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Detection of Genetic *Candida* Biofilm Isolated from Teeth Decay and Evaluated of Immunogenetic Risk Factor of Patients

A Thesis

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Biology/Microbiology

Presented By

Rafal Ahmed Obayes

Supervised by

Prof .Abber Fawzi Al-Rubaay

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(اللَّهُ نُورُ السَّمَاوَاتِ وَالْأَرْضِ ۚ مَثَلُ نُورِهِ كَمِشْكَاةٍ فِيهَا مِصْبَاحٌ ۖ الْمِصْبَاحُ فِي زُجَاجَةٍ ۖ الزُّجَاجَةُ كَأَنَّهَا كَوْكَبٌ دُرِّيٌّ يُوقَدُ مِنْ شَجَرَةٍ مُبَارَكَةٍ زَيْتُونَةٍ لَا شَرْقِيَّةٍ وَلَا غَرْبِيَّةٍ يَكَادُ زَيْتُهَا يُضِيءُ وَلَوْ لَمْ تَمْسَسْهُ نَارٌ ۚ نُورٌ عَلَىٰ نُورٍ ۗ يَهْدِي اللَّهُ لِنُورِهِ مَنْ يَشَاءُ ۚ وَيَضْرِبُ اللَّهُ الْأَمْثَالَ لِلنَّاسِ ۗ واللَّهُ بِكُلِّ شَيْءٍ عَلِيم).

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Rafal (2023)

Dedication

To the big heart in universe and the smile of my life my mother and father

To my support in life ..... my family

I dedicate the fruit of my humble effort.....

Rafal (2023)

Abstract

The study aimed to typing the dental caries based on microscopically and genotyping *Candida* spp., also detection of virulence genes based on genetic assays by two gene *ALS1,HWP1* and evaluated the role of polymorphism in two risk genes *MBL2* and *IL-10* and their relationship with teeth decay. This study conducted in period from September 2022 to February 2023.

The population samples group of patients with teeth decay in age (10-50 or more) years old Sampling included whole decay teeth and brush samples and blood from the same cases and compared with healthy group. Study parameters includes culturing and genetically studies covered by PCR banding and sequence methods and polymorphism detection for two genes *MBL2* and *IL-10*.

The results of genotyping *Candida* was shown Genotyping based ITS typing, diagnosed six *Candida spp*. genetically, they were *C. parapsilosis* = 516bp; 2 *C. albicans* = 535bp, 3-*C.tropicalis*= 521bp; 4,5,9-13 *C.famata* = 633bp, 6-8-*C.gilliermandii* =603bp, 14-*C.holmii* =717bp and filamentous fungus 750bp. The virulence factor phenotypically shown germ tube formation in *C.albicans* only, and positive biofilms on glass. And genetically two virulence gene *ALSI* in 3/10 and 5 isolates shown positive *HWP1* gene appeared positive in many isolates.

The Immunogenetic study was revealed that the primer pair successfully amplified partial sequence of *MBL2* gene promoter. The PCR products of target of *MBL2* shown 614bp. And sequence analysis sown present SNP rs7096206 G>C. *IL10* Tetra arms PCR show polymorphism in study population of rs1800896 T>C were located on the part of chromosome 1,and

at the sequence location number NC_000001.11, by comparing the observed DNA sequences of these local samples with the retrieved DNA sequences the approximate positions and other details of the retrieved PCR fragments were identified.

The polymorphism of *MBL2* shown The odd ratio was higher in genotype GC with OR= 1.56(0.4-5.7) with P value 0.5 and CC with OR=1(0.20-4.7) with P value 1 , and the allele frequency was higher in with C allele 16(40%) in patient group with high value of OR=1.24(0.5-3.1) , P.value = 0.6, while the allele frequency low 14(35%) in control group.

The rs1800896 T>C SNP allele and genotype frequencies showed significance varieties between patients and control. The subsequent SNP (rs1800896 T>C) was seen to have three genotypes (CC, CT, and TT) in patients, while in controls, just CC and CT genotypes were noticed.the IL-10 SNPs polymorphism the presence of CC genotype of rs1800896 and TC genotype in rs1800896 of *IL-10* allele was fundamentally higher in control group than patients group . Moreover, the strategic relapse model showed a diminished danger for patients improvement among people with CC genotype The Odd Ratio was higher in genotype CC with OR= 1.2(0.2-4.9) with P value 0.8 for each, and the allele frequency was with C allele 17(28.3%) in patient group with value of OR=0.7(0.3-1.6) , P.value = 0.3, while the allele frequency of CC 18(46.6%) in control group

This study succeeded in typing dental caries detection vrulance factors indication the pathogencity of *Candida* spp., in teeth decay. The *MBL2* and *IL-10* function plays as Innate recognition of microbes is a critical first step in the defense against infection. To activate antimicrobial immunity. *MBL2* and *IL-10* coding mutations are associated with multiple immune-related diseases, underscoring the physiologic importance of *MBL2* and *IL10* signaling in immunity.

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List of Abbreviatios

Abbreviation	Meaning
ALS	Agglutinin – like sequence gene
BECs	Buccal Epithelil Cells
bp	Base pair
C.albicans	Candida albicans
CP	Chronic periodontitis
DNA	Deoxy ribonucleic acid
D.W.	Distilled water
ECM	extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Hwp1	Hyphal wall protein 1
IL-10	Interleukin -10
ITS	Internal transcribed spacer
MBL2	Mannose-binding lectin
µl	Micro liter
MS	mutans streptococci
nm	Nano meter
OD	optical density
OR	Odd Ratio
PCR	Polymerase chain reaction
rpm	Rotation per minute

SDA	Sabouraud's dextrose agar
SNP	Single nucleotid polymorphism
SNV	single-nucleotide variant
Spp.	Species
TBE Buffer	Tris-borate-EDTA buffer solustion
TE	Tris –EDTA buffer solustion

1.1 Introduction

Dental caries, additionally called cavities and teeth decay, are each not unusual place and preventable. Dental caries can seem in forms: Occlusal caries: Interproximal caries. Dental caries was considering one of the popular human health problems that need health service. All the age groups are severing from dental decay. Microorganism wreck down any leftover meals that's nevertheless in our mouths and secretes acid. These acids assault the enamel, weaken the teeth, and may motive gum disease. Most cases are defined as a microbial or parasitic infections that causes destruction of teeth and tissue around the tooth .some pathogenic can transmissible among peoples (Bostanci & Belibasakis, 2012).

Tooth decay is quite possibly the most widely recognized disease of the oral cavity, influencing particularly child somewhere in the range of 5 and 12 years, and it is thought of as the most intermittent irresistible sickness around the world (Linossier *et al.*, 2011); it is an irresistible neurotic interaction, multifactorial, restricted, post-eruptive and contagious, that at long last obliterates hard dental tissues (Gamboa *et al.*,2004). The commencement and improvement of this problem is connected to the presence of bountiful microorganisms which shift contingent upon the phase of the injury (Figueroa *et al.*,2016). Oral microbes colonizing teeth are coordinated in biofilms which exists in a powerful equilibrium with the creature invulnerable protections (Reyes-Gasga ,2013). Notwithstanding the presence of bacterial communities related with fermentative digestion, an increment in dietary carbs, especially sucrose (Figueroa *et al.*,2016), result in further corrosive creation that might surpass both the limit of the spit to eliminate corrosive finished results furthermore the killing force of the salivary/plaque

support framework, delivering extra fermentation of the biofilm (Forssten *et al*,2010).

Different bioagents associated with teeth decay; Bacteria, fungi and protozoa. *Streptococcus mutans*, a significant pathogenic specialist of dental caries, (Nakano *et al*., 2006).

Candida albicans has been detected in root carious lesions. The present day examine aimed to discover the motion of this fungal species at the microbial ecology and the pathogenesis of root caries. Here, via way of means of reading *C. albicans* in supragingival dental plaque gathered from root carious lesions and sound root surfaces of root-caries topics in addition to caries-unfastened individuals, we discovered extensively extended colonization of *C. albicans* in root carious lesions. (Lozano Moraga *et al*.,2017).

The microbial interaction and cross-kingdom feeding between *Candida albicans* and oral bacteria such as *Streptococcus, Actinomyces*, and *Fusobacterium* species have been suggested being closely associated with the pathogenesis of oral infectious diseases for years (Bamford *et al*.,2009; Diaz *et al*.,2012) Cross-kingdom interactions between *C. albicans* and *Streptococcus oralis* enhance biofilm virulence on mucosal surfaces(Morales & Hogan,2010).

Mannose-binding lectin (*MBL*) is a protein molecule inherent to the immune system, in which the activation of lectin (ubiquitous carbohydrate-binding protein) domains are found in relation to collagenous structures (Fujita 2002; Turner, 2003) *MBL* in sufficiency is one of the most common human immunodeficiencies and increases first from three single-point mutations in exon 1 of the *MBL2* gene

Variations in this gene can be associated with teeth decay (Alyouse *et al.*, 2017).

Interleukin-10 (*IL-10*) is an anti inflammatory cytokine that has essential roles withinside the dental caries. The *IL10* polymorphisms in the promoter vicinity of *IL-10* gene had been related to diverse *IL-10* expressions. The articulation profile of *IL-10* in periodontal conditions presently can't seem to be explained (Mannino *et al.*,2015).

Polymorphism related with *IL-10*, which could manage gene articulation and protein work, has been generally performed to distinguish the basic pathophysiology of caries. Many single nucleotide polymorphisms have been accounted for to assume significant parts in directing *IL-10* advertiser action, one of which is arranged at position -592 (rs1800872) and is connected with the translational beginning site. (Toker *et al* 2018) showed that the *IL-10* rs1800872 polymorphisms in population.

Candida participates actively in the pathophysiology of the occurrence and advance of infection, to its virulence factors. One group of virulence factors causes colonization to take place, or the initiation of an infection, whilst the other group helps to spread the infection (Deorukhkar,2017).

Although the majority of cases of invasive yeast infection are attributed to *Candida albicans*, there are increasing rates of infection by non - *C. albicans* species in various parts of the world (Pfaller & Diekema, 2007). Conventional methods used by reference centers for the identification of medically important yeasts have been progressively replaced by PCR-based methods and proteomics (Leaw *et al.*,2006).

1.2 Aim of the Study

This study attempt to Identifiy of oral tooth decay and their virulence gene of pathogenesis and evaluate the polymorphism in *MBL2* and *IL10* in patient undergoes dental decay. In order to performed our aim we following the main objectives :

1-Accruing wash teeth samples from patients undergo any dental decay complications under physician , isolation and identification of Candida spp. Associated with dental decay.

2- Extraction *Candida* genomic DNA for identification genetically and evaluate the present of virulence gene.

3-DNA extraction of Blood samples, saliva and cultures of patients for each.

4-Evaluation *MBL2* gene and *IL-10* polymorphism in patients with teeth decay.

5- Bioinformatics analysis molecular data based on genius primer software and BioEdit software.

2.1Oral health

Teeth are considered as important part of digestive system healthy appearance face and shin smile. Oral hygiene is an integral part of personal hygiene practices and should not be neglected. While it is necessary to follow rigorous oral hygiene procedures, it is also essential to keep our toothbrushes clean and free of infection, regularly (Otukoya & Doshi 2018). First and foremost, one should wash hands with soap and water, or sanitize with ABHR before touching the toothbrush. patients should have personal oral hygiene products that include a new soft toothbrush, toothpaste, and a mouthwash/gargle that should be discarded after the condition is improved (Hua *et al.*,2016). They should store their oral health products separately and disinfect their brush regularly. Powered toothbrushes and water-pik/oral irrigators may have the potential to produce more aerosols than manual toothbrushes and should be avoided. There is a need to develop professionally driven need-based standard oral hygiene programs (e.g., soft triple head toothbrushes, and associated suction toothbrushes) in critically ill patients with disability and limited manual dexterity (Brkić & Pavlić, 2017).

2.2 Dental decay

The microbial etiology of dental caries has been the subject of different assessments that have navigated numerous years, on the off chance that not many years. The composing maintains an obvious occupation for the *mutans streptococci* (MS), especially in the past phases of caries headway. Nevertheless, there is still conversation over how much other plaque species influence the responsibility of the MS, similarly as expansive attestation that there are pathways to caries that are independent of the MS (Takahashi & Nyvad, 2008). In something like one case, strain expressness rather than species disposition has been

proposed. The "low pH streptococci" is a task given to strangely destructive skilled types of the non-MS oral species *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus gordonii, and Streptococcus anginosus*, but additional species have been suggested likewise (Matsui and Ccitkovitch 2010 ; Okahashi *et al.*,2013).

Dental caries occur because of demineralization of enamel and dentine by organic acids produced by bacteria in dental plaque through the metabolism of sugars derived from diet (Zaborskis *et al.*, 2005). Dental caries is one of the most common chronic diseases among children. The main bacterial agents in caries development are *Streptococcus mutans* and *Lactobacillu* , *Actinomyces* . Reducing these microorganisms causes a significant decrease in dental caries (Tanner *et al.*,2011).

2.3 Microbiology of caries

Many microorganisms and parasites are suspected in formation or development.decay.disease.*Streptococcus mutans* or.other.cariogenic stre ptococci, such as *S. sanguinis* could be potentially the causative agent of an oral infection if present in salivary substances from an infected individual mixed with food or drinks (Dewhirst *et al.*,2010). (Cephas *et al.*, 2011).

Dental caries or tooth decay is strongly associated with *S. mutans*, which is normally present in dental plaque of humans (Needleman , 2015). It is defined as demineralization and destruction of hard tissue of the teeth, enamel, dentin, and cemented ,Occurrence of a dental caries largely depends on the time of the tooth surface exposure to acidic by-products fermented by the bacteria. A lesion presenting as a chalky white spot on the tooth, also known as microcavity, is the early sign of demineralization of the tooth enamel (Matsui *et al.*, 2010).

A dull-brown lesion represents an active caries, which eventually leads to cavities or holes on the teeth and swelling on the gums. Common clinical symptoms of the disease are pain and discomfort when chewing food.difficulty.in.facial.movement,.sensitivity.of.tooth,.jaw.pain, discolor ation on tooth surface, inflammation on the face, and mild fever (Rickard *et al*.,2003). Dental caries can also manifest with bad breath and foul tastes. Such pyogenic oral infectious processes can be acute or chronic. During an acute carious state, the dental caries spreads laterally causing a rapid early deterioration of the pulp tissues.

Candida albicans has been detected in root carious lesions. The present day examine aimed to discover the motion of this fungal species at the microbial ecology and the pathogenesis of root caries. Here, via way of means of reading *C. albicans* in supragingival dental plaque gathered from root carious lesions and sound root surfaces of root-caries topics in addition to caries-unfastened individuals, we discovered extensively extended colonization of *C. albicans* in root carious lesions. (Lozano Moraga *et a*l., 2017).

2.4 Candida and virulence factor

Candida spp. can cause infections in human.However, five pathogens cause the most invasive infections: *C.albicans, C.glabrata, C.tropicalis, C. parapsilosis, and C. krusei,* but the most common pathogen in the clinical setting is *C. albicans* (Pappas *et al.*, 2018) , *Candida spp*. participates actively in the pathophysiology of the occurrence and advance of infection, thanks to its virulence factors. One group of virulence factors causes colonization to take place, or the initiation of an infection, whilst the other group helps to spread the infection (Deorukhkar & Roushani, 2017), *C. albicans* is present in the form of yeast in the human microbiome. The transition from yeast to the

hyphal form is a transition into a pathogenic form (Mayer *et al.*,2013 ;Tsui *et al.*,2016) .

Factors that contribute to the pathogenic potential of *C. albicans* are the expression of proteins important for adhesion and invasion. The process of adhesion is affected by various factors, such as the types of protein in the cell wall, and the physical and chemical properties of the cell surface. Adhesins of *C. albicans* recognize ligands such as proteins, fibrinogens, and fibronectins and bind to them (Deorukhkar & Roushani,2017), Since adhesins such as *Als3* and *Hwp1* are mainly expressed during hyphae creation, they play an important role in the adhesion of *C. albicans* to the host cells (Deorukhkar & Roushani,2017).

