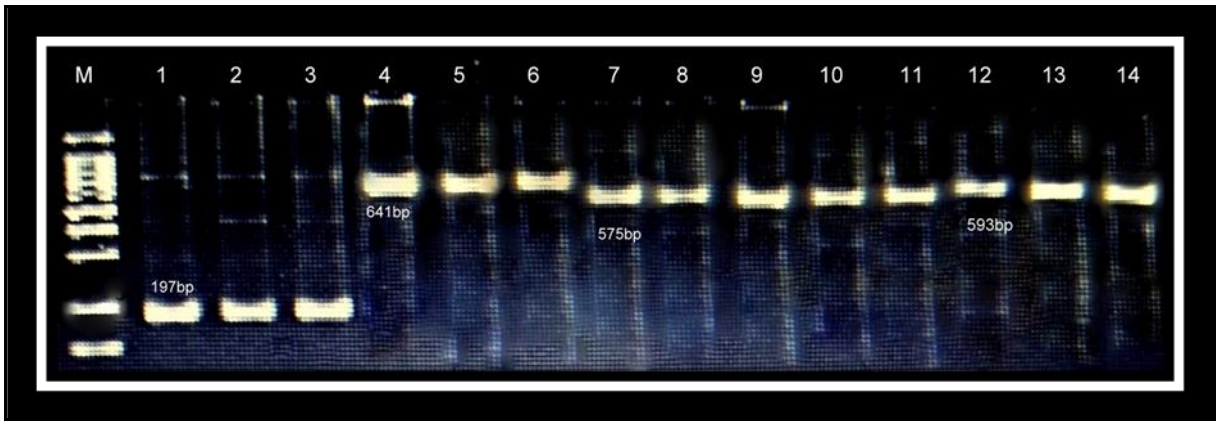


Figure (1) Electrophoresis pattern of multiplex PCR product of some bacterial genes which identified each species of bacteria, 1,2,3 *P. gingivalis* (197bp), 4,5,6 *T. forsythensis*, 7,8,9,10,11*P. intermedia* (575bp), 12,13,14 *A . actinomycetemcomitans* (593bp).

In the current study, the high number of bacteria in patients may be belonged to the suitable environment that produced in periodontal pocket ,that mean the destruction of alveolar bone and periodontal ligament, lead to bleeding of gingiva tissue and formation of pocket and these condition makes suitable place for bacterial growth specially anaerobic bacteria, The leading cause of periodontitis is the deepening of gingival pockets due to inflammation brought on by the presence of undisturbed plaque [18].While the number of bacteria in healthy people was low and this is predictable result because there is no suitable environment. Darveau*et al*,1997 showed that the number of bacteria in pocket in healthy people was 10^2-10^3 CFU , and most of organisms were gram positive , such as *Actinomyces*spp. and *Streptococci* spp.[19].While other species such as *Porphromonas* spp. and *Provetella* spp. were in low levels and these results were coincided with Ximenez-Fyvie*et al*., 2000that found *Porphromonas* spp. and *Provetella*spp represented small portion in their study[20].

Many study were indicated that the presence of the tree red complex periodontopathogens, include :*P . gingivalis*, *T. forsythia*, and *Treponemadenticola* in addition to *A. actinomycetemcomitans*, considered the most etiologic factors in periodontitis[21]. Slots and Rams ,1992 reported that the most organismsdetected from dental plaque are Gram positive, facultativelyanaerobic bacteria, particularly *Actinomycesspp.*and*Streptococci*[22].

