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MASTER'S THESIS

ASSOCIATION BETWEEN INTERLEUKIN 17A AND INTERLEUKIN 17F GENE POLYMORPHISMS AND PERIODONTAL PATHOGENS IN CHRONIC PERIODONTITIS AMONG LIBYANS

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منشورات البرنامج الوطني لتطوير المجلات العلمية

The Libyan Academy **School of Basic Sciences**

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Association between Interleukin 17A and Interleukin 17F Gene Polymorphisms and Periodontal Pathogens in Chronic Periodontitis among Libyans.

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الأكاديمية الليبية

مدرسة العلوم الأساسية

قسم علوم الحياة

شعبة الأحياء الدقيقة

ظاهرة تعدد أشكال النوكليوتيدات المفردة في إنتر لوكين17 (أ & ف) وارتباطه بمرض التهاب اللثة المزمن وأكثر أنواع بكتريا مصاحبة للمرض بين الليبيين.

بحث مقدم لمدرسة العلوم الأساسية بالأكاديمية الليبية كجزء من متطلبات الحصول على درجة الإجازة العالية في علم الأحياء الدقيقة.

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Declaration

I hereby declare that this thesis is the result of my own work unless otherwise stated.

Eshraq Alsherif

Dedication

This thesis is dedicated to my mother (peace be up on her) and my father who have encouraged me to continue my education and provided support throughout my life.

To my two lovely sisters Hana and Aisha who stood with me in difficult times.

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List of abbreviations:

Aa	*Aggregatibacter actinomycetemcomitans*
Ab1–42	Amyloid beta 1–42
APCs	Antigen-Presenting Cells
B2	Binding Buffer
BOP	Bleeding On Probing
BTRC	Biotechnology Research Centre
CAL	Clinical Attachment Level
CD4	Cluster of Differentiation 4
CGF	Crevicular Gingival Fluid
CP	Chronic Periodontitis
Cr	*Campylobacter rectus*
CT	Capture Buffer
DC's	Dendritic Cells
DNA	Deoxyribose Nucleic Acid
DNTPs	Deoxy ribonucleotides Triphosphate
DS DNA	Double strand DNA
EBV	Epstein Barr virus
Ec	*Eikenella corrodens*
Etbr	Ethidium Bromide

HC	Healthy Control
HCMV	Human cytomegalovirus
HMT ID	Human Microbial Taxon ID
HOMD	Human Oral Microbiome Database
HSV	Herpes Simplex Virus
IL 17	Interleukin 17
IL 17 R	Interleukin 17 Receptor
IL 25	Interleukin 25
IL 6	Interleukin 6
IL 8	Interleukin 8
LPS	Lipopolysaccharide
LS	Lysis Buffer
MAF	Minor Allele Frequency
MAPK	Mitogen-Activated Protein Kinase
MPO	Myeloperoxidase
NF-kB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated
OC	Orange Complex
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PD	Probing Depth
PDI	Periodontal Disease Index
Pg	*Porphyromonas gingivalis*
PGE2	prostaglandin E2
Pi	*Prevotella intermedia*
PK	Proteinase K
PLBW	Preterm Low Birth Weight

PTB	Preterm Birth
RANKL	Receptor activator of nuclear factor kappa B ligand
RC	Red Complex
RPM	Rotation per Minute
RS	Reference SNP
RT-PCR	Real Time PCR
RTX	Repeats in toxin
SNP	Single-nucleotide polymorphism
TBE	Tris - Borate – EDTA
Td	*Treponema denticola*
TE	Tris-EDTA
Tf	*Tannerella forsythia*
Th17	T helper 17 cells
UV	Ultraviolet Light

الملخص:

خلفية الموضوع: التهاب اللثة المزمن ينجم عن بكتريا مسببة لأمراض اللثة، وأيضا يتأثر هدا المرض بالعوامل الوراثية والبيئية والمسبب الرئيسي لها هو البيو فليم التي تقوم بإفرازه مجموعة من البكتريا المسببة لأمراض اللثة، وتشير الدراسات الحديثة إلى أن السيتوكينات المضادة للالتهابات مثل إنترلوكين (IL-17) تلعب دورا بارزا في التسبب في مرض التهاب اللثة المزمن.

الغرض من الدراسة: دراسة العلاقة بين تعدد الأشكال الجينية إنترلوكين17 (أ، ف) ومرض التهاب اللثة المزمن وكذلك دراسة العلاقة بين ثمانية أنواع من بكتيريا ومرض التهاب اللثة المزمن بين الليبيين.

المواد وطرق البحث: تكونت الدراسة من عدد 100 متطوع من المواطنين الليبيين، 50 رجلاً و50 امرأة بين عمر (25 -65) عاما. تم إجراء فحص الأسنان لكل مشارك، تم تشخيص أمراض اللثة وفقًا للمعايير السريرية لمؤشرات أمراض اللثة (BOP، CAL، PD،PDI) وتم أخذ عينات من الحمض النووي عن طريق مسحة من الفم لتحليل الاختلافات في الجينات للإنترلوكين 17(أ، اف) المرتبطة بمرض التهاب اللثة المزمن. وكذلك أخذت عينة من الطبقة البيولوجية (بيوفيلم)

الموجود في الجيوب اللثوية للكشف عن ثمانية أنواع من البكتريا الموجودة بالفم.

تم تحليل النمط الجيني بواسطة تفاعل البلمرة المتسلسل من ثم تحديد تسلسل النوكليوتيد الدقيق في جزء معين من الحمض النووي للكشف عن تعدد الأشكال الجيني للإنترلوكين 17(أ، اف)، بينما تم التعرف على عدد ثمانية أنواع من بكتريا مسببة لأمراض اللثة بواسطة تفاعل البلمرة المتسلسل المتعدد والترحيل الكهربائي للهلام.

النتائج: تم العثور على متغير جديد <C. *34G A في IL17F حوالي 14.6% من المرضى وارتباطه مع مرض التهاب اللثة المزمن (p-value = 0.010), تم الكشف عن الأنماط الوراثية (GG،AG) في IL17A (rs2275913) في المرضى الذين يعانون من التهاب اللثة المزمن، أيضا شوهد النمط الوراثي (CT) IL17F

(rs763780) في الفئة المصابة بالمرض ولكن لا يوجد علاقة ارتباط مع مرض التهاب اللثة المزمن (p-value = 0.334) وجود ارتباط قوي بين ثمانية انواع من البكتريا و مرض اللثة المزمن (p-value =0.0001) وزيادة معدل انتشارها في الفئة المصابة بالمرض.

الاستنتاج : توجد علاقة قوية بين ثمانية انواع من البكتريا مع مرض التهاب اللثة المزمن .هناك حاجة إلى مزيد من الدراسات الجينية واسعة النطاق لتوضيح الرابطة بين IL17A ومرض التهاب اللثة المزمن وأيضا على متغير جديد c.* 34G> A في IL17F وعلاقته بي مرض التهاب اللثة المزمن في حجم عينة أكبر.

Abstract

Background: Chronic periodontitis (CP) is triggered by periodontal pathogens and influenced by genetic and environmental factors. Recent studies suggest that anti-inflammatory cytokines such as interleukin 17 (IL-17) plays a prominent role in the pathogenesis of CP. This study aims to investigate the association between CP and interleukin IL- 17 (A and F) gene polymorphisms. We also aim to evaluate the association between eight sub-gingival pathogens and CP among Libyans.

Materials and Methods: The study consisted of 100 Libyan individuals

between the ages of 25 and 65 years including 50cases and 50controls. DNA was extracted from buccal swabs and paper points for sub-gingival pathogen samples. IL 17 (A&F) genotyping was performed by PCR followed by Sanger sequencing. Specific 16S rRNA primers for each pathogen were applied in a multiplex PCR reaction and visualized by agarose gel electrophoresis for detection of sub-gingival pathogens.

Results: A novel variant c.*34G>A in IL17F was found in 14.6% of patients and it was associated with CP (p-value = 0.010). IL17A GG and AG

(rs2275913) genotypes were detected in patients with CP.

IL-17F CT (rs763780) showed no association with CP (p-value = 0.334).

Significant association between the eight sub-gingival pathogens and CP (p-value =0.0001) and high prevalence of sub-gingival pathogens in CP group.

Conclusion: There is a strong association between eight sub-gingival pathogens and CP. More extensive genetic studies with a larger

sample size are needed to further the association between interleukin-17 A and CP and the relation between novel variant c.*34G>A and CP among Libyans.

Chapter 1. **INTR ODUCTION**

1.1 Background:

The periodontium is the tissues that support and surround the teeth structure, the word comes from the Greek terms *peri*-, meaning "around" and -*odont*, meaning "tooth" and it consists of four components: Gingiva, Periodontal ligament, Cementum and Alveolar bone as shown in figure (1.1)[1]

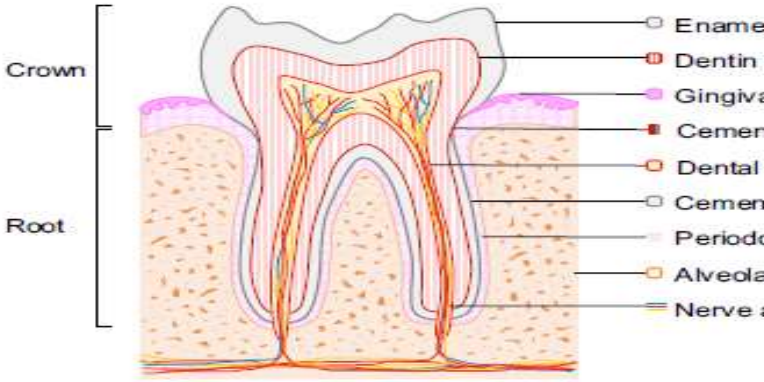
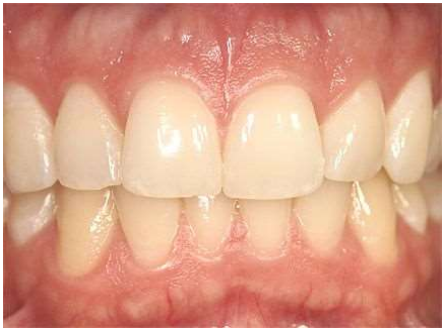


Figure 1.1 Tooth structure and components of periodontium [2] .

Chronic periodontitis (CP) is an inflammatory disease of supporting tissue of the teeth 'the periodontium' causing progressive destruction in periodontal ligament, alveolar bone, and pocket formation [3] . Clinical view of both healthy Periodontium and chronic

Periodontitis patient as shown in figures (1.2) and (1.3).



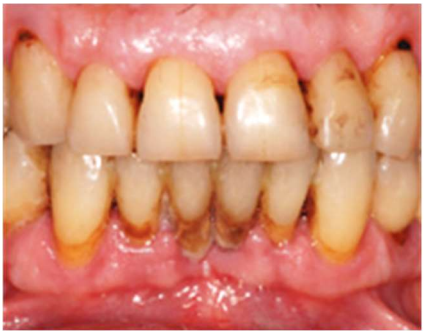


Figure 1.2 Healthy Periodontium [4] .

Figure 1.3 Chronic Periodontitis [4].

CP is a silent disease; chronic gingival inflammation and bone destruction are often painless, it has few symptoms in the early stages, in many individuals the disease has progressed significantly before they even know and seek treatment, symptoms may include the following:

1. Redness or bleeding of gingiva while brushing teeth, using dental floss or biting on hard food (e.g., apples).
2. Gingival swelling.

3. Halitosis (bad breath), and a persistent metallic taste in the mouth.
4. Gingival recession, resulting in apparent lengthening of teeth.
5. Deep pockets between the teeth.
6. Loose teeth and finally in the later stages; tooth loss [5,6].

1.2 Epidemiology of CP:

Periodontitis is the most common chronic inflammatory disease seen in

humans, affecting nearly half of adults in the United Kingdom of whom 60% are over 65 years old; it is a main public health problem, regardless of the higher level of awareness and dental care in developed countries, periodontitis is widely spread among their populations [3].

In 2009-2012 the United States had periodontitis with different forms (mild, moderate and severe) affecting as many as 47.2% of adults representing 64.7 million people. Prevalence rates up to 70.1% are associated with old age [7].

A longitudinal cohort study conducted in South Korea found the incidence of tooth extraction is increasing, and at a higher rate in patients with periodontal disease. In 2002, 50.6% of tooth extraction cases were caused by periodontal disease, and this increased to 70.8% In 2013 [8].

In 2014, Kassebaum et al. published a paper estimating the global burden of oral conditions from 1990 to 2010, periodontitis was estimated to be the sixth most prevalent disease globally, affecting 743 million people

worldwide, incidence of severe periodontitis in 2010 was 701 cases per 100,000 person years [9,10].

In Libya, the information about prevalence or the awareness level of the periodontal diseases is limited. Few of the conducted studies, showed a little information regarding periodontal health. In 2013 a cross-sectional study was conducted among 1,255 participants aged between 18-34 years in Sabha reported that only 4.7% had healthy periodontium [11].

In 2017 another cross-sectional study conducted in Libya, showed varying

degrees of periodontitis severity, half of the patients (50%) were moderate, 21% were severe, and 15% had a mild form of CP [12].

The International Classification of Functioning, Disability and Health, has described health-related domains that impact capacity and performance of a person with tooth-supporting tissue distraction, loss of periodontal attachment and alveolar bone and finally tooth loss, all these will lead to activity limitations, difficulties in chewing, speaking and smiling, participation restrictions, also personal

and professional relationships may be affected [13].

The risk for CP was raised from 5–15 fold in smokers, being proportional to the duration and amount of smoking "smoking appears to impact biofilm formation from the moment of its development, changing it from a health-compatible community to a pathogen-enriched community, predisposing the individual to periodontal disease." [14].

1.3 Pathogenesis of CP:

Colonization of the gingival crevice occurs initially by bacterial interactions

with the tooth and later by inter-bacterial interactions, leading to the formation of an organized, cooperating community called the biofilm [15].

It is evident that CP has multifactorial etiologies, stemming from the development of biofilm (bacterial colonization) on the tooth surface and gingiva, initially these bacterial deposits induce a gingival inflammation (gingivitis) which is completely reversible, continued along with the supporting tissues of the teeth, progressive attachment loss, pocket formation and bone loss (periodontitis),

finally leading to tooth loss, as shown in figure (1.4) [16,17]. Social and behavioural factors, and genetic or epigenetic factors, all are modulated and controlled by the underlying immune and inflammatory responses of the host [16].

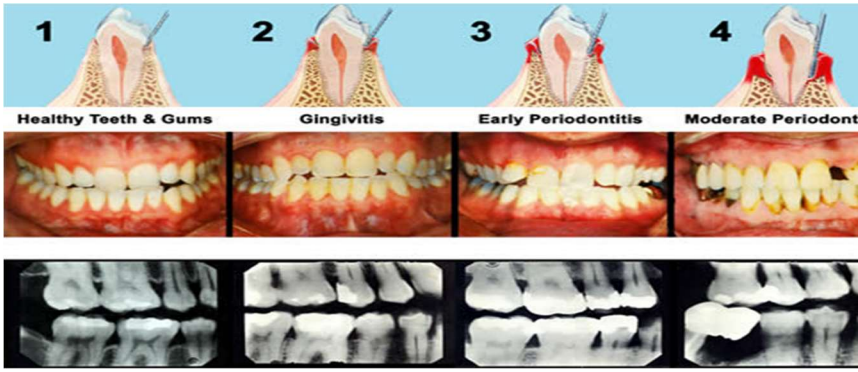


Figure 1.4 Stages of periodontal disease [18].

The host inflammatory immune reaction begins after the recognition of the bacterial pathogens by antigen-presenting cells (APCs), such as dendritic cells (DCs). DCs have a strong capability of catching antigens, which enables them to stimulate T cells. In CP, activation of DCs occurs after contact with lipopolysaccharide (LPS) or by immune complexes produced by periodontal pathogens [19–23].

Over 700 bacterial species have been identified in the human oral cavity, about 400 of these species have been identified in the periodontal pocket,

whereas about 300 species have been found in other oral sites including the tongue, oral mucous membranes, dental carious lesions, and endodontic infections [24].

The intricacy of the subgingival microbiota has been recognized through microscopic examination by van Leeuwenhoek in 1683, who was the first to observe that subgingival plaques are comprised of a large complex mixture of bacterial species [25].

Indeed, it has been estimated that 400 or more species are present inside this

area, since that time several studies have evaluated and estimated the composition of plaque using microscopy, culture and more recently DNA probe techniques [26]. The anaerobic culture methods may fail in identifying all the organisms due to the extremely slow growth (time consuming) or very specific growth requirements of some oral pathogens, which inhabit the subgingival microflora [26].

Several alternative methods have been developed for the detection of oral pathogens, such as the *16S rRNA* gene detection by conventional PCR or

Multiplex PCR for the oral pathogens that are uncultivable and are difficult to identify, these methods can eliminate the ambiguity in the diagnostic microbiology [26].

In recent years, highly sensitive microbiological detection techniques such as real-time PCR can be used to identify and quantify oral bacteria in subgingival samples [26,27].

Socransky et al (1998) proposed that oral diseases could be better understood by focusing on the consortia of organisms rather than on individual

pathogens, they identified five sets of bacteria or complexes that were repeatedly found together in periodontitis as shown in figure (1.5) [17].

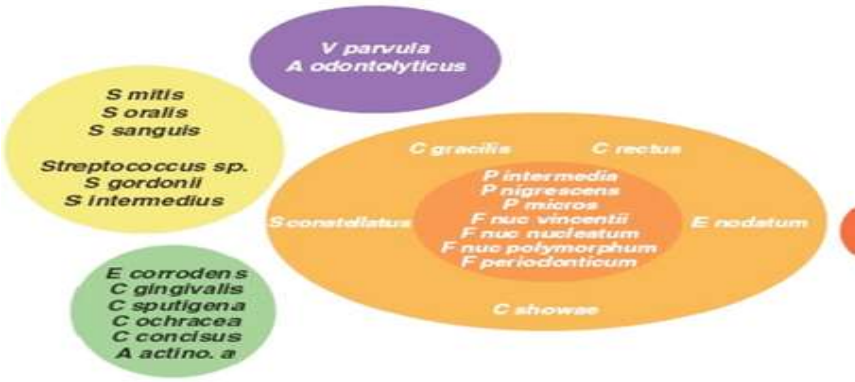


Figure1.5 Bacterial Complexes [17].

The majority of subgingival microorganisms is considered to be a normal flora, only several species have

been implicated as periodontal pathogens. Socransky et al in 1998 suggested that the most pathogenic complex including *Porphyromonas gingivalis (Pg), Treponema denticola (Td),* and *Tannerella forsythia* (*Tf*) termed the Red Complex (RC), was strongly associated with CP, they are also often associated with each other and with diseased sites and may inhibit innate host defence functions [17,28,29].

Other recognized pathogens called Orange Complex (OC), including *Prevotella intermedia (Pi)*, it is the one preceding (RC) in colonization and

proliferation. Additionally, *Prevotella nigrescns* (*Pn*), and *Campylobacter rectus* (*Cr*) also increase the depth of periodontal pockets and considered as periodontal pathogens[30]. Also there is Green complex (GC) including *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Eikenella corrodens* (*Ec*) which are often considered important in periodontal disease [24,30] .

Subgingival microbiota was detected less frequently in shallow pockets (up to 4 mm); they were found to increase in quantity in pockets of 4 to 6 mm depth

and were in the highest levels in pockets of over 6 mm depth [31].

However, there is sufficient evidence that the periodontal pathogens *P.gingivalis*, *T.denticola* and *T.forsythia* are detected more frequently in deep periodontal pockets (> 5 mm) than in shallow ones (< 4 mm) [31].

Understanding of CP has increased significantly with extensive analysis of the dental plaque associated with either clinically healthy or diseased sites, periodontitis has been characterized as

a polymicrobial oral disease and another issue has been noted a microbial shift in an oral cavity from mostly Gram-positive in healthy periodontium to the mostly Gram-negative diseased site [29].

1.3.1 Red complex (RC)

1. *Porphyromonas gingivalis (Pg):*

It is a Gram-negative, anaerobic, rod-shaped, non-motile pathogenic bacteria, which is black pigmented and it has a strong association with the incidence and severity of CP [32].

2. *Tannerella forsythia (Tf)*:

It is a Gram-negative, anaerobic pathogenic bacteria, rod-shaped, and was originally isolated in the 1970s by Dr. Anne Tanner from dental plaque collected from patients with CP [33].

3. *Treponema denticola* (Td):

It is a Gram-negative, obligate anaerobic, spiral-shaped pathogenic bacteria, it is present in a complex microbial community within the oral cavity. It has a strong association with the incidence and severity of CP [34].