2.5 Virulence factors

2.5.1 Germ tube

In diagnostic mycology, the basic work up for yeast identification starts with a germ tube test. Germ tube formation was first reported by Reynolds and Braude and hence the germ tube test is also known as Reynolds-Braude Phenomen on.(Deorukhkar.*et.al*.,2012)

this is a rapid method for identifying *C. albicans* and *C. dubliniensis* by its ability to produce short slender tube like structures which is called the germ tubes when it is incubated in serum at 37°C. Distinguish between species belonging to the genus *Candida,* If *C. albicans* isolates produce the germ tube when incubated with the human serum at a temperature of 37°C for three hours and is important as a diagnostic character to distinguish it from other types of *Candida (*miln ., 1996) , as Campbell *et al.*,1998 mentioned that *C. dubliniensis* and has the ability to produce the germ tube and has the ability to produce the germ tube In this test the observer must be able to differentiate between the germ tube and

the pseudohyphae. The elongated daughter cells from the mother cell without constriction at their origin are referred to as germ tubes whereas constriction at the origin of the mother cells is called the pseudohyphae (Kim *et al.,* 2002) A criterion for germ tube positivity is observation of minimum five germ tube in the entire wet mount preparation. Negative results are confirmed by examining at least 10 high power fields for the presence of germ tubes (Deorukhkar *et al*., 2012).

2.5.2 Biofilm and Adhesion behavior

Adhesion is a necessary step for *Candida spp*. to colonize and invade a host ecosystem. The oral cavity presents many niches for *Candida* colonisation, and yeasts are able to adhere to many ligands such as epithelial and bacterial cell surface molecules، extracellular matrix (ECM) proteins and dental acrylics. Salivary molecules، including basic proline-rich proteins, adsorbed to many oral surfaces, also promote *Candida* adherence (Cotter & Kavanagh, 2000).

Adherence to host tissue is the first step in the pathogenic process of *Candida* infections: once first contact with the host surface has been made, enzymes facilitate adherence by damaging or degrading host cell membranes and extracellular proteins (Cotter & Kavanagh, 2000),The mechanisms of adherence of *Candida* species to many cell types or surfaces are complex and aspects of adhesion are unclear, although it is known that adherence is achieved through a combination of specific (ligand receptor interaction) and nonspecific mechanisms (electrostatic forces, aggregation, cell surface hydrophobicity (Cotter & Kavanagh, 2000). Specific adherence is mediated by a number of target proteins located on the endothelial cell or within the ECM. With respect to the latter، fibronectins are adhesive glycoproteins located in the ECM interstitium, which have a high molecular weight and are involved in cell

adhesion and cell migration (Castellani *et al*., 1986; Cotter & Kavanagh, 2000). Transmembrane integrins present on the surface of *C. albicans* are thought to mediate adherence to ECM (Hostetter, 1994) ,by recognising ligands that contain amino acid sequences such as the Arginine-Glycine-Aspartic acid (RGD) sequence (Varner & Cheresh, 1996).

Candida albicans also binds to RGD sequence-containing proteins، such as fibronectin, laminin and collagen types I and IV, through integrin-related structures (Klotz *et al*., 1994) , Adherence of *Candida* to epithelial cells is also mediated by cell-wall mannoproteins, particularly the protein portion (Cotter& Kavanagh, 2000), as pre-treatment with a variety of proteolytic enzymes inhibits adherence significantly (Douglas, 2003) , Interestingly, most of the genes that encode proteins whose expression correlates with hyphal formation, also encode cell-wall mannoproteins (Calderone & Gow, 2002).

Whilst nine *ALS* (agglutinin-like sequence) genes that encode cell-surface glycoproteins had been originally detected in *C. albicans* (Hoyer & Hecht, 2000؛ Hoyer, 2001; Hoyer & Hecht, 2001),the homologous nature of two of these (*ALS3- ALS8*) has been recently shown (Zhao *et al*., 2004). these proteins are thought to play roles in adhesion with the host. A novel mechanism that *C. albicans* hyphae use to adhere specifically to buccal epithelial cells (BECs) has been shown (Staab *et al*.,1999) by the isolation of a Hwpl protein encoded by a *HWP1* gene expressed in germ tube and hyphal forms of *C. albicans*. It has been demonstrated that the NH2-terminus of this protein is a substrate for mammalian transglutaminase, a cross-linking enzyme found in human epithelial and endothelial tissue (Staab *et al*., 1999),It has also been shown that the *C. albicans* integrin-like Inti gene/protein is implicated in adhesion. The Inti protein is a transmembrane protein encoded by the

INTI gene, the deletion of which causes a partial reduction in adhesion to epithelia, loss of virulence and inhibition of hyphal formation (Calderon & Gow, 2002).

Finally, the ability of *Candida* spp. to form biofilms on the surfaces of medical devices, such as catheters and oral prostheses, has been studied by several investigators (Chaffin *et al*., 1998; Cannon & Chaffin, 1999; Chandra *et al*., 2001؛ Ramage *et al.*, 2001; Calderone & Gow, 2002; Garcia-Sanchez *et al.*, 2004). Biofilms seem to confer drug resistance to their inhabitants as the drugs have poor penetration to cells within the biofilms (Kuhn *et al.*, 2002a; Kuhn *et al.*, 2002b). Microscopy has been used to determine cellular composition during the development of the biofilm (Baillie & Douglas, 1999; Douglas, 2003).

2.6 Detection of virulence gene

Although the *C. albicans* genome project provided many clues about DNA fragments that represent *ALS* genes, only three of the *ALS* family Open reading frame(ORFs) were assembled correctly in Assembly 19 (Braun *et al* .,2005) ,Characterization of the gene family mainly relied on efforts outside of the genome project, and these efforts dominated the initial literature on *ALS* research (Hoyer *et al*.,1995 ; Hoyer *et al* .,1998 ; Zhao *et al* ., 2003).

The *ALS* gene family consists of several genes including the *ALS*1-*ALS*7 and *ALS*9 genes which play a role in the production of cell surface glycoproteins, leading to increased adhesion to host cells (Hoyer & Cota, 2016),The *ALS* genes encode glycosylphosphatidylinositol (GPI)-linked cell surface glycoproteins. Of the eight *Als* proteins, the hypha associated adhesin *Als*3 is especially important for adhesion (Phan *et al*.,2007; Murciano *et al.*, 2012; Zordan and Cormack. 2012). *ALS* gene family that

encodes cell wall glycoprotein is related to adherence to host surfaces. *ALS* genes are a family of adhesions recognized to play a role in adherence and early biofilm formation. Since biofilm formation contributes to drug resistance, *ALS* genes appear to be responsible for fluconazole resistance (Zhao *et al.*, 2004 ; Li *et al.*, 2007 ; Hoyer *et al.*, 2008;Du *et al.*, 2012).

ALS1 and *HWP1* genes and virulence factors such as germ tube formation and length. (Cheng *et al.*, 2006),The agglutinin-like sequence (*ALS*) gene family is the largest gene family known in *C. albicans* and is considered one of the important factors in the adhesion of the organism and the formation of biofilm (Hoyer ,2001).

Another protein affecting the adhesion and regulation of biofilm in *C. albicans* is the hyphal wall protein produced by the *HWP1* gene (Nobile *et al.,* 2006) . *HWP1* protein (hyphal wall protein1 gene) is a mannoprotein-linked glycosyl phosphatidylinositol that, similar to the protein encoded by the *ALS* family of genes, plays an important role in *Candida* adhesion (Staab *et al.,* 2004) ,Several studies have shown that the *HWP1*-producing gene is expressed in the early stages of biofilm formation (Romeo *et al.,* 2009 ;Javaheri *et al.,* 2016). The hyphal wall protein (*HWP*1) is a main adhesin protein, commonly expressed on the germ tube and hyphal surface of *Candida* species as a substrate attach covalently to host cells transglutaminases and cross-links this genus to epithelial cells of mucosa (Chaffin, 2008 ; Romeo & Criseo, 2008). the previous reports have demonstrated that this gene is highly expressed after transition from blastoconidia to germ tube and hyphae in *C. albicans, C. dubliniensis, and C. africana* species (Padovan *et al.,* 2009) .

Formation of biofilm is a property of *C. albicans* pathogenesis. Most infections caused by *C. albicans* are related to the creation of a biofilm on the surface of the host or on abiotic surfaces (implants), which leads to high morbidity and mortality (Tsui *et al* .,2016) Because *C. albicans* can transition from yeast to hyphae morphologically, its biofilm is a complex structure of different morphological forms (Priya & Pandian, 2020).

which does not form germ tube and pseudohyphae; therefore, *HWP1* gene is not expressed in *C. glabrata* isolates (Criseo *et al*., 2015). *Hwp1* (Hyphal Wall Protein 1gene) is a hyphae specific cell wall protein required for proper hyphal growth and virulence in systemic candidiasis and is encoded by a single copy gene (Staab *et al.*, 1999 ;Tsuchimori *et al* .,2000).

2.7 Phenotypic identification of *Candida* spp.

All *Candida* strains isolated from colony in SDA medium were sub-cultured in Brilliance Candida agar (Oxoid) and incubated aerobically at 30°C. The plates were checked at 24, 48 and 72 hours. Brilliance Candida agar was used for differentiating among species of *Candida* since they produced different colored colonies (Ellepola & Morrison., 2005) .

The precense of two chromogens in this medium (5-bromo-4-chloro-3 indolyl N acetyl ß- D-glucosaminide and 5-bromo-6-chloro-3-indolyl phosphate p-toluidine salt) helped in identify for the presence of two target enzymes, the hexosaminidase and the alkaline phosphatase, respectively. The precense of either enzymes allows for the differentiation of *C. albicans and C. tropicalis* from other species of *Candida,*within.48.hours.,The,green,colour,of,*C.albicans,*and,*C.dublinien sis* is caused by the same chromogenic reaction as well as the dark blue

color of *C. tropicalis* For others species, it is quite difficult to differentiate based on color. For example, the colors of *C. glabrata*, *C. kefyr, C. parapsilosis, C. lusitanae* could be beige, yellow or brown. Moreover, this medium can help recognize mixed strains showing different color, that could not be achieved by culture on SDA medium (Freydiere *et al* ., 2001).

The CHROMagar *Candida* differential medium is commonly used to isolate and identify presumptive *C. albicans, C. dubliniensis، C. tropicalis and C. krusei* , based on colony colour and appearance, , the product identifies *C. albicans* by growth as light to medium green colonies, *C. tropicalis* by growth as steel blue colonies accompanied by purple pigment diffusion into surrounding agar, and *C. krusei* by growth as large, fuzzy, rose-colored colonies with white edges, all after incubation for 48 h at 37°C , Recent reports have suggested that the dark green appearance of *Candida dubliniensis* can also be reliably distinguished from that *of C. albicans* , The identification of *C. glabrata* remains controversial. Several authors maintain that *C. glabrata* is readily identifiable on CHROMagar *Candida* with its characteristic dark pink to purple coloration ,Routine use of chromogenic media carries the potential for cost savings in the clinical microbiology laboratory (Yücesoy *et al*., 2003 ; da Costa *et al*., 2009).

On chromogenic media like the CHROMagar medium, the colonies of *C. albicans* are green and consistent. This macroscopic observation allows a primary identification or a presumption of the microbial species at the origin of the infection. The identification of the clinical isolate is confirmed by a variety of additional tests, which begin with microscopic observation of the germ's morphological and structural characteristics. The elements most often observed are the presence and arrangement of

blastoconidia، pseudohyphae, hyphae, germ tubes, and chlamydospores (Pincus Orenga *et al* ., 2007) .

2.8 Genotyping Band size based typing method:

Barcoding genotyping *Candida spp*., familiar used the ITS region as barcoding region in fungi. To overcome the limitations of standard phenotypic diagnosis and identification methods, culture- and "expert-free" methods capable of identifying fungi directly from biological specimens are needed. Sequence-based identification has proven to be more accurate than conventional methods in diagnostic clinical mycology (Ciardo *et al.,* 2006; Balajee *et al*., 2007).

Internal transcribed spacer (ITS) is the spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome , there are two ITSs in eukaryotes: ITS1 is located between 18S and 5.8S rRNA genes, while ITS2 is between 5.8S and 28S (in opisthokonts, or 25S in plants) rRNA genes (Lafontaine and Tollervey,2001).DNA barcoding is a global initiative that aims at streamlining species identification through analysis of short DNA sequence markers (Hebert *et al*., 2003).

The internal transcribed spacer (ITS) region has been accepted as the standard barcode marker for fungi. However, a thorough study of ITS sequences in the International Nucleotide Sequence Database (INSD: GenBank, EMBL and DDBJ) revealed that this region is not equally variable in all groups of fungi (Nilsson *et al.,* 2008),the Internal Transcribed Spacer itself consists of two parts, ITS1 and ITS2, which are separated from each other by the 5.8S subunit nested between them. Like the flanking 18S and 28S subunits, the 5.8S subunit contains a highly conserved DNA sequence, as they code for structural parts of the

ribosome, which is a key component in intracellular protein synthesis (Begerow *et al.*, 2010) .

Among the applied molecular techniques, DNA barcoding is one of the most promising and efficient methods, as it enables rapid identification of species and recognition of cryptic species across all fungal genera. As such, DNA barcoding has recently been established as the gold standard identification technique for fungal species and has been proven to be more accurate than conventional identification techniques (Ciardo et al., 2006; Balajee *et al.*, 2007), barcodes are standardized, easily amplified, universal short DNA sequences (500–800 bp), which are divergent at the species level enabling rapid identification by comparison with a validated reference sequence collection. To ensure consistency of identification, barcodes should be unique to a single species, and stable within each species (Hebert *et al.*, 2003).

The advantage of the ITS region is, that it can be easily amplified from most fungal taxa, using universal primers, with the most commonly used ones being the ITS1, ITS2, ITS3, ITS4, and ITS5 (White et al., 1990). The primary fungal DNA barcode region identifies up to 75% of the estimated ~700 pathogenic fungal species (Zhou *et al.*, 2014), It has typically been most useful for molecular systematics at the species to genus level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for example, small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within the ITS region,In addition to the universal ITS1+ITS4 primers (White *et al.,*1990),therefore, generally, either the ITS1 or the ITS2 region is used in ecological studies aiming at the characterization of fungal communities , Primers that will be used in should be able to efficiently amplify their

target DNA regions in the presence of high concentrations of non-target DNA and contaminants (Kosch & Summers ,2013).

2.9 Mannose-binding lectin (*MBL2*) gene

This gene encodes the soluble mannose-binding lectin or mannose-binding protein found in serum. The protein encoded belongs to the collectin family and is an important element in the innate immune system. The protein recognizes and binds to mannose and N-acetylglucosamine on many microorganisms, including bacteria, yeast, and viruses including influenza virus, HIV and SARS-CoV. This binding activates the classical complement pathway. Deficiencies of this gene have been associated with susceptibility to autoimmune and infectious diseases (Bodamer *et al* ., 2006).

The high prevalence of dental caries in children worldwide is a major oral health problem which requires early intervention. Dental caries is mainly caused by the action of acids produced by bacteria in addition to many other factors. Recent genetic studies have reported that a number of genes are associated with the susceptibility to dental caries (Alyosef *et al* .,2017).

The majority of these genes are associated with inflammation, increased susceptibility to infection, and dentine matrix formation. Using the TaqMan assay and direct DNA sequencing, the prevalence of 6 single-nucleotide polymorphisms (SNPs) in *MMP9*, *MBL2*,and *MMP2* genes was determined in 102 children with caries and in 100 age-matched caries-free controls. Out of the 6 SNPs tested in the 4 selected genes, only rs11003125 in the *MBL2* gene was shown to be associated with a high prevalence of caries in our cohort. In addition, polymorphsim SNPs tested revealed that certain haplotypes, namely GT of rs11003125G and

rs7501477T and GT of rs7096206G and rs7501477T, were found to be associated with a high prevalence of dental caries in our cohort, while haplotype AG of rs17576A and rs7501477G was found to have a protective effect against dental caries. In conclusion, the data indicate that rs11003125 in the *MBL2* gene was shown to be associated with a high prevalence of caries in our cohort, and 2 haplotypes are also involved in the increased susceptibility to dental caries (turner , 2003).

2.10 Teeth decay and genes polymorphism

Growth factors and cytokines in development of many human diseases, (Rankine *et al.*, 2006). Many clinical rules are regularly lacking for estimating the level of defenselessness to future periodontal infection movement or deciding the suitable treatment plan for each individual (Giannobile *et al.*,2009), various polymorphisms of different qualities have been evaluated concerning the clinical results after non-careful periodontal treatment, yet the absence of systemically strong examinations uncovered the requirement for extra investigations(Chatzop oulos and Doufexi .,2016).

Individuals helpless to periodontal sickness movement following non-careful periodontal treatment might profit from adjunctive fundamental anti-toxins or other extra therapies. The utilization of hereditary tests to anticipate the danger of future periodontal illness annihilation might make it conceivable to individualize treatment (Personalized/Precision Medicine) with the plan of lessening the occurrence of periodontally compromised teeth and tooth misfortune (Owens ,2015).

