# ISOLATION AND CHARACTERIZATION OF BUCCAL CELL DNA OBTAINED FROM MOUTHWASH SAMPLES OF HEALTHY, TOBACCO USERS AND CANCER PATIENTS.

*A Dissertation submitted to*

**THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY, CHENNAI.**

**In partial fulfillment for the award of degree of**

**MASTER OF PHARMACY**
***(PHARMACEUTICAL BIO-TECNOLOGY)***

**Submitted by**

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***Under the supervision of***

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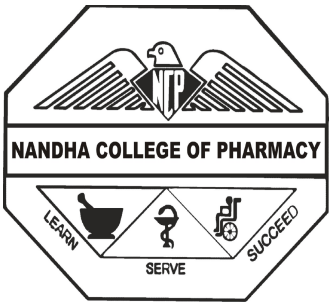


**Nandha College of Pharmacy**

**Erode – 638 052, (TAMILNADU)**

**MARCH– 2008.**

# CERTIFICATE

This is to certify that the work embodied in this thesis entitled, "**ISOLATION AND CHARACTERIZATION OF BUCCAL CELL DNA OBTAINED FROM MOUTHWASH SAMPLES OF HEALTHY, TOBACCO USERS AND CANCER PATIENTS,"** submitted to the Tamil Nadu Dr. M.G.R. Medical University, Chennai was carried out by N.V. Britya, in the Department of Pharmaceutical Biotechnology, **Nandha College of Pharmacy, Erode-52** for the partial fulfillment for the award of degree of Master of Pharmacy in Pharmaceutical Biotechnology under my direct supervision.

This work is original and has not been previously formed the basic for the award of other degree, diploma, associateship, fellowship or any other similar title and the dissertation represent entirely an independent work on the part of the Candidate.

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# DECLARATION

The work presented in this thesis entitled **"ISOLATION AND CHARACTERIZATION OF BUCCAL CELL DNA OBTAINED FROM MOUTHWASH SAMPLES OF HEALTHY, TOBACCO USERS AND CANCER PATIENTS."** was carried out by me in the Department of Pharmaceutical Biotechnology, Nandha College of Pharmacy, Erode under direct supervision of Mr. T. Saravanan, M.Pharm., Asst.Professor, Department of Pharmaceutical Biotechnology, **Nandha College of Pharmacy, Erode-52.** This work is original and has not been submitted in part or full for the award of other degree or diploma of any other university.

Place: N.V. Britya

Date:

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# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| α | alpha |
| β | beta |
| bp | base pairs |
| DNA | deoxyribonucleic acid |
| dATP | 2'-deoxyadenosine 5'-triphosphate |
| dCTP | 2'-deoxycytidine 5'-triphosphate |
| dTTP | 2'deoxythymidine-5'-triphosphate |
| dGTP | 2'deoxyguanosine-5'-Triphosphate |
| EtBr | Ethidium bromide |
| EDTA | Ethylene Diamine Tetra Acetate |
| gDNA | genomic Deoxyribonucleic acid |
| HCl | Hydrochloric acid |
| KCl | Potassium chloride |
| Kb | Kilo base |
| Kbp | Kilo base pair |
| ml | Milliliter |
| µM | Micro mole |
| µg | Microgram |
| µl | Micro litre |
| mM | Milli mole |
| $MgCl_2$ | Magnesium chloride |

| | |
|---|---|
| $MgSo_4$ | Magnesium sulphate |
| NaCl | Sodium chloride |
| ng | Nano gram |
| PCR | Polymerase chain reaction |
| rpm | Revolutions per minute |
| RFLP | Restriction Fragment Length Polymorphism |
| RAPD | Random Amplification of Polymorphic DNA |
| RNA | Ribonucleic acid |
| RE | Restriction enzyme or Restriction endonucleases |
| STR | Short Tandem Repeats |
| SNP | Single Nucleotide Polymorphism |
| SDS | Sodium Dodecyl sulphate |
| Taq | *Thermus aquaticus* |
| TE | Tris Ethylene diamine tetraacetate |
| TBE | Tris borate |
| TAE | Tris acetate and EthyleneDiamineTetraAcetate |
| TPE | Tris Phosphate |
| % | Percentage |

# 1. *INTRODUCTION*

Deoxyribonuclei acid (DNA) is a molecule present in all living things, including bacteria, plants, and animals. DNA is the information encoded in genetic material in other words it carries genetic information that is inherited or passed down from parents to offspring. It is responsible for determining a person's hair, eye and skin color, facial features, complexion, height, blood type, and just about everything else that makes an individual unique. DNA is responsible for establishing and maintaining the cellular and biochemical function of an organism[1]. DNA synthesis is called replication[2].

**1. STRUCTURE OF DNA**[3]

DNA is a double helix, with bases to the center (like rungs on a ladder) and sugar-phosphate units along the sides of the helix (like the sides of a twisted ladder). DNA is a polymer and stores biological information digitally in units called nucleotides and these specify which proteins an organism will make, as well as when and where protein synthesis will occur. Each nucleotide consists of a deoxyribose sugar, a phosphate and a nitrogenous base. The strands are complementary as deduced by Watson and Crick from Chargaff's data, A pairs with T and C pairs with G, the pairs held together by hydrogen bonds. Double ringed purine is always bonded to a single ring pyrimidine. Purines are Adenine (A) and Guanine (G). Pyrimidines are Cytosine (C) and Thymine (T). In DNA the sugar is deoxyribose. The bases are complementary, with A on one side of the molecule the other side is T and similarly with G and C[4]. The DNA regions which encode proteins are called genes.

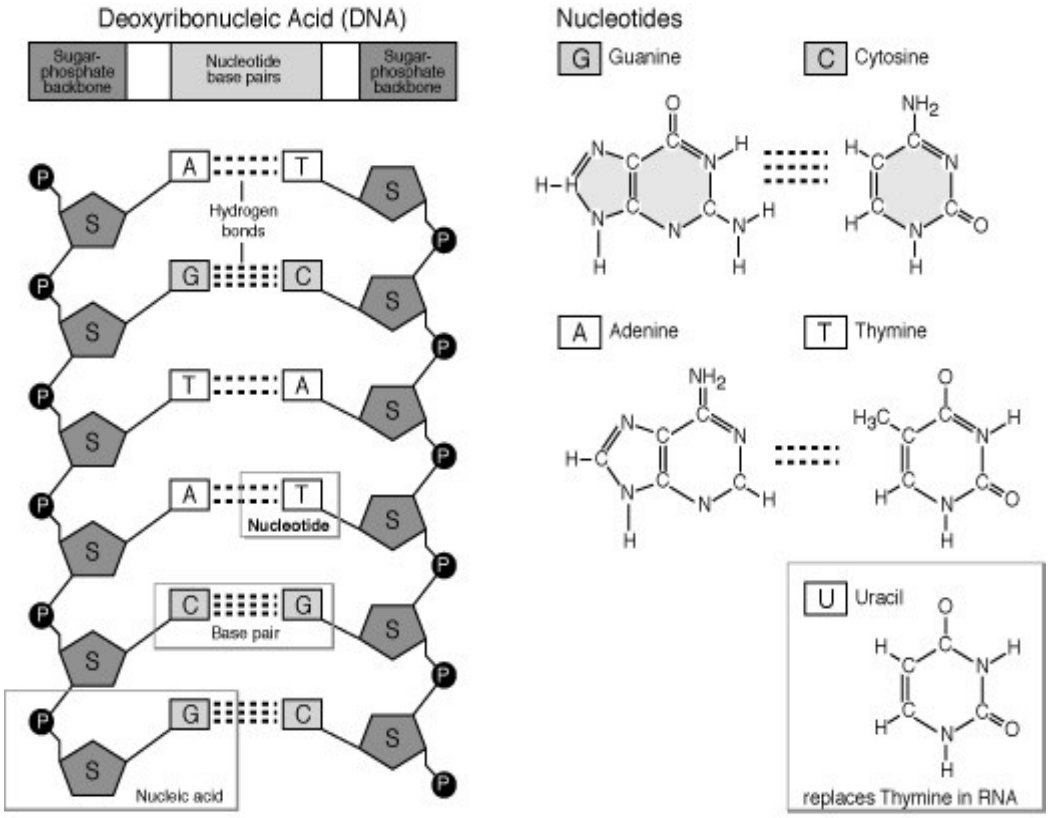


Figure 1. STRUCTURE OF DNA

## 1.1 LOCATION OF DNA

Within cells of an organism, DNA molecules are assembled into chromosomes, organelles that package and manage the storage, duplication, expression and evolution of DNA. In the chromosomes of a cell, DNA occurs as fine spirally coiled threads, that in turn coils around another, like a ladder. The total length of all DNA in the Cell's nucleus would be 3km. The entire collection of chromosomes in each cell of an organism is its genome[5]. Human cells contain 23 distinct kinds of chromosomes carrying approximately $3\times10^9$ base pairs and roughly 100,000 genes. The structure of the DNA helix is preserved by weak interactions (i.e.

hydrogen bonds and hydrophobic interactions established between the stacked base), it is possible to separate the two strands by treatments involving heating, bringing to alkaline pH[6].

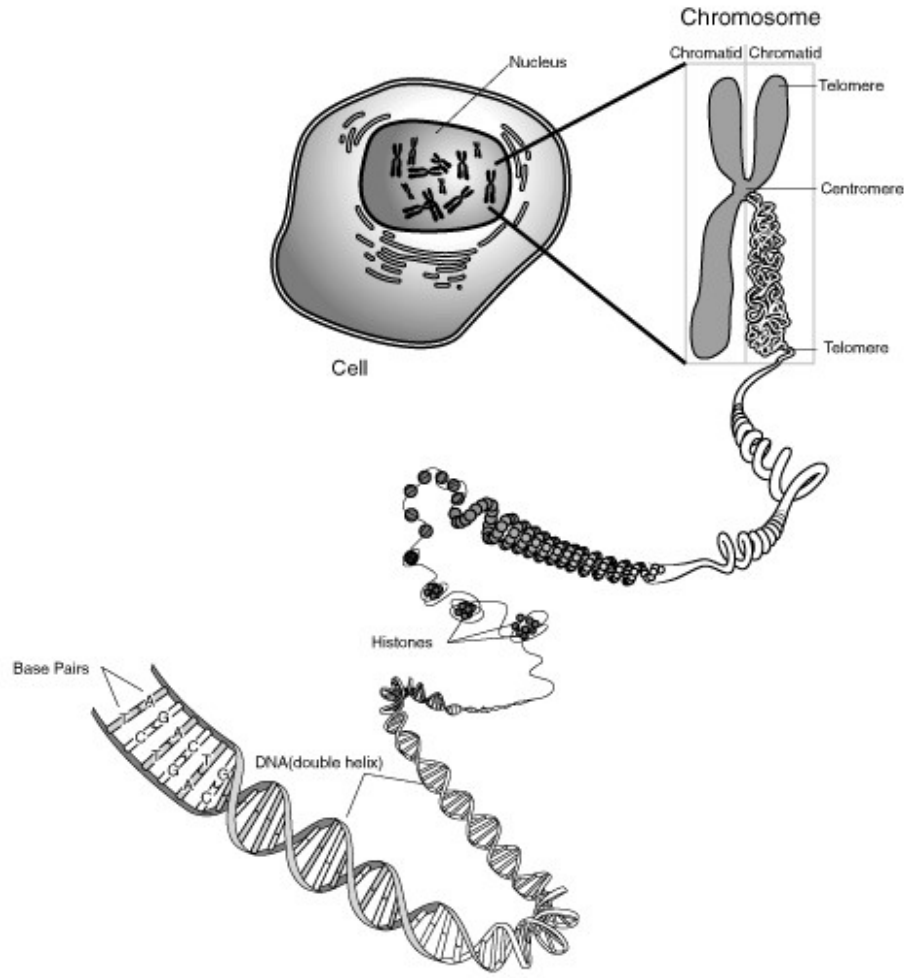


Figure 2: Location of DNA

As the Human Genome Project provides the foundation for understanding the genetic basis of common disease (1) population based genetic studies will provide the information needed for the practical application of genetic risk factors to chemical and public health practice. To this end, researchers have begun collecting specimens for

