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Association of *S. epidermidis* infection and *TNF*-α gene polymorphism of acne patients in Thi-Qar province/ Iraq

Baydaa Shahead Muter[1,*] Zainab Dakhil Degaim[1]
[1]Microbiology Department-College of Medicine -University of Thi-Qar, Iraq
*Corresponding Authors: Baydaa Shahead Muter, Microbiology Department-College of Medicine -University of Thi-Qar, Iraq. Email: medmicbm20m3@utq.edu.iq

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ABSTRACT

The present study was aimed to identication of *S. epidermidis* from acne patients, and related with the genetic polymorphism of *TNF-α* gene in the same patients. The study were included 150 acne specimens from acne patients; The patients were diagnosed clinically by a dermatologist. As well, 75 venous blood samples were collected from same patients; and 25 samples from healthy persons as control in private clinical lab of Thi-Qar province/Iraq, during August-September, 2022. Only 60 isolates (40%) were identified as *S. epidermidis*. The identification of *S. epidermidis* isolates done by microscopic examination, morphological characterization on MSA, blood agar and chrom agar, biochemical test, then accurate identification with Viteks system. The results of genetic polymorphism of 30 samples (20: acne patients, 10: control) showed statistically non-associated between C302T SNP and acne disease. Besides, the rs1799724, C302T, three polymorphic patterns were detected, CC, CT and TT. The homozygous CC pattern was detected in the majority of samples in cases (16/20) and controls (7/10). Unusually, the homozygous TT status was not detected in both cases and control. Along, the sequencing chromatogram detected delation mutation (A nucleotide) in the position 309A of 12 studied samples (5: control, 7: patients) in comparsion with reference sequencing. Also, the existing results of allele frequency of *TNF*-α gene, and the odd ratio (OR= 1.5) showed that in case of the 302 C/T SNP polymorphism was risk indicator of developing acne vulgaris; so the risk more increased in the homozygote wild type (CC).

INTRODUCTION

Staphylococcus epidermidis (*S. epidermidis*) was a coagulase-negative staphylococci (CoNS) that was a permanent part of the normal human microbiota and was typically found on skin and mucous membranes. Since *S. epidermidis* grows in huge numbers on human skin and mucous membranes, it might cause infections in specific circumstances (1). *S. epidermidis* had the largest proportion, and played the significant part among coagulase-negative Staphylococci (CoNS) that was the most important reason of clinical infections (2). The significance of *S. epidermidis* in balancing the epithelial microflora and acting as a store of resistance genes (3). Acne was caused in part by inflammation, and pro-inflammatory cytokines played a role in this progression. Tumor necrosis factor alpha (TNF- α) was a signalling protein encoded by a gene with a high degree of genomic variation. Single nucleotide polymorphisms in the TNF- α gene had been related to an increased risk of developing an inflammatory illness over time (4,5).

Polymorphism in the DNA sequence was characterised as existing between individuals, groups, or populations. The variations in DNA can arise from a number of different mechanisms, including single-nucleotide polymorphisms (SNPs), sequence repeats, insertions, deletions, and recombination. It might be the result of chance processes, or may have been induced by external agents such as viruses or radiation (6). SNPs were the most common form of genetic variation in the genome. SNPs had been extensively used as molecular markers in human disease genetics (7). TNF- α is one of the primary pro-inflammatory cytokines that initiates and regulates the cytokine cascade during an inflammatory response. (8). TNF- α gene transcripts were found to be considerably increased in acne lesions (9). Based on the high infection rates of acne, which infected the majority of individuals, particularly during adolescence, and approximately 85% of adolescents and young adults were affected by the disease; the current study aimed to identify *S. epidermidis* from acne patients and identify the genetic polymorphisms of TNF- α and acne disease.

MATERIAL AND METHODS

Above 150 samples from acne patients were collected, during the period of August-September of 2022, from private clinical lab in Thi-Qar province, Iraq. Also, 75 venous blood samples collected in tubes containing (EDTA) from the same patients, as well as, 25 blood samples from healthy persons to use as controls. Data was collected from patients' faces (forehead, cheek, and chin). Cleansing the sample site with 70% ethanol. Transport media was utilised to collect the samples; closed comedones and papules were extracted by making a scratch in the surface of the lesion with a lancet and then drawing out the contents with mild palm pressure (10).