2.11. Interleukin 10 (*IL-10*) gene

The gene encoded *IL-10* is situated on chromosome 1q31-q32. Polymorphisms in the advertiser district of the *IL-10* quality can influence the statement of *IL-10* cytokine which prompts changes in provocative

processes (Gibson *et al*.,2001) There are some clashing outcomes with respect to the relationship between *IL-10* polymorphisms and CP (Moore *et al*., 2001). *IL10* has a huge impact in aggravation and is viewed as a supportive of fiery cytokine that is mitigating cytokine that down-controls the favorable to incendiary insusceptible reaction of monocytes and macrophages (Yoshie et al.,2015). *IL-10* is a separation factor that animates B cells to discharge IgG, IgA and IgM which smothers the neurotic safe reaction driving the age of CD4+ T cells (Groux *et al*.1997). A critical expanded danger of constant periodontitis has been related with *IL-6*−572G/Cand *IL-10*−592 C/A quality polymorphisms particularl in Caucasian Europeans (Albuquerque *et al*.,2012; Song Song *et al*.,2013).

Interleukin-10 (*IL-10*) is a mitigating cytokine that plays significant parts.in infections.The *IL10*-1082,-819,and 592.polymorphisms in the advertiser district of *IL-10* quality have been related with different *IL-10* articulations. The point of this review was to research the relationship between these quality polymorphisms with persistent periodontitis in an example of Iranian populaces from Iran (Heidari *et al*.,2013). Interleukin-10 gene advertiser polymorphisms have been related with interleukin-10 diminished creation, along these lines assuming a part in the pathogenesis of periodontitis and interleukin-10 single nucleotide polymorphisms at positions - 1087(G/A) and - 597(C/A) are related with summed up persistent periodontitis and limited forceful periodontitis (Kinane and Hart 2003).

2.12 A single-nucleotide polymorphism (SNP)

A single-nucleotide variant (SNV) is a general term for single nucleotide change in DNA sequence. So a SNV can be a common SNP or a rare mutation, and can be germ line or somatic (Goya *et al* ., 2010)

.Single nucleotide polymorphisms (SNPs) are polymorphisms that are caused by point mutations that give rise to different alleles containing alternative bases at a given position of nucleotide within a locus. Due to their high abundance in the genome, SNPs already serve as the predominant marker type. Many sequencing techniques and bioinformatics methods are applied for SNP development and identification, such as random shotgun sequencing, PCR amplicon targeted sequencing, RNA sequencing, and bioinformatic mining of ESTs databases (Liu and Cordes ., 2004).

The SNPs are the most common form of variation in the genome and they are extensively used to study genetic differences between individuals and populations. These SNPs may contribute to changes in the genomic sequence, either in the coding (exons), intergenic, or noncoding (introns) region (Ahmad *et al.*, 2018),SNPs are considered the most useful biomarkers for disease diagnosis or prognosis due to their common frequency, ease of analysis, low genotyping costs, and the possibility to carry out association studies based on statistical and bioinformatics tools (Srinivasan *et al.,* 2016).

SNPs may also have a great influence on the immune response towards pathogenic challenges and diseases outcome, contributing in a range of susceptibility to infections among the individuals. Thus, the SNP may have a protective role, may influence the rate of diseases progression or even the type of cellular immune response evoked by pathogens (Hill, 2001; Skevaki *et al.,* 2015). like single nucleotide polymorphisms (SNPs), greatly influence innate immune responses towards pathogenic challenges and disease outcome; therefore a range of susceptibility to infections appears among people, with some of them being predisposed to certain infections, while others being protected (Hill., 2001) .SNPs

pinpoint differences in our susceptibility to a wide range of diseases (Calippe *et al* ., 2014).

SNP it two type SNPs in the coding region are of two types: synonymous SNPs and nonsynonymous SNPs. Synonymous SNPs do not affect the protein sequence, while nonsynonymous SNPs change the amino acid sequence of protein and SNPs in non-coding regions can manifest in a higher risk of cancer, and may affect mRNA structure and disease susceptibility, Non-coding SNPs can also alter the level of expression of a gene, as an eQTL (expression quantitative trait locus) ,(Lu *et al* ., 2015) .

SNPs in coding regions: synonymous substitutions by definition do not result in a change of amino acid in the protein, but still can affect its function in other ways (Kimchi-Sarfaty., 2011), Nonsynonymous substitutions : missense – single change in the base results in change in amino acid of protein and its malfunction which leads to disease (Al-Haggar *et al* ., 2012).nonsense – point mutation in a sequence of DNA that results in a premature stop codon, or a nonsense codon in the transcribed mRNA, and in a truncated, incomplete, and usually nonfunctional protein product (Cordovado *et al* ., 2012).

Thus, SNPs have gained importance as major drivers in disease-association studies in the recent era. In mammals, on the past decade it has been seen an enormous progress in identifying hundreds of thousands SNPs to identify associations with complex clinical conditions and phenotypic traits associated with hundreds of common diseases (Welter *et al*., 2014; Wijmenga and Zhernakova, 2018).

Genetic variation occurs within and among populations, leading to polymorphisms. Single nucleotide polymorphisms (SNPs) are the most

common type of genetic variation. A SNP is a variation of a single nucleotide at a specific position in the genome, some of which may contribute to changes in a gene, either in the coding (exons) or non-coding (introns) regions, or the regions between genes (Daly .,2004).

SNPs are able to be identified with high confidence more recently because of much deeper sequencing coverage provided by the next-generation sequencing technologies in comparison with the Sanger sequencing (Liu *et al.*, 2011),SNPs are now the markers of choice for genetic studies because they are the most abundant genetic variations widely distributed in the genome, and are generally bi-allelic polymorphisms that are amenable to automated genotyping. SNPs are efficient for genome-wide association studies (GWAS) because linkage disequilibrium can be detected with high-density SNPs when dealing with complex traits (Liu *et al.*, 2014).

Single nucleotide polymorphisms (SNPs) are polymorphisms that are caused by point mutations that give rise to different alleles containing alternative bases at a given position of nucleotide within a locus. Due to their high abundance in the genome, SNPs already serve as the predominant marker type. Many sequencing techniques and bioinformatics methods are applied for SNP development and identification, such as random shotgun sequencing, PCR amplicon targeted sequencing, RNA sequencing, and bioinformatic mining of ESTs databases (Liu and Cordes, 2004).

The genomic distribution of SNPs is not homogenous; SNPs occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and "fixing" the allele (eliminating other variants) of the SNP that constitutes the most favorable genetic adaptation

Other factors like genetic recombination and mutation rate, can also determine SNP density (Nachman ., 2001).

3. Materials and Methods

3.1: Materials

3.1.1: Instruments and Equipment

The instruments and equipment were used in current study Table -3-1

Table -3-1-: The Instruments and Equipment used in current study.

Type of equipment	Manufacturing company
Autoclave	Labtech -(KOREA)
Compound light microscope	Olympus -(Japan)
Digital camera	Sony –(Japan)
Electrophoresis unit	Labnet -(Taiwan)
Incubator	Memmert -(Germany)
Inoculating loop	Japan
Macro Centrifuge	Hitachi
Micro centrifuge	Hitachi -(Germany)
Micropipette	Eppendorf -(Germany)
Microwave	Germany
Nanodrop2000	Thermo (USA)
Refrigerated	Concord -(Lebanon)
Sensitive balance	Precisa - (Switzerland)
Thermo cycle (PCR)	Agilent -(Germany)
Tips	Sterile /UK
Tube shaker	Labinco (Netherland)
U.V Light	Korea
Vortex	Hiedolphy -(Germany)
Vortex Centrifuge	Germany
Water bath	Memmert -(Germany)

3.1.2: Biological and Chemical Materials:

The biological and chemical materials used in the current study Table -3-2-**Table -3-2-: Chemical Materials** and **Biological Materials**

No.	**Chemicals** and **Biological Materials**	**Company/country**
1.	Agarose	Bio basic-Canada
2.	DNA Ladder (DNA marker 100bp)	Fisher –Garantie
3.	Ethanol 99% and 70% alcohol	Flukachemika Switzerland
4.	Glycerol	B.D.H , England
5.	Tris Borate-EDTA–Buffer solution (TBE)10x	Bio basic-Canada
6.	Tris-EDTA Buffer solution (TE)	Promega-USA

Table -3-3-: Madia utilized in this study

No.	Type of media	Company	Country
1.	Sabouraud agar	Himedia	India
2.	CHROMO Agar	Biomax	UK
3.	Agar agar	Oxiod	England

3.1.3: Materials of Polymerase Chain Reaction .

Table-3-4-: All polymerase chain reaction materials used in the current study.

Material	Origin
Ethidium bromide	Promega (USA)
Glycerol	Hi media (India)
TE-buffer	Promega (USA)
Green Master Mix	Promega (USA)
Molecular Marker	Bioneer

Table -3-5-: Genomic DNA purification kit and Buffer.

NO.	Genomic DNA purification kit and Buffer
1	Proteinase K
2	Cell lysis Buffer
3	Nuclei lysis Buffer
4	Binding Buffer
5	Wash **1** Buffer
6	Wash **2** Buffer
7	Nuclease free water

3.1.4: Primers

All primers were used in the current study were listed in Table -3-6-

Table-3-6-:Primers for Immunogenic genes used in the current study.

Immunogenic genes			
Primer	**Sequence (5' → 3')**	**Size**	**Reference**
Specific primer	**MBL2F:5'-CCTGGTTCCCCCTTTTCTCC-3'** **MBL2R: 5'-AGATGGACCCGAAGAGGACA-3**	614bp	Designed in this study
IL0	F1:TCCAGATATCTGAAGAAGTCCTG R1: TACCTATCCCTACTTCCCCC F2:CTACTAAGGCTTCTTTGGGAA R2:CAGTGCCAACTGAGAATTTGG	Main band(420 bp), C allele (285bp) T allele 233 bp	Kurdistani et al.,2015

Table -3-7-:Primer for Diagnostic genes used in the current study.

Diagnostic gene			
Primer	**Sequence (5' → 3')**	**Size**	**Reference**
Universal primer	**ITS5: GGAAGTAAAAGTCGTAACAAGG** **ITS4: TCCTCCGCTTATTGATATGC**	500-780bp	White et al.,1990 study

Table -3-8-:Primers for Virulance genes used in the current study

Virulance genes			
Primer	**Sequence (5' → 3')**	**Size**	**Reference**
ALS1	F:GACTAGTGAACCAACAAATACCAGA R:CCAGAAGAAACAGCAGGTGA	315bp	İnci et al.,2013
HWP	F:ATGACTCCAGCTGGTTC R:TAGATCAAGAATGCAGC	503bp	İnci et al.,2013

3.2 Patients and Control Groups:

Fifty specimen were collected extracted teeth from patients suffering from severe teeth decay from different dentist clinics in the city of AL- Hilla, and were diagnosed by special physician, the control group was composed of 50 randomly healthy persons without teeth decay. All do not up take any antibiotic before three days ago, both with age ranged (10-50 or more) years during the period from September 2022 to February 2023. Also, 3ml of blood specimen were collected in EDTA tubes from same patient for molecular study, specimen be saved under -20 in frozen stat.The genotype distributions of SNPs in genes by used the PCR-sequences for interleukin 10 and sequence analysis polymorphisms for MBL2 after using PCR.

3.3 Method

3.3.1:Preparation of Culture Media

The media were listed in Table (3-3) for isolating and diagnosing fungi and yeasts according to the instructions of the manufacturer company

installed on the packages or according to the references of the scientific references and pH modification where necessary using the KOH and HCL the diluted dose were measured using pH strip. All the media were sterilized in a temperature of 121 ° C, under pressure of 15 PSI for 15 minutes. these media is :

3.3.1.1: Sabouraud's Dextrose Agar (SDA)

This medium was prepared with 65 mg of sabouraud's dextrose desolved in 500 ml of distilled water and complete to 1000 ml then placed in a hot plate and magnetized to a boiling point. After that 250 mg/ml of chloramphenicol was added, then mixed and sterilized. This medium is used for culturing pathogenic fungi and yeast isolates (Odds,1991).

3.3.1.2:CHROMO Agar

This medium was used for rapid identification of *Candida* spp., especially from non-sterile sites. The CHROM agar is prepared by disolving 47.7 mg of CHROM agar in 1000 ml of D.W. and heated to the point of effervescence (for yeast cultivation), as instructed by the manufacturers. It was then poured into a plastic 9 cm petri dishes. The media is used for researching and diagnosing the *Candida* spp. Their appearance is based on color (Nadeem *et al.*, 2010).

3.3.2:Morphological Examination

After appearance growth as well as examining colonies of *Candida* spp., from respect colour, shape and texture .

3.3.3: Microscopic Examination

Fungi isolates are examined microscopically, the tiny portion of the colony is taken by loop, loaded on a glass slide containing a drop lactophenol cotton blue. Slides examined under magnification 10X, 40X and 100X as described by (Pitt and Samson, 2000; Rosso ,2013; Rai, 2016).

3.3.4: A- Genomic DNA extraction kit (Favrogen) from *Candida* spp.

1. Tiny portion of colony of *Candida* was transferred to a 1.5 ml microcentirfuge tube for each.
2. added 200ul of FATG buffer to the cells and re-suspend the cells by vortex.
3. The cells were resuspend in 50 µl of lyticase solution were added , mixed well by vortexing, the samples were incubated at 37 oC for 30 min.
4. A 200ul of FATB buffer to the cells and re-suspend the cells by vortex.
5. A total of 20 µl was added of Proteinase K (10 mg/ml) and mixed well by vortexing. Incubated at 60 ° C for 15 min, vortex 30 seconds for every5 minutes incubation.
6. A 200 µl of ethanol (96-100%) was added and mixed well by vortexing for 10 seconds.
7. A spin Column was placed in Collection Tube. The sample mixture (including any precipitate) was transferred carefully to spin Column. Centrifuged at 11,000 rpm for 30 second then the spin Column was placed to a new Collection Tube.
8. A total 400 µl of W1 Buffer was added to the spin Column. Centrifuged at 11,000 rpm for 30 seconds and discarded the flowthrough. The spin Column was placed back to the Collection Tube.
9. A 600 µl of Wash Buffer was added to the TG Mini Column. Centrifuged at 11,000 rpm for 30 seconds and discarded the flowthrough, and the spin Column was placed back to the Collection Tube, centrifuged at full speed (12000 rpm) for an additional 3 min to dry the column.
10. The spin Column was placed to a Elution Tube.

11. A 50 ~100 µl of Elution Buffer was added to the membrane center of the spin Column. Stranded spin Column for 3 min. centrifuged at full speed (12000 rpm) for 1 min to elute total DNA.

12. Total DNA was stored at 20°C.

3.3.5: A- Genomic DNA extraction kit (Favrogen) from frozen blood

1. A 180Microleter of frozen blood were transferred to a 1.5 ml for each. microcentirfuge tube.

2. added 200ul of FBAG buffer to the cells and re-suspend the cells by vortex.

3. The cells were resuspend in 40 µl of proteinase was added for each. solution were added , mixed well by vortexing, the samples were incubated at 60°C for 30 min.

4. A 200 µl of ethanol (96-100%) was added and mixed well by vortexing for 10 seconds.

5. A spin Column was placed in Collection Tube. The sample mixture (including any precipitate) was transferred carefully to spin Column. Centrifuged at 11,000 rpm for 30 second then the spin Column was placed to a new Collection Tube.

6. A total 400 µl of W1 Buffer was added to the spin Column. Centrifuged at 11,000 rpm for 30 seconds and discarded the flowthrough.

7. A 600 µl of Wash Buffer was added to the spin Column. Centrifuged at 11,000 rpm for 30 seconds and discarded the flowthrough, and the spin Column was placed back to the Collection Tube, centrifuged at full speed (12000 rpm) for an additional 3 min to dry the column.

8. The spin Column was placed to a Elution Tube.

9. A 50 ~100 µl of Elution Buffer was added to the membrane center of the spin Mini Column. centrifuged at full speed (12000 rpm) for 1 min to elute total DNA.

10. Total DNA was stored at 4°C or -20oC.

3.4:The Estimation of DNA Concentration and Purity

The DNA concentration of samples was estimated by using the Nano drop by putting 2.5 µl of the extracted DNA in the machine to detect concentration in ng/µl and the purity detected by noticing the ratio of optical density (OD) 260/280 nm to detect the contamination of samples with protein. The accepted 260/280 ration for purifying DNA was between 1.7-1.9 (Sambrook, 2006).