1.3.2 Orange complex (OC)

1. *Prevotella intermedia (Pi)*:

It is a Gram-negative, obligate anaerobic, rod-shaped pathogenic bacteria involved in periodontitis, and commonly isolated from dental abscesses, where obligate anaerobes predominate [35].

2. *Prevotella nigrescens (Pn)*:

It is a Gram-negative, obligate anaerobic, rod-shaped and non-motile pathogenic bacteria, it is part of the normal oral flora but leads to disease when it infects the local tissue. *P.*

nigrescens could increase the incidence of periodontal diseases and play a role in the pathogenesis of CP [36].

3. *Campylobacter rectus (Cr)*:

It is a Gram-negative, facultative anaerobic, bacillus-shaped and motile pathogenic bacteria. *C.rectus* is associated with the initiation and progression of periodontal disease [37].

1.3.3 Green complex (GC)

1. *Aggregatibacter actinomycetemcomitans (Aa)*:

It is a Gram-negative, facultative anaerobic, non-motile pathogenic bacteria. *A. actinomycetemcomitans* is associated with the initiation and progression of periodontal disease and interference with host defence mechanisms [38].

2. *Eikenella corrodens (Ec)*:

It is a Gram-negative, facultative anaerobic, pleomorphic bacillus pathogenic bacteria. It is found predominantly in subgingival plaque in

patients with advanced periodontitis and may also cause extra oral infections [39] .

Recently sseveral studies have demonstrated a positive association between human cytomegalovirus (HCMV), Epstein Barr (EBV), Herpes Simplex Virus (HSV) and CP [40,41] .

In the following table a summary of eight subgingival pathogens and their association with periodontal diseases, clinical response to elimination, immune response and virulent factors table (1.1)

Table 1.1 Periodontal pathogens and their association with periodontal diseases, the clinical response to elimination, immune response and virulence factors [42–45].

Complex	Bacterial strain	Association	Elimination	Immune response	Virulent factors
Red	*P.g*	+++	+++	++	proteolytic, capsule
	T.d	++	+++	+	motile, proteolytic
	T. f	++	+	+	Proteolytic
Green	*A.a*	+++	+	+++	leukotoxin, invasion
	E.c	+	+	+	adherence to buccal epithelial cells
Orange	*P.i*	++	+	+	Proteolytic
	C.r	++	+	/	motile, leukotoxin invasion
	P.n	++	+	+	Colonization , trigger immune system

(+) strength.

(/) Immune respond is unkn

Previous studies showed a correlation between CP and many systemic diseases, subgingival microorganisms play a critical role in etiology, for example, (*Aa*) has been responsible for some systemic infectious diseases, such as endocarditis, meningitis, osteomyelitis, glomerulonephritis and arthritis. In addition, subgingival microorganisms are the most important factor implicated in preterm birth (PTB) [46,47]. There is a relationship between oral and vaginal microflora and preterm low birth weight (PLBW), related species are (*Aa*), (*Pg*), (*Tf*), (*Td*) and (*PI*). (>50% of women) in oral samples were (*Pg*), (*Tf*), (*Td*) , (*Pi*) in the preterm group. Studies showed the amount of (*Pg*) in subgingival plaque of pre-term women was higher than that of term women. 24.4% of all the pregnant subjects presented periodontal pathogens in their vaginal swabs [46–49].

(RC) involved as an etiological factor in oesophageal adenocarcinoma and oesophageal squamous cell carcinoma, although evidence is limited to cross-sectional studies. In addition, (*Tf*) has been identified in atherosclerotic lesions and also has been isolated from women with bacterial vaginosis [48–50].

A recent research discovered that (*Pg*) with their toxin called gingipains was identified in the brain of Alzheimer's disease patients. Oral (*Pg*) infection in mice resulted in brain colonization and increased production of Aβ1–42, a component of amyloid plaques, blocked Aβ1–42 production, reduced neuroinflammation, and rescued neurons in the hippocampus, gingipain inhibitors could be valuable in treating *P. gingivalis* brain colonization and neurodegeneration in Alzheimer's disease [51].

In vivo and in vitro studies demonstrate the importance of the fimbriae of *P. gingivalis* to host cell entry and to promote atherothrombotic lesions in experimental models. Hemagglutinin A (HagA) expressed by *P. gingivali* have the capability to adhere and enter human coronary artery endothelial cells and make damages [52].

E. corrodens also has been linked to a variety of disease states, including abscess, endocarditis, meningitis, osteomyelitis, keratitis, conjunctivitis and cellulitis [45].

1.4 Cytokines:

They are small proteins and signaling molecules, which play an important role in cell to cell communication in immune responses, and stimulation of the movement of cells towards sites of inflammation, infection, and trauma, as shown in figure (1.6) [53].

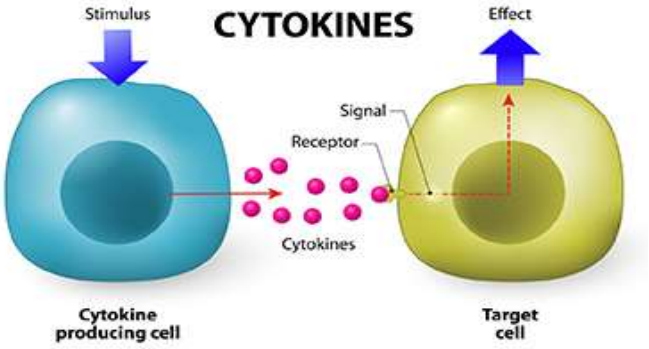


Figure 1.6 Cytokine mechanism [54].

They are a large group of low molecular weight proteins, peptides or glycoproteins that are secreted by specific cells of the immune system. Cytokine is a general name; specific names include lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities), and interleukins (cytokines made by one leukocyte and acting on another leukocytes) as shown in figure (1.7) [53].

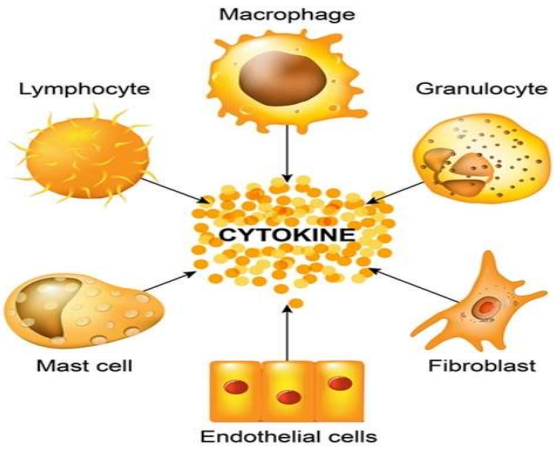


Figure 1.7 Cytokines are produced by different cells [55].

1.5 Interleukin 17:

It is a pro-inflammatory cytokine secreted by activated T cells after transformation to T helper 17 cells (Th17), the IL-17 family contains six members, IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (or IL-25), and IL-17F, and five receptors, IL-17 R(A,B,C,D) and SEF. Interleukin-17A is the most homologous to IL-17F and the genes encoding them are proximally located on the same chromosome (6p12) as shown in figure (1.8) [45] .

Figure 1.8 IL- 17 A and IL- 17 F Gene location in human [58].

After activation, DCs become mature, and stimulated to produce various cytokine patterns, like (IL-17), which will define the selective migration of CD4 T-helper subsets and the subsequent production of characteristic cytokines as shown in figure (1.9)[23].

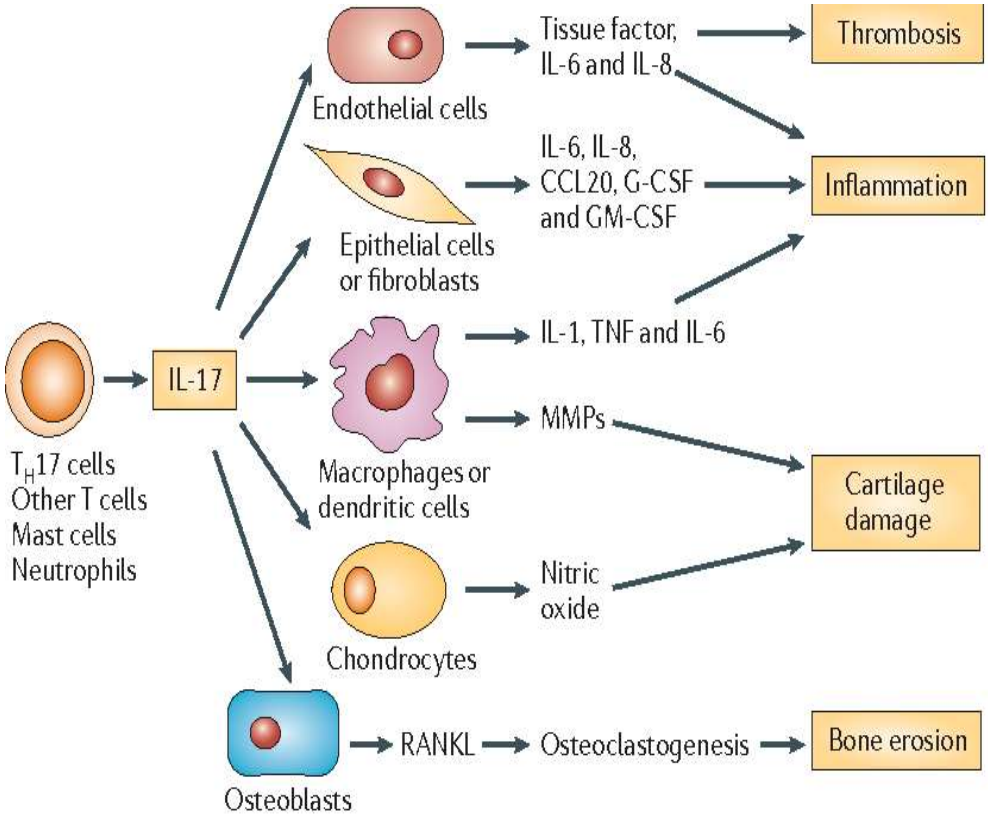


Figure 1.9 Functions of IL-17 and its role in inflammation and matrix destruction [59].

Signaling downstream of IL-17R mediates NF-kB and MAPK, leading to the production of pro-inflammatory cytokines and chemokines and subsequent myeloid cell recruitment to the inflamed tissue. Although the signalling events induced by IL-17A/F are not fully understood, several key signaling molecules have been successfully identified. The IL-17A activity is similar to IL-17F but significantly stronger. IL-17 cytokine can stimulate fibroblasts, epithelial and endothelial cells, to produce IL-6, IL-8 and prostaglandin E2 (PGE2) [57,60,61].

It is well established that IL-17 activity contributes to various aspects of inflammation. The IL-17-mediated release of IL-6 and IL-8 from mesenchymal cells leads to fever (caused by IL-6) and the accumulation of neutrophils in blood and tissue (caused by IL-8). Also, it stimulates the expression of RANKL in osteoblasts to activates the osteoclasts, which can induce bone resorption mediated by these cells [59,62,63].

1.5.1 Interleukin 17 A gene:

It spans a region of 4252 bp, consisting of three exons, untranslated region UTR, coding region and two introns. Exons 1, 2, and 3 are 72 bp, 203bp and 1584 bp respectively, the two introns are 1144 bp and 1249 bp in length as shown in figure (1.10) [64].

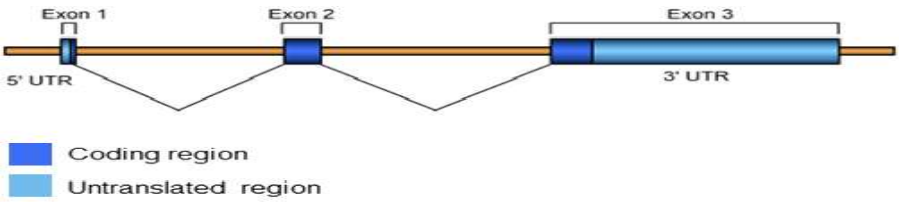


Figure 1.10 Interleukin 17A gene [64].

1.5.2 Interleukin 17 F gene:

It spans a region of 7.86 kb composed of three exons, untranslated region UTR, coding region and two introns. Exons 1, 2, and 3 are 141 bp, 221,488 bp (238 bp coding region plus 250 bp 3' UTR) in length, the two introns are 5446 bp and 1561 bp in length as shown in figure (1.11) [58].

Figure 1.11 Interleukin 17F gene [58].

1.6 Single nucleotide polymorphisms in chronic periodontitis:

It is a variation at a single position in a DNA sequence, a single nucleotide that is replaced with any of the other three kinds of nucleotides (Adenine, Guanine, Cytosine, Thymine) that occurs at a specific position in the genome, where each variation is present to some significant degree within a population with a frequency above 1% [65].

SNP within candidate genes may be related to changes in protein expression, structure and function which may lead to variations in phenotypic expression. It can also increase susceptibility to a wide range of human diseases [65–67].

Hence SNPs are very important genetic markers for investigating inter-individual differences in drug response and common diseases, they have been also found to have a significant effect on the production and/or functioning of cytokines [66].

CP inflammatory disease, caused by Gram-negative bacteria in the periodontal pockets. Many reviews have been published in recent years supporting the evidence that genetic influence an individual's predisposition for the initiation and progression of CP. Early identification

of risk factors for the development of periodontitis may also form the basis for more focused and cost- effective preventive approaches [68,69].

IL-17A and IL-17F Gene Polymorphisms in chronic periodontitis:

Allelic variations in cytokine genes and factors affecting their release have caused phenotypic differences in cytokine response among individuals and this is important for the individual's susceptibility to disease, the progression of disease or response to treatment, the determination of allelic variants of genes may be used to assess the risk of disease [70].

Many researches have demonstrated the presence of IL-17 in periodontal tissues, crevicular gingival fluid (CGF), saliva, and plasma of patients with periodontal disease [71,72].

Recent findings suggest that genetic factors, such as Interleukin IL- 17 gene polymorphisms, are important in the pathogenic process of CP, genetic variation affecting the expression or activity and may influence the susceptibility and severity of periodontitis [53,56,73,74].

1.7 Problem Statement:

CP is a multifactorial disease characterized by loss of the tissues supporting the teeth 'the periodontium' leading to progressive destruction in periodontal ligament, pocket formation and alveolar bone loss, it is the most important cause of tooth loss among adults, it is the most prevalent type of periodontitis triggered by periodontal pathogens and influenced by genetic and environmental factors [3,75].

IL 17 gene polymorphisms may create phenotypic differences and allelic variants in the interleukin response which is important for an individual's susceptibility to chronic periodontitis, progression of the disease or response to treatment [69,76].

The present study is focused on the genetic variants of IL-17A and IL-17F and investigating the effect of these variants on the susceptibility to CP. In Libya, only clinical diagnosis was established for CP in dental clinics, in the present study the aims are to investigate a possible association between eight subgingival pathogens and the susceptibility to CP, also to compare the prevalence of eight subgingival pathogens between CP group and HC group in Libyan population to develop a microbial diagnosis for periodontal diseases and support clinical diagnosis.

Chapter 2. **LITERATURE REVIEW& AIMS OF THE STUDY**

2.1 Literature review:

Regarding the pathogenesis, many clinical studies have been conducted worldwide providing evidence of associations between bacterial species and CP. Also regarding the prevalence and distribution of eight subgingival pathogens between CP and HC groups in subgingival plaque, comparing data from different populations and geographic regions, it has become apparent that there are substantial differences in the composition and proportion of the subgingival microbiota as shown in tables (2.1), (2.2).

Table 2.1 Comparison between different populations in subgingival pathogens profile in patient with CP [40,77–83]

Population	**Brazilian**	**Iranian**	**Swiss**	**Spanish**	**Chinese**	**Indian**	**Japanese**	**Turkish**
Year	2008	2010	2010	2012	2012	2012	2013	2013
Author	Vito A Fraga et al.	Chalabi et al.	Norbert Cionca et al.	Puig silla et al.	Huang et al	Mahalakshmi Krishnan et al	Tomita et al	Ertugrul et al
Sample size	30	80	51	86	84	300	50	26
	CP=30	CP = 40	CP = 51	CP = 33	CP=60	CP=128	CP=20	CP=13
Age	17-55	≥ 40	25-70	25 -50	21–52 years	20-60 years.	>39 years	>35 years
Type of sample	subgingival plaque taken by a sterile paper points	subgingival plaque taken by a sterile Gracey curette	subgingival plaque taken by a sterile paper points	subgingival plaque taken by a sterile paper points	subgingival plaque taken by a sterile paper points	subgingival plaque taken a sterile cotton pellet	subgingival plaque taken by sterile paper points	subgingival plaque taken by a sterile cotton pellet
Method	PCR	Multiplex PCR	RT -PCR	PCR	RT -PCR	PCR	RT -PCR	PCR
Result	*Pg (73%)* *Tf (33%)* *Aa (23%)* *Pi (87%)* p-value = 0.005	*Pg (81%)* *Td(89%)* *Tf (96%)* *Aa (19%)* *Pi (72%)* p-value = 0.001	*Pg (73%)* *Tf (33%)* *Pi (87%)* *Aa (23%)* p-value =0.001	*Pg (67%)* *Td (49%)* *Tf (70%)* *Aa (33%)* p-value =0.05	*Pg (95%)* *Pi (98%)* *Aa (20%)* p-value =*0.05*	*Pg (81%)* *Td(89%)* *Tf (96%)* *Aa (19%)* *Pi (16%)* *Ec(16%)* *Cr(17%)* *Pn(13%)* p-value =*0.01*	*Pg (75%)* *Tf (85%)* *Aa (25%)* p-value = *0.05*	*Pg (50%)* *Tf (73%)* *Td (58%)* *Aa (8%)* *Pi (46%)* p-value = *0.05*

To be continued Table 2.1 Comparison between different populations in subgingival pathogens profile in patient with CP [84–89].

Population	**Yemenis**	**Italian**	**Moroccan**	**Iranian**	**Congolese**
Year	2014	2014	2015	2018	2018
Author	Al-hebshi et al	Gatto et al.	Hanane Chahboun1 et al	Atarbashi-moghadam et al	Em Kalala-Kazadi et al
Sample size	20	352	120	23	12
	CP=20	CP=352	CP= 20	CP=23	CP=12
Age	30–50 years	>35 years	≤ 35 years	>50 years	14-72 years
Type of sample	subgingival plaque taken by sterile paper points	subgingival plaque taken by sterile paper points	subgingival plaque taken by sterile paper points	subgingival plaque taken by a sterile Gracey curette	subgingival plaque taken by sterile paper points
Method	RT -PCR	RT –PCR	Culture	PCR	PCR
Result (prevalence)	*Pg (98%)* *Aa (68%)* *Tf (100%)* *Td (100%)* p-value = *0.0063*	*Pg (78%)* *Td (82%)* *Tf (87%)* *Aa (19%)* *Pi (66%)* p-value = *0.0001*	*Pg (60%)* *Tf (45%)* *Aa (25%)* *Pi (90%)* *Ec(15%)* *Cr(15%)* *Pn(90%)* p-value = *0.016*	Pg (43%) *Aa (43%)* *Cr (78 %)* p-value = *0.001*	*Pg (92%)* *Tf (92%)* *Td(100%)* *Pi(75%)* p-value = *0.001*

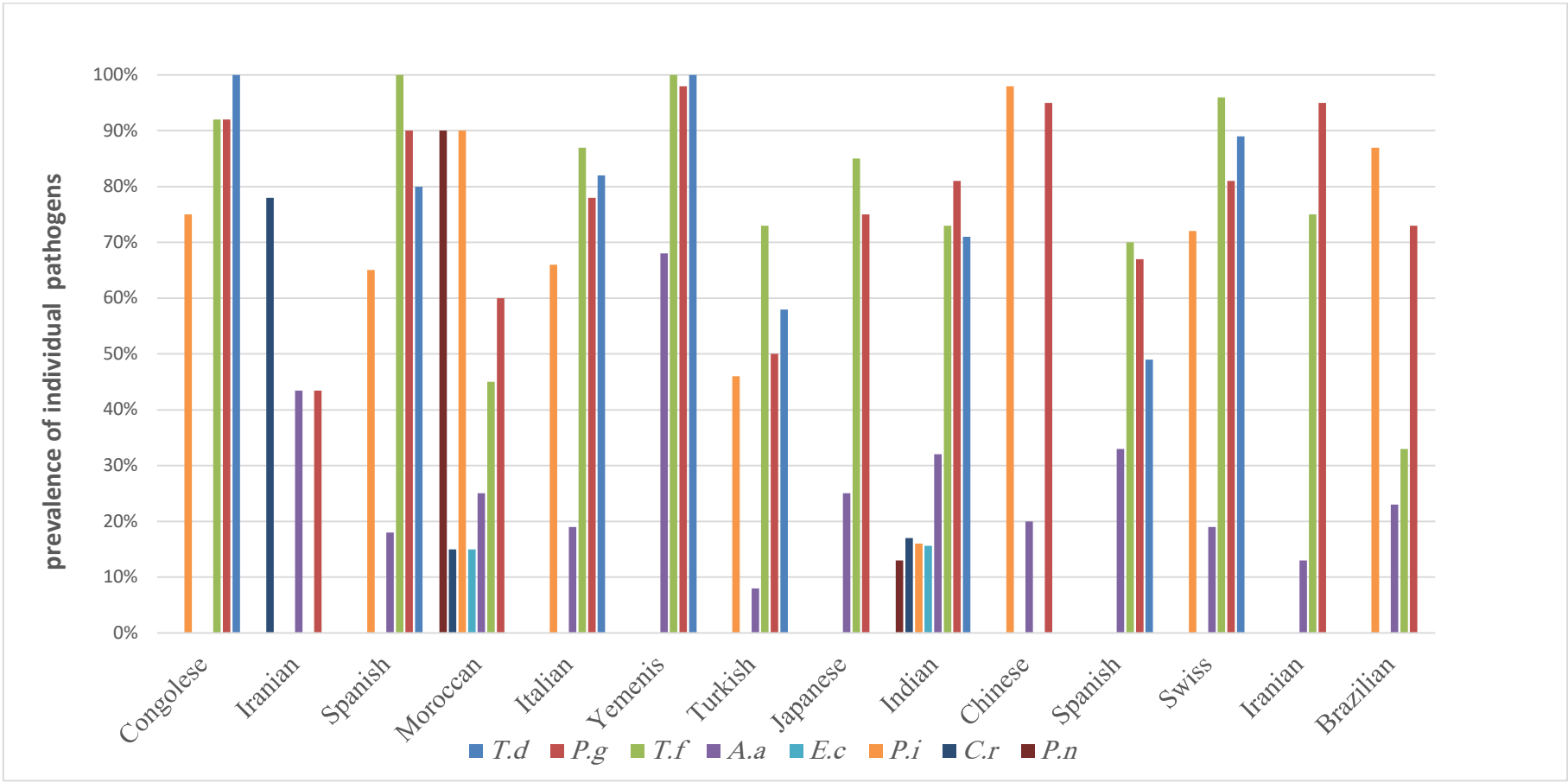


Figure 2.1 Prevalence of the eight subgingival pathogens in different populations.

