DOI: 10.7860/JCDR/2022/56558.16648

Review Article

Dentistry Section

# Development of Nucleic Acid Based Diagnostic Methods and their Implications in Oral Medicine Practice

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## ABSTRACT

Disease identification methods based on nucleic acids are commonly employed in medical diagnostic procedures. Nucleic acid detection is a vital technique that identifies specific nucleotide sequences, hence indicating the presence of a pathogen or the onset of an unhealthy condition. The techniques based on them are constantly advancing and there is a certain drive in the scientific community to devise tests that are more rapid, reliable, and economical. Advances in nucleic acid based diagnostics, for instance Polymerase Chain Reaction (PCR) and sequence alignment studies, have transformed the way clinical laboratories presently detect pathogens, allowing for rapid and effective response to infection therapy at an early phase. Nucleic acid detecting might actually be a linkage between oral and systemic diseases since persistent inflammation in the oral cavity is related with systemic diseases. The capacity to distinguish genetically between closely related disorders is especially crucial for bacterial or viral infections, which may rapidly evolve and produce new strains. Another advantage of infectious disease nucleic acid biomarkers being exogenous is that they may be discovered in the body shortly after infection. In principle, this allows for early detection and can be helpful in slowing or stopping disease transmission. This article reviews the evolutionary milestones of its current applications, emerging applications in oral diagnosis and future trends. Over the last decade, molecular biology has evolved at an accelerated pace, enabling the detection and management of a varied range of oral diseases. Understanding the numerous sequencing methodologies and genetic anomalies can assist in clinical application for improved disease diagnosis and prognosis.


**Keywords:** In-vitro diagnosis, Oral diseases, Oral microbiology, Oral premalignant disorders, Polymerase chain reaction

## INTRODUCTION

Traditional microbiology laboratory techniques are usually time-consuming, and this severely influences the outcome of the patient awaiting a definitive diagnosis to facilitate therapy. The delays are mostly during culturing the organisms, where each one would have a preferential growth medium and a varied pace of growth. Nucleic Acid Tests (NAT) provide a much needed respite in terms of speed of diagnosis with appreciable reliability [1]. In the oral cavity, over 1000 diverse species have been recognised, and research utilising next generation Deoxyribonucleic Acid (DNA) sequencing methodologies advocate that bacterial diversity may be considerably greater [2]. Nucleic acid methodologies have also proved useful in finding disease-related patterns and profiling bacterial populations [3]. This article emphasises various nucleic acid technologies that have been widely employed in research on the oral microbiota.

## NUCLEIC ACID TECHNOLOGIES

Nucleic acid techniques have transformed the area of medical microbiology due to their numerous advantages and uses in comparison to conventional techniques. Molecular techniques for microbial identification can be applied directly to clinical samples to identify the unexpected (open-ended analysis) or to target certain species (closed-ended analysis) [4]. Large quantities of clinical samples may be screened for the presence of target species using DNA-DNA hybridisation arrays (checkerboard and microarrays), specialised single-primer Polymerase Chain Reaction (PCR), nested PCR, multiplex PCR, and quantitative real-time PCR. Fluorescence In Situ Hybridisation (FISH) can be used to identify, quantify, and map the spatial distribution of target species within tissues. Recent advances in Next-Generation Sequencing (NGS) of DNA have made it a valuable tool for microbial identification and community profiling [5].

## HISTORICAL EVENTS OF IMPORTANCE

The history of NAT is the by-product of some of the most remarkable achievements of humankind, which have received accolades the world over. The first step in this direction and arguably the most important one has been the discovery of the duplex DNA structure and complementarity rules by James Watson and Francis Crick in the year 1953. The Nobel Prize for their discovery was awarded in the year 1962. In 1956, Arthur Kornberg, an American biochemist isolated the DNA polymerase enzyme which is a powerful tool in molecular biology research and applications (Nobel Prize in 1959) [6]. This was followed by Joseph G. Gall and Mary-Lou Pardue from Yale University who published a paper describing in situ hybridisation in 1969 using radioactive probes, the modifications of which are used in routine diagnostics today [7]. 1970 heralded another Nobel Prize (1975) winning discovery of the Reverse Transcriptase enzyme by Howard Temin, David Baltimore and Renato Dulbecco [8]. In 1970, the technique to detect specific DNA sequences in a complex genome called Southern Blotting was invented by Sir Edwin Southern at the Medical Research Council Mammalian Genomic Research Council in Edinburg [9].