3.5: Dissolving of Primers

All primer pairs used in this study were dissolved using Nuclease Free Water (Promega). Firstly, the primer stock tube prepared and then the working solution would prepare from primer stock tube. According to the instruction provided by primer manufacturer the Nuclease free Water were added to get 100 picomol/microliter concentration of primer stock solution. The working solution prepared from stock by dilution 10picomole/microliter primer stock solution with 90 microliter of Nuclease free Water to get 100 picomol/microliter (work solution).

3.6: The Reaction Mixture

Amplification of DNA was carried out in a final volume of 25 µl reaction mixture for detection of IL10 and MI polymorphism amentioned in table (3-7) as mentioned adding (1.5µl) from each diluted primer.

Table 3-9: Contents of the Reaction Mixture (Promega) of PCR for *MBL* genes and IL10 gene polymorphism.

No.	Contents of reaction mixture	Volume µl
1.	PCR PreMix	12
2.	Outer Forward primer	1.5
3.	Outer revers primer	1.5
4.	Patient DNA template	2
7.	Nuclease free Water (Promega)	8
Total volume		25

3.7: Thermal Cycling Conditions

The PCR reaction as shown in Table (3-8).

Tables -3-10-: Thermal cycling conditions for Primers gene under interest:

Primer pair for ITS			
Step Type	**Temperature °C**	**Time**	**Cycling**
Initial Denaturation	**95**	**3** min.	**1**
Denaturation	**95**	**40** Sec.	**30**
Annealing	**53**	**40** Sec.	
Extension	**72**	**40 Sec.**	
Final Extension	**72**	**3 min.**	**1**
Hold	**4**	**α**	**4**

IL10			
Step Type	**Temperature °C**	**Time Sec.**	**Cycling**
Initial Denaturation	**95**	**3** min.	**1**
Denaturation	**95**	**40** Sec.	**30**
Annealing	**47**	**40** Sec.	
Extension	**72**	**40 Sec.**	
Final extension	**72**	**3 min.**	1
Hold	**4**	**α**	**4**

MBL2			
Step Type	**Temperature °C**	**Time Sec.**	**Cycling**
Initial Denaturation	**95**	**3 min.**	**1**
Denaturation	**95**	**40 Sec.**	**30**
Annealing	**55**	**40 Sec.**	
Extension	**72**	**40 Sec.**	
Final extension	**72**	**3 min.**	**1**
Hold	**4**	**α**	**4**

ALS1 & HWP			
Step Type	**Temperature °C**	**Time Sec.**	**Cycling**
Initial Denaturation	**95**	**3 min.**	**1**
Denaturation	**95**	**40 Sec.**	**30**
Annealing	**55**	**40 Sec.**	
Extension	**72**	**40 Sec.**	
Final extension	**72**	**3 min.**	**1**
Hold	**4**	**α**	**4**

3.8: Assurance of the IL10 genotype:

The genotypes of IL10 SNP (NCBI SNP CLUSTER ID: rs1800871, rs1800872) were analyzed by polymerase chain reaction (PCR) according to the conventions depicted before Govatati et al., (2012). PCR enhancement was performed in a programmable warm cycler angle PCR framework (Labnet USA). The PCR result of 588 bp was electrophoresis by 1.5 % agarose gel pre-staining with ethidium bromide and afterward sequenced (Macrogen, Korea).

3.9:Tetra Arms PCR protocol:

This technique was followed to determination the alleles of IL10 gene. Arms PCR performed for detection mutation, it produces two different alleles for a gene; one mutant and another normal. The Allele-specific

PCR has the power to detect a single specific allele. The four primers followed Kurdistani *et al.*,2015, their sequence shown in table(3-6).

3.10:Detection of Amplified Products by Agarose Gel Electrophoresis

The amplified products of PCR were separated by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.agarose gel was prepared by dissolving 1.5g of agarose powder in 100 ml of 1X TBE buffer (pH:8) in flask then placed in microwave for 2minutes ,and waiting for cool to 50^{o}C and adding about 1.2µl of ethidium bromide stain concentration (0.5mg/ml) (Sambrook and Russell, 2001).

The first well we put the 4µl of DNA ladder(100-2500bp). The electric voltage 100 volt at 45min (Sambrook and Russell, 2006).

3.10.1: Primers Design:

PCR for molecular identification of MBL2 were performed using specific primer pair which was designed in this study First one amplified partial sequence of MBL2 gene was tested by graphic location of the subjected gene in the gene bank of National Center for Biotechnology Information (NCBI) .

3.10.2: DNA Sequencing of PCR amplicons

The resolved PCR amplicons were commercially sequenced at the sequencing company (Macrogen Inc. Seoul, South Korea). Only clear chromatographs obtained from ABI sequence files were further analyzed, ensuring that the annotation and variations are not because of PCR or sequencing artifacts. By comparing the observed DNA sequences of local bacterial samples with the retrieved neighboring DNA sequences of the NCBI Blast engine, the virtual positions and other details of the retrieved PCR fragments were identified.

3.10.3: Interpretation of sequencing data

The sequencing results of the PCR products of different samples were edited, aligned, and analyzed as long as with the respective sequences in the reference database using Bio Edit for multiple alignment Sequence based on Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). And based on Genius primer software, the observed variations in each sequenced sample were numbered in PCR amplicons as well as in its corresponding position within the referring genome.

3.10.4: Checking the novelty of SNPs

The observed SNPs were submitted to the dbSNP database to check their originality. Each particular SNP was re-positioned according to its place in the reference genome subsequently, the determination of the presence of previous SNP was performed by viewing its corresponding dbSNP position. Then, all dbSNP positions for all observed SNPs were documented respectively.

3.11: Statistical Analysis

Chi square test was used to test the deviation from Hardy-Weinberg Equilibrium (HWE) of SNPs by comparing the observed and expected frequencies. The association of different genotypes with the risk of teeth decay was estimated by calculation of odds ratios (OR) with 95% confidence intervals (CI). Statistical significance was set at $p < 0.05$. based on SPSS software.

4.1. Collection and culturing of different samples

A total 50 samples from patients with severe tooth decay were obtained from various hospitals and diagnosed by a specialized doctor; the control group was made up of 50 randomly selected healthy individuals who did not have tooth decay as shown in table (4-1). All of them have not taken an antibiotic in the past three days, and their ages range from (10 to 50 or more) years old between the septemper 2022 to february 2023. Sample distribution according to gender and age groupswere shown in tables (4-2 and 4-3). The result of statistical analysis of Specimen distribution according to age groups in studed population. show that significant in age 10-15 years olde (p value =0.020), age 21-25 years olde (p value =0.033) and age 51 or more years olde (p value =0.002). Also the results of Specimen distribution according to gender in studed population (p value = 0.689).

Table (4-1): specimen distribution according to study groups.

Study groups	No.	%
Control	50	50
Patient	50	50
Total	100	100

Table (4-2): Specimen distribution according to gender in studed population.

Study groups	Control	Patient	Total	p-value
	No.(%)			
Male	23(46)	25 (50)	48(48)	0.689
Female	27(54)	25 (50)	52(52)	
Total	50(50)	50(50)	100(100)	

Table (4-3): Specimen distribution according to age groups in studed population.

Study groups	Control	Patient	Total	p-value
	No.(%)			
10-15	12 (24)	3(6)	15 (15)	0.020*
16-20	4 (8)	3(6)	7 (7)	0.705
21-25	11(22)	3(6)	14 (14)	0.033*
26-30	5(10)	7(14)	12 (12)	0.564
31-35	5(10)	3(6)	8 (8)	0.480
36-40	5(10)	5(10)	10 (10)	1.000
41-45	3 (6)	3(6)	6(6)	1.000
46-50	3 (6)	8(16)	11 (11)	0.132
51or more	2(4)	15(30)	17 (17)	0.002**
Total	50(100)	50(100)	100 (100)	

Oral microbiome composition is suspected to be affected by additional variables including host genetics (Gomez *et al.*,2017) geography (Gupta *et al.*,2017), diet (Ribeiro *et al.*,2017), age (Gomez and Nelson, 2017; Lira-Junior *et al.*,2018) and cohabitation (Ross et al.,2017). The excessive occurrence of dental caries in different ages is a prime oral fitness hassle which calls for early intervention (Moca *et al.*,2021). The high decay infections in old ages may correlated with the first everlasting molars and a correlation among the growth in carious incidence and the growth in age based on results of Al-Samadani and Ahmad (2012). also these results consistence with both of Bernabé (2014) and Al-Malik and Rehbini (2006) when they concluded that caries tiers growth with age and

stay a main trouble withinside the person population and excessive carious degree withinside the everlasting dentition. Dental caries is particularly because of the movement of acids produced through microorganism similarly to many different factors. Recent genetic research has mentioned that some of genes are related to the susceptibility to dental caries. The majority of those genes are related to inflammation, elevated susceptibility to infection, and dentine matrix formation (Loesche 1996).

The comparative studies between European, African, Asian, and American populations discovered microbial variation between populations, and other studies describe ethnicity-specific clustering within the United States (Mason *et a*l.,2013; Blekhman *et al*.,2015) . The effect of diet on oral microbiome composition was assessed through the discovery of bacterial shifts that occurred as human societies transitioned from a hunter-gatherer diet to more carbohydrate-rich diet associated with farming (Adler *et al.*,2013). Diets associated with Western industrialized societies have been shown to lead to poor oral health conditions, with bacterial relative abundances being affected by high sugar content; however, the full effects of the modern high carbohydrate and sucrose diet are still being elucidated (Goodson *et al*.,2017).

Some studies have suggested that women may have a more diverse oral microbiome than men while others have found no significant differences. Additionally, certain health conditions, such as diabetes and periodontal disease, have been associated with changes in the oral microbiome (Clough,2011;Wade,2013).

As individuals age, their oral microbiomes change and periodontal pathogens increase in abundance, leading to higher oral disease

susceptibility (Papaioannou *et al.*,2009 ; Gomez and Nelson,2017). Oral microbiomes of children are likely influenced by the increasing independence in oral health habits, transition to permanent dentition, and progression to an adult-like diet (Ribeiro *et al.*,2017).

4.2. Prevalence of isolates among teeth decay Samples

Identification of all teeth decay causative microorganisms was performed by classical microbiological methods. The analysis of clinical samples obtained from teeth decay can provide valuable information for the diagnosis.

The results on Figure (4-1) Show the distribution of microorganism teeth decay source of according to type of microbe fungi source (31% while the yeast (69%) .The Figure (4-2) Distribution of yeast associated with dental caries specimen according to study group yeast in patient (69%) while in healthy (30%) . The Figure (4-3). Distribution of fungi associated with dental caries specimen according to study group fungi in patient (47%) while in healthy (52%) . Figure (4-4). Distribution of yeast associated with dental caries specimen according to gender in study group yeast in male (51%) while yeast in female (48 %) . Figure (4-5). Distribution of fungi associated with dental caries specimen according to gender in study group fungi in male (43%) while in female (56 %).

The microbiology of dental decay is complex and can be influenced by research is needed to fully understand the relationship between these factors and the distribution of microorganisms in teeth decay samples. Some studies have suggested that there may be gender differences in the prevalence and severity of dental caries. For example, one study found that adolescent girls had a higher prevalence of dental caries than boys, while another study found that adult women had a higher incidence of root caries than men. However, other studies have found no significant

gender differences in the prevalence or severity of dental caries (Martinez-Mier Eand Zandona,2013; Shaffer *et al.*,2015).

The results in figure (4-6) and table (4-4) Yeast *Candida* spp. were 96% while the Yeast *Rhodoturella* 4% and the figure (4-7) and table (4-4) *Aspergillus* 93% of infections the *Pencillium* was 6% .

Fungi are often associated with oral disease (de Carvalho *et al.*, 2006, Dagistan *et al*. 2009, Canabarro *et al.*, 2013). Fungi are highly prevalent and diverse within the healthy oral cavity (Ghannoum *et al*., 2010).

Persoon *et al.*, (2017) *found that the Candida* and, to a lesser extent, *Saccharomyces*, *Aspergillus* and *Rhodotorula* were the most frequently isolated fungal genera within root canal infections. This is similar to studies into general fungal disease Brown *et al.*, (2012) .

Also Carvalho *et al.(* 2015) performed a study on 2-5 years old children in Brazil showed that the outbreak of *candida albicans* in cervical decay of ECC group (60.4%) was significantly more than the caries free group (14.3%) and proximal caries (12.5%) and these findings were similar to our results .

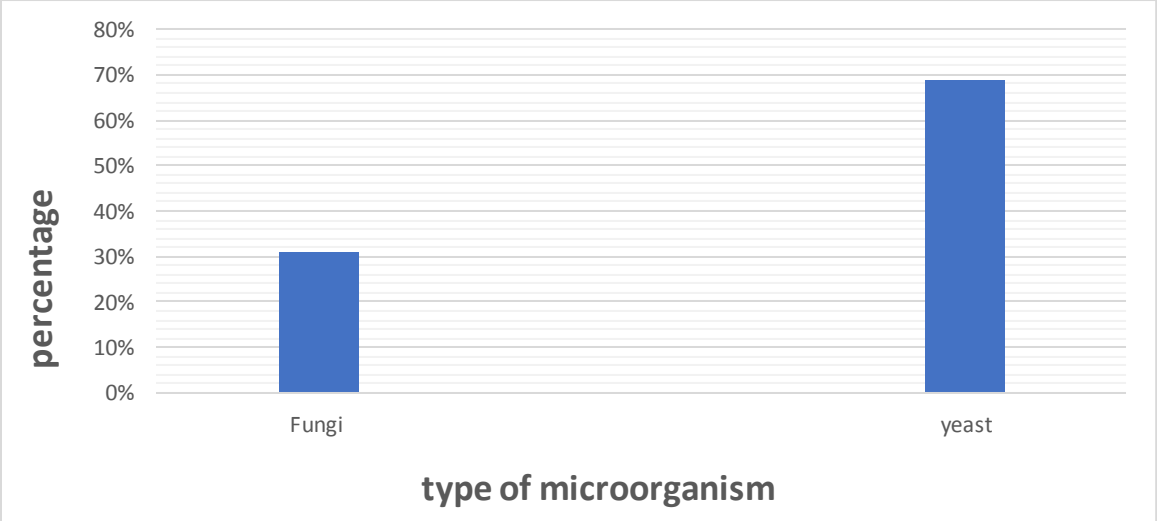


Figure (4-1) the percentage of microorganism distribution associated with dental caries Specimen.

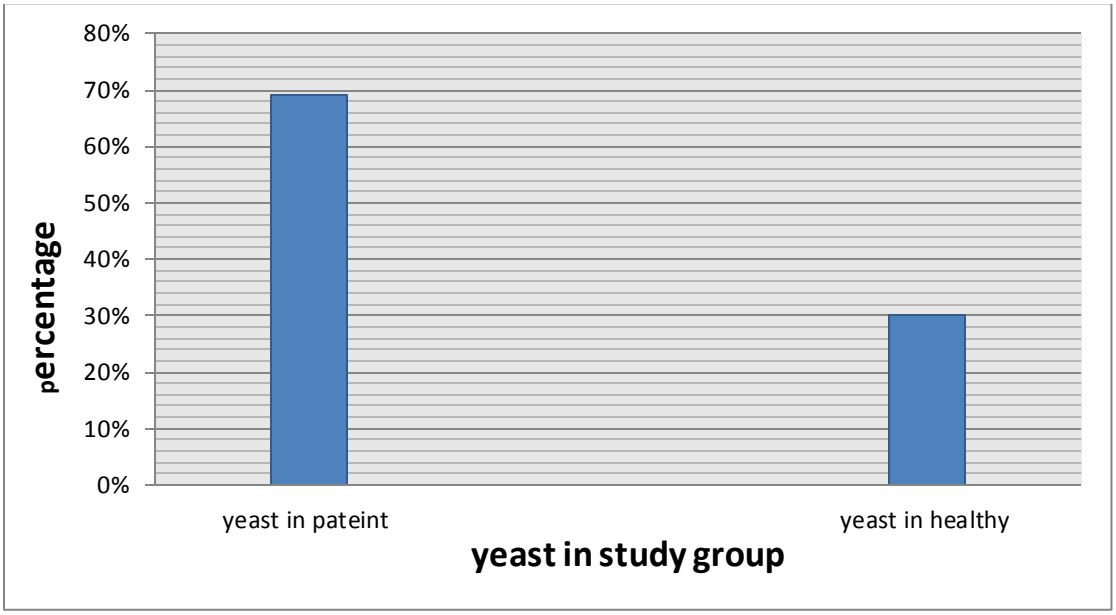


Figure (4-2) Distribution of yeast associated with dental caries specimen according to study group.