molecular analyses in epidemiologic studies and surveys in order to identify genetic risk factors for disease (2) Genomic markers including RFLP, STR, insertion-deletion polymorphism, SNP and groups of markers inherited together on one chromosome as haplotypes and being used to locate disease associated genetic loci and studies of the association between these loci to disease are elucidating the genetic basis for disease. Once risk associated genotypes are identified the validity of genetic testing for screening and clinical practice must be assessed[7]. Interest in genome-wide association studies to identify susceptibility alleles for cancer is growing, and several are currently planned or under way. A critical requirement for such large scale studies is the amount of DNA available from a sample. Traditionally whole blood is the tissue of choice, as the yield of DNA is quite high (typically 10-15μg/ml). However obtaining a blood sample is an invasive procedure that requires training in phlebotomy, and moreover blood samples require refrigerated storage and must be processed within a week or so of collection. These constraints limit the suitability of blood collection for some populations which in turn has led to a search for alternative sources of DNA. The ideal source should meet the following requirements (1) be non-invasive easy and quick to carry out (2) allow relatively long storage of samples at ambient temperatures before DNA extraction ( a critical required for field studies) with minimal loss of DNA quality and finally (3) yield a sufficiently large amount of DNA for a wide variety of applications. Alternatives that have been investigated to date, includes dried blood spots, plucked hairs and cheek swabs. However the yield of DNA is much less than these alternatives than with whole blood, cheek swabs constitute the highest yielding noninvasive alternative to whole blood as cheek swabs provide app 5-15μg of DNA[8]. Although the feasibility of collecting buccal cell samples as an alternative to venous blood samples as a source of genomic DNA has

been shown, the validity of using DNA from buccal cells for genome wide scans has not been assessed[9].

Different sample types used in DNA extractions include[10]

Whole blood, Buffy coats, Blood clots, Serum, Plasma, Cell pellets, Mouthwash, Buccal swabs, Cytobrushes, Saliva, Bronchial alveolar lavage, Mouse tails, Plants.

Other solid tissues that can be used for DNA extraction include Breast, Prostrate, Kidney, Brain, Placental, Heart, Muscle.

Other sample types include Nails, Paraffin embedded tissue, Polyps, Urine, Feces and sputum. These sample types create great difficulty in isolating DNA.

Normally DNA can be extracted by three methods

1) Non-organic

2) Column Based

3) Organic.

Obtaining high quality genomic DNA is critical for epidemiological studies that aim to evaluate the role of genetic factors in human disease susceptibility. Blood samples are an excellent source of large amounts of genomic DNA. However, epidemiological studies often need alternative sources when study subjects are reluctant to provide a blood sample, when only a self-administered collection protocol is logistically or economically feasible or as backup source of DNA in studies that collect blood samples[11].

## 1.2. BUCCAL CELLS:

Buccal cells are the cells from the inner lining of the mouth, or cheek. These cells are routinely shed and replaced by new cells. As the old cells die, they accumulate in the saliva in the mouth and can be easily be collected by a simple procedure using mouthwash[12]. The mean number of epithelial cells per 1ml of saliva is about $4.3x10^5$, whereas the number of nucleated cells in 1ml of whole blood is about $4.5\text{-}11x10^5$. Moreover the turnover of epithelial cells is quite extensive in mouth, as the surface layer of epithelial cells is replaced on average every 2.7h suggesting that there is likely to be intact gDNA in saliva samples[8].

### 1.2.1 ADVANTAGES OF BUCCAL CELLS:

Collecting buccal cells enables researchers to better understand the way people process substances that affect cancer and other diseases and to determine why some people who are exposed to certain substances develop diseases, whereas others exposed to the same substances do not. The material in the buccal cell samples, combined with information on occupational, environmental, and dietary factors, allows researchers to get a more complete assessment of what is affecting the health of human population. The buccal cell sample is being collected to study the differences in genes that may be related to how people process disease-causing substances and how the effects of diet, lifestyle, environment, race and ethnicity, age, and other factors may be related to these genes.

### 1.2.2 CHARACTERISTICS OF BUCCAL CELLS

1. Research has shown that sublingual cells correlate well with deep body tissue such as heart tissue taken during bypass surgery and skeletal muscle biopsies.

2. Buccal cells have high correlation between altered mineral levels and path physiological conditions in multiple medical syndromes.

3. Sublingual cells provide new and important information as to the status of cardiac mineral electrolytes in patients with cardiovascular disease.

4. Sublingual epithelial cells offer a rapidly renewing, homogenous cell population that reflects current total body intracellular mineral status.

5. Buccal cells have a high cytoplasm to nucleus structure facilitating mineral analysis. Cells contain full metabolic biochemical pathways.

6. Blood and urine levels of minerals and ions do not necessarily reflect what is happening in the working cellular tissues.

7. Cells contain about 99% of the body's magnesium and potassium, while serum contains only 1% of the total.

8. Low intracellular tissue levels of mineral electrolytes may exist wile the serum levels appear normal.

9. Buccal cells are safe, easy to obtain and use as a smear on specially prepared slides.

   Fixed specimens have a long life and do not deteriorate in transit[13].

Genomic DNA is identical whether it comes from blood cells or cheek cells. Buccal cell is viable alternative to isolation from blood. Buccal cell DNA is used for many diagnostic applications such as epidemiologic studies and paternity testing[14]. There are several advantages to buccal cell DNA isolation over blood. First no needles, are involved, so it is less invasive and painless. It is well suited for young subjects. Buccal cells provide less of a potential hazard to the people who handle samples[15].

Repeated sampling is not always feasible by blood. With the growing interest in large scale genomic studies epidemiological studies have become very important in trying to elucidate gene-environment interaction in individuals prone to multigenic diseases like cancer and cardiovascular diseases. As already known exfoliated buccal cells are a good source of DNA and sample collection in such cases is non-invasive and can be self-administered. The average yield is about 30µg and is sufficient for more than 300 PCR amplifications. It has been observed that good quality high molecular weight genomic DNA can be obtained from exfoliated buccal cells in the early morning mouthwash samples and that the DNA yield from similar samples decreases during the day, with very low yields obtained in the late evening. This was due to very few exfoliated cells being present in the oral cavity at the time[16]. Oral epithelial cells are constantly exfoliated and may be captured through gentle scraping of the oral mucosa or by oral rinsing[17].

One published study reported the quantity, quality, and stability of hDNA collected with mouthwash. Specimens yielded a median quantity of at least 32µg DNA (2-94) if specimens were held for up to 5 days, with yields declining to 21µg (5-56) at 30 days. Polymerase chain reaction success rates were greater than 94% and

high molecular weight DNA (>23kb) was found in all but 1 out of 24 specimens. Yields were greater when specimens were collected before brushing teeth[7]. Self Collection of oral epithelial cells at a subject's home or hospital under instruction from medically trained or untrained personnel is an attractive and efficient approach for many epidemiologic studies[18]. Methods for collecting buccal epithelial cells include cotton swabs, cytology brushes, wooden sticks, and mouthwashes, with mouthwashes giving greater yields. Several mouthwashes have been tested 3% sucrose, 5% glucose, saline, water and commercial mouthwash. In addition, clinical validation studies have shown that DNA diagnostic results from patient-matched buccal cells and whole blood are comparable. At least 2 main approaches to DNA purification have been used with mouthwash specimens; (1) a rapid boiling method, yielding low-quality DNA, and (2) a lengthy phenol-chloroform method using toxic reagents[19].

| **Method of collection** | **Median** | **Range** | **SD** |
|---|---|---|---|
| Blood | 95 | 10-283 | 54 |
| Mouthwash | 11 | 4-78 | 13 |
| Buccal cytobrush | 8 | 4-39 | 7 |

(Table 1). According to Neuhas[20]Total DNA yields (μg) for each sampling method and Standard Deviation is shown.

| Specimen type | DNA yield | Advantages | Disadvantages |
|---|---|---|---|
| Blood spots | 12-42 ng/µl (adults)<br>43-78 ng/µl (neonates) | Small sample size<br>Ease of collection<br>Low cost storage<br>Offers a source for study of exogenous or endogenous compounds other than DNA<br>Genotyping generally requires 10 ng/genotype and with current technology as llittle as 2.5 ng per SNP so that scores to hundreds of genotypes could be obtained from one blood spot | Low DNA yield may not be suitable for whole-genome amplification.<br>Non renewable<br>Smaller amplicons |
| Blood cells<br>Whole blood anticoagulated or blood clots<br>Buffy coat | 100-400 µg/10ml<br>200 µg/ml | Relatively low-cost storage<br>Yields large quantities of high quality of genomic DNA<br>Offers a source for study of exogenous or endogenous compounds other than DNA | Invasive sample collection<br>Non renewable |
| Transformed lymphocytes | $10^6$cells = 6 µg | Renewable source of DNA<br>Yields large quantities of High-quality genomic DNA | Labor-intensive preparation<br>High cost storage<br>Does not offer a source for study of exogenous or endogenous compounds other than DNA or RNA |
| Buccal cells | 49.7 µg mean; 0.2-134 µg range (total mouthwash DNA)<br>1-2 µg/cytobrush and swab.<br>32 µg median; 4-196 µg range human DNA in mouthwash | Noninvasive and easy sample collection. Genotyping generally requires 10 ng/genotype and with current technology as llittle as 2.5 ng per SNP for genotyping for getting more genotypes from buccal cell specimen. | Low DNA yield.<br>Highly variable yield.<br>Does not offer a source for study of exogenous or endogenous compounds other than DNA or RNA.<br>Bacterial contamination must be addressed. |

(Table 2) : Comparison of Specimens for DNA Banking for Epidemiologic studies

## 1.3. MUTATION[1]

Mutations are heritable changes in base sequences that modify the information content of DNA and can alter the amino acid sequence of the protein encoded by the gene.

### 1.3.1 Forward mutation:

A mutation that changes the wild type allele of a gene to a different allele is called a forward mutation.

### 1.3.2 Reverse mutation or reversion:

Mutation can also cause a novel mutant allele to revert back to wild type.