Isolation and identification

For identification a *S. epidermidis*, the collected samples were inoculated onto manitole salt agar (TM/India), which is a selective and differential medium for the isolation, purification, and identification of Staphylococci; and those isolates were inoculated onto Blood agar, Chrom agar (HI media/ India), for detecting their shape, colour, and pigments on those medium; all plates were incubated at 37ºC for 24 h, then a single pure isolated colony of *S. epidermidis* was transmitted to BHI agar medium for the preservation and to carry out other biochemical tests that confirmed the identification of isolates. The *S. epidermidis* identified under the microscope examination after inculated on different culture media, biochemical tests. For precise identification of *S. epidermidis* done with the Viteks compact system which included 43 tests.

Extraction of genomic DNA from blood samples

To extraction the genomic DNA from blood samples were processed using the Human gSYNCTM DNA Extraction Kit per the manufacturer's instructions protocol. The amplification of the TNF-α gene was done using the primer designed in this study depending to: NCBI pick primer, F:CAAAGGGAGCAAGAGCTGTG; R:AGGGACAAGCCTGGGACA. The final volume of reaction tubes is 50µl, consist of 10µl Master Mix, 1µl of each forward and reverse of the primer for the this gene, 5µl of DNA template and the volume was completed by adding nuclease free water. The thermocycling program of PCR was mentioned in Table (1).

Table (1): Program of *TNF-α* gene

Step	Temperature, °C	Time	Cycle
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	30
Annealing	60	30 sec	
Extension	72	30 sec	
Final extension	72	5 min	1

DNA Sequencing of PCR amplicons

Thirty of PCR products of TNF-α gene were chosen for sequencing of one direction, forward, according to the sequencing company's instruction manuals (Macrogen Inc. Geumchen, Seoul, South Korea). The blast algorithm is based on the Basic Local Alignment Search Tool analysis (www.ncbi.nlm.nih.gov/BLAST). Only clear chromatographs acquired from ABI sequence files were examined further, verifying that the annotation and variances are not the result of PCR or sequencing errors. Using BioEdit sequence Alignment Editor Software Version 7.1 (DNASTAR, USA), the sample sequences were

modified, aligned, and compared to the reference sequences (11).

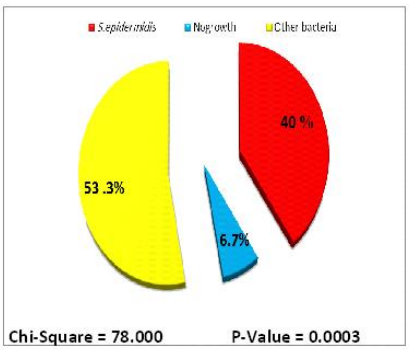
Checking the rs1799724, C302T SNP of *TNF-α* gene

The identified SNP has been submitted to the dbSNP database for validation. Every specific SNP was highlighted agreeing with its place in the reference genome. Hence, the presence of the prior SNP was established by checking its corresponding entry in dbSNP. The dbSNPs ID of the detected SNP was then recorded.

RESULTS AND DISCUSSION

Bacterial isolation and identification

From 150 samples were collected from acne patients in Thi-Qar province. Only 60 (40%) isolates of *S. epidermidis* were non-mannitol fermenter and looked as white colonies on the MSA medium, while colonies appeared as small, and blue colonies on chrom agar, as shown in Fig(1). Whereas (80/150; 53.3 %) of isolates identified as other bacterial species, and (10/150; 6.7%) werenot growth on this medium; as it illustrated in Fig. (1). Statistically, there were a significant difference between *S. epidermidis* and other Staphylococci at (P≤ 0.05).



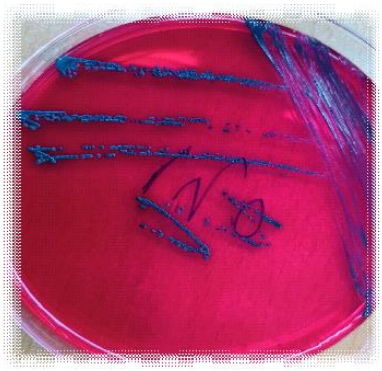


Fig. (1): A: Percentage of bacterial isolates from acne patients, B: The appearance of *S. epidermidis* on A- chrom agar

The entirely isolates of *S. epidermidis* were identified by biochemical tests, those isolates were given results according to the characteristics in Table (2).