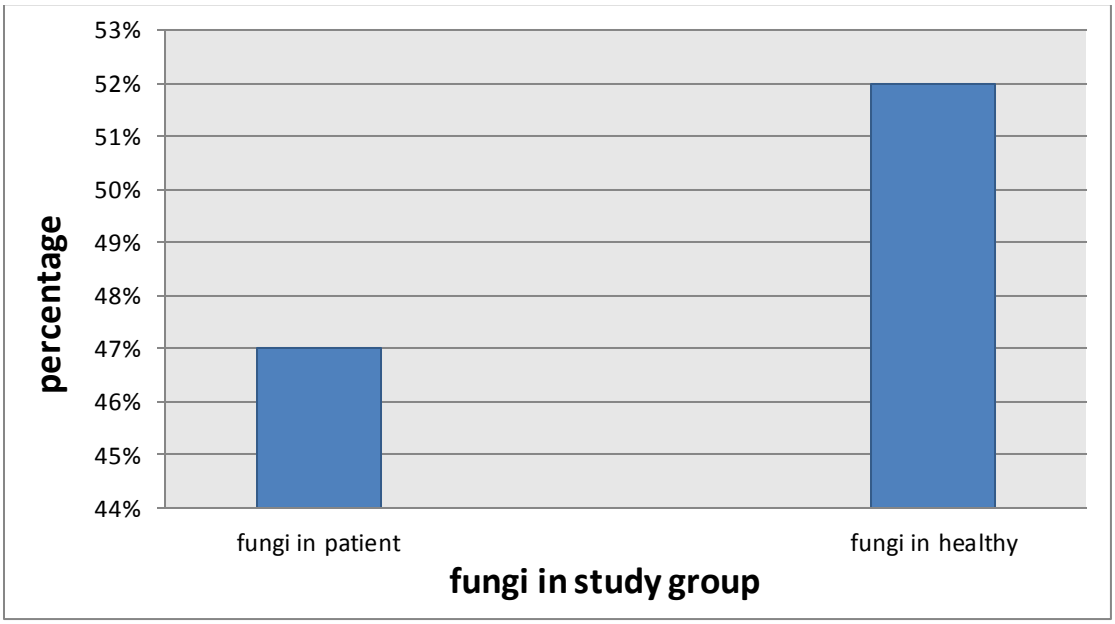


Figure (4-3). Distribution of fungi associated with dental caries specimen according to study group.

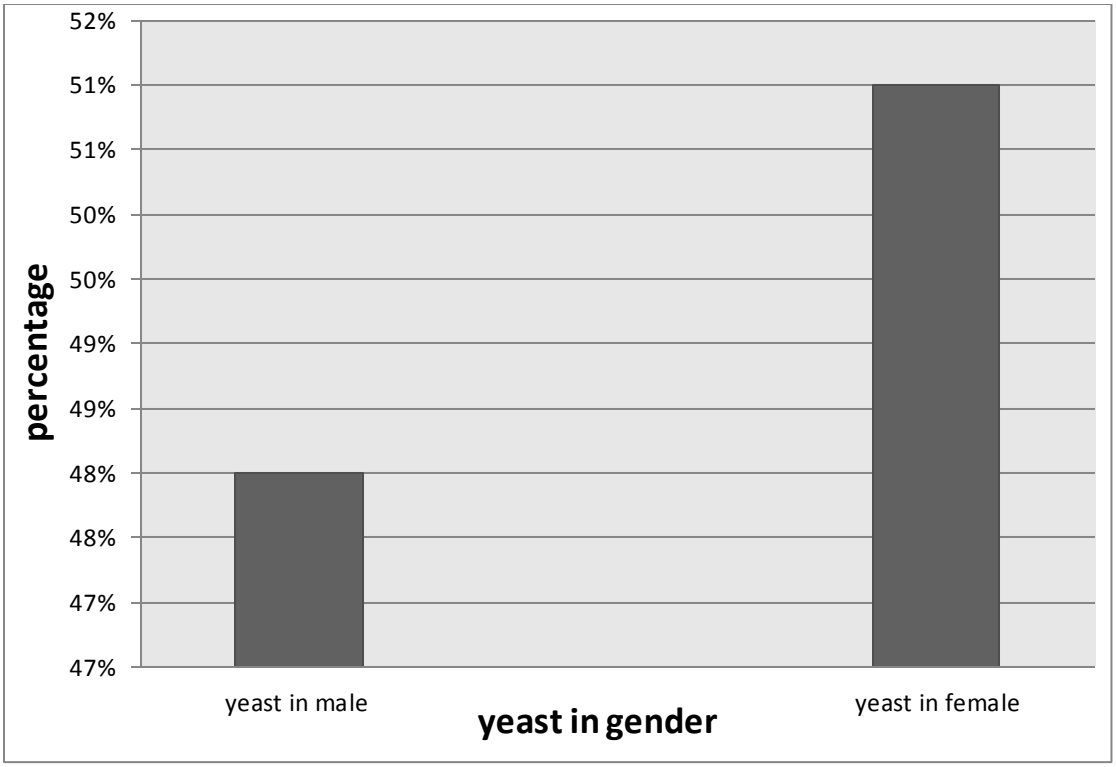


Figure (4-4) Distribution of yeast associated with dental caries specimen according to gender in study group.

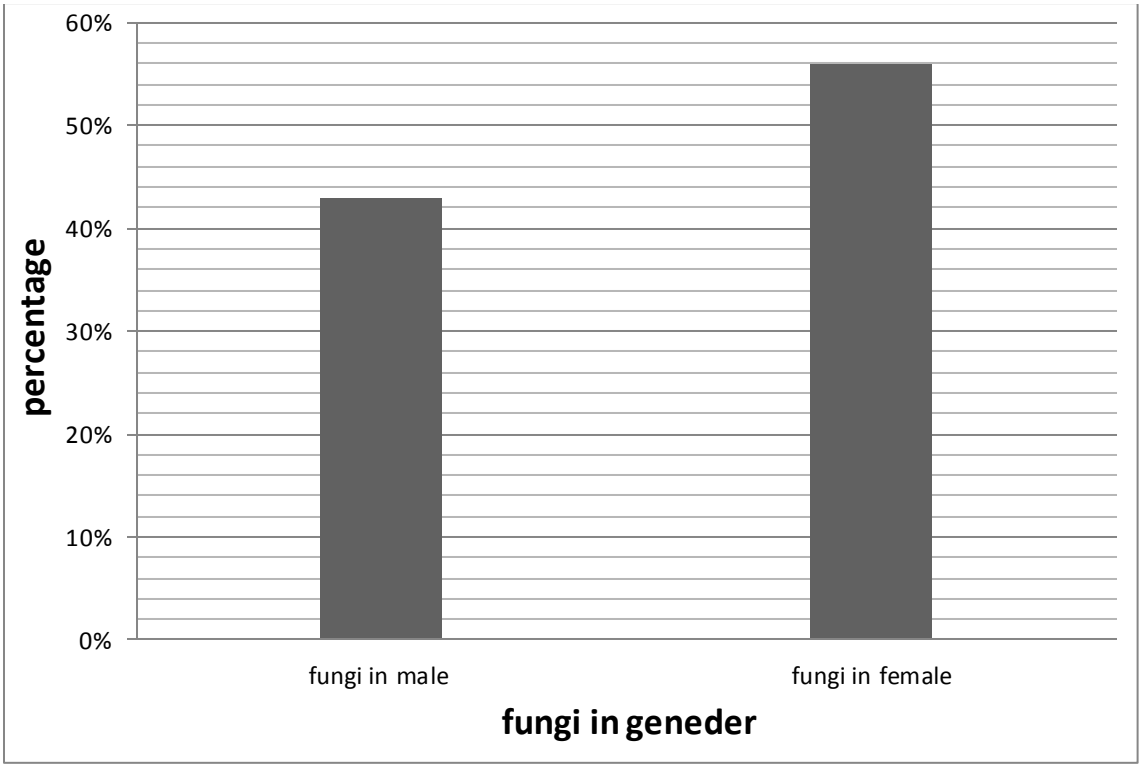


Figure (4-5). Distribution of fungi associated with dental caries specimen according to gender in study group.

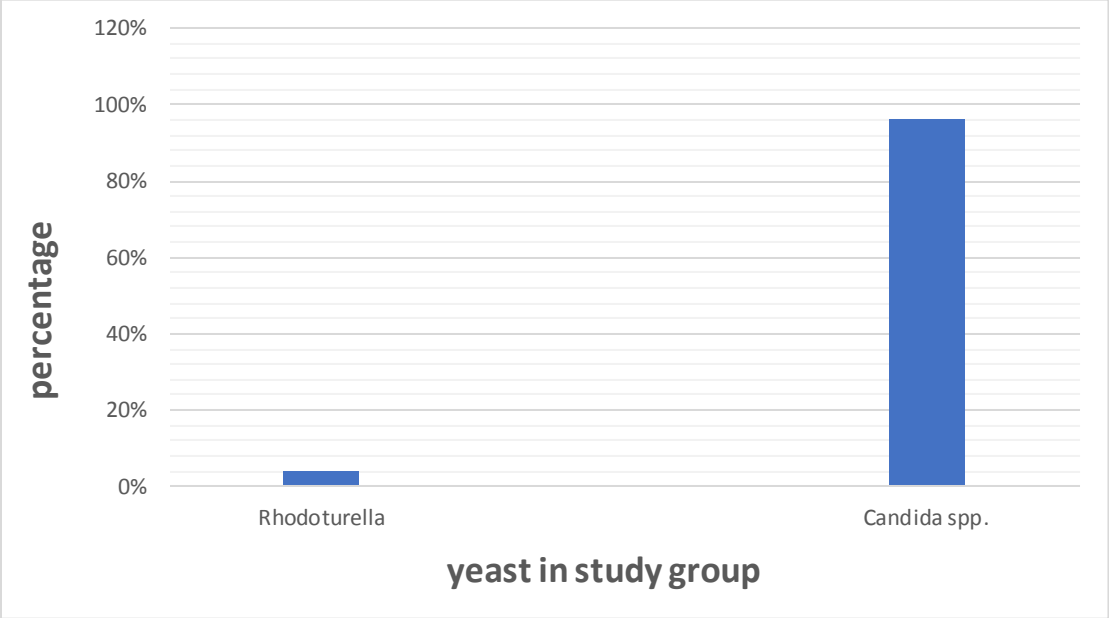


Figure (4-6). Prevalence of yeast in teeth decay specimen in studed population.

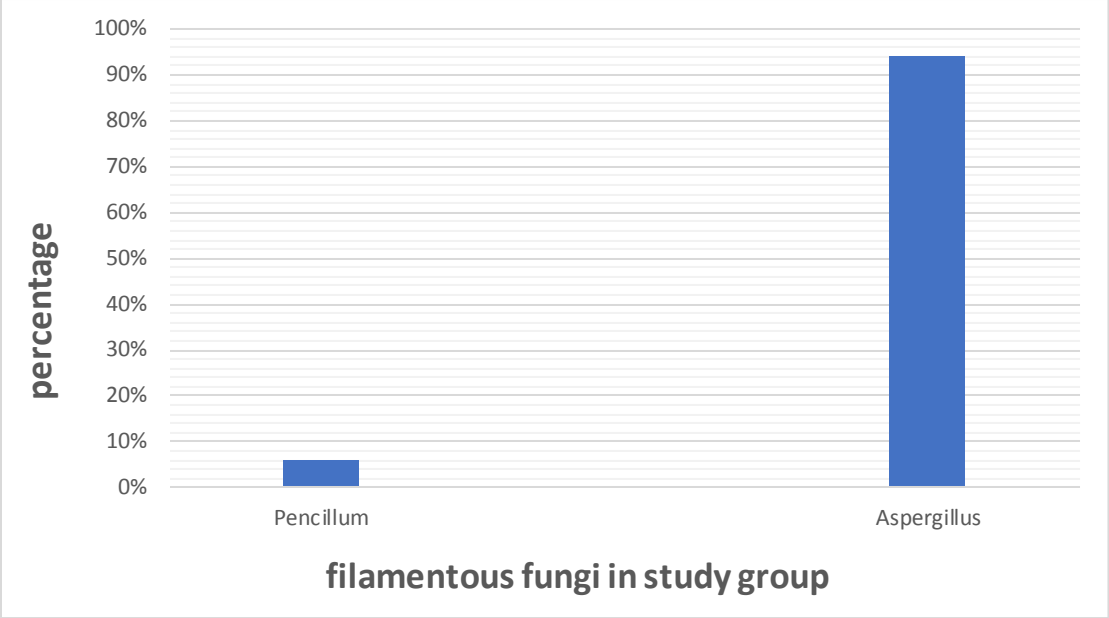


Figure (4-7). Prevalence of filamentous fungi in teeth decay specimen in studed population.

Table (4-4): Prevalence of fungi in teeth decay specimen in studed population.

The microbe	Type of microbe	No. of isolate	Percentage of isolate (%)
Yeast	*Candida spp.*	67	96 %
	Rhodoturella	3	4 %
filamentous fungi	*Aspergillus*	43	93 %
	Pencillium	3	6 %

4.2.1. Isolations and identification of *Candida* spp. on SDA:

The results of isolation *Candida* spp. correlated with dental plaques shown variation in abundance of colony frequent per plate. Some patient shown high colony numbers, others shown few colonies The case No. 15 for example, shown large numbers of colonies at the same time the colony size were large compared with colony size in other cases 15 and 20. Case 15 shown small with high frequent colonies compared with others cases Figure (4-8).

Our result revealed prevalence of many *Candida* spp. correlated with dental caries , the most common *C.albicans, C.parapsilosis* and *Candida tropicalis*. These results agree with the Eidt *et al*.,(2020). They show that Prevalence of *Candida* spp. withinside the oral hollow space ranged from 7.7 % to 78 %. Prevalence of dental caries in people harboring *Candida* spp. ranged from 27.2%–100% and become better than in people now no longer harbouring *Candida* spp. (Khadija *et al* .,2019).

Figure(4-8): Variation of *Candida* spp., colonies appearance in numbers and sizes on SDA incubation condition 48h.at 30°C.

4.2.2. Preliminary identification of Candida spp.on CHROMagar

The results of preliminary identification of *Candida* spp. based on based on Nadeem *et al.*,2010. The chromogenic reaction with substrate of medium, more *Candida* spp. isolated from teeth plaques shown apple green color, this color on CHROMagar considered as *C.albicans*, some isolates shown pale-rose and blue color as *C.parapsilosis and C.tropicals* respectively, as in Figure (4-9).

The results of CHROMagar as preliminary identification of *Candida* spp. was shown this can without difficulty pick out 4 species of *Candida* on the idea of colonial color change substrate, and as it should be differentiate among them. *C. albicans, C. tropicalis, C.parapsilosis and C. krusei*. In spit of goodness results of CHROMagar, but it's still low productivity for perfect diagnosis for all *Candida* spp. our results was consistent with Nadeem *et al.*,2010. They referee to the specificity and sensitivity of CHROMagar *Candida* for *C. albicans* calculated as 99%, for *C. tropicalis* calculated as 98%, and C. krusei it's 100%.

This study confirmed that some species of *Candida* isolated from teeth decay, have virulence gene like *ALS* and *HWP*, the genetic detection of virulence gene of biofilm formation considered more reliable tool

compared with conventional test such as crystal violate or suffranin stained *Candida* suspension on glass. The result of presence virulence gene in *Candida* spp. was consistent with many studies done in Egypt and barazil (Shrief *et al*., 2019; Goulart *et al.*,2018) .

In Egypt, the biofilm ability become determined in 58% of *Candida albicans* isolates from the chemotherapy patients. Also, the superiority of *ALS1* and *HWP1* genes a few of the biofilm-superb *C. albicans* become 56.9% (Shrief *et al*., 2019) (Nobile *et al.*,2006). Inci et al. confirmed the presence of *ALS1* and *HWP1* genes in 83.1% and 11.3%, respectively, of biofilm-generating isolates (Inci *et al.*,2013).

The result of study performed in Brazilian ladies confirmed that the frequency of *ALS1* and *HWP1* genes withinside the *C. albicans* gathered from vaginal discharge become 73.6% and 21%, respectively (Goulart *et al.*,2018). Differences withinside the kind of medical samples and strategies used for analysis might also additionally motive versions withinside the frequency of genes in diverse research. There are many reviews which have been worried with the expression of *ALS* and *HWP* genes. The outcomes of our look at and numerous different research have proven that the frequency of the presence of those genes and their expression in medical isolates can suggest the function of those genetic markers in selling the adhesion and biofilm formation in *Candida* species, particularly *C. albicans*.