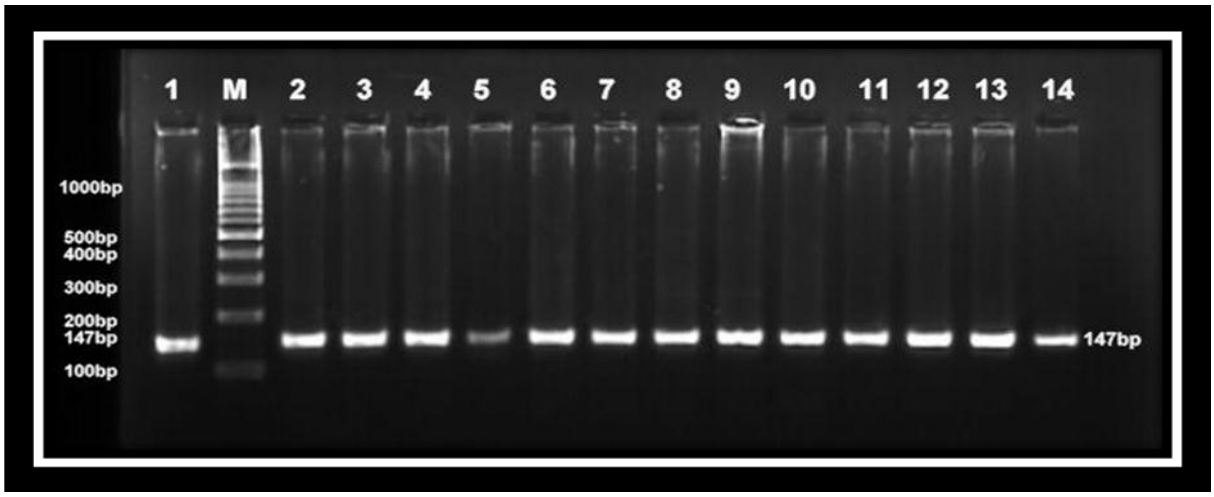
Peptostreptococcus is a Gram-positive anaerobic coccus that is widespread distributed in the normal human flora so that it was isolated from all healthy control 30 subjects and all periodontitis patients. Its role in oral disease is less well detected, although it has been included in periodontal disease, gingivitis and root canal infections[23].

Staphylococci are not usually seen in the cavity of mouth, and when this occurs, they are considered to belong to the transitory microorganisms. Individuals with periodontitis classified as possible reservoirs of the opportunistic bacteria in the cavity of oral. Antibiotics uses for treatment of periodontitis or other infections by microorganism may increase the number microbes ex. *Staphylococcus spp*. in the cavity of oral of periodontitis subjects[24].

Molecular study of chronic periodontitis

Tumor necrosis factor-308 (TNFα -308) genotyping PCR

PCR product of TNFα -308gene was amplified by using specific primers. The PCR product (band) was 147bp figure(2).



Figure(2) electrophoresis pattern of PCR product of TNFα -308gene, PCR product 147bp, M: molecular DNA ladder the optimum annealing temperature was 63^{o}C.

Detection of genotype of TNF α -308 gene polymorphism associated with chronic periodontitis by using PCR-RFLP

Genotypes of TNFα-308 gene polymorphism with allele frequency between healthy control and chronic periodontitis subjects were detected by using PCR-RFLP technique. Results from figure (4.8) appeared that the genotypes of TNFα-308 were represented AA homozygote (147bp), AB heterozygote (147bp, 126bp and 21bp) and BB homozygote (126bp and 21bp)**.**