### 1.3.3 Substitution:

This occurs when a base at a certain position in one strand of the DNA molecule is replaced by one of the other three bases. During DNA replication a base substitution in one strand will cause a new base pair to appear in the daughter molecule generated from that strand. There are two types

i) Transition: In this a purine replaces the other purine or one pyrimidine replaces the other.

ii) Transversion: Purine replaces a pyrimidine or vice versa.

### 1.3.4 Deletion:

Occurs when a block of one or more nucleotide pairs is lost from a DNA molecule.

### 1.3.5 Insertion:

The addition of one or more nucleotide pairs.

### 1.3.6 Others:

Hydrolysis, Radiation UV and Oxidation can alter the information stored in DNA.

### 1.3.7 Depurination:

The hydrolysis of a purine base A or G from the deoxyribose phosphate back bone occurs 1000times an hour in every human cell. Because the resulting apurinic sites cannot specify a complementary base the DNA replication process sometimes introduces a random base opposite the apurinic site causing a mutation in the newly synthesized complemetary strand 3 quarter of the time.

### 1.3.8 Deamination:

The removal of an amino group can change cytosine to uracil, the nitrogenous base found in RNA but not in DNA, and already known U always pairs with A rather than G deamination follwed by replication may alter a C-G base to T-A pair in future generation of DNA molecules[5]. Damaged DNA could mean the failure of important cell processes, or could even lead to cancer and early death[21].

## 1.4. DNA Repair mechanisms

The following structural changes occur in DNA during mutation:

- Pyrimidine dimers, in which two adjacent pyrimidines on a DNA strand are coupled by additional covalent bonds and thus lose their ability to pair.
- Chemical changes of single bases, such as alkylation or deamination, thus causing changes in the pairing properties of the DNA.
- Crosslinks between the complementary DNA strands, which prevent their separation in replication.
- Intercalation of mutagenic agents into the DNA causing frameshift mutations.
- Single strand breaks.
- Double strand breaks[22].

**DNA repair** refers to a collection of processes by which a cell identifies and corrects damage to the DNA molecules that encode its genome. In human cells, both normal metabolic activities and environmental factors such as UV light can cause DNA damage, resulting in as many as 1 million individual molecular lesions per cell per day. Many of these lesions cause structural damage to the DNA molecule and can alter or eliminate the cell's ability to transcribe the gene that the affected DNA encodes. Other lesions induce potentially harmful mutations in the cell's genome, which affect the survival of its daughter cells after it undergoes mitosis. Consequently, the DNA repair process must be constantly active so it can respond

rapidly to any damage in the DNA structure. The rate of DNA repair is dependent on many factors, including the cell type, the age of the cell and the extracellular environment. A cell that has accumulated a large amount of DNA damage, or one that no longer effectively repairs damage incurred to its DNA, can enter one of three possible states:

- an irreversible state of dormancy, known as senescence
- cell suicide, also known as apoptosis or programmed cell death
- unregulated cell division, which can lead to the formation of a tumor that is cancer.

### 1.4.1 DNA REPAIR AND CANCER:

Inherited mutations that affect DNA repair genes are strongly associated with high cancer risks in humans. Hereditary nonpolyposis colorectal cancer (HNPCC) is strongly associated with specific mutations in the DNA mismatch repair pathway. BRCA1 and BRCA2, two famous mutations conferring a hugely increased risk of breast cancer on carriers, are both associated with a large number of DNA repair pathways, especially NHEJ and homologous recombination. Cancer therapy procedures such as chemotherapy and radiotherapy work by overwhelming the capacity of the cell to repair DNA damage, resulting in cell death. Cells that are most rapidly dividing - most typically cancer cells - are preferentially affected. The side effect is that other non-cancerous but rapidly dividing cells such as stem cells in the bone marrow are also affected. Modern cancer treatments attempt to localize the DNA damage to cells and tissues only associated with cancer, either by physical means

(concentrating the therapeutic agent in the region of the tumor) or by biochemical means (exploiting a feature unique to cancer cells in the body)[23]. ....,...

Worldwide, tobacco kills one human being every six seconds.

- That works out to 560 people every hour, 13,440 people per day and 49 lakh people per annum.

- Tobacco kills 15 times as many people as suicides, murder or manslaughter[45]

**1.4.2 TOBACCO CHEWING AND SMOKING:**

Smoking and tobacco chewing is probably the most obvious factor for adverse mortality and is perhaps less obvious is that smoking inflicts extensive DNA damage. Tobacco smoke contains over two hundred chemicals known to cause cancer, known as carcinogens. The precise mechanism whereby cancer starts is not fully understood. These DNA mutations are permanent and forever increase the likelihood for developing lung cancer. Tobacco smoke also contains chemicals in a group called the polycyclic aromatic hydrocarbons which can lead to specific genetic mutations in a gene known as 'p53'. 'p53' plays an important role in suppressing tumours and significantly, mutations in this gene are present in around half of all major human tumours[24]. Tobacco smoking is the most important and well documented cause of cancer currently known. Epidemiological associations have been found for lung, mouth, pharynx, oesophagus, kidney, bladder, pancreas and cervix cancer. The relationship with cancer of the mouth, pharynx, oesophagus and lung is easily explained by their direct contact with smoke. In the light of the fact that over 90% cancers involve epithelial cells and that DNA damage is considered a crucial mechanism in cancer development, the evaluation of DNA damage in buccal

epithelial cells may thus provide a good biomarker of early damage in target tissues[21]. Single gene errors account for more than 4,000 known hereditable diseases, and the list is growing rapidly. A person's risk for diseases from cystic fibrosis to Huntington's disease now can be determined by looking at the DNA.

Given the emphasis on the role of genetics in cancer development and prevention, simple and cost-effective methods are needed to obtain DNA for large-scale studies. The methods for buccal cells are of two types: dry procedures that use a cytobrush or other implements for scraping of the oral mucosa, and wet procedures that involve swishing liquids in the mouth and spitting into a collection vessel. The advantages of the swish method appear to be higher average DNA yields, longer DNA fragments, and possibly higher percentages of human DNA[25].

Studies suggest that buccal cell samples remain stable at room temperature for up to 2 weeks before DNA extraction and they can be successfully analyzed even after exposure to warm or cold temperatures for 7 days. However evidence from a study of mouthwash collections suggests that samples should be extracted within 5 days, if possible, to maximize human DNA yields. Extracted DNA samples can be preserved for several years when store at -20$^{o}$C.

Among adults studies suggest that mouthwash collections provide buccal cell DNA of higher quantity and purity than cytobrushes, with the alcohol content serving as a preservative to retard the growth of bacterial and fungal contaminants. However mouthwash collection is not an option for infants or toddlers or for adults from societies unaccustomed to its use[26].

## 1.5. AGAROSE GEL ELECTROPHORESIS

Agarose is a linear polymer composed of alternating residues of D- and L-galactose joined by alpha- (1-3) and beta-(1-4) glycosidic linkages. The L-galactose residue has an anhydro bridge between the three and six positions.

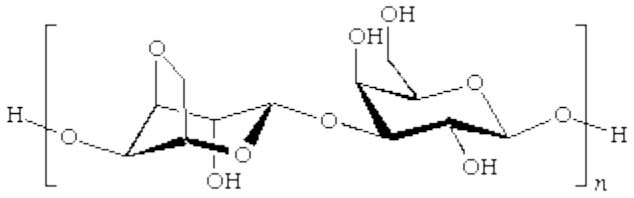


Figure 3: Structure of Agarose

Chains of agarose form helical fibers that aggregate into supercoiled structures with a radius of 20-30nm. Gelation of agarose results in a three-dimensional mesh of channels whose diameters range from 50nm to 200nm.

### 1.5.1 THE RATE OF MIGRATION OF DNA THROUGH AGAROSE GELS

The following factors determine the rate of migration of DNA through agarose gels

**(i) The molecular size of the DNA:**

Molecules of double-stranded DNA migrate through gel matrices at rates that are inversely proportional to the $\log_{10}$ of the number of base pairs. Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules.

**(ii) The concentration of agarose:**

A linear DNA fragment of a given size migrates at different rates through gels containing different concentrations of agarose. There is a linear relationship between the logarithm of the electrophoretic mobililty of the DNA and the gel concentration.

**(iii) The conformation of the DNA:**

Superhelical circular (form I), nicked circular (form II), and linear (form III) DNAs migrate through agarose gels at different rates. The relative mobilities of the three forms depend primarily on the concentration and type of agarose used to make the gel, but they are also influenced by the strength of the applied current, the ionic strength of the buffer, and the density of superhelical twists in the form I DNA. Under some conditions, form I DNA migrates faster than form III DNA; under other conditions, the order is reversed. In most cases, the best way to distinguish between the different conformational forms of DNA is simply to include in the gel a sample of untreated circular DNA and a sample of the same DNA that has been linearized by digestion with a restriction enzyme that cleaves the DNA in only one place.

**(iv) The presence of ethidium bromide in the gel and electrophoresis buffer**:

Intercalation of ethidium bromide causes a decrease in the negative charge of the double stranded DNA and an increase in both its stiffness and length. The rate of migration of the linear DNA dye complex through gels is consequently retarded by a factor approximately 15%. The most convenient and commonly used method to visualize DNA in agarose gels is stainig with the flouresent dye ethidium bromide which contains a tricyclic planar group that intercalates between the stacked bases of

DNA. Ethidium bromide binds to DNA with little or no sequence preference. At saturation in solutions of high ionic strength, approximately one ethidium molecule is intercalated per 2.5 bp. After insertion into the helix, the dye lies perpendicular to the helical axis and makes van der Waals contacts with the base pairs above and below. The fixed position of the planar group and its close proximity to the bases cause dye bound to DNA to display an increased fluorescent yield compared to that of dye in free solution. UV radiation at 254nm is absorbed by the DNA and transmitted to the dye radiation at 302nm and 366nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590nm in the red-orange region of the visible spectrum. Because the fluorescent yield of ethidium bromide-sequence-DNA complexes is 20-30 fold greater than that of unbound dye, bands containing as little as 10ng of DNA can be detected in the presence of free ethidium bromide (0.5 μg/ml) in the gel. Ethidium bromide can be used to detect both single and double stranded nucleic acids. However, the affinity of the dye for single stranded nucleic acid is relatively low and the fluorescent yield is comparatively poor. In fact, most fluorescence associated with staining single stranded DNA or RNA is attributable to binding of the dye to short intrastand duplexes in the molecules.