Figure(4-9): Preliminary identification of *Candida* spp., on CHROMagar medium, *C.albicans* shown apple green color, *C.parapsilosis* shown pale-rose color, *C.tropicals* shown blue color.

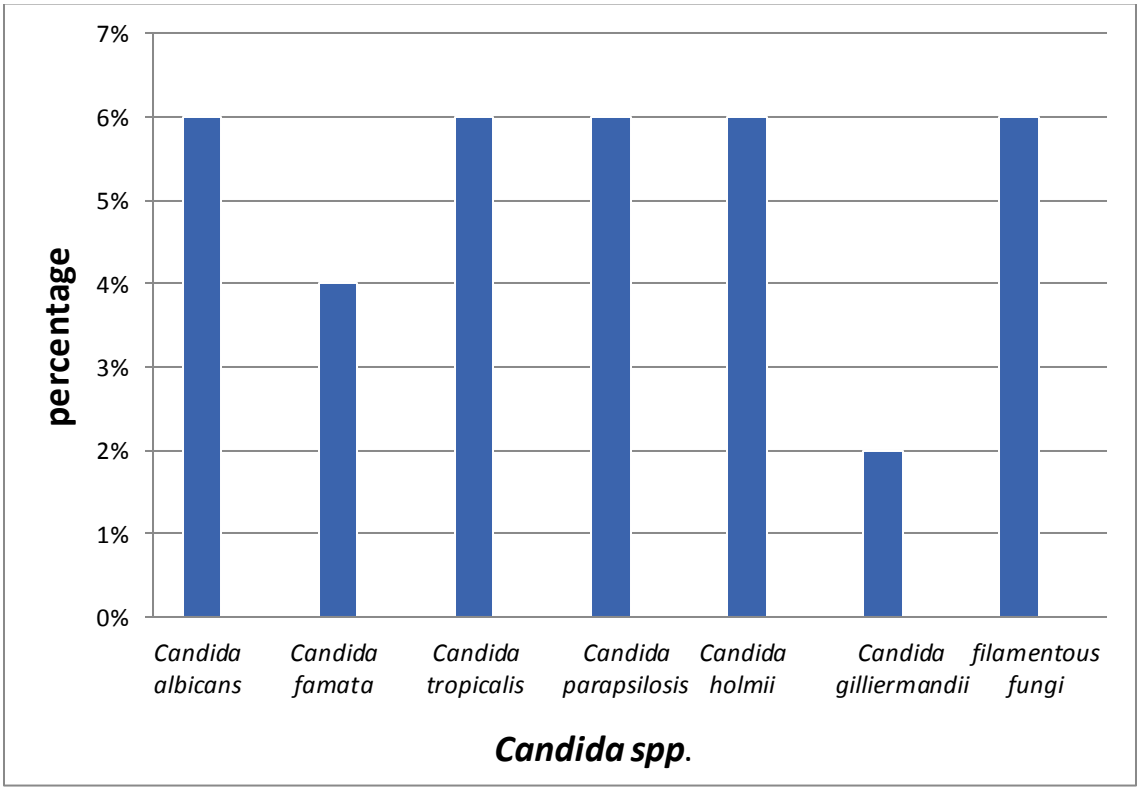


Figure (4-10). Distribution of clinical specimen source of according to isolates of *Candida* Spp. in studed population.

4.2.3. Genotyping *Candida* spp. based on ITS barcod.

The results of amplification ITS1-5.8S –ITS2 and flanking of primer pairs ITS5/ITS4 showed variation in the amplicons sizes of 15 isolates of *Candida* spp isolated from teeth plaques of patient samples population under interest. Based on the amplicon size key of Fujita *et al.*, (2001) diagnosed six *Candida* spp. genetically, they are *C. parapsilosis* = 516bp; 2 *C. albicans* = 535bp, 3-*C.tropicalis*= 521bp; 4,5,9-13 *C.famata* = 633bp, 6-8-*C.gilliermandii* =603bp, 14-*C.holmii* =717bp and filamentous fungus 750bp. (4-8). These results indicate the presence of different fungal species based on their specific PCR product sizes, allowing for genotyping and identification of the organisms.

The conventional techniques for identification *Candida* spp are regularly primarily based totally at the exam of phenotypic characters. PCR method may be time-eating and the reliance at the variable expression of phenotypic traits can cause inconsistent results (Miyakawa *et al.*,1993) and therefore genotype-primarily based totally processes can be preferable. The phenotypic identity of yeasts and filamentous fungi based on cultures takes approximately five to 10 days. The ITS region considered as good barcoding region for direct identification most *Candida* SPP., the results of this study was agree with (Fujuta.,2002).

Shi *et al.*, (2016) examined each denture sample for the presence of Candida and other fungus and bacteria using PCR (targeting ITS), using oral samples from 20 denture-wearing patients (10 with denture stomatitis and 10 healthy denture wearers). Only 50% of the samples from the healthy group had a positive identification for *Candida*, compared to 90% of the samples from the group with stomatitis.

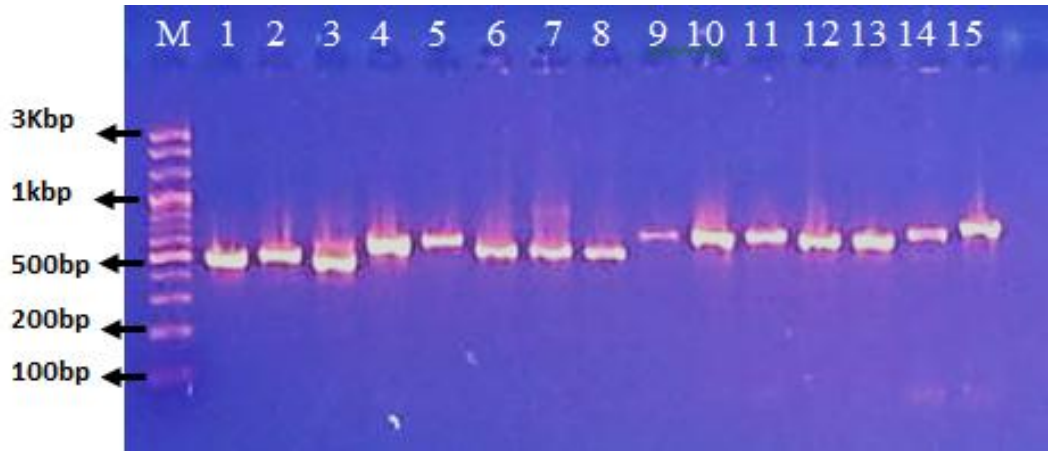


Figure (4-11): Gel electrophoresis profile of PCR product genotyping by ITS5-ITS4 primers; 1- *Candida parapsilosis* =516bp; 2 *C. albicans* =535bp, 3-*C.tropicalis*=521bp ; 4,5,9-13-*C.famata*=633bp, 6-8-*C.gilliermandii* =603bp, 14 *C.holmii*=717bp and filamentous fungus 750bp.

4.2.4.Genetically detection of virulence factor biofilm gene in *Candida* spp.

Molecular detection of virulence factors of *Candida* spp. The isolates identified by conventional methods as *Candida* spp. were confirmed by using spesific gene. *ALS1* were detected in 3 (30%) each of these isolates Figure(4-11). Only three isolates were included *ALS1* gene Figure (4-12) . The pathogenicity of *Candida* species is increased with factors such as the ability to produce hyphae, binding and adhesion, extracellular enzyme production, and biofilm formation (Mayer *et al.*,1013; Rodríguez-Cerdeira et al.,2019). The agglutinin-like sequence (*ALS*) gene family is the largest gene family known in *C. albicans* and is considered one of the important factors in the adhesion of the organism and the formation of biofilm (Hoyer,2001). The *ALS* gene family consists of several genes including the *ALS1-ALS7* and *ALS9* genes, which play a role in the production of cell surface glycoproteins, leading to increased adhesion to host cells (Hoyer&Cota ,2016). *ALS1* and *ALS3* genes affect adhesion to host epithelial cells and endothelial cells (Nailis *et al.*,2009). Shrief *et*

al.(2019) reported a frequency of 77% for the *HWP1* gene and 65% for the *ALS1* gene of *C. albicans* isolated from blood and urine cultures. They also described that 58% of *C. albicans* were capable of biofilm production .

Most infections caused by *C. albicans* are related to the creation of a biofilm on the surface of the host or on abiotic surfaces (implants), which leads to high morbidity and mortality (Tsui *et al.*,2016). Because *C. albicans* can transition from yeast to hyphae morphologically, its biofilm is a complex structure of different morphological forms (Priya *et al.*,2020).

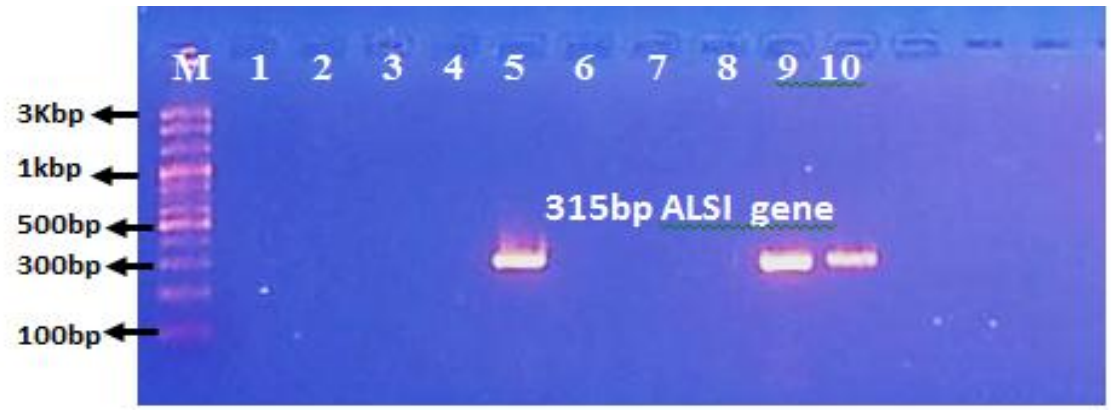


Figure (4-12): Illustrated of *ALS* genes in *C. famata* isolates shown positive PCR product of *ALSI* gene= 315bp for *C. famata* in isolates: 5,9-10:*ALS1* gene shown negative result(no bands) in other *Candida* spp.). (M: Marker (100 bp for each step.

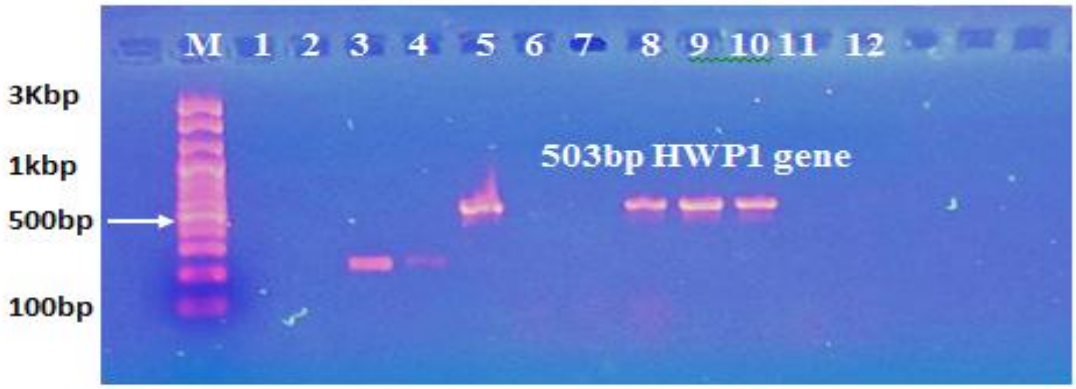


Figure (4-13): Illustrated of *HWP1* genes in *C. famata* isolates shown positive PCR product of ALSI gene= 315bp for *C. famata* in isolates: 5, 8-10: *HWP1* gene shown negative result(no bands) in other *Candida* spp, two extra band were observed in lane 3 and 4 with size 250bp. .). (M: Marker (100 bp for each step.

4.3. Immunogenic detection of allele risk in genes *MBL2* and *IL10*:

The profile gel-electrophoresis 21 genomic DNA whole blood samples Figure (4-14) was shown huge bright bands of DNA extracted from whole blood of human patients undergo teeth plaques symptoms.

The excessive occurrence of dental caries in different ages international is a prime oral fitness hassle which calls for early intervention. Dental caries is particularly because of the movement of acids produced through microorganism similarly to many different factors (Aas *et al.*,2008).. Recent genetic research has mentioned that some of genes are related to the susceptibility to dental caries. The majority of those genes are related to inflammation, elevated susceptibility to infection, and dentine matrix formation.

The development of dental caries is known to result from interactions between the presence of oral bacteria, host characteristics, dietary choices and length of exposure time (Kutsch and Young,2011). The host's genetic makeup appears to play an important role the development of dental caries (Shuler.2001). There is evidence that cytokines are important in

regulating and controlling the inflammatory response to bacterial infection (Floege and Gröne,1995; Miller *et al.*,2009). Genetic and immunological differences between hosts may also be an important risk factor for dental caries. Although the role that cytokines play in the aetiology and mechanism of dental caries remains unclear (Soell *et al.*,1994).

Figure (4-14): Gel-electrophoresis illustration quality of DNA extracted from teeth plaques patients group, 1-21 patient samples, M=molecular marker 100bp for first step.

4.3.1. The amplification of targeted region of *MBL2* gene :

The results shown success the primer pair efficiency to amplification region 52185415-52186396 as target DNA region of *MBL2* gene included, the amplification region with flanking primers 614bp Figure (4-15).

Two human *MBL* genes (*MBL-1* and *MBL-2*) are closely positioned on the long arm of chromosome 10 at q11.2–21 (Hansen and Holmskov,1998). There are few studies investigating the relationship between *MBL-2* gene polymorphisms and dental caries (Pehlivan *et al.*,2005). *MBL-1* is a pseudo gene and only MBL-2 encodes a protein product. Three polymorphic sites have been identified in exon1 of the *MBL-2* gene, at codon52 (CGT to TGT), codon54 (GGC to GAC) and codon57 (GGA to GAA) (Kilpatrick, 2002).

In study done by Yuan *et al.*, (2013) investigated the relationship between *MBL-2* gene polymorphism and children with severe early onset caries. The frequencies of three polymorphic sites in the *MBL-2* gene vary between different ethnic groups.

This *MBL2* gene encodes the soluble mannose-binding lectin (*MBL2*). The protein encoded belongs to the collecting family and is an important element in the innate immune system. The protein recognizes and binds to mannose and N-acetylglucosamine on many microorganisms , bacteria and viruses including influenza virus, HIV and SARS-CoV. This binding activates the classical complement pathway. Deficiencies of this gene have been associated with susceptibility to autoimmune and infectious diseases (Nedovic *et al.*,2014),

The *MBL2* gene is placed on chromosome 10, and is accountable for the synthesis of *MBL2* protein (Turner 2003). may also have an effect on *MBL2* gene expression and adjust the synthesis of *MBL2* protein. These polymorphisms had been investigated in numerous diseases, wherein innate immunity performs an crucial position, specifically in bacterial infections, tuberculosis, rheumatic fever, sepsis, lupus erythematosus (Bohlson *et al.*, 2007).

The cells of innate immunity specific sample popularity receptors that, after activation, are worried withinside the maturation of antigen-offering cells generating numerous mediators that play an crucial position in immune response. Mannose binding lectin 2 (*MBL2*) belongs to the sample-popularity soluble receptors and is accountable for the supplement machine activation through the lectin pathway(Ibernon M. *et al.*, 2014). *MBL2* hence has an crucial position withinside the defence towards numerous microorganism activating macrophages, and different

cells worried withinside the phagocytosis of pathogens. Previous research have indicated that *MBL2* additionally performs crucial position in ischemia–reperfusion after kidney transplantation, in kidney allograft rejection, and in long time kidney allograft function (Ibernon *et al.*, 2014).

Based on sequencing results of *MBL2* gene , one single-nucleotide polymorphisms (SNP) in *MBL2* become decided in 30 patients with caries and in 30 age-matched caries-unfastened controls. The SNP rs7096206 G>C examined with inside the on MBL2 gene. The *MBL2* gene becomes proven to be related to a excessive occurrence of caries in our cohort. In addition, In conclusion, the facts imply that rs7096206 G>C with inside the *MBL2* gene become proven to be related to a excessive occurrence of caries in our cohort, and a pair of haplotypes also are concerned with inside the elevated susceptibility to dental caries. Alyousef *et al.,(*2017).