Table (2): Biochemical tests of *S. epidermidis*

No	Biochemical test	Result of *S. epidermidis*
1	Catalase	Positive
2	Citrate	Negative
3	Coagulase	Negative
4	DNase	Negative
5	Indol	Negative
6	MR	Negative
7	Novobiocin sensitivity	Positive
8	Urease	Positive
9	VP	Positive

The results of current study recorded that *S. epidermidis* was (60/150; 40%) isolated from acne samples. The *S. epidermidis* isolates diagnosed based on microscopic examination, morphological characterization on media, also the using of many biochemical tests and Vitek system to accurate identification of this bacteria.

S. epidermidis was one of vital bacteria that causes acne (12); also, It was abundant bacterial colonizers of healthy human skin; many evidence suggested that the colonization through specific *S. epidermidis* strains might to assistance or hurt the skin barrier (13). Similarity, *S. epidermidis* was normal flora of the skin, more frequently isolated from inflammatory sites of patients with acne vulgaris (14). One of the substantial skin commensals was *S. epidermidis* which, proliferate quickly through puberty and involved in the progress of acne, and it was involved in superficial-infections within the sebaceous part (15). The occurrence of *S. epidermidis* (40%) isolated from acne samples was incorporated with results of other studies which recorded the *S. epidermidis* isolated from acne patients had similar percentage like: (16,17) in Korea and Jordan were (36% and 36.5%), respectively. While other studies documented high percentage of this bacteria that isolated from acne samples, performed in Visakhapatnam, Egypt and Indonesia (18,19,20) documented that 53.48%,61%, and 74.41%, respectively. The current results were disagreed with (21) whom recorded the *S.*

epidermidis isolated from acne patients was (8.8%), and this microorganism had a importance in the acne pathogenesis. Other study like (22) recorded *S. epidermidis* isolated from acne lesions was (53%) that isolated aerobically.

DNA Sequencing of the 582 bp region within the *TNF-α* gene

The percentage of *TNF-α* gene of blood samples which collected from acne patients was (100/100;100%) that detected by PCR technique; also the size of *TNF-α* gene which are about 582bp, as shown in Fig. (2).

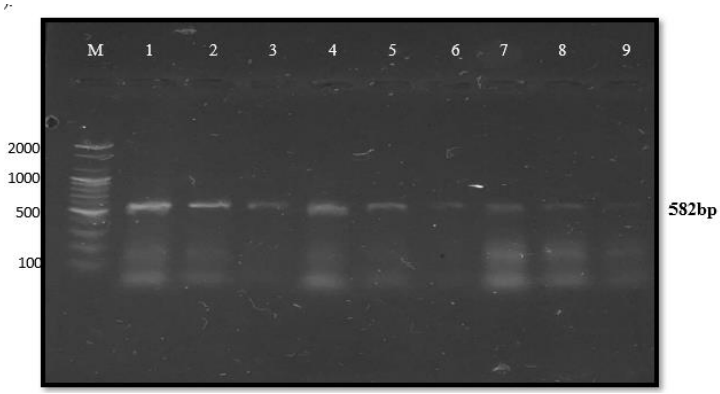


Fig. (2): Agarose gel electrophoresis of *TNF-α* gene amplification, where M: ladder, 1-9: positive results**.**

This was validated by sequencing reactions after NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was run on these PCR amplicons. On comparing the sequenced samples to the reference target sequences for the purported 582bp amplicons, the NCBI BLASTn engine showed a 98% sequence similarity. For the purpose of comparing the derived DNA sequences to the existing sequences in these tested samples (GenBank accession number (NC 000006.12) . Upon comparing the studied samples to the relevant reference sequences, the alignment results showed the presence of two nucleic acid variants (CC and CT) in both cases and controls, as shown in Fig. (3).

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250       260       270       280       290       300       310       320
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
RF  ACAGCAATGGGTAGGAGAATGTCCAGGGCTATGGAAGTCGAGTATGGGGACCCCCCCTTAACGAAGACAGGGCCATGTAG
1   ..........................................................C.....I..........
2   ..........................................................C.....A..........
3   ..........................................................C.....I..........
4   ..........................................................C.....A..........
5   ..........................................................C.....A..........
6   ..........................................................C.....I..........
7   ..........................................................C.....A..........
8   ..........................................................C.....A..........
9   ..........................................................C.....I..........
10  ..........................................................C.....I..........
11  ..........................................................C.....A..........
12  ..........................................................C.....I..........
13  ..........................................................C.....A..........
14  ..........................................................C.....A..........
15  ..........................................................C.....I..........
16  ..........................................................C.....A..........
17  ..........................................................C.....A..........
18  ..........................................................C.....I..........
19  ..........................................................C.....A..........
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Fig. (3): Alignment of of 30 samples with with the consistent reference sequences of the 582 bp PCR fragments of the TNF-α gene. The "ref." indicates to the NCBI referring sequence.. The yellow- shades indicated to Heterozygosity for the detected variant. The deletion of the detected variation is shown by the red colouring