In 1977, Frederick Sanger and others from the Medical Research Council Laboratory of Molecular Biology from Cambridge published the technique of genome sequencing with chain terminating inhibitors called the dideoxy chain termination method of sequencing. The technique described by them makes use of DNA polymerase and inhibitors that terminate the chain in specified regions on the amplified DNA segment. They demonstrated it using the DNA of the bacteriophage ΦX174 [10]. The technique has been in use for an excess of 30 years now and almost all the new techniques of sequencing have been based on the "Sanger Method". His invention paved the way for many important genomic studies worldwide, the most important of them being the sequencing of the human genome. Frederick Sanger was awarded his second Nobel Prize for this invention in the year 1980 [11].

An American biochemist named Kary B Mullis stumbled upon the idea of amplifying a DNA double helix molecule exponentially to generate a high magnitude of copies of the original in 1985. It was known as PCR. The method was patented in 1987 and a Nobel Prize was awarded to Kary B Mullis in 1993. The PCR has revolutionised NATs in both diagnostic and research laboratories. It is now a preferred rapid diagnostic method for infectious diseases. The PCR is used in diagnosing genetically inherited diseases, for forensic analyses and in evolutionary biological studies [12]. Based on the earlier inventions of hybridisation techniques, restriction endonucleases and PCR, a technique called FISH was developed in 1986. This would allow the analyses of genetic components in chromosomes, cells or tissues and is now an extensively used procedure in cancer diagnosis [13]. Microarrays and gene chips found larger applications in molecular biology and pharmaceutical research. The area of Nucleic Acid-based diagnosis is till today a constantly evolving branch of science [Table/Fig-1].

| Milestone | Year |
|---|---|
| Friedrich Miescher isolated DNA in the form of chromatin | 1869 |
| Watson and Crick discovered structure of DNA as double helix | 1953 |
| Arthur Kornberg discovered the first DNA polymerase | 1969 |
| Frederick Sanger put forward new method of DNA sequencing- known as the dideoxy chain-termination method, or Sanger sequencing | 1980 |
| Kary Mullis amplified a DNA double helix molecule- polymerase chain reaction | 1985 |
| First viral gene transfer in humans achieved ex vivo | 1990 |
| Mostafa Ronaghi introduced a new DNA sequencing technique called pyrosequencing | 1996 |
| First human chromosome 22 was sequenced | 1999 |
| Kuong Truong and team developed the technique known as Fluorescence In Situ Hybridisation (FISH) | 1999 |
| Next Generation Sequencing (NGS) process allowed for the first time - an entire genome to be sequenced at once | 2005 |
| Sequencing-by-synthesis technology that detects hydrogen ions when new DNA is synthesised | 2011 |
| Nanopore Sequencing-sequence single molecules of DNA and RNA without the need for amplification | 2016 |
| Nucleic Acid Amplification Test, or NAAT- viral diagnostic test for Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) | 2020 |

**[Table/Fig-1]:** Brief history of the development of nucleic acid diagnostics.

## DEVELOPMENT OF CURRENT METHODS USED IN DIAGNOSTICS

### Amplification Techniques

The PCR procedure is focused on the in vitro replication of DNA using automated denaturation, primer annealing, and extension cycles (thermocyclers). As a result, an exponential amplification of the genomic area bordered by the primers occurs, conferring PCR's remarkable sensitivity in identifying the target DNA [14]. In theory, a PCR reaction should be able to produce billions of copies of a target DNA with only a single template to start with. Since, it was invented by Karry B. Mullis, this technique has been changed to make it more versatile and useful in a wide range of situations [15].