There are two human *MBL* genes but *MBL-1* is a pseudogene and only *MBL-2* encodes a protein product. *MBL-2* comprises four exons with exon 1 encoding a signal peptide, a cysteine-rich region and part of the glycine-rich collagenous region. Exon 2 encodes the remainder of the collagenous region and exon 3 encodes an α-helical coiled-coil structure which is known as the ‘neck’ region (Turner , 2003)

MBL has been proven to bind to a extensive variety of micro-organisms (Neth *et al.,* 2000) and in a few instances there is a great correlation with recognised structural functions. Nevertheless, it isn't feasible to extrapolate from the mounted biochemistry and make predictions approximately interactions with character micro-organisms.

The acquisition of designated information of the quality structure of *MBL* has been paralleled through a chain of courses reporting huge binding of this collectin to precise microorganisms. Such reviews have offered proof of MBL binding to person pathogens, consisting of human immunodeficiency virus kind 1 and influenza A virus (Ezekowitz *et al.*,1989), and to the yeasts *C. albicans* and *C. neoformans* (Schelenz *et al.*,1995).

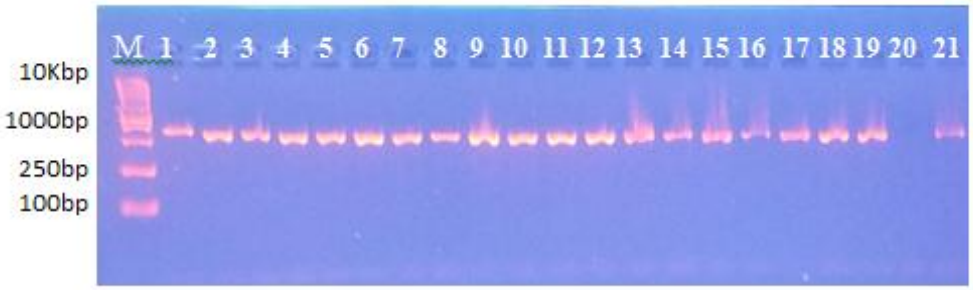


Figure (4-15): Gel electrophoresis profile of target DNA region of MBL2 gene for patient group amplification region with flanking primers, 1-21 patient PCR products 614bp, M=molecular marker, first step100bp.

4.3.1.1. Location of Mannose binding lectin 2(MBL2) gene:

The MBL2 gene located on Chr: 10q21.1 with length7405 nt . This gene encodes the soluble mannose-binding lectin or mannose-binding protein found in serum. The protein encoded belongs to the collectin family and is an important element in the innate immune system. The protein recognizes and binds to mannose and N-acetylglucosamine on many microorganisms, including bacteria, yeast, and viruses including influenza virus, HIV and SARS-CoV. This binding activates the classical complement pathway. Deficiencies of this gene have been associated with susceptibility to autoimmune and infectious diseases.

MBL2 is a gene encoding serum agglutinin secreted by the liver, which plays an important role in the natural immune defense system; the protein contributes to complement activation, opsonophagocytosis, inflammatory response regulation, and apoptosis (Shimomura-Kuroki *et al.*,2018). The *MBL2* gene contains four exons and three introns; it is located on chromosome 10(Kilpatrick, 2002). *MBL2* is a candidate susceptibility gene for infectious diseases: the association

between *MBL2* gene polymorphisms and caries susceptibility was first reported by Pehlivan *et al.*,(2005) who analyzed two SNPs (codons 54 and 57) using polymerase chain reaction restriction fragment length polymorphism assays; however, they did not find any significant associations. Subsequently, Olszowski *et al.*,(2012) studied the relationships of *MBL2* (rs1100325 and rs1800450) gene polymorphisms and caries in children; they found that allele G was a risk factor for caries. Other studies have revealed similar results (Alyousef *et al.*,2017, Mokhtari *et al.*,2019).

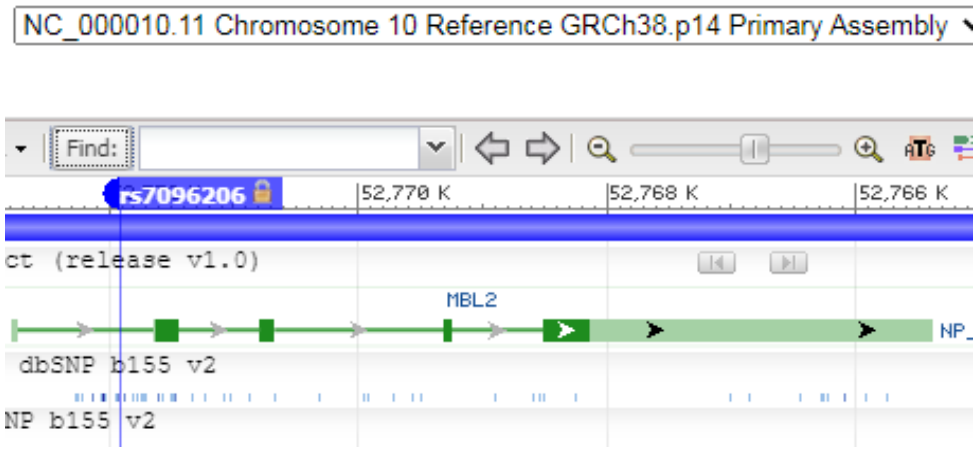


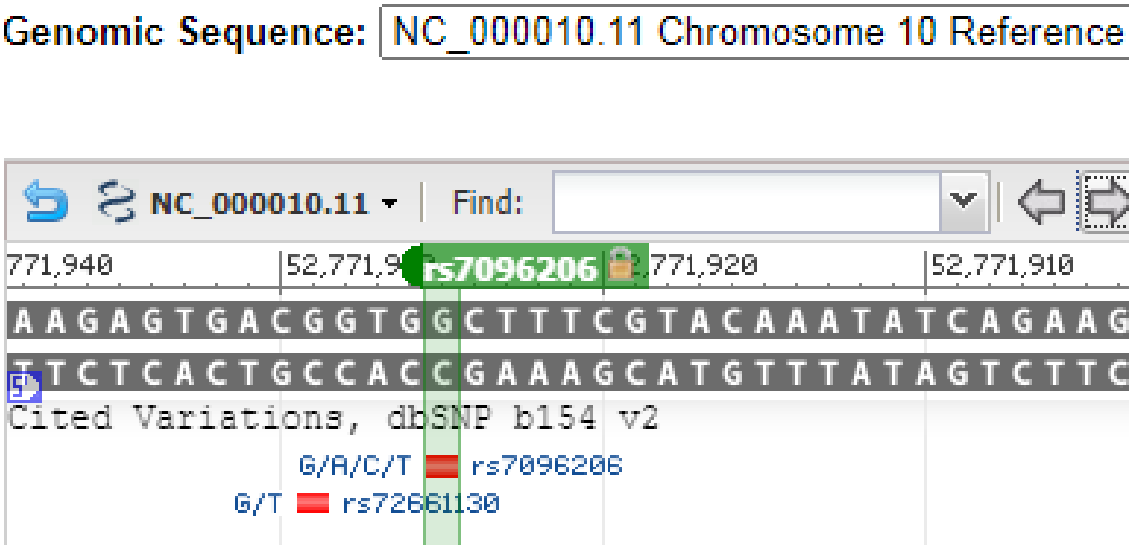
Figure (4-16): Illustration MBL2 location in Chr:10, and site SNP rs7096206 G>C.

4.3.1.2. Detection of SNP Validity with NCBI:

MBL2 genes were identified in the Ensembl database, the gene in the Splice region variant and Upstream gene variant data were selected

4.3.1.3. Validity of rs7096206 G>C:

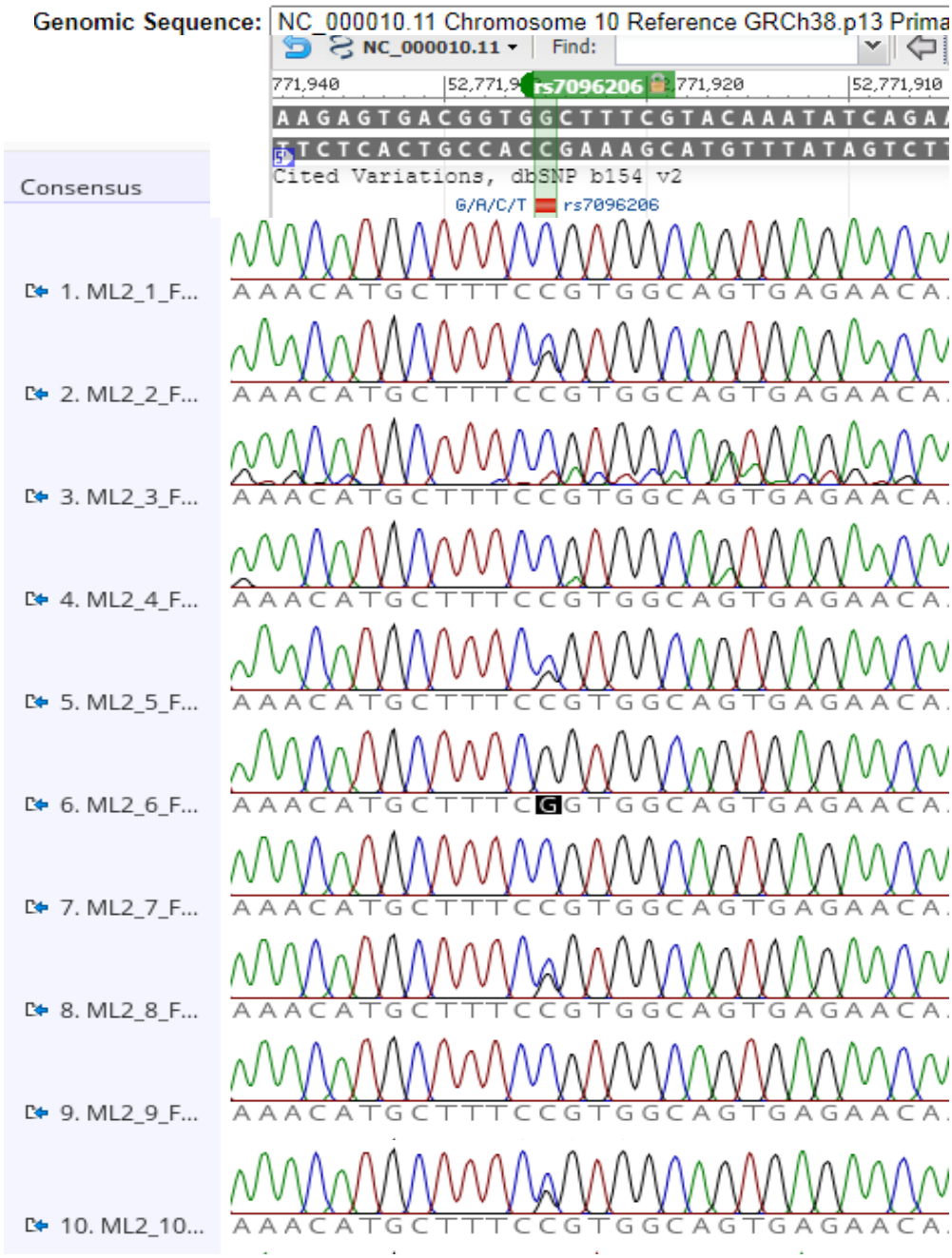
To conformed the validity of SNP: rs7096206 G>C, the site SNP was at 52771925 on Chromosome 10, a combination of SNP site of rs7096206 G>C Homozygous allele G mutant to allele C, Figure (4-17).



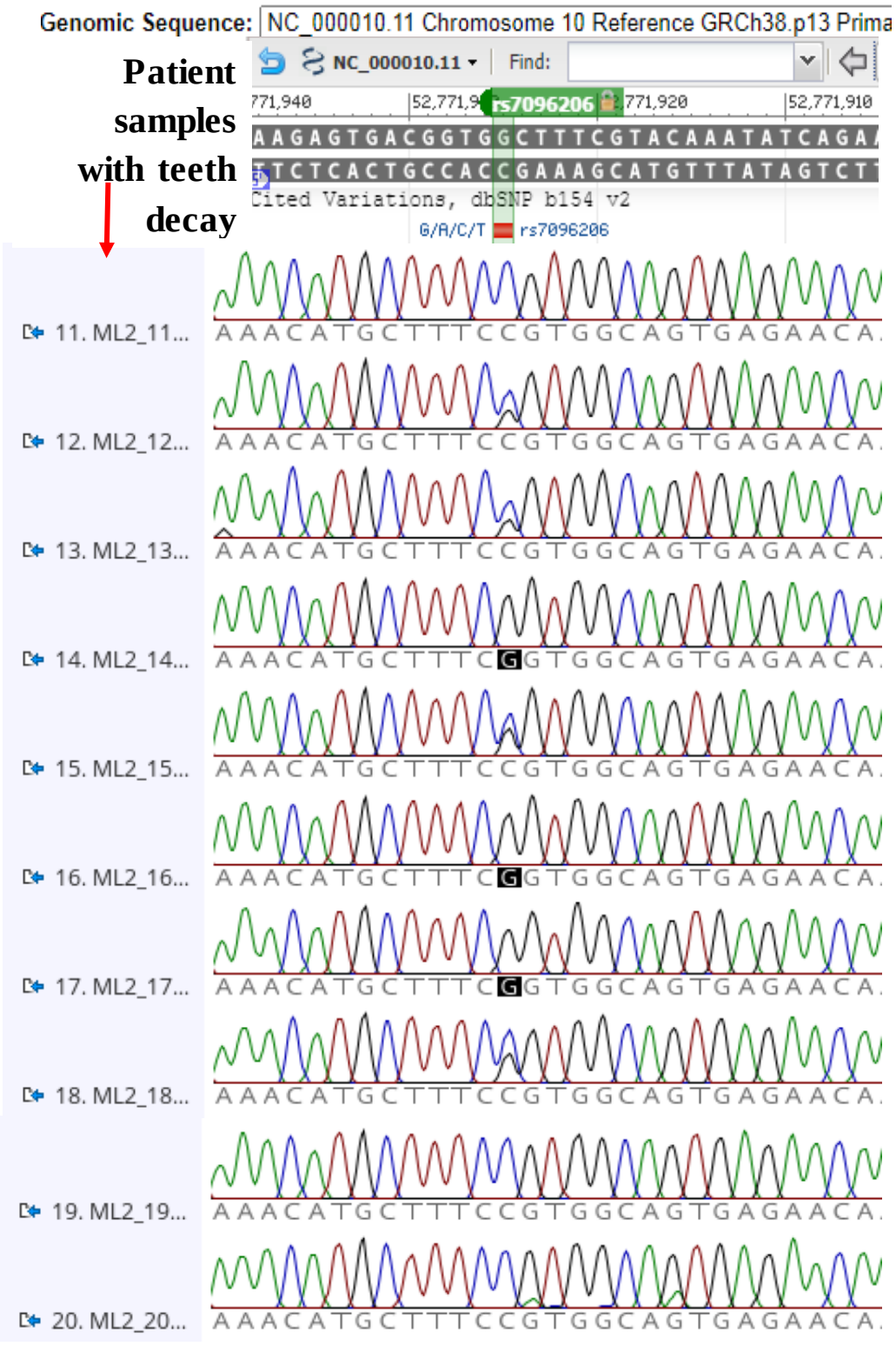
Figure(4-17): Location MBL2 validity of SNP rs7096206 0n site 52771925 on Chr:10

4.3.1.4. The multiple alignment of chromatograms of targeted region:

The results of targeted region shown one SNP observed: rs7096206 G>C, The rs7096206 G>C shown high frequent, this result paved away to considers this SNP(rs7096206 G>C) to be correlated risk allele C with Teeth decay disease Figure (4-18).



Figure(4-18): The multiple alignment of chromatograms of partial sequence of Chr:10, shown SNP rs7096206 G>C correlated with patient disease(1-10 cases). Alignment performed by Geneious prime software for *MBL2*.