Ethidium bromide is prepared as a stock solution of 10mg/ml in water, which is stored at room temperature in dark bottles or bottles wrapped in aluminum foil. The dye is usually incorporated into agarose gels and electrophoresis buffers at a concentration of 0.5 μg/ml. Although the electrophoretic mobility of linear double-stranded DNA is reduced by 15% in the presence of the dye, the ability to examine the agarose gels directly under UV illumination during or at the end of the run is a great advantage. However, sharper DNA bands are obtained when electrophoresis is

carried out in the absence of ethidium bromide. During restriction digestion the agarose gel should be run in the absence of ethidium bromide and stained after electrophoresis is complete. Staining is accomplished by immersing the gel in electrophoresis buffer or water containing ethidium bromide for 30-45 minutes at room temperature. Destaining is not usually required. However, detection of very small amounts (<10ng) of DNA is made easier if the background fluorescence caused by unbound ethidium bromide is reduced by soaking the stained gel in water or 1mm $MgSO_4$ for 20 minutes at room temperature**.**

**(v) The applied voltage:**

At low voltages, the rate of migration of linear DNA fragments is proportional to the voltage applied. However, as the strength of the electric field is raised, the mobility of high-molecular weight fragments increases differentially. Thus, the effective range of separation in agarose gels decreases as the voltage is increased. To obtain maximum resolution of DNA fragments >2kb in size, agarose gels should be run at no more than 5-8V/cm.

**(vi) The type of agarose**:

The two major classes of agarose are standard agaroses and low-melting temperature agaroses. A third and growing class consists of intermediate melting/gelling temperature agaroses, exhibiting properties of each of the two major classes.

**(vii) The electrophoresis buffer**:

The electrophoretic mobility of DNA is affected by the composition and ionic strength of the electrophoresis buffer. In the absence of ions electrical conductivity is minimal and DNA migrates slowly, if at all, In buffer of high ionic strength electrical conductance is very efficient and significant amounts of heat are generated, even when moderate voltages are applied. In the worst case, the DNA denature.

### 1.5.2 TYPES OF ELECTROPHORESIS BUFFER:

Several different buffers are available for electrophoresis of native, double-stranded DNA. These contain Tris-acetate and EDTA (pH 8.0; TAE) (also called TE buffer), Tris borate (TBE) or Tris-phosphate (TPE) at a concentration of 50mM (pH 7.5-7.8). Electrophoresis buffers are usually made up as concentrated solutions and stored at room temperature. All these buffers work well, and the choice among them is largely a matter of personal preference. TAE has the lowest buffering capacity of the three and will become exhausted if electrophoresis is carried out for prolonged periods of time.

When this happens, the anodic portion of the gel becomes acidic and bromophenol blue migrating through the gel toward the anode changes in color from bluish-purple to yellow. This change begins at pH 4.6 and is complete at pH 3.0. Exhaustion of TAE can be avoided by periodic replacement of the buffer during electrophoresis or by recirculation of the buffer between the two reservoirs. Both TBE and TPE are slightly more expensive than TAE, but they have significantly higher buffering capacity. Double stranded linear DNA fragments migrate

approximately 10% faster through TAE than through TBE or TPE the resolving power of TAE is slightly better than TBE or TPE for high molecular weight DNAs and worse for low-molecular weight DNAs. This difference probably explains the observation that electrophoresis in TAE yields better resolution of DNA fragments in highly complex mixtures such as mammalian DNA.

Gel loading buffers are mixed with the samples before loading into the slots of the gel. These buffers serve three purposes They increase the density of the sample, ensuring that the DNA sinks evenly into the well; they add color to the sample, thereby simplifying the loading process and they contain dyes that in an electric field, move toward the anode at predictable rates, Bromophenol blue migrates through agarose gels approximately 2.2 fold faster than xylene cyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5x TBE at approximately the same rate as linear double stranded DNA 300bp in length, whereas xylene cyanol FF migrates at approximately the same rate as linear double-stranded DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5-1.4%.

Agarose gels are cast by melting the agarose in the presence of the desired buffer until a clear, transparent solution is achieve. The melted solution is then poured into a mold and allowed to harden. Upon hardening, the agarose forms a matrix, the density of which is determined by the concentration of the agarose[4].

| Agarose concentration in gel (%w/v) | Range of separation of linear DNA (kb) |
|---|---|
| 0.3 | 5-60 |
| 0.6 | 1-20 |
| 0.7 | 0.8-10 |
| 0.9 | 0.5-7 |
| 1.2 | 0.4-6 |
| 1.5 | 0.2-3 |
| 2.0 | 0.1-2 |

Table 3: Range of separation in Gels containing Different amounts of standard agarose.

The DNA fragments that results from restriction enzyme cutting are easily separated and displayed by electrophoresis through agarose gels.

## 1.6. POLYMERASE CHAIN REACTION

PCR is an invitro method for the enzymatic synthesis of specific DNA sequences using two oligonucleotides primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing and the extension of the annealed primers by DNA polymerase results in the exponential accumulation of a specific fragment whose termini are defined by the 5'ends of the primers. Because the primer extension products synthesized in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR yields about a million fold ($2^{20}$) amplification. PCR was invented by Karry Mullis was

originally applied in Human Genetics department at Cetus to the amplification of human β-globin DNA and to the prenatal diagnosis of sickle-cell anaemia. PCR was first proposed in early 1970's by H. Ghobind Khorana and put in practice by Kary Mullis and co-workers at Cetus[4].

An important property of PCR particularly in diagnostic application is the capacity to amplify a target sequence from crude DNA preparations as well as from degraded DNA templates.

### 1.6.1 ESSENTIAL COMPONENTS OF PCR:

- ⇨ A thermostable DNA polymerase to catalyze template dependent synthesis of DNA. Taq Polymerase (0.5-2.5U).

- ⇨ A pair of synthetic oligonucleotide to prime DNA synthesis. Primers should be selected with a random base distribution, and with a GC content similar to that of fragment being amplified. Primers with stretches of polypurines polypyrimidines or other unusual sequence should be avoided. In particularly avoiding primers with 3'end overlaps will reduce the incidence of primer dimer. Concentration 0.05-0.5μM of primer is acceptable. Primer dimer is an amplification artificant observed when many cycles of amplification are performed on a sample containing very few initial copies of template. It is a double stranded fragment whose length is very close to the sum of two primers and appears to occur when one primer is extended by the polymerase over the primer. High concentration of primers favour mispriming which may lead to nonspecific amplification

- ⇨ dNTPs 200-250 μM of each dNTP are recommended for Taq polymerase in reactions containing 1.5mM $MgCl_2$.

- ⇨ Buffer to maintain pH : Tris-Cl (pH 8.3-8.8). Monovalent cations: Standard PCR buffer contains 50 mM KCl and works well or amplification of segments of DNA >500 bp in length. 1.5 mM $MgCl_2$ is optimal (200 μM each dNTP). Generally excess $Mg^{2+}$ may results in the accumulation of non-specific amplification products and insufficient $Mg^{2+}$ will reduce the yield.

- ⇨ Template DNA: In case of mammalian gDNA up to 1.0 μg of DNA is utilized/reaction an amount that contains approximately 3x $10^5$copies of a single copy autosomal gene.

- ⇨ Thermostable DNA polymerases: This is isolated from two classes of organisms the thermophilic and hyperthermophilic eubacteria Archaebacteria. Sometimes cocktails are preferred.

PCR in short is denaturation of the template by heat, annealing of the oligonucleotide primers to the single stranded target sequence and extension of the annealed primers by a thermostable DNA polymerase[56]. Process usually involves the following:

### 1.6.2 Denaturation:

dsDNA template denature at a temperature i.e., determined in part by their G+C content. The higher the proportion of G+C, the higher the temperature required to separate the strands of template DNA. The longer DNA molecules the longer time required to separate. If temperature is short or time is short, only AT rich regions of the template DNA will be denature. When the temperature is reduced later in the PCR cycle the template DNA will reanneal into a fully native condition. This is carried out at 94-95 $^{0}$C which is the highest temperature the enzyme can endure for 30 or more cycles. Higher temperature may be required to denature template that are rich in G+C content. DNA polymerases isolated from Archae are more heat tolerant than Taq.

### 1.6.3 Annealing:

If Annealing temperature is too high the oligonucleotide primers anneal poorly, yield is also low. If temperature is low nonspecific annealing of primers may occur, resulting in unwanted amplification.

### 1.6.4 Extension of oligonucleotide primers:

72-78 $^{0}$C is the optimum temperature for extension. The polymerization rate of Taq polymerase in approximately 2000 nucleotides/min and carried for 1 min for every 1000 bp of product. Result of PCR is not altered by using 3 times longer extension time.

**Standard reaction**

The standard PCR is typically done in a 50 or 100 µl volume an in addition to the sample DNA contains 50 mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 µg/ml gelatin, 0.25 µM of each primer, 200 µM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP) and 2.5 U of Taq polymerase.

### 1.6.5 RAPD-PCR[46]

**RAPD** stands for random amplification of polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified are random. No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain where exactly it binds. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared (it is not suitable for forming a DNA databank). Due to the fact that it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD is used to characterize, and trace, the phylogeny of diverse plant and animal species.

## 1.7. POLYMORPHISMS

Variation whatever may be its cause and however it may be limited, is the essential phenomenon of evolution. The readiest way, then of solving the problem of evolution is to study the facts of variation.---William Bateson (1894). The term

polymorphism has been defined as a 'Mendelian trait' that that exists in the population in at atleast 2 phenotypes, neither of which occurs at a frequency of less than 1%. Some DNA polymorphisms are neutral single base pair changes detected by virtue of the consequent introduction or removal of a restriction enzyme recognition.

These are variations in DNA sequence between individuals. There are about 60,000 polymorphisms in human genome[27]. RFLPs are not rare being distributed throughout the genome at a frequency of between 1/200 and 1/1000 bp. Not unexpectedly, the vast majority of polymorphisms occurs in introns or intergenic regions rather than within coding sequences and may thus be expected to be neutral with respect to fitness. Those polymorphisms that occur either in coding regions or in the promoter region may however affect whether the structure or function of the gene product or the expression of the genes and may have the potential to be of phenotypic or even pathological significance. Restriction enzymes are named based on the bacteria in which they are isolated in the following manner:

| | | |
|---|---|---|
| E | *Escherichia* (genus) | |
| Co | coli | (species) |
| R | RY13 | (strain) |
| I | First identified Order ID'd in bacterium | |

| Enzyme | Source | Recognition sequence | Cut | Average Fragment Size (Kb) | Estimated number of sites |
|---|---|---|---|---|---|
| EcoR I | *Escherichia coli* | 5’ GAATTC<br>3’ CTTAAG | 5’---G AATTC---3’<br>3’---CTTAA G---5’ | 5 | $6x10^5$ |
| BamHI | *Bacillus amyloliquefaciens* | 5’ CCWGG<br>3’ GGWCC | 5’---G GATCC---3<br>3’---CCTAG G---5’ | 5 | $7.5x10^5$ |
| HindIII | *Haemophilus influenza* | 5’ GGATCC<br>3’ CCTAGG | 5’---A AGCTT---3’<br>3’---TTCGA A---5’ | 4 | $6x10^5$ |

Table 4: Restriction enzymes and their property

**1.7.1 RFLPS:**

In practice the DNA of many individuals of one lineage is first cleaved with a restriction enzyme which exhibits a probe. At present approximately 200 different RFLP probes for a total of 10 restriction enzymes have already been identified and employed for mapping purpose. Occasionally RFLPs may also be caused by more complex phenomena such as deletions or insertions. DNA polymorphisms offer a number of advantages for mapping genomes firstly number of DNA markers already exceeds that of suitable protein markers, secondly a DNA sequences does not necessarily have to express a protein in order to be identified by polymorphism cleavage sites. DNA polymorphisms can of course occur in any DNA sequence particularly in introns.