The PCR technique was then modified to increase its specificity. Conventional PCR technology has further derivatives which include:

#### 1. Hot PCR

Karry Mullis then came up with a technique called "hot PCR." Unwanted primer extension was seen to happen at the initial temperature rise phase prior to the first denaturation. Hot PCR is when you do not add polymerase or deoxyribonucleotide triphosphate to the mix until the first denaturation, which makes the reaction more precise. The in-vitro amplification of DNA by PCR is routinely used in diagnostic and research laboratories worldwide. The method involves a heat stable DNA polymerase, commonly used is the *Thermus aquaticus* (Taq). The DNA polymerase in association with primers and nucleotides result in multiple copies of the target DNA sequence. DNA denaturation, primer binding and primer extension is achieved through multiple cycles of heating and cooling in a thermocycler. Hot PCR involves depriving the mix of polymerase or deoxyribonucleotide triphosphates until first denaturation occurs thereby increasing the stringency of the reaction [16].

#### 2. Multiplex PCR

This was one of the first variants of PCR where multiple targets could be identified by using several sets of primers. In multiplex PCR, two or more sets of primers specific to distinct targets that yield amplicons of varying sizes are utilised in the same reaction [17]. The areas that do not amplify are identified on deletion scanning by visualising them on an electrophoresis gel.

Multiplex PCRs are used in [18]:

- → Gene deletion analysis
- → Identifying pathogens
- → Identifying mutations and polymorphisms
- → Quantitative analysis of genetic components

The disadvantages of multiplex PCRs are the unequal amplification of a specific target relative to the other targets, the formation of primer-dimers, and that it is technique-sensitive regarding the determination of the primer to template ratio [18].

#### 3. Nested PCR

This modification was made to increase the specificity of amplification. It was used for the first time by Mullis K and Faloona FA in a publication explaining the technique to amplify a β- globin gene from a human DNA segment in 1987 [19]. It involves a double amplification step wherein a pair of outer primers first amplify a "long product". A set of second primers internal to the outer primers then amplify a subset of the longer product, which is the eventual target sequence [15]. The disadvantage of this modification is that there is a large scope for contamination of the second reaction due to the remnants of the first.

#### 4. Real-Time PCR

This technique was first used by Gingeras TR et al., in 1992. The scientific team was interested in "tagging" DNA molecules to delineate their presence. It was by accident that one of the team members added ethidium bromide to the PCR mix before the amplification procedure. This gave rise to the idea that there should be a linear relationship between fluorescence and the amount of DNA after amplification [20]. The procedure was repeated with florescence readings taken after each cycle of amplification. They used a Charged Coupled Device (CCD) camera to monitor the elaboration of the DNA content during the PCR, and thus Real-Time PCR came into being [21]. The system used ethidium bromide, a radioactive substance that did not facilitate the detection of small amounts of PCR amplification products. There was also a need for a visualisation medium that was DNA specific and produced no florescence when it had no DNA bound to it. The solution was the discovery of a more sensitive florescent dye called SYBR Green I, which today can be considered an industry standard [21,22]. A Real-Time PCR test today consists essentially of a fluorescent dye that binds to the PCR product and flags its presence via fluorescence. Based on the emission intensity, the DNA content is quantified in the reaction [23]. The advantages of Real-Time PCR are numerous. It eliminates the postamplification step of analysing the product on an agarose gel and the detection of the DNA can be done in real time, i.e., as, and when the amplification takes place. This brings with it the speed and simplicity of diagnosis. The use of standardised instrumentation contributes to reproducibility. A quantitative capability is achieved by the ability to determine the amount of DNA at the start based on the number of products accumulated at a particular cycle number [3]. Gene amplification in human cancers, such as *c-erbB-2 (HER2/neu)* in salivary gland and breast tumours, *int-2* (thyroid cancers),

and *CCND1* (cyclin D1) in breast carcinomas, oral cancers, and epithelial dysplasia, has been studied using this method [24].

#### 5. Reverse Transcription PCR (RT-PCR)

RT-PCR was created to intensify RNA targets using the enzyme reverse transcriptase, which can generate complementary DNA (cDNA) from an RNA template [23].

Diagnostic testing is crucial in battling the Coronavirus Disease 2019 (COVID-19) pandemic, which is caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). SARS-CoV-2 infection is usually confirmed by RT-PCR. First, reverse transcriptase converts purified total RNA (viral RNA and host RNA) into complementary DNA (cDNA), followed by qPCR to exponentially amplify the target gene of interest from cDNA aliquots [25]. Other tumour-defining translocations which may be detected with RT-PCR include t(15;17) in acute promyelocytic leukaemia, t(8;14) in Burkitt's lymphoma, t(2;5) in anaplastic large-cell lymphoma, t(11;22) in Ewing's sarcoma and primitive neuroectodermal tumour, t(2;13) in alveolar rhabdomyosarcoma, and t(X;18) in synovial sarcoma [24].