Table 2.2 Comparison between CP and HC groups in subgingival pathogens profile of different populations [40,77,80,81,83].

Population	**Brazilian**		**Iranian**		**Chinese**		**Indian**		**Japanese**		**Turkish**	
Year	2008		2010		2012		2012		2013		2013	
Subgingival pathogens	CP	HC	CP	HC	CP	HC	CP	HC	CP	HC	CP	HC
T.d	/	/	/	/	/	/	71%	6%	/	/	77%	23%
P.g	73%	47%	95%	65%	95%	42%	81%	11%	75%	0%	77%	23%
T.f	33%	0%	75%	5%	/	/	73%	11%	85%	0%	92%	69%
A.a	23%	3%	13%	8%	20%	13%	32%	3%	25%	0%	15%	0%
E.c	/	/	/	/	/	/	16%	6%	/	/	/	/
P.i	87%	43%	/	/	98%	63%	16%	8%	/	/	77%	15%
C.r	/	/	/	/	/	/	17%	9%	/	/	/	/
P.n	/	/	/	/	/	/	13%	14%	/	/	/	/

(/) No relevant information.

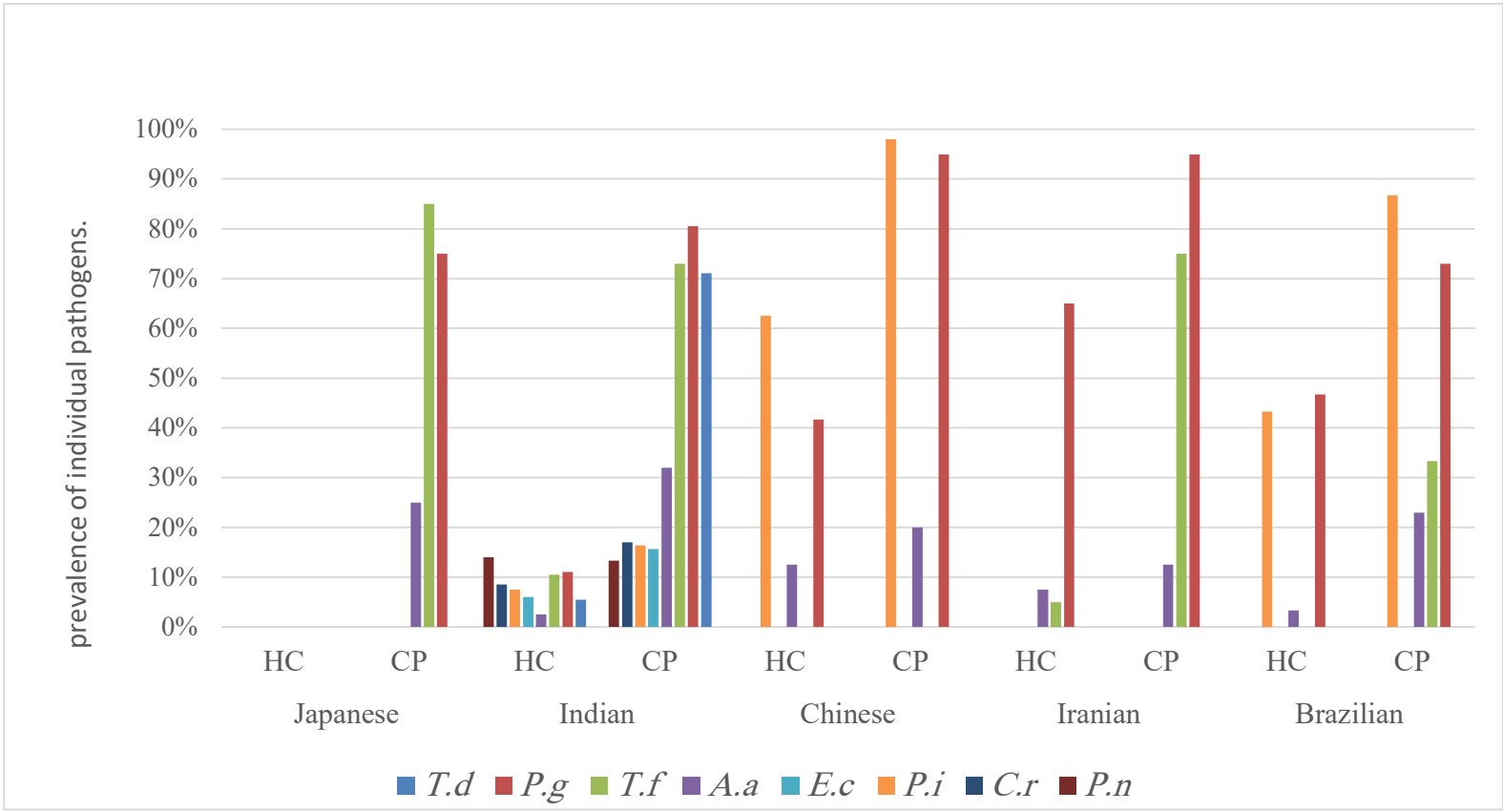


Figure 2.2 Comparison between CP and HC groups in subgingival pathogens profile of different population.

Several experimental and clinical studies have investigated gene polymorphisms of the cytokines in CP and shown that IL-17 levels are elevated in diseased human periodontal tissues and may play a destructive effect on experimental models of periodontal disease. Data from different studies all over the world investigating the association between IL-17 A and IL-17 F Gene polymorphism and CP, for example, studies were conducted in Brazil, India, Turkey and Iran were compared and reviewed in this thesis [56,68,70,76,90–92].

In Brazil there were four studies conducted on IL- 17 A and IL- 17 F gene polymorphism and degree of association with CP, the first study conducted in 2012 to investigate the association between IL- 17 A and IL-17 F Gene polymorphism and CP in Brazilian population, viewed results that showed higher frequency of AG and AA alleles among the IL-17 A (rs2275913 / G197A) genotypes of patients with CP comparing with HC group, in particular, by a substitution of the G by an A nucleotide base in the IL- 17 A gene promoter, is significantly associated with CP [76].

The presence of the allele A in IL- 17 A polymorphism was associated with worse clinical and inflammatory periodontal parameters and increased neutrophil activity (MPO activity and IL-8 levels) when compared with the GG the genotype in CP group. In contrast, the IL- 17 F (rs763780 / T7488C) due to His-to-Arg substitution at amino acid 161

(H161R) in the exon 3 region, the genotypes did not affect the clinical features of CP patients. SNPs are listed in table (2.3) [76] .

Table 2.3 IL-17(A، F) reference SNP.

Reference SNP		
IL-17A	rs2275913	G197A
IL-17F	rs763780	T7488C / His161Arg

The second study was conducted in 2013 and showed that the allelic distribution of the IL-17A a higher frequency of GG genotype was founded in CP group comparing to AA and AG genotype. No evidence showed for associations between IL-17 F and patients with CP. SNPs are shown in table (2.3) [93].

The third study was conducted in 2015 and showed that IL-17A Polymorphism, IL- 17 A, AA genotype and A allele could be associated with susceptibility to CP. No evidence was shown for associations between IL- 17 F and CP. SNPs are shown in table (2.3) [56].

The fourth study was conducted in 2016 and showed that IL-17 A Polymorphism had a higher frequency of GG genotype was founded in CP group. SNPs are listed in table (2.3) [94].

Another study that was conducted in Turkey in 2015, showed that T and C alleles IL-17F genotypes frequencies of the CP group were not significantly different from the HC group, IL-17F gene polymorphisms were not associated with CP in Turkish population. SNP are shown in table (2.3) [70].

In India two studies were conducted among Indian population, the first one's in 2013 results showed that IL- 17 F gene (rs763780) polymorphisms was not associated with CP in Indian population, SNPs are listed in table (2.3) [90].

The second Indian study in 2016, showed that IL-17A gene polymorphism, A allele are at 5 times greater risk of developing chronic periodontitis than healthy controls, IL-17A was significantly associated with CP in Indian population, type of SNP are shown in table (2.3) [91].

In Iran, one study conducted in 2013, showed that IL-17A gene polymorphism (rs10484879) is significantly associated with CP and peri-implantitis; destructive inflammatory process affecting the soft and hard tissues surrounding dental implants in Iranian population [95].

Table 2.4 Comparison between eight conducted studies to investigate the association between Interleukin-17A and Interleukin -17F Gene polymorphism and CP in in different population around the world [56,70,76,90–93,95,96].

Population	**Brazilian**				**Indian**		**Turkish**	**Iranian**
Year	**2012**	**2013**	**2015**	**2016**	**2013**	**2016**	**2015**	**2013**
Author	Corrêa et al.	Machado et al.	Zacarias *et al*.	Linhartova *et al*.	Jain et al,	Liladhar et al.	Erdemir *et al*.	Kadkhodazadeh et al.
Sample size	60	202	313	523	225	105	237	197
	CP=30 HC=30	CP=85 HC=72	CP=140 HC=173	CP=244 HC=154	CP=63 HC=101	CP=35 HC=35	CP=90 HC=35	CP= 75 HC=84
Age	>35 years	14–62 years	>30 years	/	20-60 years	20-56 years	>30 years	>30 years
Type of sample	Gingival tissue	Blood	Blood	Blood	Blood	Blood	Blood	Blood
IL- 17 A	Yes	Yes	Yes	Yes	Yes	Yes	/	Yes
IL- 17 A	rs2275913	rs2275913	rs2275913	rs2275913	/	rs2275913	/	rs10484879
IL- 17 F	Yes	Yes	Yes	Yes	Yes	/	Yes	/
IL- 17 F SNPs	rs763780	rs763780	rs763780	/	rs763780 rs2397084	/	rs763780	/
Method	PCR-RFLP	RT.PCR	PCR-RFLP	RT.PCR	PCR and restriction enzyme	PCR-RFLP	RT.PCR	Competitive Allele Specific PCR(KASP) technique
Result of IL- 17 A	+	+	+	+	/	+	/	+
Result of IL- 17 F	–	–	–	/	–	/	–	/

(+) Positive association.

(+) Negative association.

(/) No relevant information.

2.2 Aims of this study:

1) Investigation of the association between eight subgingival pathogen (*P.g, T.d, T.f, A.a, P.i, P.n, E.c, C.r*) and CP, also compare the prevalence of these subgingival pathogens between CP group and HC group in Libyan population.

2) Investigation of the association between IL- 17 A (rs2275913) and IL- 17 F (rs763780) gene polymorphisms and the susceptibility to CP in Libyan population.

Chapter 3. **MATERIALS & METHODS**

3.1 Study population:

The study was conducted in the laboratories of Genetic Engineering Department at the Biotechnology Research Centre in Tripoli. Medical records, oral examination and sample collection were conducted in dental clinics of Periodontology Department of Faculty of Dentistry, Tripoli University, Tripoli Dental Centre, Alhuria Poly Clinic Center and some private dental clinics from August 2018 to August 2019. The Bioethics committee of Biotechnology Research Centre (No. BEC.BTRC05-2018) Case-control study consisted of 100 individuals who were randomly selected from adult Libyan volunteers who live in different geographical places in Libya, divided into three geographic regions (West, East and South).

Fifty periodontally healthy individuals and 50 patients with chronic periodontitis, the age of the study population ranged between 25-65 years were included in the study. The clinical examination was performed for each participant by inspecting the soft tissues around the teeth with a probe by using University of Michigan ‘0’ probe with William’s markings at 1, 2, 3, 5, 7, 8, 9, and 10 mm increments (no mark at 4 & 6 mm), radiographic examination was performed by evaluating the patient's periapical X-ray films; to determine the amount of bone loss around the teeth.

The diagnosis for the periodontal status was established for all subjects based on Periodontal Disease Index (PDI), developed by Ramfjord (1967), which is a system designed to assess destructive periodontal disease; the scores, ranging from 0 to 6, denote periodontal health or gingivitis (scores 0–3) and various levels of attachment loss denote periodontitis (scores 4–6). Also known as (Ramfjord Index). For the PDI assessment, six teeth were evaluated: the upper left central, first premolar and right first molar; and the lower right central, first premolar, and left first molar were measured [4,97].

Probing depth (PD) and clinical attachment level (CAL) were examined at six sites (mesiovestibular, vestibular, distovestibular, mesiolingual, lingual and distolingual) of each tooth as shown in figure (3.1) CAL was measured with the distance from depth of periodontal pocket to the cemento enamel junction (CEJ). The measurement PD was determined by measuring distance from a gingival margin to the base of the periodontal pocket with a calibrated periodontal probe [98].

Bleeding on probing (BOP) widely used criterion to diagnose gingival inflammation, it is an indicator of tissue inflammatory response to bacterial pathogens, is a limited but yet useful prognostic indicator in clinical diagnosis for patients in CP [99].

Figure 3.1 periodontal examination.

After the periodontal examination, participants were divided into two different groups: chronic periodontitis group (n= 50) composed of individuals who had at least 3 sites in different teeth with PD > 3mm, CAL > 3mm, and more than 30% of BOP and Healthy control group (n =50), formed by individuals who have CAL and PD less than 3 mm, less than 30% of BOP, with at least 18 teeth in the oral cavity except third molar [98–100].

Inclusion criteria for this study were:

1. Patients diagnosed with chronic periodontitis.
2. Patients should have at least 18 teeth .
3. Patients without systemic compromise (i.e., immunologic and autoimmune disorders, diabetes mellitus, rheumatoid arthritis, inflammatory bowel diseases and psoriasis).

4. Patients who had not received any periodontal treatment during last 6 months, with attachment loss > 3mm at more than one tooth, at least 3 sites of probing depth > 3mm, more than 30% of BOP and lesions distributed at least two teeth in each quadrant.
5. Non-smoker patient in both groups.

Exclusion criteria for this study were followed:

1. Patients with aggressive periodontitis.
2. Patients who used (antibiotic – anti-inflammatory and/or anti-immunosuppressive) medications in 3 months preceding the research.
3. Systemic diseases (i.e. immunologic and autoimmune disorders, diabetes mellitus, rheumatoid arthritis, inflammatory bowel diseases, psoriasis), pregnancy and breast feeding.
4. Smoker patient in both groups.

All volunteers informed and signed written consent before participating. They filled a self-reported questionnaire containing age, ethnic background, place of residence, smoking status, immunologic and autoimmune disorders, rheumatoid arthritis, inflammatory bowel diseases, psoriasis, diabetes mellitus, hypertension, cancer, cardiac disease, pregnancy and breast feeding status. Ethical approval was obtained from the Bioethics Committee at biotechnology research Centre.

All selected patients and controls were informed about the aims of the study and that DNA samples would be analysed for variations in genes associated with CP, and subgingival samples analysed to identify the most subgingival pathogenic organism associated with CP group and the distribution of subgingival pathogen in HC group.

3.2 Subgingival samples:

The sub-gingival samples were taken from the deepest periodontal pocket from a group of individuals with CP and from normal sulcus depth in matched periodontal HC. Sub-gingival samples were collected for pathogens identification, samples were collected by gently inserting sterile paper points size 30 (META BIOMED, manufactured in China), sterilization was done by autoclave (121 ^{o}C, 15 psi, 15 min), after the supra-gingival plaque was removed before the sample was taken by using a sterile Gracey curette and sterile cotton rolls, paper point was inserted into the bottom of the pocket and removed after 10 seconds then placed in a sterile 1.5 ml micro-centrifuge tube (Eppendorf) filled with phosphate buffer saline (PBS) transported in ice and stored at -20 ^{o}C till they were assayed. Paper point sample as shown in figure (3.2).

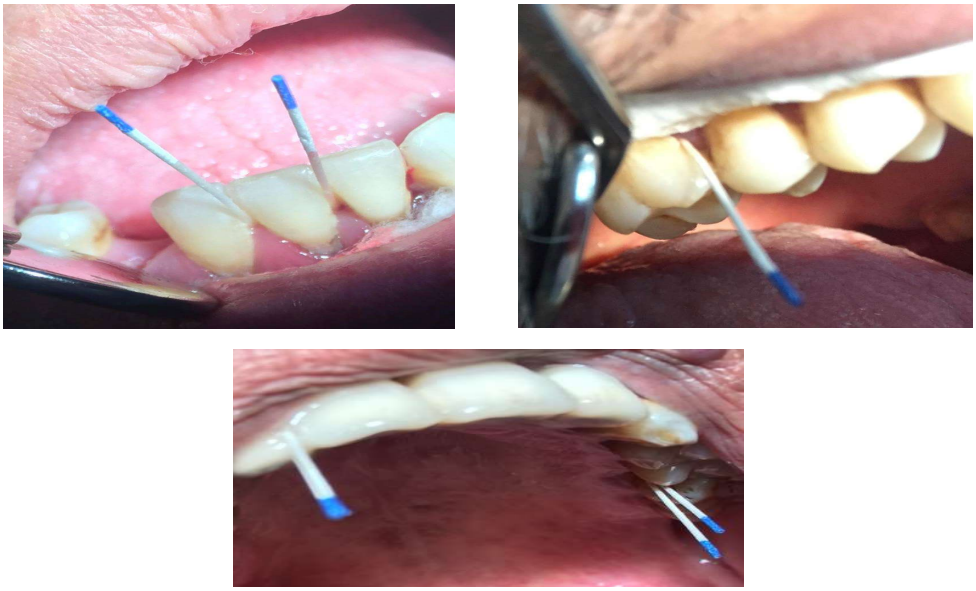


Figure 3.2 Method of paper points sample collection and site of insertions in maxilla & mandible in CP patient.

3.3 Sample preparation for PCR:

Bacterial genomic DNA was extracted from paper points size 30 (sterile absorbent paper points, META BIOMED) using a modified protocol (Ashimoto et al, 1996) as follows:

1. The paper point samples were placed in a 1.5ml centrifuge tube (Eppendorf) that contains PBS and heated to 37°C in a heat block for 10 min and mixed well on a vortex mixer.

2. 0.3 ml of the microbial suspension was washed 3 times with distilled water and spun down between each wash was at 14,000 rpm for 3 minutes.
3. The bacterial pellets were re-suspended in 100 µ nuclease -free water then boiled in a heat block for 10 min and placed on ice for further analysis by PCR.