RFLPs are especially useful for identifying genetic defects in humans and can be exploited for diagnostic purposes as long as the DNA alternations involved do not occur several times, and are associated with single genes. Most of the RFLPs known today appear to have occurred randomly and bear no relation to neighboring gene.

RFLPs were first used for characterizing mutant viruses. In humans, RFLPs were first identified in the vicinity of the globin gene and have been used for diagnosing sickle cell anaemia. It can be also used for mapping genes and hence for characterizing genetic defects even if the gene in question is completely unknown[28].

**1.7.2 Mutation specific RFLPs**[29]:

In some single gene disorder the mutation responsible eliminates a restriction enzyme recognition site. This direct approach has been used in sickle-cell disease using the Restriction enzyme Mst II.

Variation in the nucleotide sequence of the human genome is common, occurring approximately once every 200 bp. These single base pair differences in DNA nucleotide sequences are inherited in a Mendelian codominant manner and have no phenotypic effects as they usually occur in intergenic non-coding DNA. If a difference in DNA sequence occurs within the nucleotide recognition sequence of a restriction enzyme the DNA fragments produced by that RE will be of different lengths in different people. This can be recognized by the altered mobility of the restriction fragments on gel electrophoresis.

### 1.7.3 Restriction enzyme digestion:

Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called Restriction endonucleases or Restriction enzymes. Restriction enzymes are bacterial enzymes which cut (hydrolyse) DNA into defined and reproducible fragments. In bacteria, they form part of the Restriction modification defense mechanism against foreign DNA. They are basic tool for gene cloning. These special enzymes recognize specific sequences called palindromic sequence in the DNA molecule and cleave symmetrically in both strands. Restriction digestion begins by mixing the DNA and the Restriction enzyme. The actual reaction conditions vary from one enzyme to the next and include temperature, NaCl and/or $MgCl_2$, concentration, pH. All of these variables except temperature are optimized by mixing the enzyme and DNA with a buffer specific for the enzyme of choice. Once the ingredients are mixed in the reaction tube, the tube is incubated at the reaction optimal temperature for 1 hour or longer. Then finally when the digest has run for the appropriate amount of time, the reaction tube is put back on ice to prevent nonspecific degradation of DNA. The principle behind the technology rests on the possibility of comparing band profiles generated after Restriction digestion in DNA molecules of different individuals. Diverse mutations that might have occurred affect DNA molecules in different ways producing fragments of variable lengths. These differences in fragment lengths can be seen after gel electrophoresis[30].

# 2. *REVIEW OF LITERATURE*

- **Ingrid Meulenbelt *et al.,*[36] (1995),** adopted non-invasive DNA sampling and isolation method involving oral samples taken with cotton swabs. Out of 262 DNA samples isolated using mouth swabs, 257 were successfully used in PCR reactions of 20 different human loci. They also found that phenol/chloroform extractions used to isolate uncontaminated genomic DNA without yeast spores or bacteria could be used for DNA analysis other than PCR. They have used this method for genetic linkage study, various genetic population studies, and in zygosity determination of twin pairs.

- **Amy H. Walker *et al,*[37] (1999),** processed 995 buccal swabs for use in PCR based genotype assays. They processed biosamples for as long as 3 years and found no appreciable decrease in the rate of PCR success. They concluded that adequate DNA for PCR-based applications could be obtained from buccal swabs, but sampling or processing considerations might be important in obtaining optimal results.

- **Lea C. Harty *et al.,* [18] (2000),** devised a simple, non invasive, cost efficient technique for collecting buccal cell DNA for molecular epidemiology studies. Subjects brushed their oral mucosa and expectorated the fluid in their mouths which was applied to Guthrie cards pretreated to retard bacterial growth and inhibit nuclease activity. The cards were well suited for transport and storage because they dry quickly, need no processing and were compact and lightweight. They concluded that treated cards were an alternative to brushes/swabs and mouth

rinses for collection of buccal cell DNA and offer some advantages over other methods.

- **Lea C. Harty *et al.,*[17] (2000),** found that self collection of oral epithelial cells under the direction of a trained interviewer yielded similar quantities of DNA as clinic based collection by a medical technician and larger quantities of DNA were obtained from men than from women. Men may have larger buccal mucosal surface areas or may brush harder, thereby loosening more cells. Thus, self collection of DNA by using oral rinses could be a suitable method for obtaining high quality samples and achieving high participation rates.

- **Loie Le Marchand *et al.,*[38] (2001),** assessed the feasibility of obtaining buccal cell DNA by mail from participants in a large community based cohort study I in Hawaii. They found that the mean DNA yield was lower in females (41.7µg) than in males (53.4 µg) and in Japanese (37.8 µg) as compared with Hawaiians (51.9 µg) and Caucasians (54.8 µg). All samples were successfully genotyped or polymorphisms in the CYP1A1, CYP2E1, GSTM1, GSTT1 and NQO1 genes by PCR-RFLP. From these data they concluded that in situations where blood samples cannot be obtained, mail collection of mouthwash samples should be considered because it yielded substantial amounts of high quality gDNA for large number of study subjects.

- **Heather Spencer Feigelson *et al.,*[29] (2001),** used a mouthwash protocol to collect six daily buccal cell samples from 35 healthy volunteers. They determined total DNA, human specific(hDNA), degradation of DNA, and ability to amplify by PCR. However tooth brushing 1 hour before sample collection reduced the

amount of hDNA by nearly 40%. Their results suggests that buccal cells should be collected before brushing teeth and processed within 5 days of collection to maximize hDNA yield.

- **Schichun Zheng *et al.,*[40] (2001),** collected buccal samples from children ranging in age from 4 months to 15 years. They evaluated a technique that involves Whole Genome Amplification using the improved primer extension preamplification method. They reported that the standard buccal protocol failed to yield successful PCR reactions in 30-51% of specimens whereas WGA buccal protocol however produced genotyping results fully concordant with the referent blood or bone marrow DNA results for all fine loci, and is very useful for improving the efficiency and validity of PCR based genotyping in pediatric populations.

- **Ellen M. Heath *et al.,*[19] (2001),** developed a noninvasive sampling method for collecting cells for routine DNA testing in a clinical laboratory setting. Of the five mouthwashes tested, Scope brand mouthwash received the highest overall ranking. They found in a 4 week, room temperature stability study, the DNA in mouthwash samples was stable for at least 2 weeks, yields ranged from around 12-60 µg/ donor, and the DNA was of high quality and the yield was suitable for use in downstream analytical application such as Southern blotting, amplification analysis, sequencing and archiving.

- **Montserrat Garcia-Closas *et al.,*[11] (2001),** conducted a two phase study and in phase I they compared cytobrush and mouthwash samples collected by mail in two different epidemiological studies (a) cytobrush samples (n=120) from a US

case control study of breast cancer (b) Mouthwash samples (n=40) from prospective cohort of male US farmers. Findings from phase I were confirmed in phase Ii where they randomized cytobrush (n=28) and (n=25) samples among participants in the breast cancer study and compared both collection methods. The median human DNA yield determined by hybridization with a DNA probe from Phenol chloroform extracts was 1 and 1.6 µ/2 brushes for phase 1and 2, and 27.5 and 16,6 µg per mouthwash samples for phase 1 and 2 respectively. Most (94-100%) mouthwash extracts contained high molecular weight DNA(>23kb) in contrast to 55-61% of brush extracts. They concluded that a single mouthwash sample provided substantially higher molecular weight DNA than two cytobrush sample.

- **Stephanie J. London *et al.,*[41] (2001)** collected large quantities of buccal cell DNA in school children. They brushed each buccal surface with a soft toothbrush and then rinsed with 10ml of water. They preferred the toothbrush method because of the higher total DNA yields and greater success in generating PCR products. Out of 1563, samples they obtained results for SNP in the interleukin-13 gene(at 2044) by RFLP-PCR on 98.8% and in the promoter at the myeloperoxidase gene (at 463) by real time PCR on 99.7%.

- **Karen Steinberg *et al.,*[7] (2002),** have reviewed current practices for DNA Banking in Epidemiology studies and focused on 4 types of specimens namely whole blood preserved as dried blood spots, whole blood from which gDNA is isolated, immortalized lymphocytes from whole blood or separated lymphocytes prepared immediately or subsequent to cryopreservation and buccal epithelial cells. They concluded that gDNA from whole blood was the safest assurance for

most current and future molecular applications. Buccal cells were considered when noninvasive self-administered or mailed collection protocols were required.

- **Satia-Abouta *et al.*,[25] (2002),** compared the DNA yield, quality and associated costs of buccal cell DNA collected using cytobrushes and swish in self-adminstered procedures. They found a non-statistically significant higher yield from mouthwash compared with cytobrush collections (15.8 µg vs. 12.0 µg respectively). They concluded that collection of DNA with cytobrushes using simple instructions was cost effective in large scale studies and yielded sufficient quantity and quality of DNA for genotyping.

- **Tara Engeman Andrisin *et al.,*[42] (2002),** determined long term stability, quantity and quality of genomic DNA samples collected in buccal cells by the mouthwash method, for use in pharmacogenetic studies. They concluded that genomic DNA in mouthwash was stable for prolonged periods at room temperature, and was sufficient for pharmocogenetic studies.

- **Philip E. Castle *et al.*,[43] (2003),** collected buccal cells from 29 participants, by use of mouthwash rinses and were split into equal aliquots, with one aliquot irradiated by electron beam irradiation equivalent to the sterilizing dosage of U.S. Postal services and the other left untreated. They checked that irradiated aliquots had lower median DNA yields (3.7µg/aliquots) than untreated aliquots (7.6 µg/aliquots) and were more likely to have smaller maximum DNA fragment size on the basis of genomic integrity gels, than untreated aliquots. They concluded that E-beam irradiation reduced the yield and quality of buccal cell specimens and although irradiated buccal cell specimens may retain sufficient DNA integrity for

some amplified analyses of many common genomic targets, assays that target longer DNA fragments (>989 bp) or require whole genome amplification may be compromised.

- **T. Neuhas *et al.,*[20] (2004),** used Light Cycler technology in analysis of non-invasively derived DNA. DNA extracted from blood, mouthwash and buccal cytobrush samples from 100 volunteers were analyzed for the genotypes of cytochrome P450, CYP1B1, glutathione-S-transferases GSTT1, GSTM1 and GSTP1. The median amounts of DNA isolated from blood, mouthwash and buccal cytobrush samples were 95,11 and 8µg respectively.

- **Audrey F Saftlas *et al.*,[26] (2004),** tested two cytobrush collection methods to optimize total DNA yield and purity for HLA (Human Leukocyte Antigen) genotyping in mothers and infants. One was brushing the left and right inner cheeks and the second was brushing the upper and lower gutters. Mother gutter samples yielded higher amounts of DNA than cheek samples. While DNA yield from cheek and gutter collections from infants were equivalent. They also concluded that cytobrushes stored in paper envelopes yielded significantly more and higher purity DNA than brushes in plastic bags or tubes.