## Hybridisation Techniques

These sets of techniques exploit the property of complement pairing of DNA and RNA molecules [15].

#### 1. Fluorescence In Situ Hybridisation (FISH)

The Fluorescence in situ hybridisation was developed as an extension of the invention by Joseph G. Gall and Mary-Lou Pardue. The need for an advanced method arose out of the disadvantages of isotopic hybridisation. They were isotope decay, long exposure times to produce a signal on the radiological film, limited resolution of the radiographic technique, and associated health hazards associated with radioactive substances [26]. The first application of FISH was in 1980, when an RNA molecule was labelled directly to detect a DNA molecule. The synthesis of biotin labelled UTP and dUTP to be used as substrates for hybridisation in-vitro was a significant step in the refinement of this technique [27]. The milestones of the refinement of this technology are summarised in [Table/Fig-2]. FISH is used to detect bacteria directly in clinical samples and for fastidious organisms.

| Milestone | Year |
|---|---|
| Suppression hybridisation | 1987 |
| Application for RNA analysis | 1987 |
| Whole chromosome painting | 1988 |
| Single copy detection of genes | 1988 |
| Simultaneous visualisation of different spectra of fluorophores | 1986 |
| Ratio-colour coding | 1992 |
| Computed interpretation of a 5-colour scheme | 1996 |
| Combinations and Ratios (COBRA) | 1999 |
| Microfluidic FISH implementations | 2007 |
| Single cell messenger RNA sequencing | 2017 |
| Spot-based Spatial cell-type Analysis by Multidimensional mRNA density estimation (SSAM) | 2019 |

**[Table/Fig-2]:** Developmental milestones in FISH.

Commercially obtainable kits that use the FISH principle are available for *Chlamydia* spp., *Helicobacter* spp., and *Enterococcus* spp., to name a few. Tumour diagnosis has improved due to the cytogenetic analysis and comparative genomic analysis options available due to FISH [28]. This method can also be used to look for Epstein-Barr virus (EBV) particles in oral hairy leukoplakia, a condition that is common in people who are immunosuppressed [25].

#### 2. Polyamide Nucleic Acid (PNA)-FISH

PNA-FISH was first described by Peter E. Nielsen and others in 1991 [29]. The PNAs have turned out to be efficient FISH probes due to their ability to enter cells more easily and their higher specificity than DNA oligomers. AdvanDX is an example of a commercially available kit using this technique to detect *S. aureus* and *Candida albicans* directly from clinical samples that has found Food and Drug Administration (FDA) clearance [30].

#### 3. Southern blotting and microarray technology

The two techniques are interrelated, and hence they have been grouped together for the ease of elaborating on their development. Sir Edwin Southern invented the technique of Southern Blotting, which is still in use today. It is a versatile tool where the assay permits the determination of the size of the target sequence that is bound to the probe [31]. The novel idea of transferring the separated genetic fragments onto a membrane paved the way for many more innovative inventions. He also developed the idea of printing out nucleotides in an array using a conventional printer for the ease of detecting DNA targets. Technologies that emerged used solid surfaces to support DNA fragments in an array fashion for hybridisation, and fluorescence detection was done by automated plotting devices [32].

Affymetrix, a company dedicated to manufacturing microarray systems, came up with a technology that uses photolithography and solid phase DNA synthesis to construct assays [33]. Southern blotting and microarray were used at different times in the human genome project when "sequencing by hybridisation" was considered as an appropriate method for decoding DNA. The most publicised advantage of microarrays has been that they can provide for large-scale parallel hybridisation analysis. Additionally, it's a potential tool for determining drug targets, helps analyse gene expressions and is used in mutational studies [34]. The B-cell lymphoma diagnosis with microarrays has been studied and seems to have influenced the diagnosis and treatment of cancers extensively [35].