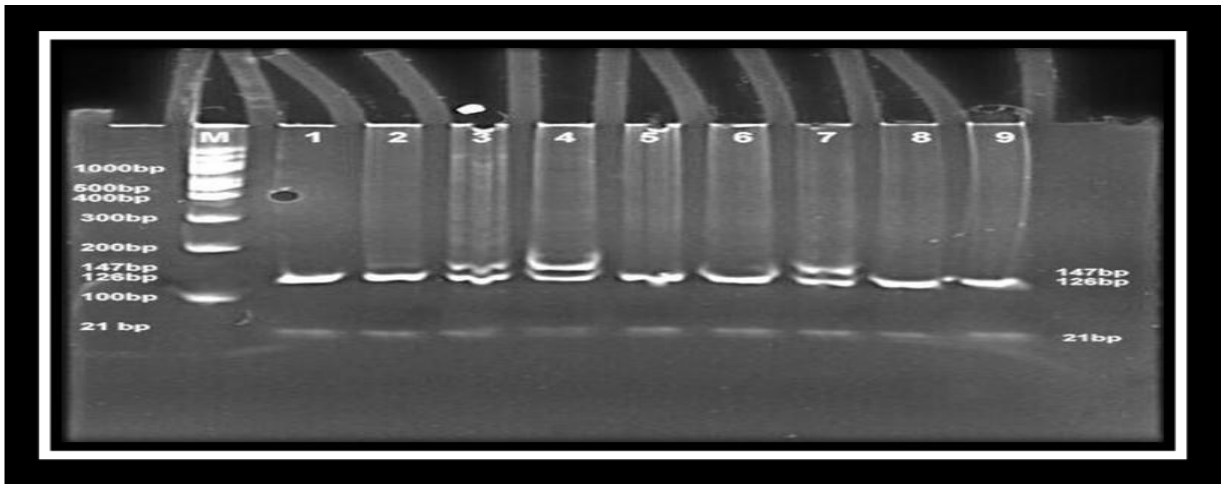


Figure (3) Electrophoresis pattern of TNFα -308 PCR-RFLP by 9% PAGE gel for PCR product AA (147bp) with restriction enzyme *NcoI*. Lane (M), DNA ladder. Lane (3, 4, 7) heterozygote (AB) genotype, Lane (1, 2, 5, 6, 8, 9) homozygote (BB) genotype.

Genotype frequencies of AA, AB , and BB of TNF-308 gene polymorphism were 12(40%), 10(33.33%) and 8(26.67%) in the healthy subjects, whereas 12(12%), 25(25%) and 63(63%) in the chronic periodontitis patients respectively **.** The differences between all genotypes in healthy and patients with chronic periodontitis were significant The *P-value* of the genotype frequency of TNFα -308 gene was (0.001) which is less than 0.05.

Allele frequency of TNFα -308 gene polymorphism associated with chronic periodontitis

Data of allele frequencies of point mutations on TNFα -308 gene in healthy control and chronic periodontitis patients were presented in table (5). For healthy groups the allele frequency of (A) variant allele was 0.57(57%), and (B) allele variant frequency was 0.43(43%) according to Hardy- Weinberg equation. Whereas for patient groups the allele frequency of (A) variant allele was 0.25(25%), and (B) allele variant

frequency was 0.75(75%) according to Hardy- Weinberg equation, PCR-RFLP results of TNF-α -308 gene polymorphism showed that the patients with genotype BB were more affected by chronic periodontitis at 4.68 times than patients with AA and AB 0.2 and 0.67respectively. P value was significant for all genotypes 0.001.

Table(5) Genotype of TNF-308 gene polymorphism with Allele frequencyin healthy control and chronic periodontitis patients

OR CI 95%	***P* value**	**Patients**	**Healthy**	**Genotype TNF-308**
0.20	0.001	12(12%)	12(40%)	**AA**
0.67	0.001 0.001	25(25%)	10(33.33%)	**AB**
4.68		63(63%)	8(26.67%)	**BB**
		100	30	**Total number**
Allele frequency				
Patients	**Control**		**Allele**	
0.25(25%)	0.57(57%)		**A**	
0.75(75%)	043(43%)		**B**	

In the this study, the detection of a polymorphism of TNF-α geneat loci -308 a DNA samples of the Iraqi population suffering or not from periodontal disease were done using PCR-RFLP technique. The results were appeared that there was an association between the polymorphism of TNF-α polymorphism gene and chronic periodontitis. odds ratio for TNF-α -308 showed that the patients with genotype BB were more affected by chronic periodontitis at 4.68 times than patients with AA and AB 0.2 and 0.67respectively**.** These results was disagreed with the results that showed by studies that were done by many researchers , they found that no correlation of TNF-α -308 gene polymorphisms with periodontal conditions in a Greek population and the odds ratio of the genotypes was 0.878 compare with odds ratio of the current study it was 4.68 , this variation in results could be belong to geographical area of the both study[25-26].