The frequency of SNP was identified by replacing the C with a T at position 302 (rs1799724. C>T). of the amplified PCR fragments, named 302 C>T, or C302T. Variation frequency was unexpectedly identified in three polymorphic patterns (CC, CT, and TT). Most cases (16/20) and controls (7/10) identified had the homozygous CC pattern, whereas fewer cases (4/20) and controls (3/10) had the heterozygous CT pattern. Inexplicably, the homozygous TT status was not identified in either the case or control group, as stated in table (3).

According to the sequencing chromatogram file were detected delation mutation (A nucleotide) in the position 309A of 12 studied samples (5: control, 7: patients) in comparsion with reference sequencing.

Hardy-Weinberg equilibrium (HWE)

It was the idea that, the genetic variation in the population would persist constant from one generation to the next in the absence of disturbing factors. To determination the HWE, the genotyping and allele frequency of TNF-α , C>T SNP were represented in tables (3), (4), and (5).

The results of HWE dependable were non-significant results (P=0.899) were found in this investigation, the CC genotype frequency was greater in acne patients (80%) than in healthy controls (70%), although this difference was not statistically significant (OR=1.7, P. value= 0.5). The TT genotype frequency, however, was not found in both groups, as indicated in the table (3).

Table (3): Genotype frequency of TNF-α 302, C>T SNP among acne and control

TNF-α 302, C>T	Case=20	Control =10	OR	P. value
CC	16 (80%)	7 (70%)	1.7	0.5
CT	4 (20%)	3 (30%)	0.6	0.5
TT	0 (0.0%)	0 (0.0%	0	

The allele frequency of TNF-α gene, C>T SNP among cases and control was statistically, no a significant relationship (p. value 0.6) between *TNF-α* gene and 302 C>T polymorphisms, Table (4).

Table (4): Allele frequency of *TNF-α*, 302, C>T SNP among acne and control

TNF-α 302, C>T	Case: 20	Control: 10	OR	P. value
C	36	17	1.5	0.6
T	4	3		

The present results of HWE of TNF-α gene, the C302T SNP among the control, recorded that closely relation between expected genotype and observed genotype, as shown in table (5).

Table (5): Hardy-weinberg equilibrium in TNF-α, 302 C>T SNP among control

Genotype	CC	CT	TT
Observed genotype	7	3	0
Expected genotype	**7.225**	**2.55**	**0.225**
X^2=0.24 Df 1 P=0.899			

- Allele C Frequency **0.85**
- Allele T Frequency **0.15**

Interpreting to distribution of TNF-α, C>T SNP in this study population under recessive and dominant model table (6) and table (7) showed non significantly results, so appeared the recessive model (CC+CT), OR=1.9, more sensitive infected in acne than dominant model.

Table (6): Distribution of TNF-α, 302, C>T SNP in this study population under recessive model

Genotype	Case: 20	Control: 10	OR	P . value
CC+CT	20	10	1.9	0.7
TT	0	0		

Table (7): Distribution of TNF-α, 302, C>T SNP in this study population under dominant model.

genotype	Case NO: 20	Control:10	OR	P . value
CC	16	7	1.7	0.5
CT+ TT	4	3		

The current results of DNA sequencing recorded C302T SNP polymorphism, or rs1799724 of TNF-α gene. This SNP was reported in ClinVar database (https://www.ncbi.nlm.nih.gov/snp/rs1799724).

TNF-α had a pro-inflammatory effect in acne lesions. Therefore, many studied gene were directed to determine the correlation between the frequency of certain SNPs in the TNF-α gene and the development of acne vulgaris (23). The SNPs were discovered predominantly in the TNF-α gene promoter and might influence of gene expression positively or negatively, perhaps resulting in protective effect from acne (24).