## Genotype-based Detection Techniques

Subtyping the organisms is an important step in disease outbreak investigation and management. It helps to provide crucial data on the source of infection and the pattern of spread, either in the community or in a nosocomial outbreak [36,37]. The 1980s saw the emergence and acceptance of 16S rRNA as an appropriate target for rapid sequence determination. This finding led to the detection of fastidious and uncultivable organisms at species level and their identification at domain level from direct clinical samples [38]. The genotypic techniques have improved over time and are now being used in diagnostic laboratories [37].

#### 1. Pulsed field gel electrophoresis

This is a variation of the conventional gel electrophoresis technique and was first described by Schwarts and Cantor in 1984 at Columbia University. This technique was developed as conventional gel electrophoresis and was not very effective in separating larger DNA fragments. The experiments were conducted using intact *S. cerevisiae* using alternately pulsed, perpendicularly directed electrical current to enable the movement of larger DNA fragments [39]. Thus, the gel had well separated fragments of DNA, enabling its effective analysis. The technology has evolved to use high resolution cameras and computer software for capturing and analysing the gel and to enable the creation of a database of Pulsed-field Gel Electrophoresis (PFGE) [37].

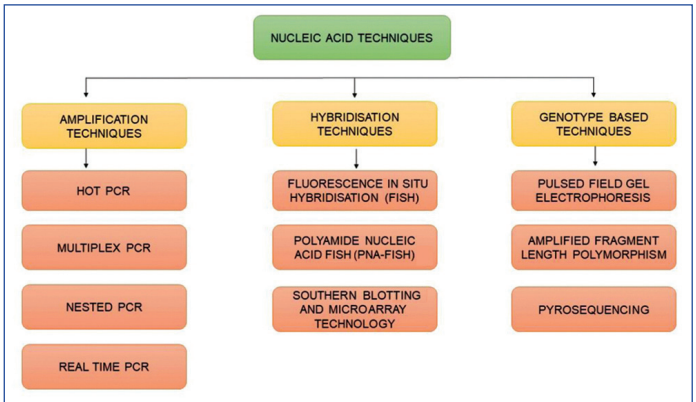
#### 2. Amplified fragment length polymorphism

This method was developed to gain the ability to screen multiple randomly spread-out DNA fragments simultaneously without any prior knowledge of the DNA sequence [40]. It was developed by a Netherlands-based company called Key Gene in the 1990s wherein it detects multiple DNA restriction fragments by means of PCR amplification [41]. The method is easy, economical and provides a high resolution visualisation of the fragments [41].

### 3. Pyrosequencing

Pal Nyren and Mostafa Ronaghi, both of the Royal Institute of Technology in Stockholm, Sweden, are credited with the invention of this method. It is based on the idea that a single strand of DNA to be sequenced is taken first and hybridised with a primer. The complementary strand is made with a polymer, one base at a time. As each base is added, an enzyme produces a light reaction that shows which one has been added. This way, the sequence of the target DNA can be worked out with the help of automated instruments [42].

The different types of nucleic acid technologies are summarised in [Table/Fig-3].




**[Table/Fig-3]:** Types of nucleic acid techniques.

## IMPLICATIONS FOR ORAL MEDICINE PRACTICE

Numerous commensal microorganisms constitute the oral microflora. Certain microbes are known to be harmful and cause common mouth illnesses, whilst others are harmless. Many infectious diseases were difficult to diagnose in the past, given the lack of material for microbiologic culture, protein analysis, or direct microscopy. Furthermore, cultural identification approaches commonly used to take a great amount of time and effort. In some circumstances, PCR has superseded traditional pathogen detection procedures. Even when the number of infectious organisms is low or slow-growing, or when the infectious agent is in a material that is not suited for culture, DNA or RNA of an infectious organism can be found in a test sample [43]. The PCR has been used to identify organisms in blood, saliva, sputum, semen, and faeces, as well as in fixed tissues [43].

Nucleic acid technologies have been utilised to identify genomic alterations in a number of malignancies, including oral cancer, as well as to identify genetic abnormalities in inherited conditions such as cleft lip and palate. Genome sequencing has enabled the identification of the full complement of DNA variants, de novo mutations, and the genes underpinning Mendelian diseases, as well as the characterisation of important structural variants that may contribute to cleft lip and palate [44].