3.4 Polymerase Chain Reaction (PCR):

Bacterial DNA was detected by using PCR to obtain multiple copies of the target bacterial gene segments. Primers were designed according to a publication by Ashimoto et al, in 1996 and ordered from Metabion (Germany) [101]. The specificity of primers sets was assessed by blasting their sequences against reference oral bacterial 16S rRNA gene sequences in Human Oral Microbiome Database (HOMD) are listed in table (3.1).

Table 3.1 Primer pairs used for subgingival bacterial detection checked with Human Microbial Taxon ID (HMT) for specificity.

Subgingival pathogen primers	Sequence	Base position	Amplicon size	HMT ID
A.actinomycetcmcomitans F	5' AAACCCATCTCTGAGTTCTTCTTC 3'	478-1,034	557	531
A.actinomycetcmcomitans R	5' ATGCCAACTTGACGTTAAAT 3'			
T. Forsythia F	5' GCGTATGTAACCTGCCCGCA 3'	120-760	641	613
T. Forsythia R	5' TGCTTCAGTGTCAGTTATACCT3'			
C. rectus F	5' TTTCGGAGCGTAAACTCCTTTTC 3'	415-1,012	598	748
C. rectus R	5' TTTCTGCAAGCAGACACTCTT 3'			
E. corrodens F	5' CTAATACCGCATACGTCCTAAG 3'	169-856	688	577
E. corrodens R	5' CTACTAAGCAATCAAGTTGCCC 3'			
P. gingivalis F	5' AGGCAGCTTGCCATACTGCG 3'	729-1,132	404	619
P. gingivalis R	5' ACTGTTAGCAACTACCGATGT 3'			
P. intermedia F	5' TTTGTTGGGGAGTAAAGCGGG 3'	458-1,032	575	643
P. intermedia R	5' TCAACATCTCTGTATCCTGCGT 3'			
P. nigrescens F	5' ATGAAACAA AGG TTTTCCGGTAAG 3'	219-1,022	804	693
P. nigrescens R	5' CCCACGTCTCTG TGGGCTGCGA 3'			
T. denticola F	5'TAATAC CGAATG TGCTCATTT ACA T 3'	193-508	316	584
T. denticola R	5' TCAAAG AAGCATTCC CTCTTC TTC TTA 3'			

Using 16S rRNA-based polymerase chain reaction which would probably offers a highly sensitive and specific detection method for bacteria in biological samples; 16S rRNA genes are present in every bacterium and are conserved within a species, the genes contain signature sequences distinguishable among different bacterial species, and it is particularly valuable for detection of oral microorganisms that cannot be cultivated. The optimal PCR conditions were determined, agarose gel electrophoresis of PCR products revealed a single band at the predicted size, to achieve the best multiplexing results primers were tested individually prior to multiplexing procedure by using conventional PCR, thermal cycling conditions, and primer sequences, reaction mix and template DNA are shown in table (3.1), (3.2), (3.3) and (3.4). For Multiplex PCR Components, reaction mix and template DNA, see table (3.5) and (3.6). Negative control was obtained by using sterile paper point size 30 not used in patient oral cavity, then put in microcentrifuge tube (Eppendorf), that contains PBS and was processed in PCR procedures.

Multiplex PCR products were analyzed using agarose gel electrophoresis and ethidium bromide staining.

3.5 Conventional PCR procedure for individual pathogens:

The eight subgingival pathogens were detected by polymerase chain reaction using specific primers and protocol described by Ashimoto et al, 1996, primers which were used are listed in table (3.1).

A mastermix sufficient for all reactions was prepared, the standard reaction mix contained: 1X (3 µl MgCl2) GoTaq™ Reaction Buffer 10µl of 5X Buffer, 1µl of dNTPs, with 1.5µl each of forward and reverse primers, 0.25 µl of GoTaq™ DNA polymerase and finally the volume of water depends on the number of samples (To 50 µl) as shown in table (3.2).

Table 3.2 Summary of PCR reaction components for individual subgingival pathogen detection.

Reagent	Final concentration	Volume
$MgCl_2$ (25mMol stock)	1.5 µM	3 µl
5X buffer	1 µM	10X
dNTPs (10 µM each)	0.2 µM	1 µl
Forward primer	0.3 µM	1.5µl
Reverse primer	0.3 µM	1.5µl
Taq DNA polymerase 5 U/ µl	1.25 µM	0.25 µl
DNA template		5µl
Nuclease- free water		Up to 50µl

1. The reaction mix was placed in PCR tubes that were placed in cooled (ice) rack, then 5 µl DNA templates ware added.
2. The PCR tubes containing the mix and DNA samples were placed in the thermocycler machine (GeneAmp® PCR System 9700, Applied Biosystems, Germany).

The PCR cycling conditions consists of about 36 cycles. Each cycle consists of three precisely time controlled and temperature controlled steps: denaturation, annealing and extension. As shown in tables (3.3) and (3.4).

Table 3.3 Summary of polymerase chain reaction cycle settings for *P.gingivalis , T.denticola, T.forsythia , E.corrodens , C.rectus.*

Step	Time	Temperature
Initial denaturation	2 min	95°C
Denaturation	30 sec	95°C
Annealing	1 min	60°C
Extension	1 min	72°C
Final Extension	2 min	72°C
Soak	Indefinite	4°C

Table 3.4 Summary of polymerase chain reaction cycle settings for *A. actinomycetemcomitans, P. intermedia,*and *P. nigrescens.*

Step	Time	Temperature
Initial denaturation	2 min	95°C
Denaturation	30 sec	94°C
Annealing	1 min	55°C
Extension	2 min	72°C
Final Extension	10 min	72°C
Soak	Indefinite	4°C

3.6 Post PCR agarose gel electrophoresis for conventional PCR:

Agarose gel electrophoresis allows separation and identification of nucleic acids based on charge migration in an electric field which is governed by the size and conformation of the nucleic acid. Nucleic acids of different sizes are thus separated.

Gel preparation:

1. A 150 ml 1X TBE buffer (90 ml distilled water and 10 ml 10X TBE Buffer for each 100 ml of buffer) was added to 3 g of agarose powder (2%) in a volumetric flask.
2. The solution was heated using a microwave oven to dissolve the powder thoroughly.
3. When the solution had cooled ~55-60° C, 3µl of ethidium bromide (EtBr) (0.5µg/ml) was added to the solution and mixed well.
4. The solution was poured slowly into the gel casting equipment and the comb was inserted to the tray and any bubbles in the solution were removed.
5. The solution was left to set for about 40 min and the comb was carefully removed.
6. 1x TBE running buffer was added to the gel tank.

7. The gel was placed with the wells facing the electrode that provided the negative current (cathode).

Loading and running the gel

1. 2µl of Blue/Orange 6X loading dye was placed in each of micro-centrifuge tube, and 5µl of PCR product was added and mixed a few times with a pipette tip, and then injected into the wells.
2. The cover on the gel rig was placed, the current was applied. The gel was run at 90 volts and 120 mA for 30 minutes.
3. Higher agarose concentration was used to resolve smaller bands from each other, and a lower percentage gel to separate larger bands.

The gel was then visualized in UV trans-illuminator (VILBER LOURMAT UV transilluminator) to examine the presence of PCR fragments at the expected length by a comparison with a 100 bp DNA ladder (Metabion, Germany).

3.7 Multiplex PCR procedure:

Qiagen multiplex PCR kit was used for multiplexing. First 10X Primer mix 2 µM each was prepared: For 100 µl primer mix 2 µl of each primer (from 100 uM stock solution) was added. When using 3 primer mix 88µl the remaining water was added to 12 µl total primers.

Procedure:

1. 2x QIAGEN Multiplex PCR Master Mix was prepared, and template DNA, PCR grade water, and primer mix were then added to individual PCR tubes. The solutions were mixed completely before thermocycling use.

2. Prepared reaction components are shown in Table (3.5).

3. The reaction was mixed thoroughly and dispensed appropriate volumes into PCR tubes.

4. DNA template was added (5 µl reaction) to the individual PCR tubes that contained the reaction mix.

Table 3.5 Multiplex PCR Components.

Component	Volume/reaction	Final concentration
Reaction mix 2x QIAGEN Multiplex PCR Master Mix	25 µl	1x
10x primer mix, 2 µM each primer	5 µl	0.2 µM
RNase-free water	15 µl	
Template DNA	5 µl	≤1 µg DNA/50 µl
Total volume	50 µl	

5. Thermal cycler was program according to the manufacturer's instructions as shown in Table (3.6)

6. PCR tubes were placed in the thermal cycler and cycling program was run.

Table 3.6 Thermal cycler program.

Step	Duration	Temperature	
Initial step	15 min	95° C	
Denaturation	30 s	95° C	**38 cycles**
Annealing	90 s	57 ° C	
Extension	90 s	72° C	
Final extension	10 min	72° C	

3.8 Post PCR agarose gel electrophoresis for Multiplex PCR:

All information and steps regarding gel preparation, loading and running gel, was previously mentioned in the section (3.6).

3.9 Sample collection:

IL- 17 A and IL- 17 F gene polymorphism sample:

Samples were collected by gently rubbing the inside of the mouth using a non-invasive, cotton-tipped buccal swab. The volunteers were asked to rinse their mouth with tap water 30 seconds before sampling of buccal swabs, to avoid the contamination as a result of food particles. For each individual, both sides of buccal mucosa were wiped with a cotton swab, for 10 seconds epithelial cells were adhered to the swab. Once collected, each swab was marked with the identification number as shown in Figure (3.3).

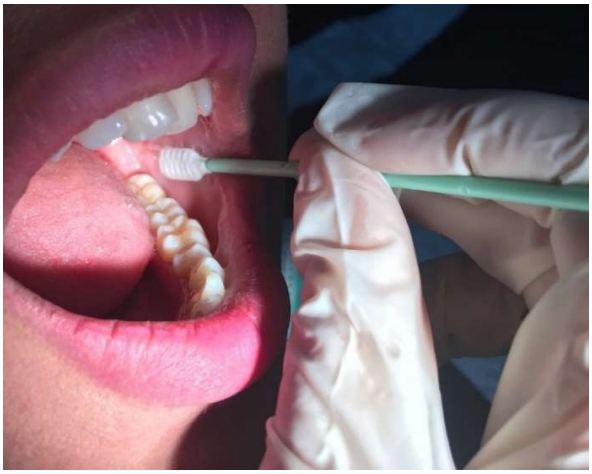


Figure 3.3 Buccal swab sample from a patient in dental clinic.

3.10 DNA Extraction:

Isohelix Buccal DNA Isolation Kit was used, for extraction of human genomic DNA, which contains:

Solution LS 25 ml, solution PK 1ml, solution CT 25 ml, and solution TE 15 ml, as shown in table (3.7).

Table 3.7 Contents of Isohelix Buccal DNA Isolation Kit.

Reagent	Volume	Storage temperature
Solution LS	25 ml	Room temperature
Solution PK	1 µl	-20 0C
Solution CT	25 ml	Room temperature
Solution TE	15 ml	Room temperature

3.11 DNA Isolation Protocol:

1. DNA stabilization was obtained by adding 500 µl LS solution to the tube containing the buccal swab.
2. 20µl PK solution was added to tube containing the buccal swab and LS solution then Vortex briefly.
3. The tube that contained the swab, LS solution and PK solution was placed in a Thermomixer on 60°C for 1 hour then Vortex briefly.
4. Then the liquid (approx. 500µl) was transferred into a 1.5 ml centrifuge tube.
5. The swab head was moved into a sterile 1.5ml centrifuge tube (Eppendorf) so that the swab head is uppermost after that the tube was spun briefly using a sterile pipette tip. Then the recovered supernatant was added to the 500µl collected previously, to increase yield.
6. 600µl CT solution was added to the tube then Vortex briefly.
7. The tube was placed in a micro centrifuge and spun at maximum speed (13,000 rpm) for 7 minutes to pellet the DNA.
8. All the supernatant was removed carefully with a pipette tip so as to not disturb the DNA pellet.
9. Then 1.5ml centrifuge tube (Eppendorf) was re-spun briefly and any remaining liquid was removed.
10. 150µl TE solution was added to the tube.

11. The solution was left for at least 5 minutes at room temperature for the DNA to re-hydrate, after that Vortex briefly.

12. Finally re-spun was done for 15 min at (13,000 rpm) undissolved debris was removed the supernatant and transferred to a sterile 1.5ml tube.

3.11.1 Measurement of genomic DNA concentration:

The concentration and purity of extracted DNA was measured by the Nano Drop Lite spectrophotometer (Thermo Fisher Scientific). It provides concentration information by using the A260 measurement and sample purity information by using the A260/A280 ratio.

Measurement procedure:

1. The appropriate application from the Home screen (DNA or RNA) was selected for DNA measurements, dsDNA.
2. The on-screen instructions were followed and a blank was established by pipetting 1 µl of the blanking buffer (TE buffer) on to the bottom pedestal, lower arm then Blank button was pressed.
3. After blank measurement was completed, the arm was raised and the buffer was wiped from both the upper and lower pedestals by using a dry laboratory wipe.

4. Blank was confirmed by pipetting a fresh aliquot of blanking buffer (TE buffer) onto the bottom pedestal, lower the arm then Blank was pressed.

5. When the measurement was completed, the upper arm was raised then the buffer was wiped from both the upper and lower pedestals using a dry laboratory wipe.
6. The sample was measured by pipetting 1 µl of sample onto the bottom pedestal, lower arm and then Measure button was pressed.
7. Upper and lower pedestals ware wiped by using a dry laboratory wipe and the instrument was prepared to measure the next sample.

3.11.2 Agarose gel electrophoresis of extracted DNA:

To determine that the DNA samples extracted from the previous procedure are suitable for PCR reaction, they were analysed by agarose gel electrophoresis.

The gel preparation:

1. A 100ml 1X TBE buffer (90 ml distilled water and 10 ml 10X TBE Buffer) was added to 0.85g (0.85%) of agarose powder in a volumetric flask.
2. The solution was heated using a microwave oven to dissolve the powder thoroughly.
3. When the solution cooled, 3µl ethidium bromide (EtBr) was added to the solution and mixed well.
4. The solution was poured slowly to the tray and the comb was inserted to the tray; any bubbles in the solution were removed.
5. After the solution was cooled and solidified, the comb was removed carefully.
6. 1x TBE running buffer was added to the tray.
7. The gel was placed with the wells facing the electrode that provide the negative current (cathode).

Loading and running the gel:

1. 2µl of Blue/Orange 6X loading dye was placed in each of the centrifuge tube and 5µl of DNA solution was added. DNA sample and the loading dye in each tube were mixed well for a few seconds with a pipette tip, they were then injected in the wells left by the comb.
2. The cover on the gel rig was placed, the current was applied, and the gel was run at 120 volts and 200 mA for 30 minutes.
3. Then the gel was removed from the gel electrophoresis machine then put in to the ultraviolet trans-illuminator machine for visualization of genomic DNA bands.

3.12 Polymerase Chain Reaction (PCR):

The IL- 17 A and IL- 17 F genes polymorphisms was performed by the separated polymerase chain reaction using specific primers described by Zacarias et al in 2015, the specificity of primers was checked by BLAS, the primers which were used are listed in table (3.8) [56].

IL-17A gene polymorphism at the promoter region (197G/A) with amplicon size 102 bp, IL-17F gene polymorphism in the third exon due to His-to-Arg substitution at amino acid 161 (H161R) with amplicon size 134 bp.

Table 3.8 Main characteristics IL- 17 A and IL- 17 F gene polymorphisms and primers' sequences.

SNP ID	Interleukin	Primers sequences	Amplicon size
rs2275913	IL- 17A F	AACAAGTAAGAATGAAAAGAGGACATGGT	102 bp
	IL -17A R	CCCCCAATGAGGTCATAGAAGAATC	
rs763780	IL- 17F F	ACCAAGGCTGCTCTGTTTCTACCAAGGCTGC TCTGTTTCT	143 bp
	IL -17F R	GGTAAGGAGTGGCATTTCTA	

A premix sufficient for all reactions was prepared. The standard final reaction mix contained 1X GoTaq™ Reaction Buffer 10µl of 5X Buffer (with 1.5 mM $MgCl_2$), 1µl of dNTPs (0.2mM each dNTP), with 1.5µl each of forward and reverse primers (0.3µM), 1.25u of GoTaq™ DNA Polymerase and finally water to 50 µl as shown in table (3.9).

3. The reaction mixes were placed in PCR tubes placed in cooled (ice) rack, then 2 µl DNA templates was added.
4. The PCR tubes containing the mix and DNA samples were placed in the thermocycler machine (Eppendorf, GeneAmp*PCR System 9700, Germany).

Table 3.9 Summary of PCR reagents used and their quantity for each sample.

Reagent	**Final concentration**	**Volume**
$MgCl_2$	1.5 µM	3µl
5X buffer	1 µM	10X
dNTPs 10Mm each	0.2 µM	1µl
Forward primer	0.3 µM	1.5µl
Reverse primer	0.3 µM	1.5µl
Taq DNA polymerase 5 U/ µl	0.5 µM	1µl
DNA template		2 µl
H_2O		Up to 50µl

The PCR process consists of 35 subsequent cycles. Each cycle consists of three precisely time-controlled and temperature-controlled steps: denaturation, annealing and extension. As shown in table (3.10)

Table 3.10 Summary of polymerase chain reaction program.

Step	Time	Temperature	
Initial denaturation	2 min	95°C	
Denaturation	35 sec	95°C	
Annealing	35 sec	IL- 17 A 59°C IL- 17 F 53°C	**35 cycles**
Extension	1 min	72°C	
Final Extension	5 min	72°C	
Soak	Indefinite	4°C	

3.13 Post-PCR Agarose Gel Electrophoresis

To ensure the successful run of the PCR program and to detect PCR product in specific length, agarose gel electrophoresis was applied. All information and steps regarding gel preparation, loading and running gel, was previously mentioned in the section (3.6).

3.14 Purification of the PCR products:

To remove excess salts, PCR primers, and dNTPs, the PCR product was purified by using PureLink® PCR Purification Kit (Invitrogen) according to the manufacturer's instructions.

Purification was done as the following protocol:

1. Before use, 48 ml Isopropanol 100% was added to 72 ml Binding Buffer (B2) to precipitate the DNA more firmly to the matrix.
2. 4 volumes of PureLink® Binding Buffer (B2) with isopropanol was added to 1 volume of the PCR product (50 µL) and mixed well, after that the mixture was placed in a PureLink® column collection tube.
3. Tubes were placed in the centrifuge and spun at 10,000 rpm speed for 1 minute.

4. Binding Buffer (B2) after the flow-through was discarded and spun column was placed into the collection tube.
5. 320 mL Ethanol 100% was added to 80 ml Wash Buffer before used.
6. 650 µL of Wash Buffer was added to spun column.
7. The spun column was centrifuged at room temperature at 10,000 rpm for 1 minute then the flow was discarded through from the collection tube and place the column into the tube.
8. After that, the spin column was centrifuged for the second time at (12,000 rpm), at room temperature for 2 minutes to eliminate any residual wash buffer then. The collection tube was discarded.
9. Spun column was placed in a clean 1.7-mL PureLink® Elution Tube supplied with the kit.
10. 50 µL of Elution Buffer was added to the center of the column.
11. Then the column was incubated at room temperature for 1 minute, after that the column was centrifuged at (12,000 rpm) for 2 minutes.
12. The elution tube contains the purified PCR product. The column was removed and discarded. The recovered Elution volume was ~48 µL.
13. The purified PCR product was stored at –20°C, kept for the cycle sequencing.

3.15 Post-Purification Agarose Gel Electrophoresis:

To ensure the successful run of the Purification proGram, agarose gel electrophoresis was applied. The same steps used in the Agarose Gel Electrophoresis after PCR were applied. The Purified products were separated at 120 Volt and 200 milliamperage, for 30 min. Then visualized under ultraviolet (UV) light and photographed with digital camera for documentation to assess the presence of fragments of the expected length by a comparison with the 100 bp molecular weight marker, DNA ladder (Metabion ,Germany).

3.16 DNA Cycle Sequencing:

The purified PCR products were cycle sequenced to determine the exact IL-17A (rs2275913) and IL-17F (rs763780) SNPs in DNA fragment, the following protocol was applied by using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). The sequencing PCR reaction was done at the Pasteur Institute in Tunis.

DNA Cycle Sequencing was done as the following protocol:

1. 1 µM of Reverse primer was prepared for the sequencing reaction mix.
2. After that, 1µl of BigDye Mix (BigDye® Terminator v3.1), 3.5µl Buffer (5X), 1µl of Reverse primer and 12.5 µL nuclease-free water added. Then they were placed in a 1.5ml centrifuge tube (Eppendorf) in a total volume of 19µl for each reaction.
3. The reaction mixture was vortexed and spun briefly, then 19µl of each reaction mix was placed in a 96-well reaction plate in a specific order, 1µl of the purified DNA samples were added. As shown in Table (3.11).