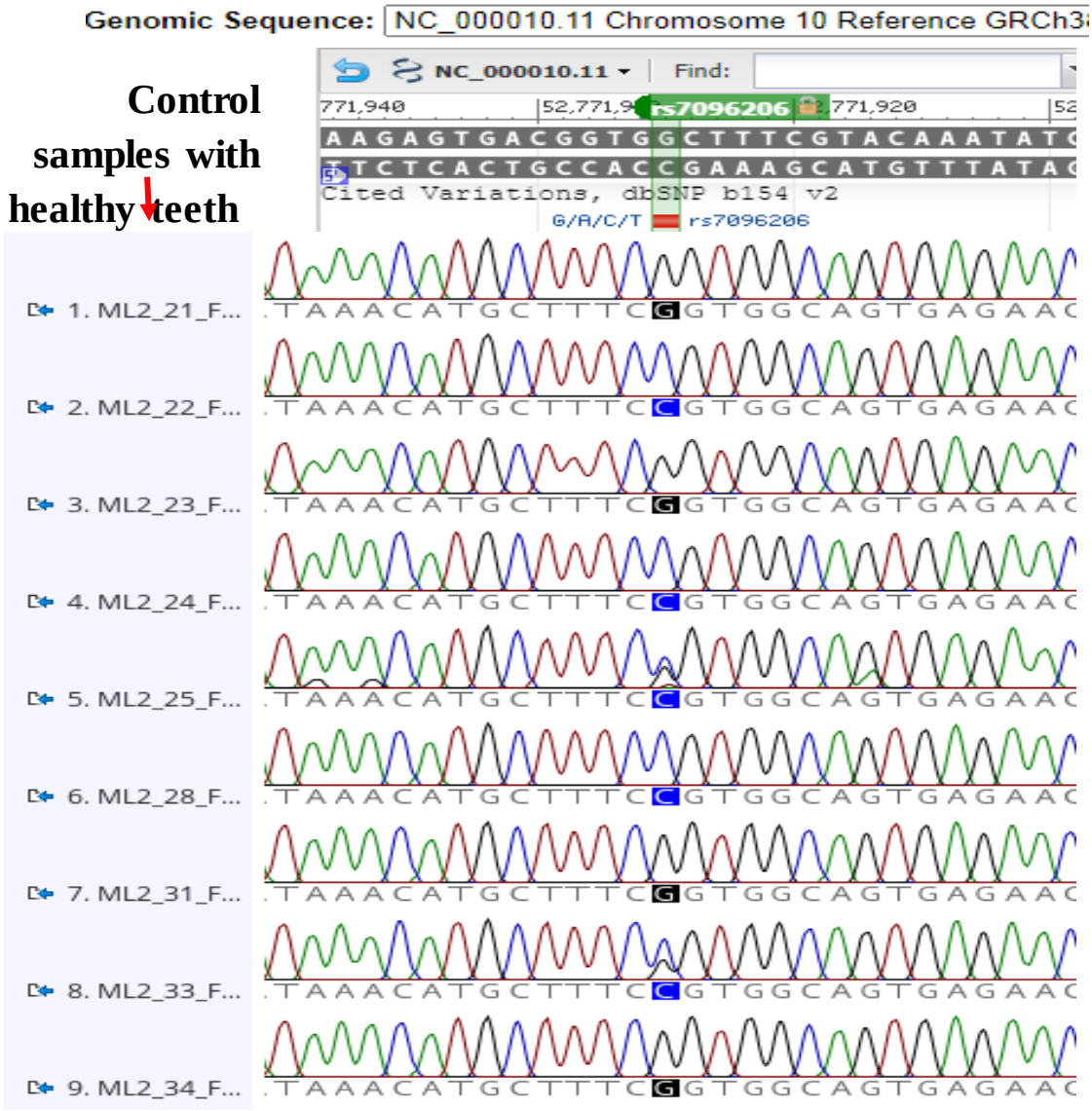


(continue (4-19) : The multiple alignment of chromatograms of targeted region shown three SNP rs7096206 G>C teeth decay patient disease (continue 11-20 cases). alignment performed by Geneious prime software for *MBL2*.

4.3.1.5. The multiple alignment of chromatograms of Healthy group:

To compare genotypes and allele frequencies of *MBL-2* between patients and controls, control individuals were evaluated.

The results of targeted region shown SNP rs7096206 G>C correlated with healthy (control group).



Figure(4-20): The multiple alignment of chromatograms of partial sequence of Chr:10, shown SNP rs7096206 G>C, 1-9 control cases. Alignment performed by Geneious prime software for *MBL2* .

4.3.1.6. Genotyping risk allele and allele frequency:

The results of multiple alignments of chromatograms were shown present three genotypes, GG, GC and CC. The allele C was considered mutant allele based on valid SNP rs7096206 G>C pointed on chromosome 10 at site at 52771924 which mutant allele G>C on upstream DNA Figure (4-18), The genotype GC and CC were shown more distribution in patients undergo teeth decay compared with control group, the values of Odd Ratio(OR) were support that C allele in both TC and CC was correlated with disease under interest and considered as risk allele. The Odd Ratio was higher in genotype GC with OR= 1.56(0.4-5.7) with P value 0.5 and CC with OR=1(0.20-4.7) with P value 1 , and the allele frequency was higher in with C allele 16(40%) in patient group with high value of OR=**1.24(0.5-3.1) ,** P.value = 0.6, while the allele frequency low 14(35%) in control group Table(4-5) .

In study done by Alyousef *et al.,* (2017) on gene polymorphisms and caries in Sudia children; they found that allele G was a risk factor for caries. Mokhtari *et al*.(2019) also found that there were on gene polymorphisms and caries in e in Iranian adults .In Xiao-Pan *et al*.(2020) study, they found there were no relationships with dental caries were found involving *MBL2* (*rs7096206C/G*) (odds ratio, 0.721; 95% confidence interval, 0.449–1.156) or *MBL2* (*rs7095891G/A*) (odds ratio, 1.076; 95% confidence interval, 0.675–1.177).

Table 4-5: *MBL2* Genotypes distribution and allele frequency of wild type allele and mutant allele of SNP: rs7096206 G>C, OR values and p values for teeth decay infection patients.

rs7096206 G>C	Case N=20		Control N=20	OR(95%CI)	P-value
genotypes	GG	8(40%)	10(50%)	Reference group	
	GC	8(40%)	6(10%)	1.56(0.4-5.7)	0.5
	CC	4(20%)	4(20%)	1(0.2-4.7)	1
Allele Frequency	G	24(60%)	26(65%)	0.65(0.26-1.6)	0.6
	C	16(40%)	14(35%)	1.24(0.5-3.1)	0.6

4.3.2. IL10 tetra-Arm PCR polymorphism:

To evaluattedthe polmorphism of the SNP *IL10* rs1800896 T>C, partial sequence shown validity site of SNP *IL10* rs1800896 T>C on gene *IL10* Figure (4-21). The targeted region was amplified by tetra arm primers(Two outer primers Forwared and reverse, and two inner primers figure (4-22),In general the *IL10* levels were additionally raised during the intense and constant disease and there was likewise a decrease in immunosuppressed patients. The aim of this study is the detection of *IL-10*- rs1800896 T>C – polymorphism.

There is evidence that cytokines are important in regulating and controlling the inflammatory response to bacterial infection (Miller *et al.*,2009). Genetic and immunological differences between hosts may also be an important risk factor for dental caries. Although the role that cytokines play in the aetiology and mechanism of dental caries remains unclear, it was found that *S. mutans*, an important factor in dental caries development, could stimulate proinflammatory cytokine production (Soell *et al.*,1994).

IL-10 is the usual silencer cytokine of the responses resistant structures, and in people it's been verified that their stages are however linked with the bounds periodontitis focuses (Rizzuti *et al.*,2015). Exploratory verification sponsor ships the reification procured in people and has installation a causal courting wherein *IL-10* stages are linked with cyan conflictingly to bone incident in animal fashions of periodontitis (Murdock *et al.*,2014), It is on this placing that you can actually derive to explicit that any detail affecting the *IL-10* stages should speculatively effect the presence, fact in addition to degree of periodontitis. As of now we had exposed that people passing at the range form of the *IL-10*-592 C/A polymorphism offered lessened stages of *IL-10* enunciation and an prolonged threat suffering from never-ending periodontitis (Claudino *et al.*,2008).

Unusually, contamination through micro organism infinitesimal creatures has been linked with an extension withinside the statement of accurate for flamable cytokines mattoirs, but what is extra *IL-10* (Gemmell *et al.*,1998) and except the polymorphisms of the *IL-10* exceptional were linked with frailty to diverse pollutions (Kim *et al.*,2002), strong creating a bidirectional affiliation among the 2 elements that as but had now no longer been explored in sufferers with wearisome periodontitis.

Regardless, the effects of the present day paintings display might which have the choice to polymorphism of *IL-10* are settled with a doubt through genotype, and that presence/bacterial stack of periodontal microorganisms no basically impacts the statement of this suppressive cytokine (Miller *et al.*,2009).

In this unique situation, our effects advise that the guideline of thumb of the polymorphism of the *IL-10* is solidly suffering from the profile innate traits of the host, and that microbiological stimulation, further as compensatory managerial units of the host, they might simply anticipate a discretionary part (Redford *et al.*,2011).

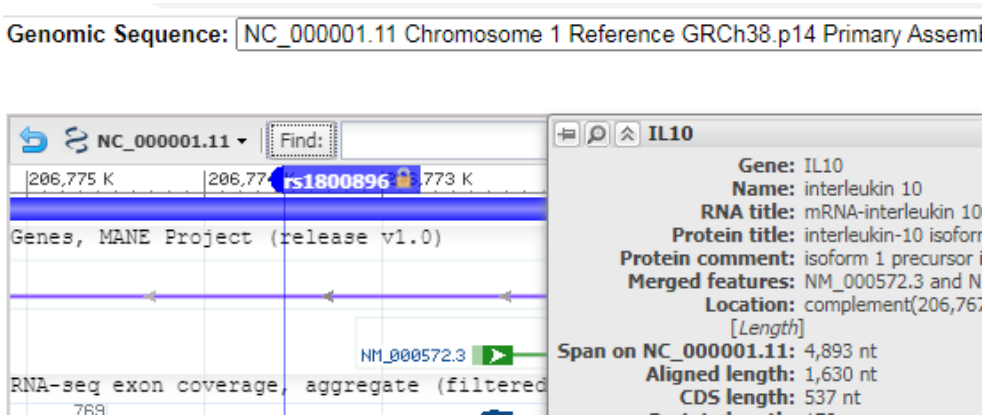
It is further as in an certainly disseminated article the multifaceted layout of *IL-10* regulatory parts, displaying that particular mobileular sorts can bring *IL-10* paying little psyche to outdoor enhancements, at the same time as that during diverse situations the pressure or nature of the improvements considerably influences the assertion of *IL-10*. From an genuinely hypothetical factor of view, the enlistment of *IL-10* through the host seems to limition the blow-returned added through the close by invulnerable response to war bacterial contamination (Redford *et al.*,2011).

only one study has evaluated the association between interleukin (IL)-1b, IL-1 receptor antagonist and IL-10 and dental caries susceptibility (Cogulu *et a*l., 2015). In the other study (Hu *et al*.,2019) individuals with dental caire the IL10 rs1800872(A/C) genotype had a lower risk of dental caries compared with those with the AAgenotype (OR 0.863,CI 0.54, 1.37).

Chatzopoulos *et al* .,2018 investigated the role of *IL-6* and *IL-10* gene polymorphisms on the risk of disease progression following active non-surgical periodontal therapy in patients diagnosed with chronic periodontal disease The results suggest that *IL-10* -592 C/A polymorphism is associated with a higher risk of periodontal disease progression following non-surgical periodontal therapy in a Caucasian population.. Patient carriers of the *IL-6* GG and *IL-10* CA or *IL-10* AA genotype were considered susceptible to periodontal disease. Polymorphisms in the regulatory regions of genes may alter the

expression of cytokines revealing an important role of genetic predictors of disease susceptibility and clinical measures More specifically, both *IL-6* -572 G/C and *IL-10* -592 C/A polymorphisms have been associated with an increased protein expression and inflammatory response (Albuquerque *et al* ., 2012).

Moreover, others observed that these single-nucleotide polymorphisms (SNPs) of *IL-10*, including −1082(−1087)A/G، 824 - 819 C/T and −592(−597)C/A, were associated with the generalized chronic periodontitis and/or aggressive periodontitis (Atanasovska-Stojanovska *et al* .,2012) (Zhong *et al.*,2012) , which elucidates the role of *IL-10* in periodontal diseases.



Figure(4-21): The validity SNP rs1800896 site on Chr:1 at position 206773553 on promoter region of *IL10* included SNPs: rs1800896 T>C.

4.3.2.1 Amplification of gene *IL10* SNP rs1800896 T>C:

To evaluattedthe polmorphism of the *IL10* SNP rs1800896 T>C, the genotyping wild and mutant alleles of Targeted region of gene *IL10* was amplified by tetra arm primers(Two outer primers Forwared and reverse, and two inner primers, the results shown three genotypes TT,TC and CC genotype. Figure (4-22).

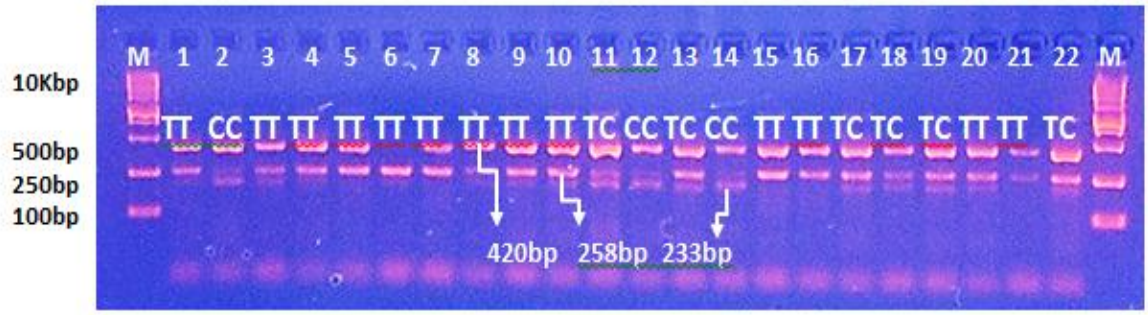


Figure (4-22): Gel profile of *IL10*, PCR tetra primer method for 22 patients as case group:13 samples showed TT genotype, 5 showed TC genotype, and CC genotype. Bands: Top Control (420 bp), middle C allele (285 bp), bottom T allele (233 bp). Lanes: M: molecular marker(100bp for first step; 1,3-10, 15-16,20-21 = TT homozygous wild allele;2,12,14 = CC homozygous mutant allele; 11,13,17-19, 22=CT heterozygous mutant allele.

4.3.2.2. Genotypes distribution and allele frequency :

The genotype CC were shown more distribution in patients undergo teeth decay compared with control group, the values of Odd Ratio(OR) were support that C allele in CC was correlated with disease under interest and considered as risk allele. The Odd Ratio was higher in genotype CC with OR= 1.2(0.2-4.9) with P value 0.8 for each, and the allele frequency was with C allele 17(28.3%) in patient group with high value of OR=0.7(0.3-1.6**) ,** P.value = 0.3, while the allele frequency of CC 18(46.6%) in control group Table(4-6) .

Table (4-6) : *IL10* Genotypes distribution and allele frequency of wild type allele and mutant allele of SNP: rs1800896 T>C, OR values and P values for teeth decay infection patients.

rs1800896 T>C	Case N=30		Control N=30	OR(95%CI)	P-value
genotypes	TT	17(56.6%)	7(23.3%)	Reference group	
	TC	9(30%)	18(60%)	0.28(0.09-0.82)	0.02
	CC	4(13.4%)	5(16.6%)	**1.2(0.2-4.9)**	0.8
Allele Frequency	T	43(71.7%)	32(53.3%)	1.4 (0.6-3.2)	0.3
	C	17(28.3%)	18(46.6%)	0.7(0.3-1.6)	0.3

5.1: Conclusions

1-The plaugs of whole teeth decay composed carried many *Candida* spp. like *C. parapsilosis* , *C. albicans* ,*C.tropicalis, C.famata* ,*C.gilliermandii* and *C.holmii*.

2- Most of *Candida* spp., carries two virulence factor: *ASL1* and *HWP1* gene.

3- The polymorphism of *MBL2* based on sequence assay shown role of allele risk correlated with patient group more than in healthy group.

4- The technique of Tetra-arms PCR was producer in appearance of *IL10* polymorphism and shown the linkage of allele risk with teeth decay.

5.2 :Recommendation

1- Evaluation the co-inhabitant of *Streptococcus mutans* and *Candida albicans* in the same in teeth plaques.
2- Mimic co-inhabitant biofilm on glass to evaluate the co operation living *Candida* and bacteria.