### Periodontitis

Periodontitis is a chronic inflammatory disease that causes periodontal tissue damage and has a negative influence on overall health. Clinical studies have shown that gingival tissues with chronic periodontitis have more Toll Like Receptor 9 (TLR9) genes and proteins. Comparing periodontitis lesions to healthy tissues, immunohistochemistry analysis indicated elevated TLR9 and DNA-dependent activator of IFN-regulatory factors (DAI) expression in periodontal lesions [44,45]. The discovery of periodontal bDNA's immunostimulatory effects and TLR9-mediated periodontal inflammation provides a new route in dentistry research, suggesting a possible treatment target for periodontal disease [44].

### Caries Risk Assessment

Dental caries is a pathological biofilm mediated condition that results in phasic demineralisation and remineralisation of dental hard tissues. Diagnosis can be made by conventional clinical methods and radiographic techniques [46]. However, recent studies have discovered the role of nucleic acids in their diagnostic aids [46,47]. Checkerboard DNA-DNA hybridisation, genomic fingerprinting, cloning and sequencing of the 16S rRNA gene, Terminal Restriction Fragment Length Polymorphism (T-RFLP) and DNA sequencing, as well as the analysis of bacterial genome data, are new molecular technologies. Additionally, cariogenic species in saliva can be measured via PCR-based bacterial identification [47].

### Fungal Infections

In some cases, like candidiasis, some fungal local infections can be diagnosed in saliva. The precise identification of *Candida* species is critical for better therapy strategies against these organisms. Because traditional identification methods cannot tell the difference between some yeast species, PCR-based molecular approaches and sequencing can be used as an alternative tool for yeast identification [48]. These methods include the analysis of the sequences of the ribosomal 18S gene, the 5.8 rDNA region, and the domain of the 26S rDNA gene. PCR, together with denaturing gradient gel electrophoresis on 26S rDNA gene sequences, has been used to monitor and characterise *Candida* in the oral cavity [49].

### Infectious Viral Diseases

Utilising immunohistochemical staining of paraffin slices, viral proteins can be detected. BZLF1 immunostains are particularly successful at confirming the presence of oral hairy leukoplakia in tongue biopsies taken from AIDS patients. According to pathologists, BZLF1 staining is restricted to the nucleus of oral hairy leukoplakia's enlarged epithelial cells, and the antibody's cytoplasmic cross-reactivity should be disregarded [50]. Though not yet routinely used in laboratories, alternative approaches to detecting these viral gene products are RT-PCR and Nucleic Acid Sequence Based Amplification (NASBA). The clonality of EBV-infected tissues in relation to the structure of EBV DNA may be determined via Southern blot analysis [51].

Herpes Simplex Viruses (HSV) (types I and II) are common causes of human oral and anogenital lesions. Compared to culture, Nucleic Acid Amplification Tests (NAATs) for the detection of HSV genomic DNA are faster and more sensitive. Recently, FDA-approved NAATs for HSV DNA detection that are less labour-intensive than laboratory-developed PCR tests have become available [52]. NAAT detection of HSV mRNA in patients is a very sensitive and specific way to find out if they have HSV-1 or HSV-2 infections [51].

### Oral Premalignant Disorders

**1. Oral leukoplakia:** The most common potentially malignant disorder is oral leukoplakia. Leukoplakia is at present defined as "a white plaque of questionable risk, having excluded (other) known diseases or disorders that carry no increased risk for cancer" [WHO 2005] [52].

Farah CS and Fox SA, in their study in 2019 concluded that reactive alterations in the connective tissue of the lesion are an early sign of the development of dysplasia in leukoplakia. The investigations also indicated that RNA sequencing may be used to determine the disease's molecular processes. Also, it was thought that as the level of dysplasia gets worse, the disease gets worse, too [53]. In 2020, Xu S et al., used Sanger sequencing to investigate the role of circular RNAs in the development of leukoplakia and discovered that circHLA-C plays a significant role in disease progression. They discovered that circHLA-C levels rise with the severity of dysplasia. Because it can be both

a possible diagnostic marker and a genetic marker, it could be used in oral leukoplakia [54].