Table 3.11 DNA Cycle Sequencing reaction components.

Reagent	Concentration	Volume
Big Dye Mix	0.125x	1 µL
Buffer	5x	3.5 µL
Template	-	2 µL
Primer	-	1µL
Nuclease-free water	-	12.5 µL
Total volume	1x	20 µL

4. The reaction plate was covered with an adhesive film then placed in the thermocycler machine. Table (3.12) shows the cycle sequencing program used. The PCR process consisted of a series of 25 subsequent cycles.

Table 3.12 Cycle sequencing program.

Step	Time	Temperature
Initial denaturation	1 min.	96°C
Denaturation	10 sec.	96°C
Annealing	5 sec.	50°C
Extension	4 min.	60°C
Soak	Indefinite	4°C

3.16.1 Extension Products Purification:

Extension products Purification was done using ethanol/EDTA precipitation protocol as follows:

1. 125 mM EDTA solution was prepared from 0.5 M EDTA, at 8 pH.

2. 70% ethanol was prepared using absolute ethanol.

3. The sequencing plate was briefly centrifuged for (5 to 10 seconds) at 10,000 rpm in a swinging bucket.

4. The MicroAmp™ Clear Adhesive Film from the plate was removed.

5. The following was added in the following order as shown in table 3.13:

Table 3.13 Extension Products Purification with ethanol/EDTA precipitation.

Component	Volume
sequencing reaction	10 µl
125 mM EDTA solution	2.5 µl
absolute ethanol	30 µl
Total volume	42.5 µl/ well

6. The plate with MicroAmp™ Clear Adhesive Film was sealed.

7. Then the plate was vortexed for 2 to 3 seconds, after that briefly centrifuged (5 to 10 seconds) at 10,000 rpm.

8. The plate was incubated at room temperature for 15 minutes, time was critical in this step.

9. The plate was centrifuged for 45 minutes at 10,000 rpm in a swinging bucket.

10. The MicroAmp™ Clear Adhesive Film was slowly removed to prevent disruption of the pellet. 4 layers of absorbent paper were placed into the centrifuge and the plate was carefully inverted onto the paper without dislodging the pellet. Then centrifuged at 185 × g for 1 minute.

11. After that 30 µL from 70 % ethanol was added to each well.

12. The plate with MicroAmp™ Clear Adhesive Film was sealed and centrifuged at 10,000 rpm (4°C) for 15 minutes.

13. The MicroAmp™ Clear Adhesive Film was slowly removed to prevent disruption of the pellet. 4 layers of absorbent paper were placed into the centrifuge and carefully invert the plate onto the paper without dislodging the pellet. Then centrifuge at 185 × g for 1 minute.

14. Finally, the plate was allowed to air dry, and it was protected from light, for 5 to 10 minutes at room temperature.

3.16.2 Capillary Electrophoresis:

Sanger Sequencing Method was applied, the run was performed on Applied Biosystem (3500 Genetic Analyzer) as the following procedure:

1. The computer, the instrument, and the Data Collection Software were started.

2. The capillary length, number of capillaries, and polymer type were selected.

3. The polymer delivery system was checked for sufficient polymer and absence of bubbles.

4. The buffer and water/waste reservoirs were prepared.

5. The 96-well reaction plate was placed into the instrument. The run was setup by using Data Collection Software. A plate record and results group with file name and folder preferences were created. Instrument protocol, including run module and dye set configurations was created, analysis protocol was created.

6. Run parameters were determined, the plate was linked and run was started.

3.16.3 Sequencing Data Analysis:

Analysis protocols using Sequencing Analysis Software pure and mixed bases were identified, a quality value for each base was assigned, failed sequence samples were detected, quality values (QV) for each base were calculated, data analysis output were imported to the Sequencher software version (5.4.6).

A new project window was created, where reference sequence with SNPs detected, sequence fragments and assembled contigs were imported, manipulated, and displayed. Reference sequence facilitates comparative sequence alignments and defines base numbering. The contigs were assembled automatically and aligned to reference sequence. Chromatograms were viewed, SNPs in reference sequence were selected, and accordingly each SNP in the samples was examined manually and wrote down.

3.17 Statistical analysis:

Summary statistics of clinical parameters; (age, gender, PDI, BOP, CAL, PD) for CP and HC groups assessed by SPSS version 25 statistical software package (SPSS Inc., Chicago, Illinois, USA).

Chi-square test was used to determine the association between the 8 sub-gingival pathogen; (P.g, T.d, T.f, A.a, P.i, P.n, E.c, C.r) and clinical parameters (BOP, CAL, PD), a p-value of less than 0.05 was considered to be statistically significant for all analyses.

A Mann–Whitney U test was used to identify the differences in clinical periodontal parameters (BOP, CAL, PD) between CP and HC.

The odds ratio was calculated to assess the association between the bacterial species. Twenty eight bacterial combinations were tested for the chronic periodontitis group, it was also used to determine whether any sub-gingival pathogens singly had coincident effects on chronic periodontitis by using cross-tabulation and Chi-square test, p-value less than 0.05 was considered to be statistically significant for all analyses.

Chi-squared test was used test to determine the association between IL-17 A &IL- 17 F gene polymorphisms and CP, a p-value of less than 0.05 was considered to be statistically significant for all analyses.

Chapter 4. **RESULTS**

4.1 Analysis of clinical parameters:

The clinical parameters; (Age, Gender, PDI, BOP, CAL, PD) of CP and HC groups were comparable in mean and standard deviation. This summarized in table (4.1).

Table 4.1 Analysis of clinical parameters by mean and standard deviation.

Clinical parameters	**CP (Mean ± SD, n =50)**	**HC (Mean ± SD, n =50)**
Age	42.98± 9.87	35.96±7.87
Gender, male : female	22 : 28 44% : 56%	28 : 22 56 %: 44%
PDI	5.40±0.49	1.90±0.67
BOP	38.58±11.85	7.34± 12.15
CAL	8.46±1.47	1.90±0.67
PD	6.08± 0.98	1.90±0.67

4.2 Distribution of chronic periodontitis in study population:

The distribution of CP and HC groups among Libyans according to PDI, this summarized in table (4.2).

Table 4.2 The distribution of chronic periodontitis and healthy control groups among Libyans according to PDI.

groups	Score (0)	Score (1)	Score (2)	Score (5)	Score (6)
HC 50	35(70%)	8(%16)	7(14%)	-	-
CP 50	-	-	-	30 (60%)	20 (40%)

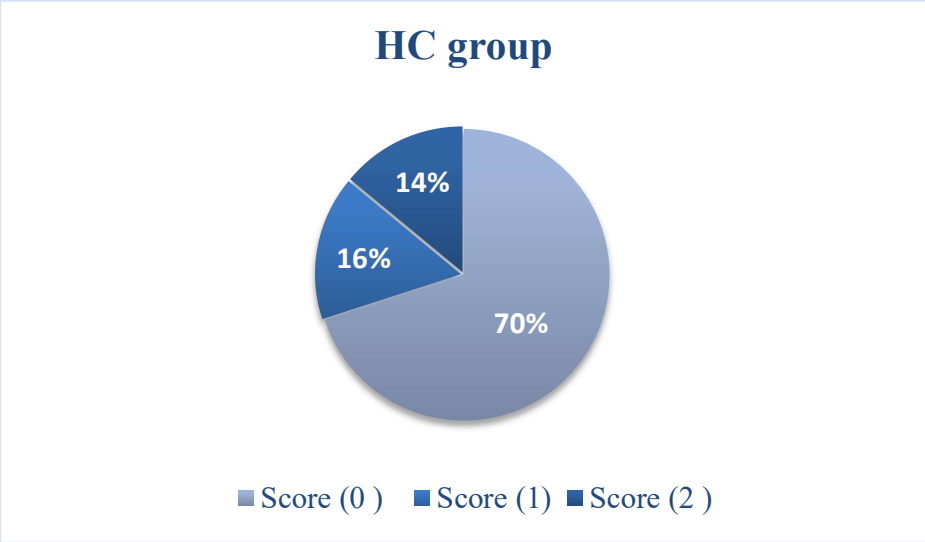


Figure 4.1 The distribution of scores in healthy control groups among Libyans according to PDI.

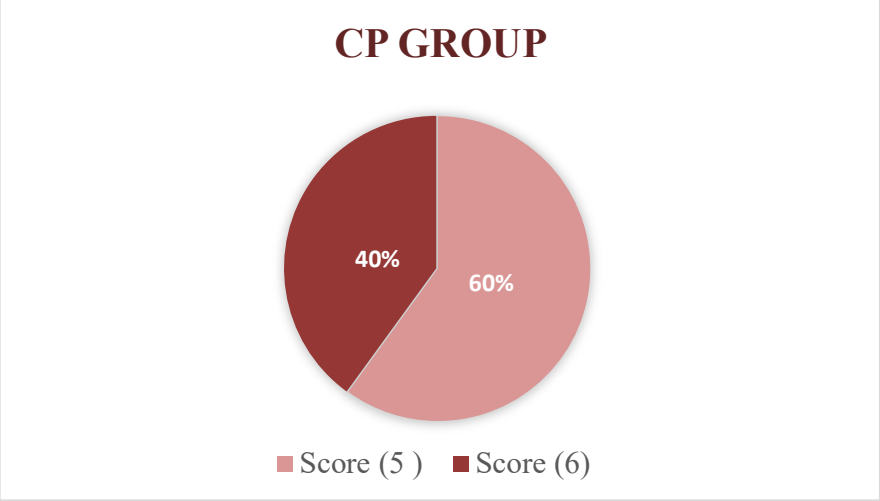


Figure 4.2 The distribution of scores in chronic periodontitis group among Libyans according to PDI.

4.3 Geographical distribution in study population:

The geographical distribution in CP and HC groups among Libyans, this is summarized in table (4.3).

Table 4.3 The geographical distribution in CP and HC groups among Libyans.

Geographical reign	CP N (%)	HC N (%)	Total 100
West	22(44)	30(60)	52
South	17(34)	8 (16)	25
East	11 (22)	12 (24)	23

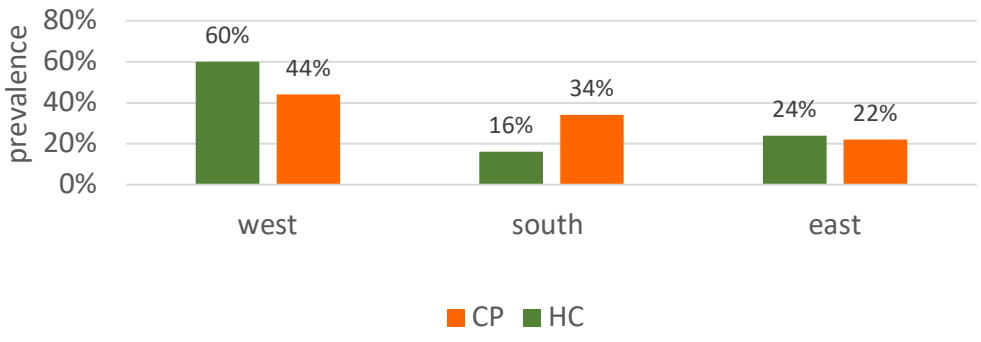


Figure 4.3 The geographical distribution in study population groups (CP and HC).

4.4 Detection of individual periodontal pathogens in chronic periodontitis by conventional PCR:

All primers in PCR amplified a band at the predicted size and no amplification of non-specific bands was observed.

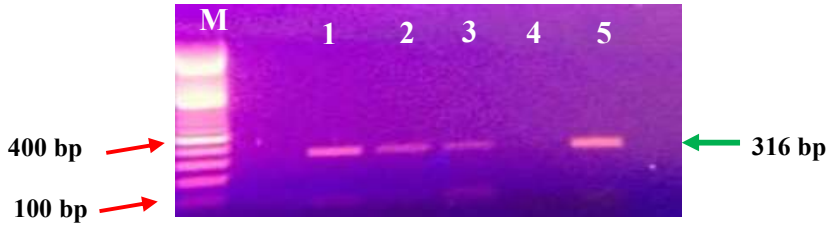


Figure 4.4 Conventional PCR bands of specific 16S RNA gene region for *T. denticola.*

PCR for *T. denticola* in CP group. Lanes 1-5 represent amplified target 16S RNA gene for *T. denticola* at expected size 316 bp. Green arrow represent the expected amplicon size of 316 bp.

Negative control for the PCR amplification represented by sample number (4) sterile paper point and PBS was used.

M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size.

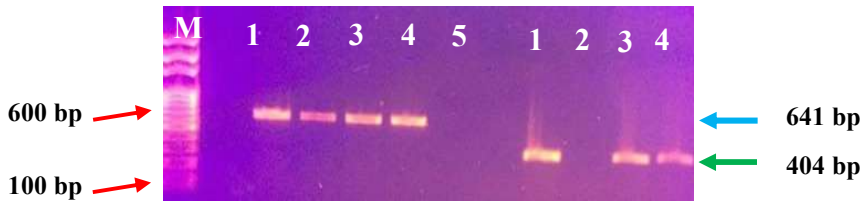


Figure 4.5 Conventional PCR bands of specific 16S RNA gene region for *P. gingivalis and T. forsythia.*

PCR for *P.gingivalis*, and *T. forsythia* in CP group.Blue and green arrows represent *P. gingivalis, and T. forsythia* at sizes 641bp and 404 bp, respectively.

Negative control for the PCR amplification represented by sample number (5,2) sterile paper point and PBS was used. M = 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size. .

4.5 Detection of sub-gingival pathogens in CP group by Multiplex PCR:

Pathogens in red, orange and green complex groups were clearly seen at the corresponding amplicon size when detected in the agarose gel.

4.5.1 Red complex:

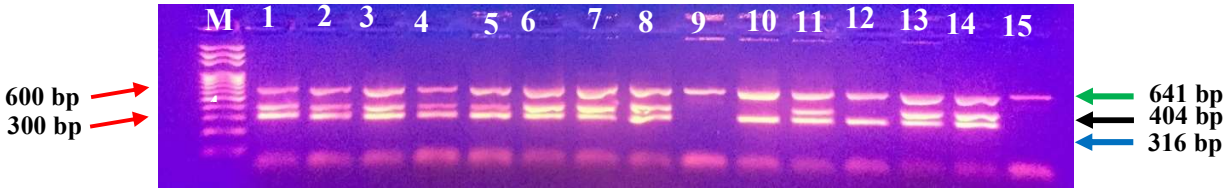


Figure 4.6 multiplex bands of specific 16S gene regions in Red complex pathogens for CP group.

Lanes 1-15 represent amplified target DNA *for T. denticola* at sizes 316 bp, *P. gingivalis* at sizes 404 bp, *T. forsythia* at size 641bp. M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of PCR amplicon size. Green arrow represent the expected amplicon size of 641bp. Black arrow represents the expected amplicon size of 404 bp. Blue arrow represent the expected amplicon size of 316.

4.5.2 Orange complex:

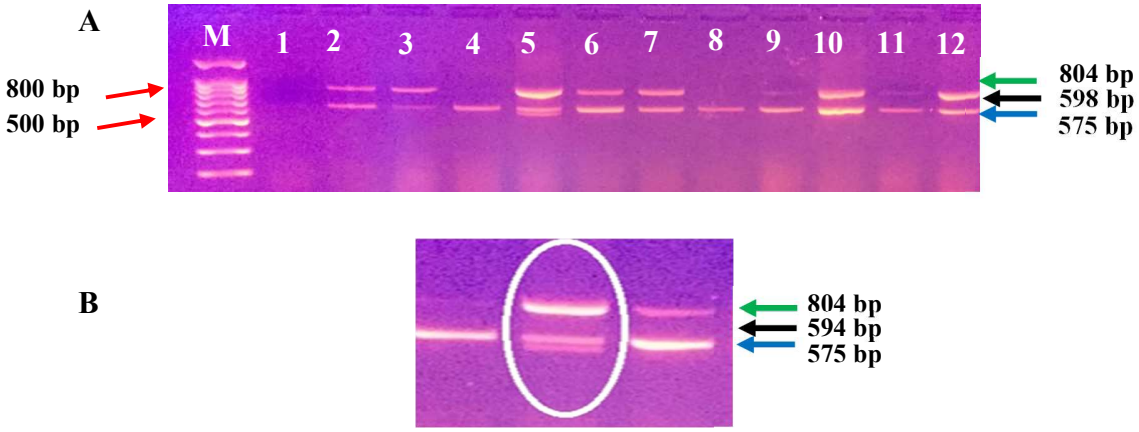


Figure 4.7 Multiplex bands of specific 16S RNA gene regions in Orange complex for CP group.

A . Lanes 1-12 represent amplified target DNA *for P.intermedia* at sizes 575 bp, for *C.rectus* at size 598, for *P.ngrescens* at size 804bp. M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of PCR amplicon size . Green arrow represents the expected amplicon size of 804. Black arrow represents the expected amplicon size of 598, blue arrow represents the expected amplicon size of 575.

B. Magnification of sample number 5.

4.5.3 Green complex:

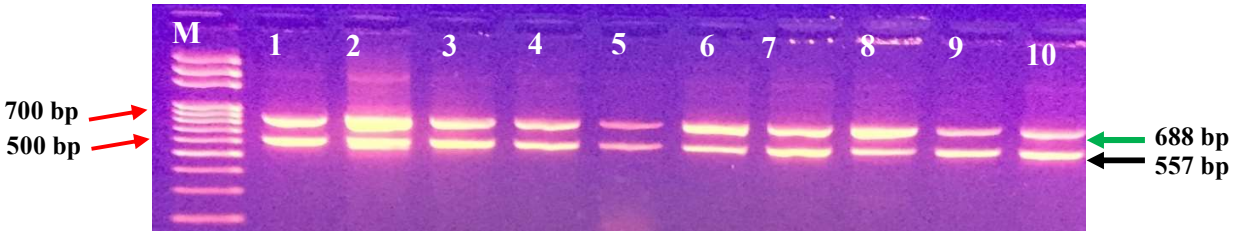


Figure 4.8 Multiplex bands of specific 16S gene regions in Orange complex pathogens for CP group.

Lanes 1-10 represent amplified target DNA for *A. actinomycetcmcomitans* at sizes 557, *E. corrodens* at size 688 bp. M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size . Red arrows corrospens sizes in the ladder for approximate estimation of PCR amplicon size. Green arrow represents the expected amplicon size of 688 bp.Black arrow represents the expected amplicon size of 557 bp.

4.6 Detection of sub-gingival pathogens in HC group by Multiplex PCR:

4.6.1 Red complex:

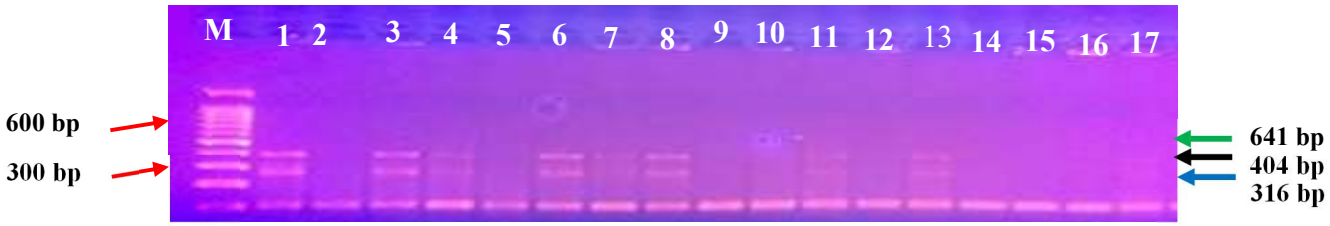


Figure 4.9 Multiplex bands of specific 16S RNA gene regions in Red complex pathogens for HC group.

Lanes 1-17 represent bands a of amplified target DNA for *T. denticola* at sizes 316, *P. gingivalis* at sizes 404 in some of the samples, a negative result for *T. forsythia* at size 641bp in all of the samples. M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of PCR amplicon size. Green arrow represents negative or invisible band of *T. forsythia* at size 641bp. Black arrow represents expected band for *P. gingivalis* at sizes 404 bp. Blue arrow represents weak band for *T. denticola* at sizes 316 bp.

4.6.2 Orange complex:

Figure 4.10 Multiplex bands of specific 16S RNA gene regions in Orange complex pathogens for HC group.

Lanes 1-16 represent amplified target DNA *for P. intermedia* at sizes 575 bp, for *C. rectus* at size 598, for *P. ngrescens* at size 804bp. M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of PCR amplicon size . Green arrow represents the expected amplicon size of 804,black arrow represents the expected amplicon size of 598, blue arrow represents the expected amplicon size of 575 bp.