- **Clarie Mulot *et al.*,[44] (2004)**, compared the gDNA obtained from buccal cells through mouthwash, cytobrush and treated cards. They analyzed the amount and quality of DNA and the influence of a lag time at room temperature to simulate delays of sample mailing. They found that mouthwash was more expensive, than cytobrush. The cytobrush method appeared to be more appropriate for them.

They also demonstrated that cytobrushes could be used for studies with very young subjects, on a large scale.

- **Dominique Quinque *et al.*,[8] (2006)**, developed a simple noninvasive procedure for saliva sample collection, DNA collection and DNA extraction. The average amount of human DNA was about 11.4 μg/ml of saliva which was more than the DNA obtained from other noninvasive samples such as cheek swabs. They determined the amount of human specific genomic DNA by TaqMan assays which varied from 5.7 to 100% of the total DNA, suggesting nonhuman DNA was present in the extracts.

- **Priya Koppikar *et al.*,[16] (2006)**, evaluated that good quality high molecular weight genomic DNA can be obtained from exfoliated cells in the early morning mouthwash samples, and that the DNA yield from similar samples decreases during the day, with very low yields obtained in the late evening. They also determined that DNA so obtained was successfully amplified by PCR, and can be used for further studies like RFLP.

# 3. AIM AND OBJECTIVE

Interest in genome-wide association studies to identify susceptibility alleles for cancer is growing, and several are currently planned or under way. A critical requirement for such large scale studies is the amount of DNA available from a sample. Traditionally whole blood is the tissue of choice, as the yield of DNA is quite high (typically 10-15 μg/ml). However obtaining a blood sample is an invasive procedure that requires training in phlebotomy, and moreover blood samples require refrigerated storage and must be processed within a week or so of collection together they may cause potential hazard to the handlers.

The present study was aimed at investigating the suitability of analyzing the genotype by using genomic DNA collected non-invasively by mouthwash in association with the RAPD-PCR approach and by studying genetic polymorphisms by RFLP. In the light of the fact that over 90% cancers involve epithelial cells and that DNA damage is considered a crucial mechanism in cancer development, the evaluation of DNA damage in buccal epithelial cells may thus provide a good biomarker of early damage in target tissues.[21]

Working with human genome, Botstein proposed the use of DNA fragments as genetic markers for monitoring segregation. The first molecular markers to be used were fragments produced by digestion of DNA with restriction enzymes. The variation in fragment size obtained from different individuals after the digestion created the class of markers called restriction fragment length polymorphism (RFLP). If we compare the restriction map of DNA from patients suffering from a disease with

the DNA of a normal people, we may find that a particular restriction site is present or absent from the patients.

Molecular markers are DNA fragments that can be used as a fingerprint in the identification or characterization of individuals. These markers have become an increasingly helpful tool in genetic research and applications to biotechnology. The basic premise behind molecular markers is that there is natural genetic variation in individuals, and many genetic sequences are polymorphic, meaning they differ among individuals. The objective of this work is to find out the variation i.e., polymorphism which would be helpful in diagnosing genetic defects. Molecular markers seek to exploit this variation to identify individuals, traits, or genes on the basis of genetic differences.

The RFLP performed was to find variation between individuals namely healthy, tobacco users and cancer patients by exposing their DNA to Restriction enzymes and running an aliquot of the resulted DNA on 1% Agarose. The DNA will be separated according to their molecular weight and bands generated would be useful in tracing out the difference. This property can be used for diagnosing genetic defects.

# 4. PLAN OF WORK

**A. Selection of volunteers and collection of mouthwash samples.**

Volunteers mainly chosen were healthy persons, tobacco chewers, cancer patients preferably with oral cancer. The mouthwash chosen were 4% sucrose solution, 0.9% saline and 2 branded mouthwashes.

**B. Isolation of genomic DNA from mouthwash samples using,**

i) simple protocol including NaCl, EDTA, NaOH, & Tris Hcl.

ii) a lengthy protocol using phenol chloroform extraction.

**C. Agarose gel electrophoresis**

1% agarose gel was used to find out the purity and yield of DNA present in the samples.

**D. Quantification of DNA by using UV spectrophotometer.**

The DNA sample was diluted in the ratio of 1: 100 with sterile water or TE buffer. The absorbance was measured at 260nm and calculated accordingly.

**E. (RAPD) Polymerase chain reaction (PCR).**

DNA samples were amplified using unknown sequence and known IFN primer to check the source and integrity of DNA obtained..

**F. Restriction Fragment Length Polymorphism (RFLP).**

DNA samples were digested using two restriction endonucleases to find out the polymorphism undergone.

# 5. MATERIALS AND INSTRUMENTS USED

## MATERIALS USED

| S.No | Chemical Name | Company |
|---|---|---|
| 1 | Sodium Chloride | LOBA CHEMI MUMBAI |
| 2 | EDTA | s d-fiNE CHEMICALs MUMBAI |
| 3 | Sodium Hydroxide | s d-fiNE CHEMICALS MUMBAI |
| 4 | Tris HCl | HI-MEDIA MUMBAI |
| 5 | Sodium Dodecyl Sulphate | s d-fiNE CHEMICALS MUMBAI |
| 6 | Proteinase K | GENIE |
| 7 | Phenol | s d-fiNE CHEMICALS MUMBAI |
| 8 | Chloroform | s d-fiNE CHEMICALS MUMBAI |
| 9 | Sodium acetate | NICE |
| 10 | Ethanol | Sakthi sugars ltd, Appakoodal |
| 11 | Agarose | HI-MEDIA MUMBAI |
| 12 | Ethidium Bromide | HI-MEDIA MUMBAI |
| 13 | Bromophenol blue | HI-MEDIA MUMBAI |
| 14 | Sucrose | LOBA CHEMI MUMBAI |
| 15 | Boric acid | INDIAN PHARMACEUTICALS CHENNAI |
| 16 | Eco RI | MEDOX CHENNAI |
| 17 | Bam HI | MEDOX CHENNAI |
| 18 | Hind III | MEDOX CHENNAI |
| 19 | PCR KIT | GENEI BANGALORE |
| 20 | Primer | GENEI BANGALORE |
| 21 | Tris base | HI-MEDIA |

# INSTRUMENTS USED

| S.No | Instrument name | Company |
|---|---|---|
| 1 | Autoclave | NEW LAB EQUIPMENT |
| 2 | Cooling Centrifuge | REMI MOTOR LTD |
| 3 | Digital Balance | SHIMADZU |
| 4 | Deep Freezer | BLUE STAR |
| 6 | Digital pH Meter | ELICO |
| 7 | Double beam UV-Visible Spectrophotometer | ELICO |
| 8 | Electronic Digital Water bath | GENUINE |
| 9 | Hot Air Oven | GENUINE |
| 10 | Laminar Air Flow | GENUINE |
| 11 | Refrigerator | Godrej |
| 12 | Agarose gel apparatus | GENUINE |
| 13 | PCR | EPPENDORF THERMAL CYCLER |

# 6. METHODOLOGY

**6.1 Selection of participants and collection of mouthwash samples:**

For this randomized crossover study, 29 participants from in and around Erode district were selected. 14 Women (Mean Age 52), 15 Men (Mean Age 49) were approached for mouthwash samples. 25 compiled the study. 4 participants ignored the study and did not provide sample.

Volunteers were classified into three categories namely

i) Normal or healthy patients who did not have any smoking or tobacco chewing habits and led a good healthy life. In this category 4 (2 men and 2 women) samples were collected.

ii) Tobacco chewers are those who were exposed to smoke or tobacco in any form and were chewing tobacco for more than 10 years with or without smoking or alcohol. In this category 16 samples were collected (11 men and 5 women).

iii) Cancer patients are those who have been diagnosed with tumours. Oral cancer patients are almost preferred but due to limited numbers other cancer like stomach and rectal cancer patients were selected around Erode and were undergoing chemotherapy and radiation treatment as inpatients in hospital. 5 participants (4 women and 1 man) were approached for their samples.

**Procedure for sample collection:**

⇨ A day before in evening volunteers was approached with a sterile leak tight cup with lid as a collection vessel containing the solution to be swished inside their mouth. The solutions used were 4% sucrose, 0.9% saline, and two commercial brands Rexidine and Listerine.

⇨ A consent form containing information to be filled regarding their Age, Gender, Tobacco chewing habits and other particulars. To protect the confidentiality of these genetic tests multiple safeguards were taken. Collection containers were identified by numbers not by names. Names of the participants will not be used in any of the reports. The study results are reported in statistical summary only[12].

⇨ They were asked to swish 15 ml of the mouthwash solution vigorously for 60 seconds the next day morning before brushing their teeth[16]. The solution should be moved from cheek to cheek without gargling as gargling introduces phlegm from throat which is not conducive to resuspension or automated tipmixing[31]. When spitting it was ensured that the entire mouthwash sample was collected into the sterile container to maximize the yield. More cells meant more DNA. The entire process required only 5 minutes. Note : No food or drink should be consumed before sample collection.

⇨ The samples were collected in the next morning and brought to lab and processed for isolation of DNA.

## 6.2. ISOLATION OF DNA:

After bringing the mouthwash sample to the lab it was processed according to two protocols to find out which protocol yielded more and pure amount of DNA.

Protocol I [M.J., Mac Pherson][32].

a) The buccal epithelial cells were collected by directly centrifuging at 10,000 rpm for 10 min at room temperature and the supernatant was discarded.

b) The pellet obtained was resuspended in 500 µl of 10 mM NaCl and 10 mM of EDTA (pH 7.5) and transferred to a screw-capped microcentrifuge.

c) This solution was centrifuged for 15 sec and the supernatant was discarded.

d) The cell pellet was again resuspended in 500 µl of 50 mM NaOH by vortexing for 10 sec.

e) The sample was incubated at 100 °C for 5min.

f) The sample was neutralized with 100 µl of 1 M Tris-HCl and vortexed for 5 seconds.

g) Again the samples were centrifuged for 15 sec to remove cell debris and supernatant was retained which contained the buccal cell DNA.

h) 5 µl of DNA prepared in this way was sufficient for a single PCR reaction.

Protocol II [Walsh, D.J., et al[33]. and Lum A., Le Marchand[34]].

a) Mouthwash sample were placed in 50 ml conical tube and centrifuged at 2700 rpm for 15 min. The supernatant was dumped and the pellet were resuspended in 25ml of T10E10 to remove residual mouthwash from the sample. The samples were centrifuged at 2700 rpm for 15 min and the supernatant was dumped.

**Composition of T10E10**

1M Tris/HCl (pH 8.0) =10ml

0.5M EDTA (pH 8.0) = 20ml

Total volume = 1000ml

b). The pellets obtained were resusupended in 700 µl of lysis buffer.