TNF-α was a strategic fragment in several biological procedures, it had a double important in acne pathogenesis. In the early stages, TNF-α was involved in the initiation of lesion formation and the regulation of innate immune events, and in the later stages, it played an imperative role in the control of the inflammatory reaction and was linked to excessive inflammation, resulting in the immunopathology of acne vulgaris(25).

TNF-α was an important regulator of innate immunity and a key inducer of the inflammatory response. Likewise, it enhanced major (MHC I) molecule expression on activated T-cells, promotes IL-2-dependent T-cell proliferation, and it was a cofactor in B-cell proliferation and immunoglobulin production (26).

The results of current study recorded that non-association between *TNF-α* 302 C>T SNP polymorphism with acne patients (P. value= 0.899). The locus of TNF-α positioned in the highly polymorphic MHC III region on chromosome 6p21.3. There were various SNPs in current gene, especially in its 5´ regulatory region (27).

The results of current study were C>T (rs1799724) polymorphism was not significantly associated with acne; this results were incorporated with study done by (24) whom documented that the C>T -863 and C>T -1031 SNPs were not related with acne vulgaris. This study was not agreed with other studies such as: -857 SNP (rs1799724) were significantly connected with acne and acne severity (28,25). The current results were not consistent with (29) documented the TNF-α -863 (rs1799724) polymorphism was highly related with acne in complete groups of acne patients .

The results of allele frequency recorded that the C allele was more frequently observed in patients (n=36) and control (n=17), while the T allele was found in low ratio of patients and controls groups. Remarkably, the -857C>T polymorphism of *TNF-α* gene, the C allele displayed a positive relationship with acne, while the T allele appeared to had a protective effect (25).

In present results of genotype distribution of *TNF-α* gene documented that the CC genotype was identified in the majority of acne samples (16; 80%), and controls (7/70%), while TT pattern was not detected in both groups. The present results were incorporated with results of (25) whom showed the genotype frequency of control individuals was (CC: 53.2%), while in acne patients was (CC: 68.3%). The homozygous (CC), and heterozygous (CT) genotype frequency were increased in patients, although the (TT) frequency was identical in both groups (29).

The current results of allele frequency of *TNF*-α gene, and depending to odd ratio (OR= 1.5) displayed that in case of the 302 C/T SNP polymorphism was risk indicator of developing acne vulgaris; so the risk more increased in the homozygote wild type (CC) SNP. Also, C>T SNP polymorphism of TNF-α gene, the study population under recessive and dominant model appeared the recessive model (CC+CT), (OR=1.9), more sensitive infected in acne than dominant model. The acne severity was likewise in relationship with the TNF-α genotypes, the percentage of CC against (CT+TT) individuals was similar in controls and acne patients; while the minor allele frequency were decreased in patients displaying more and more severe signs (25).

Various genes involved in acne develpment and acne severity were elaborate in immune and inflammatory responses as *TNF*. The polymorphisms like: -238, -308, -857, -863 and -

1031 restricted in the promoter region of *TNF*, that affect on the gene expression positively or negatively, discussing a protective or detrimental effect on acne (30).

Furthermore, frequent studies reported the significant association of the TNF-*α* T>C polymorphism (rs1799724) which detected in present results with the development of numerous diseases, such as cancer. Also, the TNF-*α* T-857C (rs1799724) polymorphisms might be related with an increased risk of cancers (31).

The present results of sequencing data detected the delation mutation (A nucleotide) in the position (309A) in studied samples (7: patients, 5: control) in comparsion with reference sequencing.

Several missense and nonsense mutations in the protein-coding area of disease susceptibility genes are responsible for causing simple monogenic hereditary diseases. Despite this, it is often the case that a collection of mutations that affect the expression rather than the structure or function of several genes causes susceptibility to complicated diseases (32).

The *TNF-α* gene mutations affect its expression and contributed to the transformed inflammatory response. Also, the -857 SNP was in relation equilibrium with -863 and -1031 mutation sites which had high risk of acne progress (25). Previously, (33, 34) suggested that the mutations in the coding region of the *TNF-α* gene had never been discovered in human diseases; however the numerous polymorphisms in the promotor region of the *TNF*-α gene (33).

There were many studied mutations detected in *TNF* gene and other genes which related with developing of acne vulgaris and other diseases, such as: Fibroblast growth factor receptor 2 (FGFR2) gene played an imperative role in the acne pathogenesis; the germline mutations of the FGFR2 gene (S252W, P253R) had been established to cause Apert syndrome (35).

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