4.6.3 Green complex in HC group:

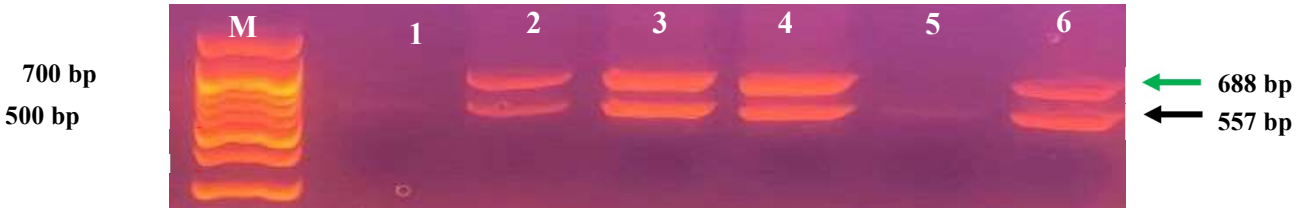


Figure 4.11 Multiplex bands of specific 16S RNA gene regions in Green complex pathogens for HC group.

Lanes 1-6 represent amplified target DNA for *A. actinomvcetcmcomitans* at sizes 557, *E. Corrodens* at size 688 bp. M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the PCR product to determine the correct size . Red arrows represent the corrospending sizes in the ladder for approximate estimation of PCR amplicon size. Green arrow represents the expected amplicon size of 688 bp,black arrow represents the expected amplicon size of 557 bp.

4.8 Prevalence and distribution of the eight sub-gingival pathogens in study population groups:

The Prevalence and distribution of the eight sub-gingival pathogens in CP and HC groups among Libyans is summarized in table (4.4).

Table 4.4 Prevalence and distribution of eight sub-gingival pathogen in chronic periodontitis and healthy control groups among Libyans.

Sub-gingival pathogens	CP n =50 (%)	HC n =50 (%)
P.intermedia	50 (100)	20 (40)
T.forsythia	48 (96)	3 (6)
T.denticola	46 (92)	10 (20)
E.corrodens	46 (92)	10 (20)
P.gingivalis	41 (82)	6 (12)
C.rectus	37 (74)	16 (32)
P.nigrescens	36 (72)	15(30)
A. actinomycetemcomitans	20 (40)	8 (16)

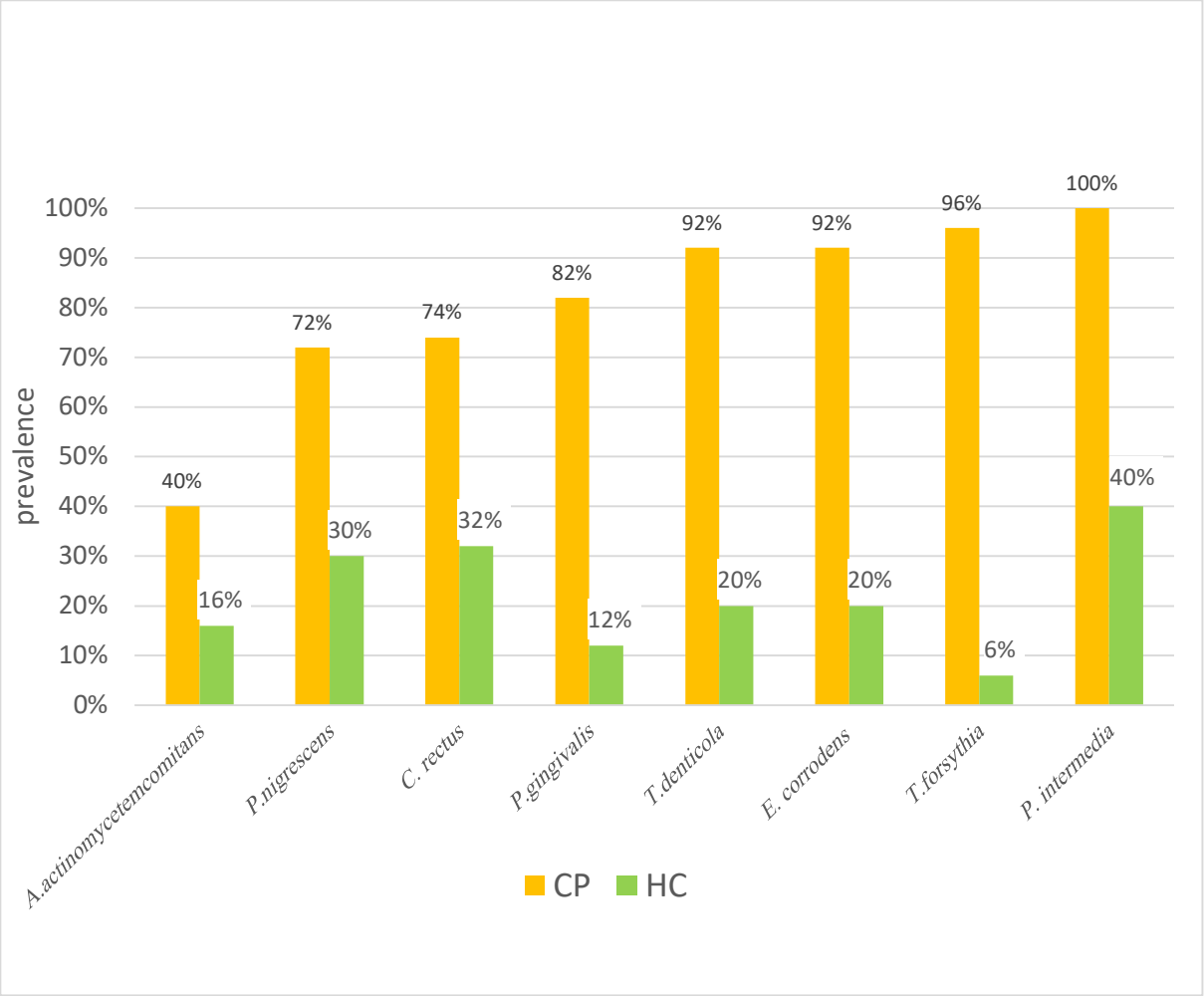


Figure 4.12 Prevalence and distribution of eight sub-gingival pathogen in chronic periodontitis and healthy control groups among Libyans.

4.9 Association between the clinical parameters and study groups:

A Mann–Whitney U test was used to estimate the association between clinical parameters and two study groups. The association was significantly high (p-value = 0.0001) as shown in Figures (4.12), (4.13) and (4.14).

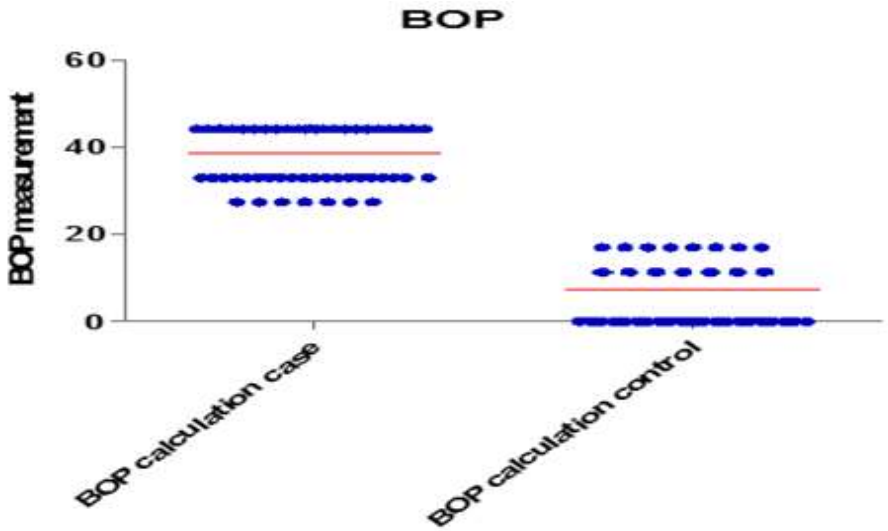


Figure 4.13 Scatter plot diagram of BOP in cases and controls, red line represents the mean.

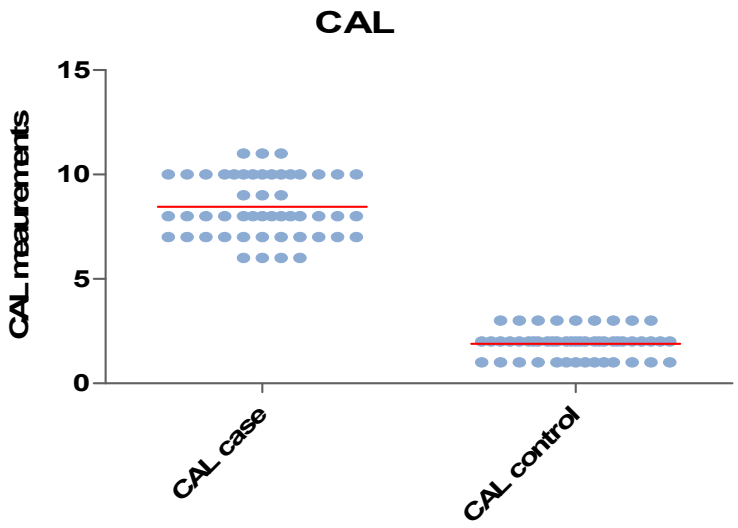


Figure 4.14 Scatter plot diagram of CAL in cases and controls, red line represents the mean.

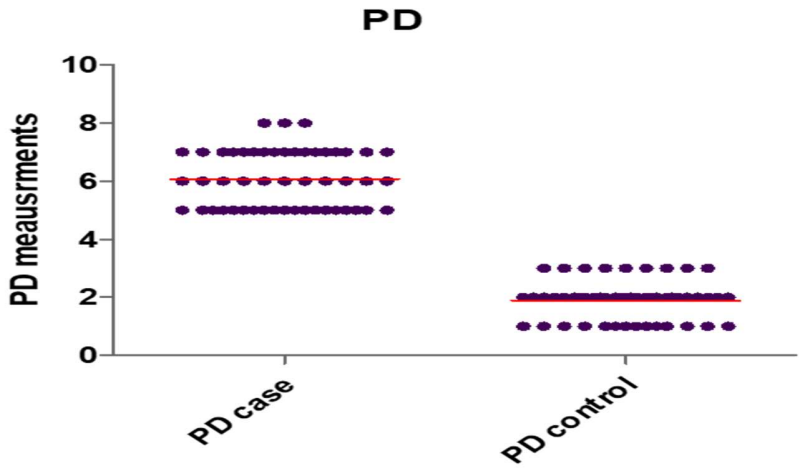


Figure 4.15 Scatter plot diagram of PD in cases and controls, red line represents the mean.

4.10 Association between the sub-gingival pathogens and CP:

Chi-squared test used to estimate the relation between eight sub-gingival pathogens and CP group, the association was significantly high (p-value = 0.0001).

Table 4.5 Association between red complex pathogens and clinical parameters (BOP).

Pathogen	BOP < 30%	BOP >30%	p-value
T.denticola	20	41	0.0001
P.gingivalis	12	35	0.0001
T.forsythia	9	42	0.0001

Table 4.6 Association between red complex pathogens and clinical parameters (CAL).

Pathogen	CAL < 3	CAL > 3	p-value
T.denticola	10	46	0.0001
P.gingivalis	6	41	0.0001
T.forsythia	3	48	0.0001

Table 4.7 Association between red complex pathogens and clinical parameters (PD).

Pathogen	PD < 3	PD > 3	p-value
T.denticola	10	46	*0.0001*
P.gingivalis	6	41	*0.0001*
T.forsythia	3	48	*0.0001*

Table 4.8 Association between green complex pathogens and clinical parameters (BOP).

Pathogen	BOP < 30%	BOP > 30%	p-value
A. actinomycetemcomitans	8	20	0.0001
E.corrodens	16	40	0.0001

Table 4.9 Association between green complex pathogens and clinical parameters (CAL).

Pathogen	CAL < 3	CAL > 3	p-value
A. actinomycetemcomitans	*8*	*20*	*0.0001*
E.corrodens	*10*	*46*	*0.0001*

Table 4.10 Association between green complex pathogens and clinical parameters (PD).

Pathogen	PD < 3	PD > 3	p-value
A. actinomycetemcomitans	*8*	*20*	*0.0001*
E.corrodens	*10*	*46*	*0.0001*

Table 4.11 Association between orange complex pathogens and clinical parameters (BOP).

Pathogen	**BOP < 30%**	**BOP < 30%**	**p-value**
P.intermedia	*26*	*44*	*0.0001*
C.rectus	*20*	*33*	*0.0001*
P.nigrescens	*17*	*34*	*0.0001*

Table 4.12 Association between orange complex pathogens and clinical parameters (CAL).

Pathogen	**CAL < 3**	**CAL > 3**	**p-value**
P.intermedia	20	50	0.0001
C.rectus	16	37	0.0001
P.nigrescens	15	36	0.0001

Table 4.13 Association between orange complex pathogens and clinical parameters (PD).

Pathogen	PD < 3	PD > 3	p-value
P.intermedia	20	50	0.0001
C.rectus	16	37	0.0001
P.nigrescens	15	36	0.0001

4.11 Association between single sub-gingival pathogen and CP in study population:

Analysis was used to determine whether any bacterial pathogen singly had coincident effects on the CP group Chi-square test and odds ratio was used, the p-value (0.0001) was significantly high.

Table 4.14 Association between single sub-gingival pathogen in CP group.

Sub-gingival pathogens	Logistic regression for CP	
	Odds ratio	p-value
T.denticola	46	0.0001*
P.gingivalis	33.4	0.0001*
T.forsythia	376	0.0001*
A. actinomycetemcomitans	3.50	0.0001*
E.corrodens	46	0.0001*
P.intermedia	73.5	0.0001*
C.rectus	6.04	0.0001*
P.nigrescens	6	0.0001*

Table 4.15 Association between single sub-gingival pathogen in HC group.

Sub-gingival pathogens	Logistic regression for HC	
	Odd ratio	p-value
T.denticola	0.250	0.0001*
P.gingivalis	0.136	0.0001*
T.forsythia	0.064	0.0001*
A. actinomycetemcomitans	0.190	0.0001*
E.corrodens	0.250	0.0001*
P.intermedia	0.667	0.160
C.rectus	0.471	0.013
P.nigrescens	0.429	0.006*

* Refers to significant p-value

4.12 Association between bacterial species in CP group:

Twenty-eight bacterial combinations were tested for the CP group by using the Chi-square test, a statistically significant odds ratio ($p<0.05$) was obtained for 18 of the 28 bacterial combinations. Significantly positive association was obtained in 9 of the 17 with a high odds ratio for any 2 species; *P.gingivalis* / *T.denticola, T.forsythia* and *P.intermedia* at (OR=4.28, 8.78, 8.78)*, A. actinomycetemcomitans / E.corrodens, P.nigrescens, P.intermedia* and *C.rectus (*OR=*8.5, 13.5, 28.5, 28.5),* another high ratio between *C.rectus/ P.intermedia* (OR=12.64) , *P.nigrescens/ P.intermedia* (OR=13.61) ,as shown in table (4.16).

Table 4.16 The odds ratio (95 % confidence intervals) of associations among species tested from (N= 50) chronic periodontitis subjects.

Sub-gingival pathogens	*Pg*	*Td*	*Tf*	*Aa*	*Pn*	*Cr*	*Pi*	*Ec*
Pg	–							
Td	4.28* (0.87-21.06)	–						
Tf	8.78* (1.06-72.52)	1.91 (0.33-10.97)	–					
Aa	0.14* (0.05-0.36)	0.06* (0.01-0.19)	0.03* (0.006-0.13)	–				
Pn	0.47 (0.17-1.25)	0.24* (0.07-0.83)	0.11* (0.04-0.52)	13.5* (2.81-64.67)	–			
Cr	0.78 (0.27-2.19)	0.24* 0.07-0.83)	0.14* (0.02-0.67)	28.5* (3.52-230.1)	1.01 (0.38-2.62)	–		
Pi	8.78* (1.09-74.29)	3.91 (0.42-36.37)	1.95 (0.17-22.79)	28.5* (3.52-230.1)	13.61* (1.69-109.10)	12.64* (1.57-101.7)	–	
Ec	2.78* (0.70-11.04)	0.91 0.21-0.91)	0.45 (0.08-2.62)	8.5* (2.20-32.83)	6.61* (1.39-31.27)	2.89 (0.86-9.76)	0.23 (0.025-2.17)	–

* Refers to significant p-value.

4.13 Genomic DNA concentration and purity:

Nano Drop Lite spectrophotometer (Thermo Fisher Scientific) was used to measure concentration of genomic DNA and purity at the 260/280 ratio, it was extracted from buccal swab samples. The mean of genomic DNA concentration was 57 ng/µl, minimum value was 2.4 ng/µl and maximum value was 203 ng/µl.

The mean A260/A280 ratio to assess the purity for extracted genomic DNA was 1.5, minimum value was 1.1 and maximum value was 2.16.

4.14 Detection of IL- 17 A gene:

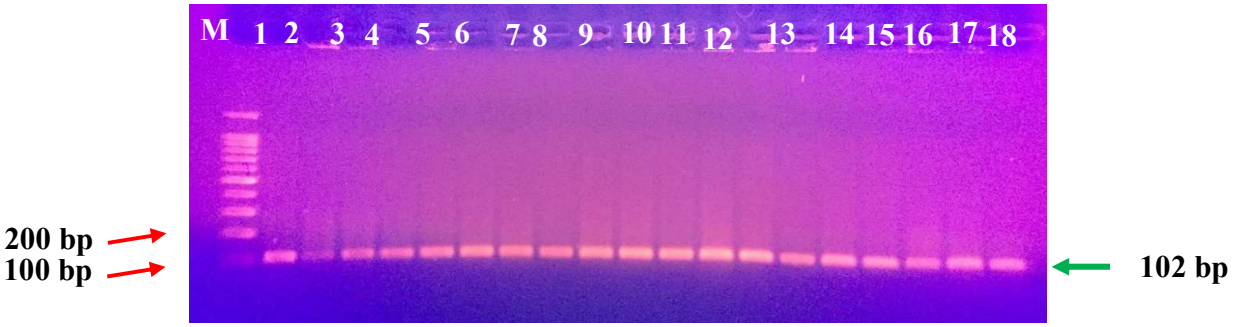


Figure 4.16 Bands of specific IL- 17 A gene regions.

Lanes 1-18 represent amplified target DNA IL- 17 A at sizes 102 bp. M= 100 bp DNA Ladder(Metabion ,Germany) was run together with the PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of PCR amplicon size. Green arrow represents the expected amplicon size of 102 bp.

4.15 Detection of IL- 17 F gene:

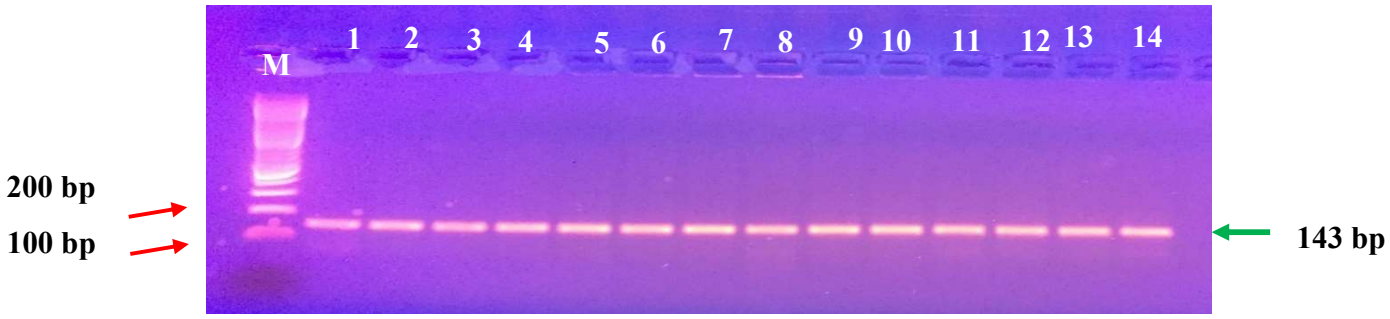


Figure 4.17 Bands of specific IL- 17 F gene regions.

Lanes 1-14 represent amplified target DNA IL- 17 F at sizes 143 bp. M= 100 bp DNA Ladder (Metabion ,Germany)was run together with the PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of PCR amplicon size. Green arrow represents the expected amplicon size of 143 bp.