**Composition of Lysis buffer**

10mM TrisHCl (pH 8.0) = 0.1576g/100ml

10mM EDTA (pH 8.0) = 0.3722g/100ml

0.1M NaCl = 0.5848g/100ml

2% SDS = 2g/100ml

The lysis buffer containing the pellet were transferred into a 2.0 ml microcentrifuge.

b) 35 µl 20 mg/ml Proteinase K was added to each sample and vortexed to mix. Then the sample was incubated at 58 °C for 2 hrs.

c) 700 µl 1:1 phenol:chloroform was added to each sample and vortexed for 10 sec and centrifuged at 14,000 rpm for 2 min. The supernatant was removed and placed in a new 2 ml microcentrifuge tube.

d) 700 µl chloroform was added to each sample and vortexed for 10 sec and centrifuged at 14,000 rpm for 2 min. The supernatant was removed and placed in a new 2 ml microfuge tube.

e) The DNA was precipitated with 60µl of 3 M Sodium Acetate (pH 6.0). It was inverted to mix and 2 volumes of 100% ethanol was added inverted to mix and left at −20 °C for 2 hr overnight.

f) The solution was spinned at 10,000 rpm and the supernatant was discarded.

g) The pellet were washed with 500 µl 70% ethanol and spinned at 14,000 rpm for 2 min and the supernatant was dumped and the pellet was suspended in TE buffer and stored at 4 °C.

**6.3. AGAROSE GEL ELECTROPHORESIS[4].**

**METHOD**

a) The edges of clean dry glass plate were sealed with tape to form a mold and were set on a horizontal section of the bench.

b) Sufficient electrophoresis buffer was prepared to fill the tank and to cast the gel.

c) A solution of 1% agarose in electrophoresis buffer was prepared at a concentration appropriate for separating the particular size fragments expected in the DNA samples.

d) The neck of Erlenmeyer flask was loosely plugged with Kimwipes and the slurry was heated in a boiling water bath or in a microwave oven until the agarose dissolves.

e) Insulated tongs or gloves were used to transfer the flask into a water bath at 55 °C. When the molten gel has cooled, ethidium bromide was added to a final concentration of 0.5 µg/ml and the gel solution was mixed thoroughly by gentle swirling.

f) While the agarose solution was cooling, appropriate comb was chosen for forming the sample slots in the gel. The comb was positioned 0.5-1.0 mm above the plate so that a complete well is formed when the agarose is added to the mold.

g) The warm agarose solution was poured into the mold and the gel was allowed to set completely and a small amount of electrophoresis buffer was then poured on top of the gel and the comb was carefully removed. The excess electrophoresis buffer was poured off and the tape was carefully removed.

h) The gel was mounted in the electrophoresis tank. Enough electrophoresis buffer was added to cover the gel to a depth of approximately 1 mm.

i) The DNA samples were mixed with 6µl of the desired 6X gel-loading buffer. The sample mixture was slowly loaded into the slots of the submerged gel using a disposable micropipette. The size standards were loaded into slots on both the right and left sides of the gel.

j) The lid of the gel tank was closed and attached to the electrical leads so that the DNA will migrate toward the positive anode. A voltage of 1-5 V/cm was applied. If the leads have been attached correctly, bubbles should be generated at the anode and cathode due to electrolysis and within a few minutes, the bromophenol blue should migrate from the wells into the body of the gel[4].

k) The gel was run until the bromophenol blue have migrated to an appropriate distance through the gel.

l) As the DNA samples or dyes have migrated a sufficient distance through the gel, the electric current was turned off and the leads, lids were removed from the gel tank and the gel was examined under UV light[4].

## 6.4. QUANTIFICATION OF DNA USING UV SPECTROPHOTOMETER[35].

### a. DNA Measurement

1. 1:100 dilution of DNA sample was prepared with TE buffer in a micro centrifuge tubes.

2. The spectrophotometer was turned on and the wavelengths were set to 260nm and 280 nm.

3. Readings were taken with blank having TE buffer and sample DNA in corresponding cuvette.

4. The ratio between 260 nm and 280 nm absorbance reading should be between 1.5 and 2.0.

### b. DNA quantification

The following calculation is made to quantify the DNA sample:

Absorbance 1 should contain 50µg of DNA

Therefore Absorbance at 260 nm contains ---------- [DNA] in µg/ µl

## 6.5. POLYMERASE CHAIN REACTION (RAPD-PCR)

RAPD stands for **R**andom **A**mplification of **P**olymorphic **D**NA.

DNA amplification is a very simple method for *in vitro* amplification of specific nucleic acids using Taq DNA polymerase and minimum two oligonucleotides specific to the DNA to be amplified.

**Materials:**

1. Taq DNA polymerase.
2. Deoxynucleotidetriphosphate mix.
3. 10X Taq polymerase buffer containining100 mM Tris HCl (pH 9.0), 500 mM KCl, 15 mM $MgCl_2$ and 0.1% gelatin.
4. Genomic sample DNA.
5. Primers Forward IFN 3F: 5' GGCACAACAGGTAGTAGGCG 3' and Reverse primer, IFN 5R: 5' GCCACAGGAGCTTCTGACAC 3'.

The reagents were added in a 500µl PCR tubes in the following order:

a) 38 µl of sterile MilliQ water or autoclaved double distilled water was added into a 0.2/0.5 ml microfuge tube.

b) 5 µl of 10X Taq polymerase assay buffer with $MgCl_2$ was added.

c) 3 µl of 10mM dNTP mix (2.5 mM each) solution was added.

d) 1 µl of sample DNA (200 ng/ml) was added.

e) 1 µl each of forward and reverse primer (250 ng/µl) was added.

f) 1 to 2 units of Taq DNA polymerase (3 Units/µl) was also added.

g) All these were mixed gently.

**PCR program:**

1) Initial denaturing at 94 °C for 9 min followed by 35 cycles of denaturing at 94 °C for 1 min.

2) Annealing at 55 °C for 1min.

3) Extension at 72 °C for 1min.

4) Final Extension at 72 °C for 10 min. Reaction mixture without DNA served as negative control[2].

5) The RAPD-PCR was carried in Eppendorf Thermal Cycler and the lid was preheated to 102 °C which prevented the addition of paraffin layer and evaporation due to varying temperature.

6) After the reaction was over the reaction mix was taken out and 10µl of aqueous layer was run on 1% agarose gel stained with ethidium bromide for 1 to 2 hours at 100 volts[56] and visualized under UV light for the desired length of DNA.

## 6.6. RESTRICTION FRAGMENT LENGTH POLYMORPHISM[30]:

**Materials required:**

Water bath or heating block at 37 °C or, Oven,

Micropipettes,

Agarose gel unit,

Tubes and tips.

**Reagents required:**

Restriction Enzyme,

Buffer 10X,

DNA samples,

Loading dye 6X,

0.5M EDTA,

Sterile distilled water.

**Protocol:**

Buffers are usually available in the marker in a concentration 10 times that at which it will be used (called 10X) and the restriction enzyme which has an activity measured in Units where 1 unit is the amount of enzyme needed to digest 1µg of DNA in 1 hour.

The following volumes of reagents were added to the reaction tube :

**Reaction Mix:**

Restriction enzyme 2-3 units – 0.5 µl

Enzyme buffer 10X − 1.0 µl

DNA sample − 1.0 µl

The volume was brought to 10 µl with sterile distilled water.

a) The water and buffer was first put into the tube and then enzyme was added (Putting enzyme into the water first was avoided as it may start to break down).

b) The DNA sample was put in last and mixed by tapping the tube with finger.

c) The reaction mix was quick spin to remove bubbles (as DNA will adhere to bubble surface and become inaccessible to enzyme).

d) This was incubated at the recommended temperature for 1 hour. Often 1 hour was sufficient for digestion but longer times are frequently used, such as overnight, to assure complete digestion by the enzyme.

e) The reaction was stopped by adding 2.5 µl 6X loading dye mix, and loaded directly onto agarose gel and run at 100 V for 1 to 2 hours and visualized under UV for DNA fragments and compared with DNA marker.

# 7. RESULTS AND DISCUSSION

### 7.1 Selection of participants and collection of mouthwash sample

| S.No | V.No. | Age | Gender | Category | Mouthwash solution type |
|---|---|---|---|---|---|
| 1 | 1 | 27 | Female | Healthy | 4% sucrose |
| 2 | 2 | 23 | Male | *Tobacco chewer* | 4% sucrose |
| 3 | 3 | 72 | Female | **Oral Cancer** | 4% sucrose |
| 4 | 4 | 24 | Male | **Oral Cancer** | 4% sucrose |
| 5 | 5 | 24 | Male | *Smoker* | 4% sucrose |
| 6 | 6 | 56 | Male | Healthy | Listerine |
| 7 | 7 | 26 | Female | Healthy | Rexidine |
| 8 | 8 | 23 | Male | Healthy | Rexidine |
| 9 | 10 | 50 | Male | *Alcoholic* | Rexidine |
| 10 | 11 | 65 | Female | *Tobacco chewer* | Listerine |
| 11 | 12 | 46 | Female | *Tobacco chewer* | Listerine |
| 12 | 13 | 67 | Male | *Smoker* | 0.9% saline |
| 13 | 14 | 70 | Male | *Smoker* | 0.9% saline |
| 14 | 15 | 38 | Male | *Tobacco chewer, Alcoholic and Smoker* | Listerine |
| 15 | 16 | 36 | Male | *Alcoholic* | Listerine |
| 16 | 17 | 27 | Female | **Stomach Cancer** | 0.9% saline |
| 17 | 18 | 60 | Female | **Oral Cancer** | 0.9% saline |
| 18 | 19 | 60 | Female | **Rectum Cancer** | 0.9% saline |
| 19 | 21 | 76 | Male | *Tobacco chewer, Alcoholic & Smoker* | Listerine |
| 20 | 22 | 58 | Male | *Smoker* | Listerine |
| 21 | 25 | 70 | Female | *Tobacco chewer* | Listerine |
| 22 | 26 | 63 | Male | *Smoker. Alcoholic* | 0.9% saline |
| 23 | 27 | 73 | Female | *Tobacco chewer* | 0.9% saline |
| 24 | 28 | 63 | Male | *Smoker, Alcoholic* | 0.9% saline |
| 25 | 29 | 65 | Male | *Smoker* | 0.9% saline |
| Volunteers No.9, 20, 23, 24 ignored the study and did not provide sample. | | | | | |

**TABLE 7.1**


Fig. No. 7.1. DNA smears.

DNA was extracted from buccal cells obtained from mouthwash samples and resuspended in approximately 0.5 ml of TE. One µl from each sample was run on a 1% agarose gel stained with ethidium bromide. The above figure showed the DNA smears of Volunteer no 2 and 3. Volunteer no 2 was a tobacco chewer and no.3 was a cancer patient. Lane no 5 and 6 shows smears of DNA. Lane 1 was loaded with DNA marker. The band had migrated approximately to 21 bp which depicted that the DNA was of high molecular weight, but a smear over a broad size range was also observed.

High molecular weight genomic DNA was isolated using a simple non-invasive method from buccal cells of 25 volunteers. The present method yielded an average of 2µg DNA from the buccal cell samples, when the sample was collected in the early morning, before brushing teeth, transferred at room temperature to the laboratory and processed within 3 hrs. Human gene specific primer sequences were used in PCR amplifications and 1 DNA samples were amplified successfully.