The current study was documented a strong risk for chronic periodontitis of TNF-α -308 for mutant BB genotype carriers according to odds ratio 4.68 ,this is the first study which indicated that there was a strong association between TNF-α -308 gene polymorphism and chronic periodontitis in Iraqi population. These results may be return to the frequent wars in this country and the impact of weapons used that could contain some chemical material, which led to a weakness in the genetic structure of the Iraqi people and increase its sensitivity to bacterial infections, which in turn led to polymorphism of TNF-α at loci -308. In a three-week period of conflict in Iraq during 2003, it was estimated that over 1000 tons of depleted uranium munitions were used[27], these may be used for water treatment in the future[28-33].

Conclusion

Periodontitis is a chronic disease that associated with multiple bacterial infection and the present of disease is associated with polymorphism of TNF-α -308 gene in Iraqi population.

Acknowledgments

The authors thanks University of Babylon, College of Sciences and Department of Biology for providing the necessary facilities during this work,our great appreciation to the staff working in college of dentistry in Babylon university, our gratitude to all the patients for their cooperation in achieving this study.

References

1. Page RC and Kornman KS., The pathogenesis of human perio-dontitis: an introduction. *Periodontol*;2000, 14:9-11.

2. Pihlstrom, B. L., Michalowicz, B. S., and Johnson, N. W., Periodontal diseases, Lancet 2005, 366, 1809-1820.
3. Goutoudi P, Diza E, Arvanitidou M., Effect of periodontal therapy on crevicular fluid interleukin-1beta and interleukin-10 levels in chronic periodontitis. *J Dent*; 2004, 32:511-20.
4. Hajishengallis, G., and Lamont, R. J. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology, Mol Oral Microbiol2012, 27, 409-419.
5. Brown, L. J., Johns, B. A., and Wall, T. P., The economics of periodontal diseases, Periodontol 2000, 29: 223-234.
6. Kuo, L. C., Polson, A. M., and Kang, T. Associations between periodontal diseases and systemic diseases: a review of the inter-relationships and interactions with diabetes, respiratory diseases, cardiovascular diseases and osteoporosis, *PublicHealth*2008, *122*, 417-433.
7. Socransky, S. S., and Haffajee, A. D., Periodontal microbial ecology, Periodontol 2000, 38: 135-187.
8. Roberts FA, McCaffery KA, Michalek SM., Profile of cyto-kine mRNA expression in chronic adult periodontitis. *J Dent Res*; 1997, 76:1833-1839.
9. Callard R, George AJ, Stark J., Cytokines, chaos, and com-plexity. Immunity; 1999, 11:507-513.
10. Joshi VM, Vandana KL., The detection of eight putative periodontal pathogens in adult and rapidly progressive periodontitis patients. Indian J Dent Res;2007, 18:6-10.
11. Koneman EW, Allen SD, Janda MW, Schreckenberger PC., The anaerobic bacteria. Color Atlas and Textbook Of Diagnostic Microbiology, 5th edition, ,Philadelphia, Lippincott JB, Ch1997, 14: 725-727.
12. Jousimies-Somer HR, Summanen PH, Baron EJ, Citron DM, Wexler HM, Finegold SM: Wadsworth-KTL anaerobic bacteriology manual.(2002). 6th ed. Belmont, CA: Star Publishing.
13. Prescott, Harley and Klein's. Microbiology 6th edition published by McGraw-Hill Science/Engineering/Math Hardcover, 2004.
14. MacFaddin, J.F., Biochemical Tests for Identification of Medical Bacteria. 3rd ed. Lippincott Williams and Wilkins, 2000, USA.
15. Forbes, B.A. ; Daniel, F.S. and Alice, S.W., Bailey and Scott's Diagnostic Microbiology . 12th ed. ,Mosby Elsevier company . 2007, USA .
16. Tellapragada C., Vandana KE, Shashidhar A., Parvati B., Asha K., Shashidhar V., and Chiranjay M., Prevalence of Clinical Periodontitis and Putative Periodontal Pathogens among South Indian Pregnant Women**.** International Journal of Microbiology 2014, Volume 2014, Article ID 420149, 5 pages.
17. Uglialoro AM, Turbay D, Pesavento PA, Delgado JC, McKenzie FE, Gribben JG, HartlD, Yunis EJ, Goldfeld AE, Identification of three new single nucleotide polymorphisms in the human tumor necrosis factor-alpha gene promoter. Oct; 1998, 52(4):359-67.
18. Van PalensteinHelderman, W. H., Joarder, M. A., Bequm A., Prevalence and severity of periodontal disease and dental cariesin Bangladesh. Int Dent J. 1996, 46: 76-81.
19. Darveau, R. P., Periodontitis: a polymicrobial disruption of host homeostasis, *Nat Rev Microbiol*2010, 8: 481-490.
20. Ximénez-Fyvie LA, Haffajee AD and Socransky SS., Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J ClinPeriodontol. 2000, 27(9):648-57.
21. Feng Z, Weinberg A., Role of bacteria in health and disease of periodontal tissues Periodontol 2000. J ; 2006, 40:50-76.
22. Rams TE, Slots J., Antibiotics in periodontal therapy: an update. PubMed PMID: 1298559., 1992, 13(12):1130- 1134.
23. Riggio MP, Lennon A., Development of a PCR assay specific for *Peptostreptococcusanaerobius*. J Med Microbiol. 2002, 51(12):1097-101.
24. Jussara CS.; Clélia Ap. de P.; Silvana SF.; José RC. ; Antonio OJ.., Staphylococcus spp. in the oral cavity and periodontal pockets of chronic periodontitis patients. Brazilian Journal of Microbiology 2004, 35:64-68.
25. Sakellari, D., Katsares, V., Georgiadou, M.,Kouvatsi, A., Arsenakis, M. and Konstantinidis, A. , No correlation of five gene polymorphisms with periodontal conditionsin a Greek population. Journal of Clinical Periodontology 2006, 33: 765–770.
26. Tervonen, T., Raunio, T., Knuuttila, M. &Karttunen, R., Polymorphisms in the CD14 and IL-6 genes associated with periodontal disease. Journal of Clinical Periodontology 2007, 34, 377–383.
27. Paul Brown (2013) . (25 April 2003) "Gulf troops face tests for cancer". theguardian.com. Retrieved 29 August 2013.