**2. Oral Submucous Fibrosis (OSF):** Oral Submucous Fibrosis (OSF) is defined as a slow progressive disease characterised by the fibrous bands in the oral mucosa, ultimately leading to severe restriction of mouth movement, including the tongue [55]. Several studies analysed the molecular profile of OSF and found that the RNA profile was drastically altered [56,57]. According to Tsai CH et al., the primary etiological agent, areca nut, causes significant elevations in MicroRNAs (miRNAs) [56].

The disease's molecular processes have been analysed in various approaches. However, a better knowledge of gene expression differences between normal and diseased tissues is needed to fully comprehend the process. The role of two biomarkers, Bone Morphogenic Protein (BMP)-7 and Transforming Growth Factor (TGF)-β, in the etiology of OSF was revealed by Khan I et al., as well as their confirmation [57]. Yang SF et al., discovered upregulation of type I plasminogen activator inhibitor in OSF specimens, which they confirmed using RT-PCR and western blot [58].

**3. Oral Lichen Planus (OLP):** It is a T-cell mediated autoimmune disorder in which cytotoxic CD8+ T cells trigger apoptosis of the basal cells of the oral epithelium. Baek K and Choi Y, through high-throughput sequencing of the 16S rRNA gene identified the bacterial communities present in OLP lesions in order to understand the involvement of these organisms in the pathogenesis of the disease [59]. In biopsy samples taken from individuals with OLP both high-throughput sequencing of the 16S rRNA gene and whole genome sequencing indicated an increase in *E. coli*, suggesting a possible role in triggering or causing the condition.

The RNA sequencing in lichen planus gave further insights into the role of oral microbiota in the genesis and progression of OLP. Numerous gene expression investigations have revealed that there are differences in expression profiles. The top five upregulated and downregulated genes in OLP were NEB, TNC, NRIP1, DLG1, PTPN22 and SGMS1, TET2, SMO, PARD3, ATP5C1, respectively [53]. Baek K and Choi Y employed high throughput sequencing of the 16 S rRNA to determine the bacterial communities present in OLP lesions in order to recognise the significance of these organisms in the pathogenesis of OLP. Both high throughput sequencing of the 16 S rRNA gene and whole genome sequencing found an increase in *E. coli* in biopsy tissues acquired from individuals with OLP indicating a potential role in the disease's initiation or progression [59].

## Oral Squamous Cell Carcinoma (OSCC)

Constant cell turnover results in an abundance of nucleic acid in the oral cavity, either as a result of phagocytosis or the release of extracellular DNA that becomes a structural component of the biofilm. Squamous cell carcinoma of the mouth is a frequent epithelial cancer that is noted for its heterogeneity. Researchers have been able to detect the genetic abnormalities seen in Oral Squamous Cell Carcinoma (OSCC) thanks to the use of NGS. There have been whole exome sequencing studies that found that TP53, CDKN2A, PIK3CA, and HRAS genes were all mutated in people who had them [60-62]. Dual specificity protein phosphatase 1, H3 Histone, Family 3A, Interleukin 1 Beta, Interleukin 8, ornithine decarboxylase antizyme 1, Spermidine N1-Acetyltransferase 1, S100 calcium binding protein P, and miR-31 levels are now proven to be upregulated in oral cancer patients, while miR-125a and miR-200a levels are downregulated [Table/Fig-4] [44,46,53,56-63].

Another noteworthy mutation discovered was in the NOTCH1 gene, which is known to influence squamous differentiation [61]. EGFR, STAT3, JAK kinases, TGF and FBKW7 are among the other genes that have been altered [61]. In OSCC differential expression