4.16 Purification of IL- 17 A PCR product:

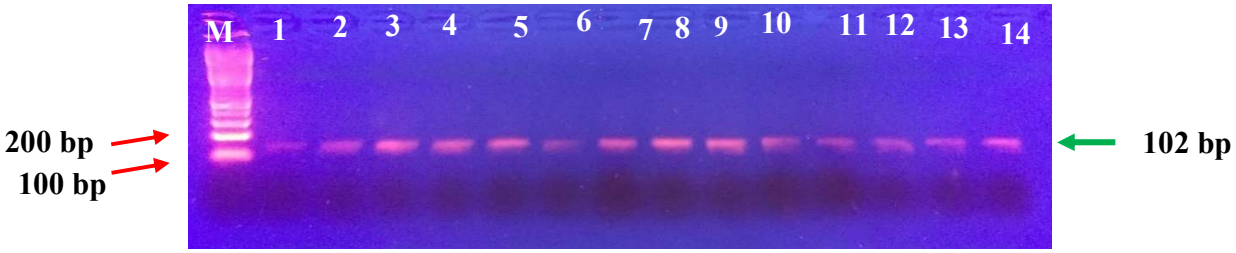


Figure 4.18 Bands of purified IL- 17 A gene PCR product regions.

Lanes 1-14 represent purified PCR product for IL- 17 A at sizes 102 bp. M= 100 bp DNA Ladder (Metabion ,Germany)was run together with the purified PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of purified product size. Green arrow represents the expected amplicon size of 102 bp.

4.17 Purification of IL- 17 F PCR products:

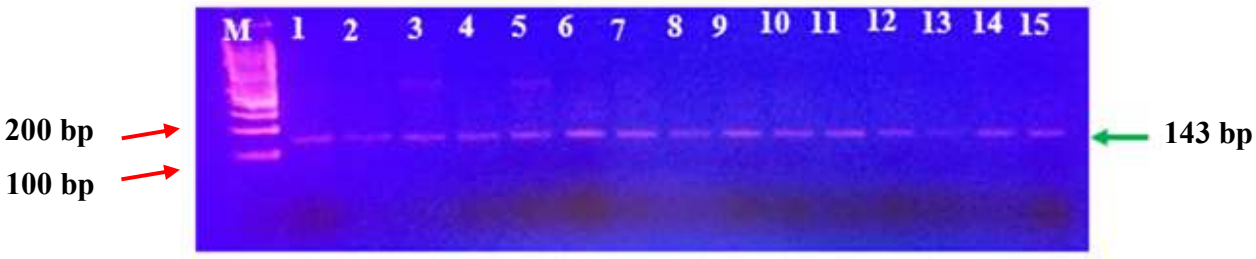


Figure 4.19 Bands of purified IL- 17 F gene regions.

Lanes 1-15 represent purified PCR product for IL- 17 F at sizes 143bp. M= 100 bp DNA Ladder (Metabion ,Germany) was run together with the purified PCR product to determine the correct size. Red arrows represent the corrospending sizes in the ladder for approximate estimation of purified product size. Green arrow represents the expected amplicon size of 143 bp.

4.18 Genotype frequency:

Genotype analysis for IL-17A and IL-17F genes polymorphism was done by Sequencher software version (5.4.6) in mRNA/genomic position c-197 g.4849 for IL-17A and mRNA/genomic position 553 IL-17F.

Table 4.17 Genotype frequency for IL-17A.

IL-17A genotype	CP N(%)
GG	3 (40)
AA	0 (0)
AG	2 (40)
Total	5

Table 4.18 Genotype frequency for IL-17F

IL-17F Genotype	CP N (%)	HC N (%)	MAF (CP)	p-value
TT(A)	32(78)	0(0)	C=15.85	0.334
CC (G)	4(10)	8(100)		
TC (R)	5(12)	0(0)		
Total	41	8		

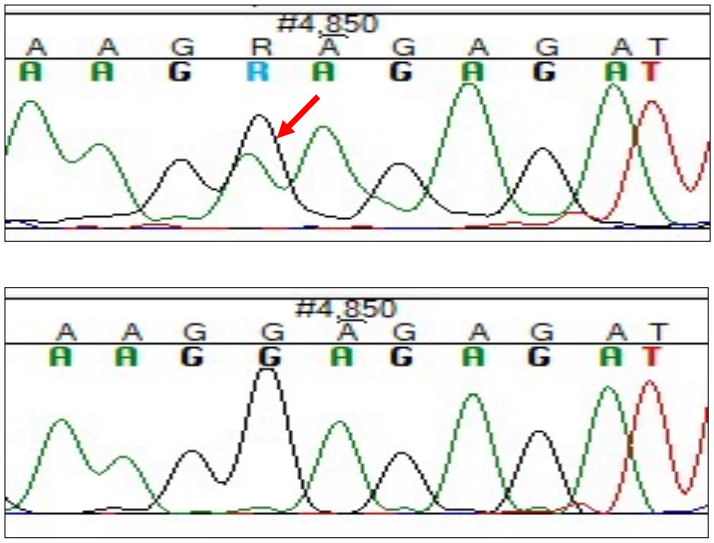


Figure 4.20 Chromatogram IL-17A (rs2275913) SNP (ambiguity code: R).

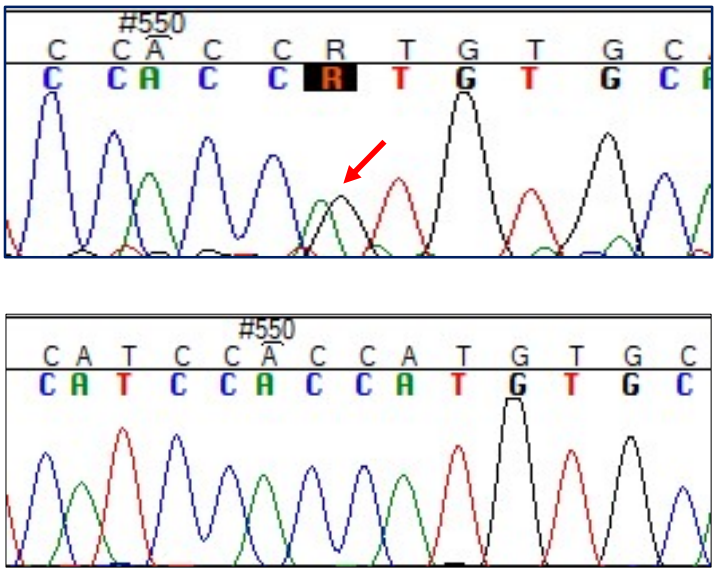


Figure 4.21 Chromatogram showing T7488C (rs763780) SNP in IL17F (ambiguity code: R).

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4.19 IL-17A allele frequency:

Table 4.19 Allele frequency for IL-17A gene (rs2275913).

IL-17F Allele	CP N (%)
G	8 (80)
A	2 (20)
Total	10

4.20 IL-17F allele frequency:

Table 4.20 Allele frequency for IL-17F gene (rs763780).

IL-17F Allele	CP N (%)	HC N (%)	p-value
T	69 (84.14)	0(0)	0.334
C	13 (15.85)	16(100)	
Total	82	16	

4.21 Novel genatic variant :

A novel variant c.*34G>A in IL17F was found in 14.6 % of CP patients, a substitution of a single nucleotide caused change in glutamic amino acid to lysine amino acid. Position on chromosome number (6) AGAAGCTGTAGAAATGCCACT, in third exon.

GAA (glutamic amino acid)

AAA (lysine amino acid)

Missense mutation is a point mutation in which a single nucleotide change results in a codon that codes for a different amino acid, glutamic amino acid was replaced by lysine amino acid.

This novel variant has not been described in any database (NCBI db SNP, ClinVar, ExAc, 1000 genome) nor reported in other studies.

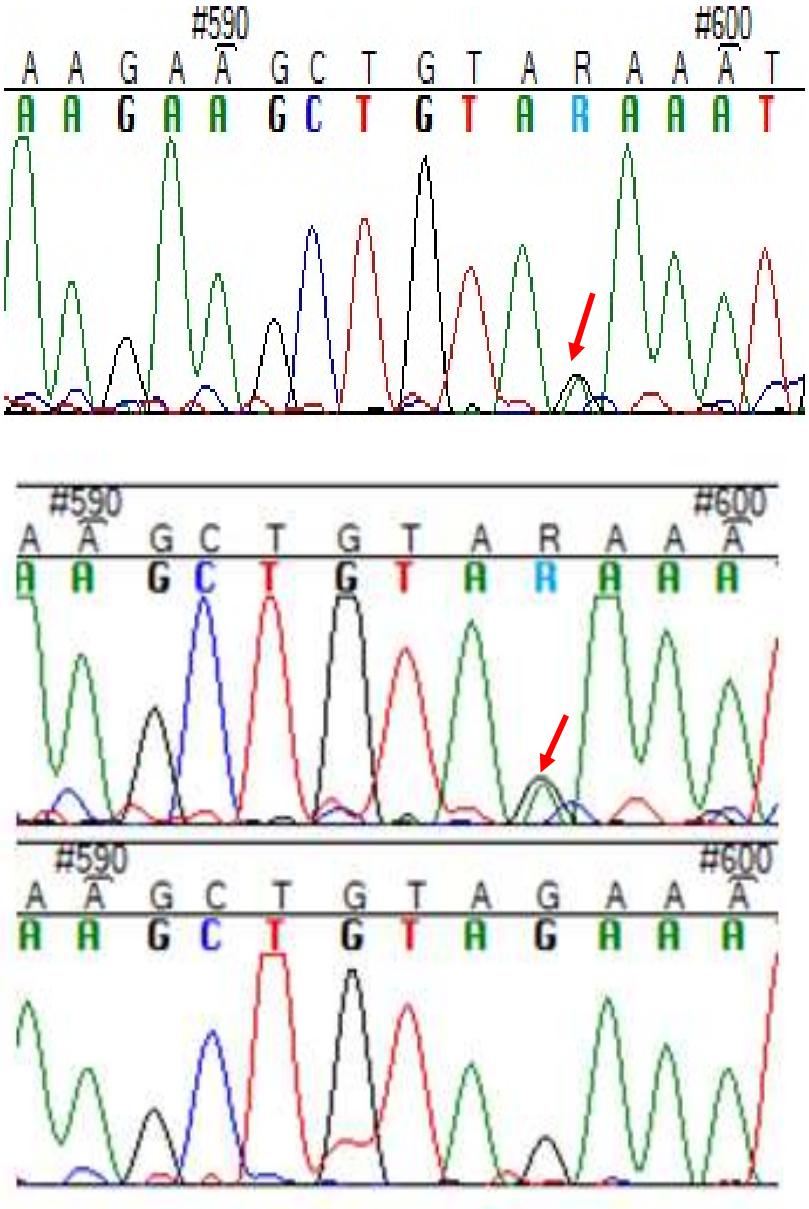

Figure 4.22 Chromatogram showing Novel genetic variant c.*34G>A in IL17F gene.

4.22 Genotype frequency for Novel genetic variant:

Genotype analysis for IL-17F gene was done by Sequencher software version (5.4.6) in mRNA/genomic position 597.

Table 4.21 Genotype frequency for Novel genetic variant c.*34G>A in IL17F gene.

IL-17F Genotype	CP N (%)	HC N (%)	MAF (CP)	p-value
AA	0(0)	0(0)	A= 7.31	0.010
GG	35(85.3)	8(100)		
AG	6(14.6)	0(0)		
Total	41	8		

4.23 Allele frequency for Novel genetic variant c.*34G>A in IL17F gene:

Table 4.21 Allele frequency for novel genetic variant c.*34G>A in IL17F gene.

IL-17F Allele	CP N (%)	HC N (%)	p-value
G	76(92.68)	16(100)	0.010
A	6(7.31)	0(0)	
Total	82	16	

4.24 Association between Novel genetic variant c.*34G>A and CP:

A novel variant was found in nine samples of total 49, Chi-squared test was used to estimate the relation between novel genetic variant c.*34G>A and chronic periodontitis, the association was significant (p-value = 0.010) as shown in table (4.22), in addition, the distribution of novel genetic variant c.*34G>A in CP was only in severe cases (PDI score 6).

Table 4.22 Association between Novel genetic variant c.*34G>A and CP.

Group	Healthy	Moderate periodontitis	Sever periodontitis	Total	p-value
AA	0	0	0	0	0.010
GG	8	20	15	43	
AG	0	0	6	6	

Chapter 5. **DISCUSSION**

5.1 Discussion:

Little information has been published about the periodontal conditions in Libyan population and their effect on periodontal health. In 2013, a cross-sectional survey was conducted among the young adults aged 18-34 years from Sebha city by Syed Wali Peeran et al. A total of 1,255 individuals, of which 80.15% were females and 19.84% were males were diagnosed with CP [11]. Within the limits of the study, it can be concluded from the clinical examination that 40.63% patients were detected with shallow pockets (4-5 mm) and 4.06% detected with deep pockets (>5 mm), only 4.7% young adults in Sebha have healthy periodontium [11].

In 2017, Elhassan et al wrote about the reasons to seek periodontal treatment in Libyan community, he tried to understand and analyse the motivation factors to seek periodontal care in the Libyan community. Variation in the degrees of periodontitis severity was found, 50% were diagnosed as moderate chronic periodontitis, followed by 21% as severe chronic periodontitis, 15% had a mild form of CP [12].

Comparing to the present study of a total of 50 individuals in CP group, which 56% were females and 44% males ware diagnosed with CP, it can be concluded from clinical examination and PDI that 60% patients were detected with pockets depth (4-6 mm) and 40% detected with deep pockets (>6 mm), there was no mild form of CP diagnosed.

This is the first microbiological study in Libya that investigates the association between eight sub-gingival pathogens and CP, detecting the prevalence of eight sub-gingival pathogens in CP group and HC group among Libyan population.

Regarding the investigation of CP pathogenesis, the relationship of the species in the different complexes (red, green and orange) to clinical parameters (BOP, CAL and PD) was examined in the present study. Eight sub-gingival pathogens (*P.gingivalis, T.denticola, T.forsythia, A. actinomycetemcomitans, E.corrodens, P.intermedia, P.nigrescens* and *C.rectus*) exhibited a very strong relationship with pocket depth, clinical attachment loss and bleeding on probing in CP group (p-value = 0.0001) was significantly high, they were detected more frequently in deeper periodontal pockets (≥ 5mm) more than in shallow ones (< 4 mm). Socransky and Haffaiee in 1998 have detected the relationship between pocket depth and members of the red complex, In addition, *P.gingivalis* and *T.forsythia have* been connected with the progression of gingivitis to

periodontitis. *T.forsythia* could be useful as an indicator bacterium of periodontal destruction in its early phase. Popova et al in 2014 have investigated and provided enough evidence to relate red complex with CP and these bacteria have been defined as key periodontal pathogens; disease progression and unsuccessful periodontal therapy depend on a present of accumulation of *P.gingivalis, T.denticola, T.forsythia* [17,33,42].

The CP was initiated through the colonization of the sub-gingival pathogens, the next step is bacterial invasion or invasion by pathogenic products into the periodontal tissues, interactions of bacteria or their substances with host cells, and this directly or indirectly causes degradation of the periodontium, resulting in tissue destruction [42,77,83]. It has been proven that *P. gingivalis* and *A. actinomycetemcomitans* can invade and cause damage to gingival tissues and lead to progression of the inflammatory process.*A. actinomycetemcomitans* is a green complex bacteria associated with the severity of the disease concerning PD and CAL, it was most frequently detected in sites with a pocket depth of ≥5 mm, this finding it was observed in multiple analyses. It is closely associated with aggressive Periodontitis but could be found in patients with chronic periodontitis [42,77,83].

Socransky and Haffaiee in 1998 have noted that *P.intermedia* was detected more frequently in deep periodontal pockets (≥5 mm) in CP patient, Popova et al, provide strong evidence of bacterial accumulation of orange complex pathogens including *P.intermedia, P.nigrescens, C.rectus* colonization may lead to an increasing progression of periodontal disease and damaging the periodontium due to stimulate immune respond [17,42].

Ashimoto et al in 1996 determine the prevalence of eight sub-gingival pathogens in American population with CP used, a 16S rRNA-based conventional PCR detection method, with the prevalence of *T.forsythia* (86%), *C.rectus* (74%), *P.gingivalis* (70%), almost close to the present study results; *T.forsythia* (96%), *C.rectus* (74%), *P.gingivalis* (82%), in addition the Ashimoto finding showed a moderate prevalence of *A. actinomycetemcomitans* (30%) and high prevalence of *E.corrodens* (80%) which was close to the finding of the current study *A. actinomycetemcomitans* (40%) and *E.corrodens* (92%), but the prevalence was slightly different in *P.nigrescens(52%), T.denticola* (54%) and *P.intermedia* (58%), it was lower than the current study finding *P.nigrescens(74%), T.denticola* (92%), *P.intermedia* (100%). Both studies showed significant association between eight sub-gingival pathogens and CP group.

Global differences in composition and prevalence of sub-gingival pathogens in patients with CP visualized in different populations in Mediterranean countries (Spain, Italy, Turkey, and Morocco), west Asia (Iran, Yamen), East Asia (China, Japan), South Asia (India), South America (Brazil), Western Europe (Switzerland) and Middle Africa (Congo). Therefore, it is plausible that differences in prevalence rates are not caused by geography solely, as well as differences among different ethnic or racial groups, variety of microbial identification methods, including culture, conventional PCR, multiplex PCR and RT PCR were used in these studies, sub-gingival sampling methods: a sterile cotton pellet, a sterile curette, a sterile paper point [102,103.]

The present study reported a high prevalence of red complex bacteria, *T.forsythia* (96%), *T.denticola* (92%) and *P.gingivalis* (82%) in CP patients, on the contrary, the prevalence of *T.forsythia*, was higher in the Yemenis population (100%), it was similar to the results in Iranian and Indian population (96%), the prevalence of *T.denticola* was higher in the Congolese and Yemenis population (100%), the prevalence of *P.gingivalis* was slightly higher in Yemenis (98%). Congolese population compared to our finding in the present study, maybe this slight variance in increasing prevalence was due to using RT PCR as a detection method in Yemenis population as opposed to the Iranian and Indian used PCR [40,83,88,104].

Also, the current study showed a moderate prevalence of *A.actinomvcetcmcomitans* (40%) close to Iranian population with prevalence(43%), on the contrary of Yemenis population, the prevalence was higher(68%) than our results [89,104].

In orange complex bacteria, the prevalence of *P.intermedia* (100%) was very close to the prevalence of Chinese (98%) and Moroccan population (90%), the prevalence of *C.rectus* was slightly higher in Iranian population (78%) than present study (74%) maybe it was related to sub-gingival sample collection method in Iranian study. The sub-gingival sample had taken by a sterile periodontal curette, it was gently inserted to the bottom of the periodontal pocket, and sub-gingival material was removed by a single stroke, in the current study samples were collected by gently inserting sterile paper points after the supra-gingival plaque was removed by using a sterile Gracey curette. The prevalence of *P.nigrescens* (90%) in Moroccan population was slightly higher than the present study (72%), all mentioned studies showed a significant association between eight sub-gingival pathogens and chronic periodontist by using Chi-squared test, the degree of the association was from significantly to high with ranged p-value (0.05-0.0001)[40,80,86].

Comparing the distribution of eight sub-gingival pathogens in CP and HC groups in different population with our results; our study reported a very high prevalence of *T.forsythia* (96%) in CP but a low prevalence in HC group (6%), similar to Japanese population with a high prevalence of *T.forsythia* (85%) in CP but a low prevalence in HC group (0%)[40,80,81,83]. Also Indian population showed a high prevalence of *T.forsythia* (75%) in CP but a low prevalence in HC group (5%) and Chinese population had a high prevalence of *T.forsythia* (73%) in CP but a low prevalence in HC group (11%) [40,80,81,83].

In the current study, *T.denticola* showed a high prevalence *(*92%) in CP compared with HC (20%) in Libyan population, similar to Indian population high prevalence (71%) in CP compared with HC (6%), *P.gingivalis* showed a high prevalence (82%) in CP compared with HC (12%) in Libyan population, similar to Indian population high prevalence (81%) in CP compared with HC (11%) and Japanese population high prevalence (75%) in CP compared with HC (0%) [81,83].

In the current study, *A. actinomycetemcomitans* showed a moderate prevalence (40%) in CP and low prevalence (16%) in HC group in Libyan population, close to Indian population the prevalence was in CP (32%) and HC (3%), *E.corrodens* showed a high prevalence (92%) in CP and low prevalence (20%) in HC group in Libyan population, on the contrary in

Indian population the prevalence was low in CP (16%) and it was very low in HC group (6%) [83].