28. Karam FF, Hussein FH, Baqir SJ, Alkaim AF. Optimal conditions for treatment of contaminated waters with anthracene by Fenton processes in close system reactor. Journal of Chemical and Pharmaceutical Science. 2016; 9(3): 1111-1115.
29. Raheem RA, Al-gubury HY, Aljeboree AM, Alkaim AF. Photocatalytic degradation of reactive green dye by using Zinc oxide. Journal of Chemical and Pharmaceutical Science. 2016; 9(3): 1134-1138.
30. Kamil AM, Mohammed HT, Alkaim AF, Hussein FH. Adsorption of Congo red on multiwall carbon nanotubes: Effect of operational parameters. Journal of Chemical and Pharmaceutical Sciences. 2016; 9(3): 1128-1133.
31. Omran AR, Baiee MA, Juda SA, Salman JM, Alkaim AF. Removal of Congo red dye from aqueous solution using a new adsorbent surface developed from aquatic plant (Phragmitesaustralis). International Journal of ChemTech Research. 2016; 9(4): 334-342.
32. Kareem A, AbdAlrazak N, Aljebori KH, Aljebori AM, Algboory HL, Alkaim AF. Removal of methylene blue dye from aqueous solutions by using activated carbon/ urea-formaldehyde composite resin as an adsorbent. Int. J. Chem. Sci. 2016; 14(2): 635-648.
33. Aljeboree, A. M. Adsorption of crystal violet dye by Fugas Sawdust from aqueous solution. International Journal of ChemTech Research. 2016; 9(3): 412-423.

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