| Oral diseases | Altered genome sequence | Authors and year of study |
|---|---|---|
| Periodontitis | Increased TLR9 and DAI expression | Rojo-Botello NR et al., (2012) [44] |
| Dental caries | Increased 16S rRNA/rDNA | Guo L and Shi W (2013) [46] |
| Oral leukoplakia | Elevation of circular RNA | Farah CS and Fox SA (2019) [53] |
| OSF | Elevation of miRNA | Tsai CH et al., (2003) [56] |
| | BMP7, TGF | Khan I et al., (2012) [57] |
| | Upregulation of type I plasminogen activator inhibitor | Yang SF et al., (2018) [58] |
| OLP | • 16s rRNA<br>• Upregulated genes- NEB, TNC, NRIP1, DLG1, PTPN22<br>• Downregulated genes- SGMS1, TET2, SMO, PARD3, ATP5C1 | Baek K and Choi Y (2018) [59] |
| OSCC | • Upregulated genes- Dual specificity protein phosphatase 1, H3 Histone, Family 3A, Interleukin 1 Beta, Interleukin 8, ornithine decarboxylase antizyme 1, Spermidine N1-Acetyltransferase 1, S100 calcium binding protein P, and miR-31<br>• Downregulation of miR-125a and miR-200a | Agrawal N et al., (2011) [60] |
| | Alteration of EGFR, STAT3, JAK kinases, TGF, and FBKW7 | Collins A et al., (2013) [61] |
| | Differential expression of miR-204-5p, miR-370, miR-1307, miR-193b-3p, miR-144-5p, miR-30a-5p, and miR-769-5p | Pederson NJ et al., (2018) [62] |
| | Mutation of miR-222-3p, miR150-5p, and miR-423-5p | Chang YA et al., (2018) [63] |

**[Table/Fig-4]:** Nucleic acid in oral health diagnosis.

of miR-204-5p, miR-370, miR-1307, miR-193b-3p, miR-144-5p, miR-30a-5p, and miR-769-5p has been discovered in murine cells [62]. Other research has shown that three miRNAs (miR-222-3p, miR150-5p, and miR-423-5p) are mutated in oral leukoplakia and OSCC suggesting that they could be useful in early detection and monitoring the progression of oral leukoplakia to OSCC [63]. The potential of miRNA in OSCC metastasis has also been investigated. According to published data, there was a substantial upregulation of 45 miRNAs in OSCC tissues compared to normal controls. Further investigation of miR-21-3p reveals that it may have a role in cell metastasis in the evolution of OSCC.

Treatment aimed at lowering miR-21-3p levels may be clinically beneficial and improve prognosis [64]. The most common variation in OSCC was discovered to be TP53 mutations, which might be exploited as a diagnostic marker [65]. A mutation in the tumour suppressor gene CDKN2A/p16 aids in various molecular processes involved in malignant transformation and disease development [66]. TNF-α in OSCC tissues has been found to promote a pro-inflammatory and proinvasive phenotype, and higher TNF-α expression leads to tumour invasion and is thus linked with a poor prognosis. Targeted therapy aimed at reducing the influence of this gene on oral cancer may be beneficial [67].

# CONCLUSION(S)

Newer technologies are aimed towards minimising output time, increasing automation and accuracy with the miniaturisation of equipment to provide point-of-care services. Although molecular techniques may not be 100% accurate, they can be said to have an average accuracy of >99%. With the growing availability of malleable diagnostic technology, NATs are always going to be an evolving branch of medicine. Saliva has become an attractive alternative to conventional invasive diagnostic procedures thanks to the effective contribution of nucleic acid technology. Saliva has several advantages over other body fluids as a diagnostic tool for oral

and systemic disorders, and it can provide an accurate diagnosis based on specific biomarkers. This developmental process will help empower the medical system to effectively understand diseases and find their appropriate treatment. There is a spur of activity to make diagnostic tests faster, more affordable, and more rapid to positively influence patient outcomes. The utilisation of molecular procedures varies widely today, but it is possible that many of today's most technically sophisticated techniques will be employed in the future decade. These methods will likely improve the understanding of diseases that affect the head and neck and the ability to render a diagnosis.

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**PLAGIARISM CHECKING METHODS:** [Jain H et al.]
- Plagiarism X-checker: Mar 26, 2022
- Manual Googling: May 27, 2022
- iThenticate Software: May 28, 2022 (23%)

**ETYMOLOGY:** Author Origin

**AUTHOR DECLARATION:**
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? No
- Was informed consent obtained from the subjects involved in the study? No
- For any images presented appropriate consent has been obtained from the subjects. No

Date of Submission: **Mar 23, 2022**
Date of Peer Review: **Apr 26, 2022**
Date of Acceptance: **May 30, 2022**
Date of Publishing: **Jul 01, 2022**