In the current study, *P.intermedia* showed a very high prevalence (100%) in CP and moderate prevalence (20%) in HC group in Libyan population, close to Chinese population the prevalence was (98%) in CP and (63%) in HC and Brazilian population the prevalence was (87%) in CP and (43%) in HC, *C.rectus* showed a high prevalence (74%) in CP and moderate prevalence (32%) in HC group among Libyan population in the present study, on the contrary in Indian population the prevalence was low in CP (17%) and it was very low in HC group (9%), *P.nigrescens* showed a high prevalence (72%) in CP and moderate prevalence (30%) in HC group among Libyan population in the present study, on the contrary in Indian population the prevalence was low in CP (13%) and HC group (14%) [77,80,83].

The odds ratio was calculated to assess the association between the bacterial species. Twenty eight bacterial combinations were tested for the CP group, a statistically significant odds ratio ($p<0.05$) was obtained for 18 of the 28 bacterial combinations for any two species.

Comparing to our result with the Indian population in 2012, a statistically significant odds ratio ($p<0.01$) was obtained for 19 of the 28 bacterial combinations for any two species. Also, Ashimoto et al showed a similar

result in their study in 1996, the odds ratio analysis of bacterial combinations was statistically significant ($p<0.01$) for 17 of the 28 bacterial combinations for any two species [83,101].

In the current study, a significantly positive association was obtained in 9 of the 17, with a high odds ratio for any two species, such as the relationship between the red complex bacteria; it showed a strong positive association between (*P.gingivalis / T.denticola)* and (*P.gingivalis / T.forsythia*) (OR=4.28, 8.78). The relationship between the orange complex bacteria (*P.intermedia, P.nigrescens* and *C.rectus*) showed very strong positive association between *C.rectus / P.intermedia* (OR=12.64), *P.nigrescens / P.intermedia* (OR=13.61). Besides, there was an obvious association between red and orange complexes bacteria, there was a strong positive association between *P.gingivalis* and *P.intermedia* (OR=8.78).

The relationship between the two green complex bacteria (*A. actinomycetemcomitans* and *E.corrodens*) showed a strong positive association (OR=8.5), also, the relationship between the green and orange complex bacteria showed a very strong positive association between *A. actinomycetemcomitans / P.nigrescens, P.intermedia* and *C.rectus* (OR= 13.5, 28.5, 28.5).

Comparing our results to Ashimoto et al in 1996, similar positive results were obtained but with lower OR values, they were obtained between T. *denticola / P. gingivalis* (OR=3.44)*, P. gingivalis / P. intermedia* (*OR=* 5.85), *P. intermedia / C. rectus* (OR=3.33)*, A. actinomycetemcomitans / P.nigrescens* (OR= 3.57) [105].

Comparing to Mahalakshmi et al in 2012, similar positive results but with lower OR values positive, they were obtained, they were between, *A.actinomvcetcmcomitans / E.corrodens(OR=4.54),*

A.actinomvcetcmcomitans / P.nigrescens (OR=4.73),

P.intermedia / C.rectus (*OR=3.05*)[83].

High odds ratio between organisms may indicate a symbiotic relationship in periodontal pockets. A pathogen may more readily colonize sub-gingival sites already occupied by other organisms, due to gingival inflammation or growth factors produced by other organisms. However, some organisms may merely colonize together in periodontal lesions since they both produce destructive disease without interacting with each other [101].

IL- 17 is a pro-inflammatory cytokine that stimulates T cells, fibroblasts and osteoclasts for bone resorption, and takes part in dendritic cell maturation. It Produces and secretes a wide spectrum of inflammatory factors such as IFγ, tumour necrosis factor- α (TNF- α), IL- 6 and IL- 8.

IL- 17 levels in saliva, gingival crevicular fluid and plasma were observed to be significantly higher in periodontal disease [57,60,61].

Most genetic research in periodontitis has focused on gene polymorphisms that play role in immune regulation or metabolism, such as cytokines, cell-surface receptors, chemokines, enzymes and others that are related to antigen recognition [75].

Polymorphism is defined as a variant with a frequency above 1%. The term "polymorphism" which has been used widely often leads to confusion because of incorrect assumptions of pathogenic and benign effects, respectively. According to the American College of Medical Genetics and Genomics standards and guidelines in 2015, the term polymorphism is replaced by "variant" with the following modifiers: (i) pathogenic, (ii) likely pathogenic, (iii) uncertain significance, (iv) likely benign and (v) benign [67,106].

In 2015, a study was conducted to investigate the association between vitamin D receptor (VDR) polymorphism and patients who had CP, it has confirmed the importance of genetic factor (vitamin D receptor) and susceptibility to disease, it may be used to assess the risk of CP in Libyan population [107].

This is the first study to investigate the association between IL- 17 A and IL- 17 F gene polymorphism with CP in the Libyan population. Indeed, the SNPs selected for this study had been previously associated with the occurrence of CP in different populations, such as Brazilian, Turkish and Indian. All of the mentioned studies with different population gene polymorphisms may create phenotypic differences in the cytokine response among individuals and that is important for an individual's susceptibility to disease, the progression of disease or response to treatment, by detection of genotype frequencies between CP and HC groups, IL-17A (rs2275913) gene polymorphism was found to be associated with CP. The presence of the A allele (AA or AG) genotype, was associated with increasing the risk of CP, also clinical and inflammatory periodontal parameters in CP was increased, in Indian population subjects with A allele are at 5 times greater risk of developing CP than HC, other studies showed similar results, such as the studies of Correa et al, Zacarias et al and Liladhar et al [56,76,91] .

Other studies showed that the presence of the G allele (GG) genotype was associated with increasing the risk for chronic periodontitis,the studies that showed similar results such as Saraiva et al and Linhartova et al [93,94].

With the limitation of the present study, only five samples of IL-17A genes from CP patients was completely cycle sequenced and showed clear results, three of them ware GG genotype and two of them AG genotype, which detected in patients diagnosed with severe chronic periodontitis, a larger sample size is needed for more details about analysis and association.

IL- 17 F (rs763780) gene polymorphism, was found not associated with chronic periodontitis among different populations (Brazilian, Turkish and Indian), genotype and allele frequencies of IL-17F (rs763780) polymorphism were similar in both periodontitis and periodontally healthy individuals, there was no relationship between the severity of the disease and genotype frequencies [56,70,76,90,93].

The present study agreed with previous studies among different populations such as Brazilian, Turkish and Indian, there was no association between IL- 17F (rs763780) gene polymorphism and CP among Libyan population p-value (0.334).

Chapter 6. **CONCLUSIONS**

6.1 Conclusions:

We found highly significant association between eight sub-gingival pathogen and chronic periodontitis patients (p-value = 0.0001) in Libyan population. *T.forsythia, T. denticola, P.gingivalis*, *P.intermedia* and *E.corrodens* were frequently detected in the deepest pocket (≥ 6) and in periodontal tissue breakdown (CAL= ≥5).

Also a highly significant association between novel variant c.*34G>A and CP among Libyan population (p-value = 0.010).

There was no association between IL- 17 F (rs763780) gene polymorphism and CP among Libyan population. More extensive genetic studies with a larger sample size are needed to clarify the association between interleukin-17A (rs2275913) gene polymorphism and CP, also the relation between novel variant c.*34G>A and CP among Libyan population.

Chapter 7 .RECOMMENDATIONS

1.1 Recommendations:

1. In the future studies quantitative method (Q PCR) need to be used to know bacterial load.

2. Determination of well-known subgingival pathogens in the periodontal pockets, that allows the evolutionary potential and progression of the periodontal disease to be estimated.

3. Prediction of the disease progression would allow targeted preventive therapy in periodontology.

4. The availability of chairside diagnostic Test Kits will aid in early diagnosis and treatment, to diagnose “active disease” as soon as it occurs, rather than months later.

5. Using 16S ribosomal DNA (rDNA) associated with sequencing method to determine the bacterial diversity in the human subgingival plaque, also to determine species identity or closest relatives by comparison with sequences of known species.

6. Perform a larger sample size for genetic analyses of IL-17A gene polymorphism and novel variant c.*34G>A in IL17F in CP patients.

7. The use of DNA sequencing method is generally preferred over other methods like restriction enzymes when screening the entire gene and hence detecting more SNPs in IL-17A and IL-17F gene especially when studying new populations.

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APPENDIX

Appendix 1 Questionnaire& consent form in Arabic language.

الاستبيان

الاسم ثلاثي ----------تاريخ الميلاد---------- العمر-------
الجنس----------مكان السكن----------القبيلة ---------
فصيلة الدم----------لون البشرة----------**رقم الحالة**----------المهنة ومكان العمل----------
-تاريخ التسجيل----------رقم الهاتف----------

آخى العزيز اختى العزيزة: نشكركم خالص الشكر لاشتراككم معنا في هذه الدراسة والتي تهدف إلى تحديد علاقة **تعدد أشكال النوكليوتيدات المفردة في جين انتر لوكين 17(أ &اف) بمرض التهاب اللثة المزمن وأكثر أنواع بكتريا مصاحبة للمرض بين الليبيين** .

الرجاء الإجابة على جميع هذه الأسئلة:

1- هل تعاني من مرض السكري ؟ (نعم) (لا) ----------
2- هل تتناول أدوية للسكري؟ (انسولين) (اقراص).....
3- هل تعاني من مرض ارتفاع ضغط الدم؟ ومنذ متى ؟ (نعم) (لا) ----------
4- هل تعاني من الآم متكررة بالبطن؟ (نعم) (لا) ----------
5- هل تعاني من امراض التهابات في الأمعاء ؟ (نعم) (لا) ----------
6- هل تعاني من التهاب المفاصل (الروماتيزم) ؟ (نعم) (لا) ----------
7- هل تعاني من داء الصدفية ؟ (نعم) (لا) ----------
8- هل تعاني من امراض القلب/الجلطة؟ (نعم) (لا) ----------
9- هل تناولت مضادات حيوية خلال 3اشهر الماضية ؟ (نعم) (لا) ----------
10- هل تناولت مضادات الالتهابات خلال 3اشهر الماضية ؟ (نعم) (لا) ---------- --
11- هل تعاني من امراض مناعية او اضطرابات المناعة الذاتية ؟ وما هي ؟ (نعم) (لا) .....
12- هل تناولت أدوية مثبطة للمناعة خلال 3 اشهر الماضية ؟ (نعم) (لا) ----------
13- خاص بالسيدات: هل أنتي حامل او مرضعة ؟ (نعم) (لا) ----------

اقر أنا---------- بأنني اسمح بأخذ العينة اللازمة لهذه الدراسة طالما أنها في إطار المتعارف عليه طبيا.

التوقيع ----------

تمنياتنا للجميع بالصحة والعافية

Appendix 2 Patient Data sheet.

PART 1: GENERAL DATA

Participant's name: _____ Date Examined: _____

Participant's Code: _____ Age: _____

Marital Status: □ S □ M □ W

Sex: □ Male □ Female

Education Attainment: □ High school or lower □ College or higher

Occupation: _____

PART 2: MEDICAL HISTORY

Smoking history: □ Smoker (_____ pack years) □ Non-smoker

Comorbidities:

□ Diabetes mellitus
□ Hypertension
□ Autoimmune disease
□ Liver disease
□ Cardiac disease
□ psoriasis
□ Inflammatory bowel diseases
□ Rheumatoid arthritis
□ Renal disease
□ Others (specify)
□ Bronchial Asthma

PART 3: CLINICAL DATA

BP: _____ Wt (kg): _____ Ht (cm): _____

Pertinent Physical Examination Findings:

_____
_____
____

PART 4: PERIODONTAL EXAMINATION

Total Number of Teeth Present: _____

Total Number of Teeth Lost: _____

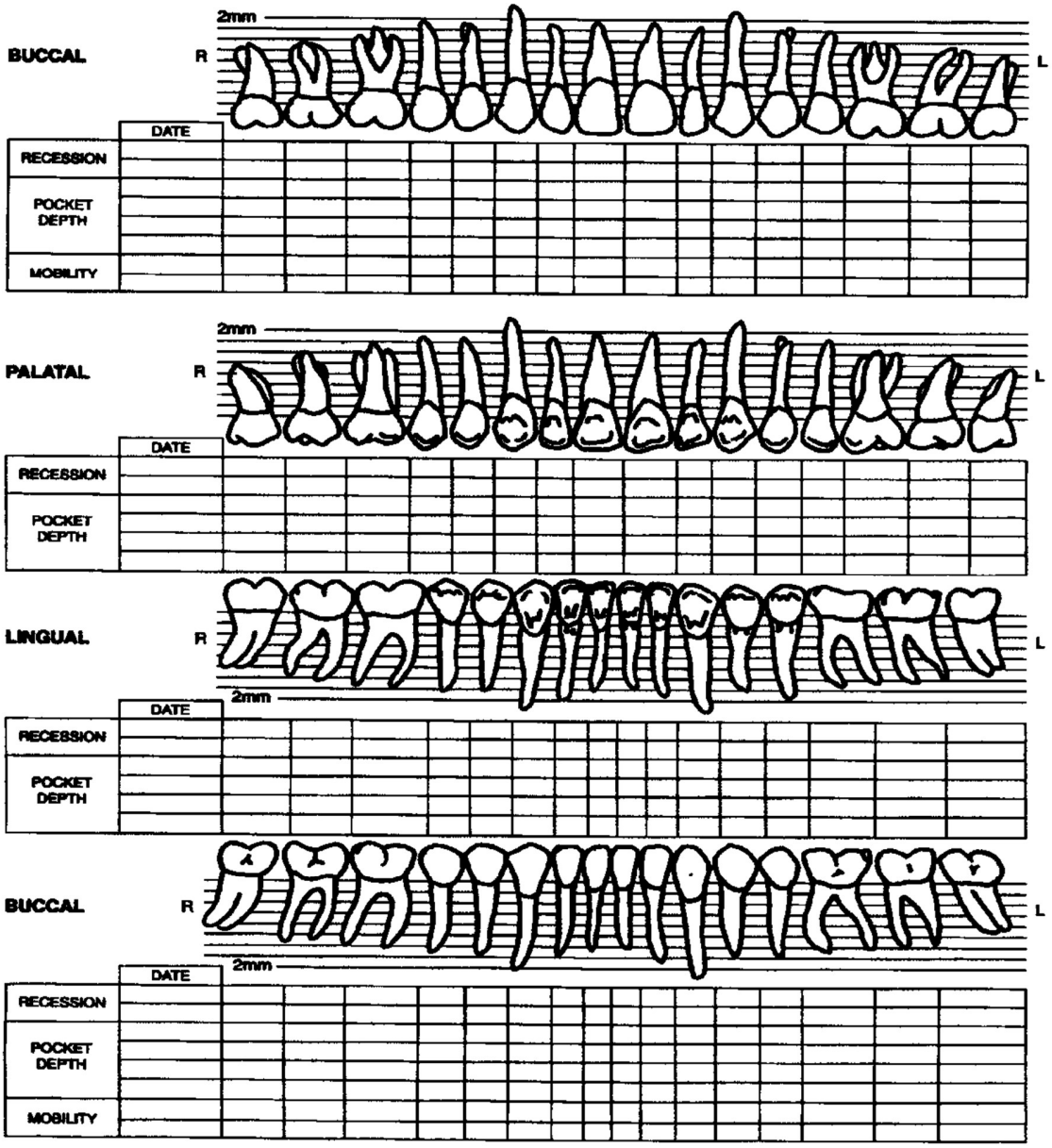
Periodontitis _____ Present _____ Absent

Severity of Periodontitis _____ Mild _____ Moderate _____ Severe

Other Findings: _____

Recommendation:_____

Appendix 3 Patient Data sheet.



Appendix 4 Additional informative sheet about the study in Arabic language.

الرسالة التوضيحية لدراسة علاقة تعدد أشكال النوكليوتيدات المفردة في انتر لوكين 17(أ &ف) بمرض التهاب اللثة المزمن وأكثر أنواع بكتريا مصاحبة للمرض بين الليبيين

انتم مدعوون للمشاركة في هذه الدراسة البحثية، مشاركتك في هذا البحث طوعية تماما و لك الحق ان تقرر المشاركة في هذه الدراسة إن رغبت او عدم المشاركة. من المهم جدا أن تفهم اسباب القيام بهذا البحث وعلى ماذا سيشتمل. الرجاء قراءة المعلومات التالية بعناية و يمكنك مناقشة اي فقرة مع فريق البحث.

ما الغرض من هذا البحث؟
ان الغرض من هذه الدراسة هو أن ندرس الطفرات (التغييرات) و الاختلافات الجينية (المادة الوراثية) لمرض التهاب اللثة المزمن ومدى انتشارها و فهم النمط الوراثي لهذا المرض في ليبيا وأيضا معرفة اكثر أنواع بكتريا المضرة المصاحبة لهذا المرض في ليبيا .

لماذا يُطلب منك أن تشارك في هذه الدراسة؟

كل المصابين بمرض التهاب اللثة المزمن وكذلك ندعو جميع الليبيين الاصحاء ابتداءً من سن 25 سنة للمشاركة في هذه الدراسة.

ماذا سيحدث أثناء الدراسة؟
إذا وافقت على المشاركة في هذا البحث سيطلب منك توقيع نموذج الموافقة المستنيرة و تعبئة الاستبيان المرفق. عملية البحث تشمل اخذ عينة ,وهي عبارة عن مسحة من اللعاب و اخري من الجيوب اللثوية .
ستتم الدراسة على المادة الوراثية المستخلصة من اللعاب و الجيوب اللثوية واجراء الفحوصات ذات العلاقة بهدف الدراسة المذكور.

ماذا سيحدث للعينات الخاصة بي؟
سوف يتم تخزينها في مجمدات منخفضة الحرارة و تخزينها بدون كتابة اسمك عليها بعد اعطاءها رقم مرجعي خاص.

ما هي الفوائد المحتملة إذا كنت تشارك في الدراسة؟
إن المعلومات التي سيتم الحصول عليها في هذه الدراسة سوف تساعد الباحثين في فهم الاسباب المحتملة لمرض التهاب اللثة المزمن و فيما اذا كان وجود طفرات او تغييرات في المادة الوراثية قد يكون سببا في هذا المرض وكذلك تهدف ايضا الدراسة الى معرفة اكثر أنواع البكتريا الممرضة المتواجدة عند مريض التهاب اللثة المزمن في ليبيا.

ما هي المخاطر المحتملة إذا كنت تشارك في الدراسة؟
لا يوجد أي خطر أو ضرر.

كيف سيتم الحفاظ على سرية المعلومات الخاصة بي؟
سوف نقوم بعناية بحماية المعلومات التي تطلعنا عليها بشأنك وعائلتك. وما نتعرف عليه من المسائل الطبية وتاريخ ونتائج عينات اللعاب والجيوب اللثوية سيتم وصفه فقط بالطريقة التي لا تعرّف بك. ولحماية خصوصيتك، سوف يتم تسجيل النتائج مع رمز سري. سوف يتم تسجيل اسمك فقط في نموذج الموافقة. سيتم الإبقاء على الرمز السري المعين في ملف مغلق ومحمي بعناية. سيتم تخزين الملفات الإلكترونية أو الورقية من هذا البحث في خزائن مقفلة والوصول اليها يتم فقط من قبل الباحث الرئيسي للدراسة والأفراد المرخص لهم.

المشاركة الطوعية / الانسحاب
ان المشاركة طوعية تماما. يمكنك سحب موافقتك في أي وقت. إذا اخترت عدم مشاركتك في الدراسة أو انسحابك في وقت لاحق من هذه الدراسة, يمكنك الاتصال بالباحث الرئيسي.

Appendix 5 Ethical approval.

Libya State
Ministry of Higher Education & Scientific Research
Authority of Natural Science Research and Technology
دولة ليبيا
وزارة التعليم العالي والبحث العلمي
هيئة أبحاث العلوم الطبيعية والتكنولوجيا
Biotechnology Research Center مركز بحوث التقنيات الحيوية

الرقم الإشاري: .....

التاريخ : .....
الموافق: .....

Bioethics Committee at Biotechnology Research Center (BEC-BTRC)

Approval Letter

Ref No; BEC-BTRC05-2018

Dear applicant, ***Dr. Nabil Enattah***

Referring to your request for ethical approval for the research project entitled: **(Association Between Interleukin 17A and Interleukin 17F gene polymorphous and periodontal pathogens in chronic Periodontitis among Libyan).**

The bioethics committee at BTRC is pleased to inform you that your proposal has met the standard of bioethics, and have given it's ethical approval for your project for 12 months. It is important to follow the guidelines for bioethics, compliance with the following:

1. Proceed with project according to the study proposal plan.
2. Ensure safe disposal of the samples after the completion of the research study, or to be stored in a safe place.

This approval was given for research purpose under the law obligations

Dr. Adam Elzaghied

Chairman of Bioethics Committee

Biotechnology Research Center
General Director of BTRC

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