A number of different protocols have been reported for extraction of DNA from normal healthy individuals in case-control studies. Many generally observe reluctance on the part of healthy volunteers to donate blood, in such studies. Also drawing blood from individuals requires a skilled technician, sterile needles, syringes, and controlled conditions for later processing when the donors might be suspected to be carriers for pathogenic viruses like hepatitis and HIV. Also transport of blood samples is often risky.


Fig.No. 7.2 DNA from Buccal cells.

This agarose gel electrophoresis showed a dull band of high molecular weight. Lane no 3 which belongs to a healthy volunteer no. 1. Lane no. 1 is loaded with marker.


Fig.No. 7.3 DNA from Buccal cells

Figure 7.3 showed the agarose gel electrophoresis of cancer patients (volunteers no.17, 18, 19, Lane no 3, 4, 5, ) and one tobacco chewer (volunteer no. 21, Lane no 6).Lane 1 was loaded with DNA marker. A single high molecular weigh band denotes a good quality DNA.

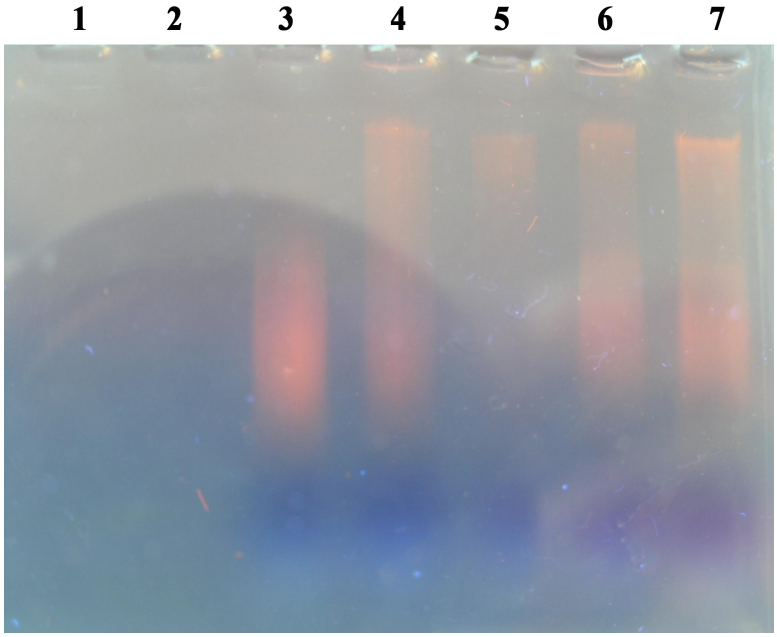


Fig. No. 7.4 DNA from buccal cells.

Figure 7.4 showed DNA smears of (volunteers 8,10,15, 16, 22, ) which in this order corresponds to healthy, alcoholic, tobacco chewer, alcoholic, smoker, tobacco chewer, smoker, tobacco chewer, smoker & alcoholic and smoker. Lane no.3, 4,5,6,7 showed dull smear which indicates the presence of DNA.

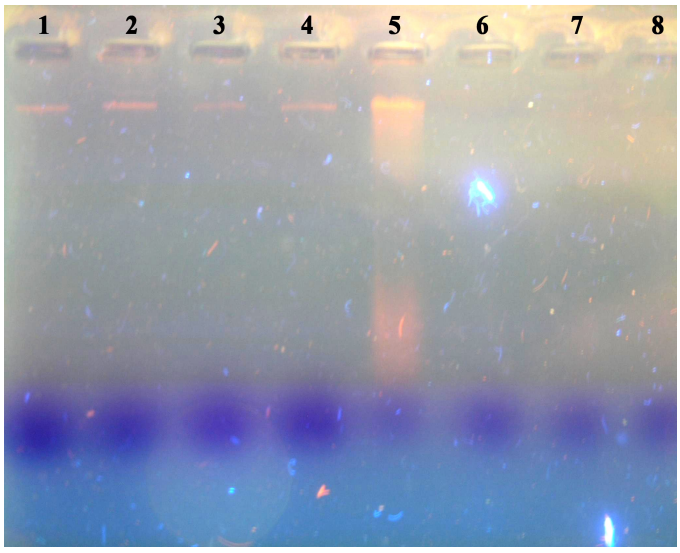


Fig.no. 7.5. DNA from mouthwash sample

The bands of volunteers no 25, 26, 27, 28, 29 are shown in Lane 1, 2, 3, 4, 5. These bands are single which denotes the presence of good quality high molecular weight DNA. The patients were tobacco chewer, smoker, tobacco chewer, smoker and a smoker.

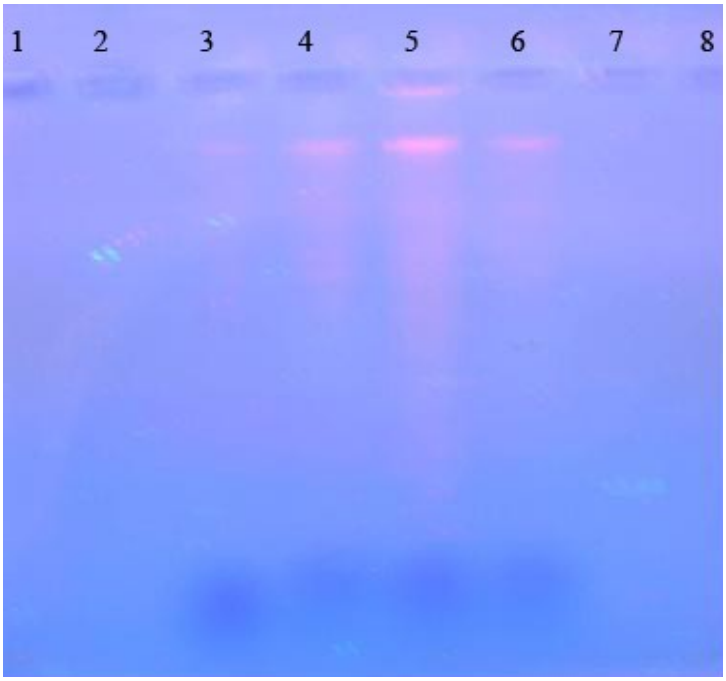


Fig. No. 7.6 DNA from buccal cells

Figure 7.6 showed the band of (volunteers 4, 5, 6, 7) which corresponds to cancer, smoker, and 2 healthy patients. Lane no 3, 4, 5, 6 shows a pink dull band.

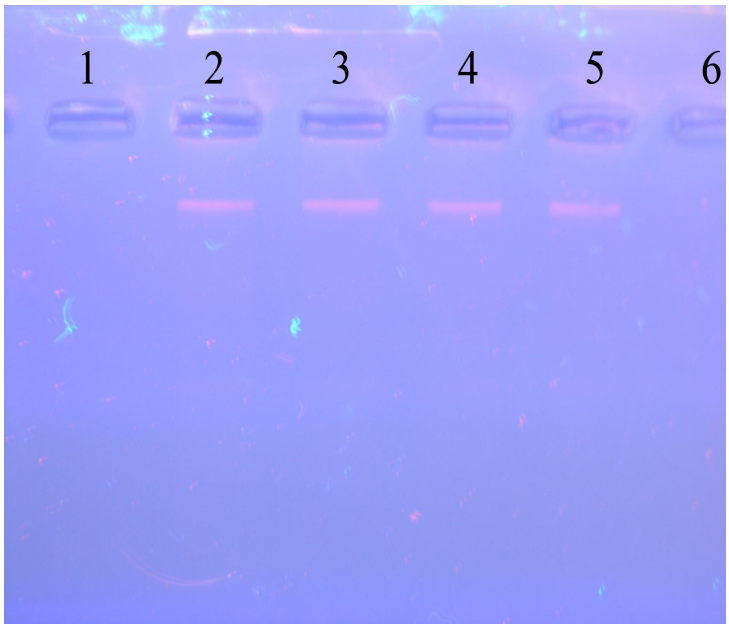


Fig.No. 7.7 DNA from buccal cells.

Figure 7.7 showed agarose gel photos of (volunteers no 11, 12,13, 14) which corresponds to 2 tobacco chewers and 2 smokers.

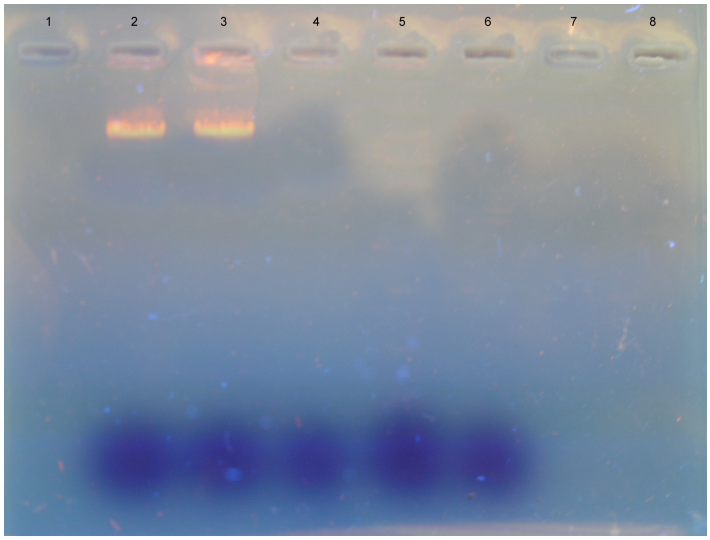


Fig.No. 7.8. PCR results of 1 DNA sample amplified with IFN primers.

PCR Photo of 1 DNA sample, in lane 2 is the parent DNA and in lane 3 is the amplified DNA. The samples no 17 was taken and amplified with IFN primers.

| S.No | Absorbance at 260 | Absorbance at 280 | Absorbance ratio at 260/280 | YIELD in µg |
|---|---|---|---|---|
| 1. | 0.0357 | 0.0299 | 1.1 | 1.7 |
| 2. | 0.0423 | 0.0382 | 1.1 | 2.1 |
| 3. | 0.0431 | 0.0396 | 1.0 | 2.1 |
| 4. | 0.0428 | 0.0382 | 1.1 | 2.1 |
| 5. | 0.0452 | 0.0353 | 1.2 | 2.2 |
| 6. | 0.0481 | 0.0254 | 1.8 | 2.4 |
| 7. | 0.0357 | 0.0299 | 1.4 | 1.8 |
| 8. | 0.0362 | 0.0237 | 1.5 | 1.8 |
| 9. | 0.0372 | 0.0249 | 1.4 | 1.8 |
| 10. | 0.0477 | 0.0251 | 1.7 | 2.3 |
| 11. | 0.0376 | 0.0212 | 1.7 | 1.8 |
| 12. | 0.0358 | 0.0214 | 1.6 | 1.7 |
| 13. | 0.0368 | 0.0234 | 1.5 | 1.8 |
| 14. | 0.0396 | 0.0295 | 1.6 | 1.9 |
| 15. | 0.0512 | 0.0342 | 1.4 | 2.5 |
| 16. | 0.0492 | 0.0384 | 1.2 | 2.4 |
| 17. | 0.0276 | 0.0198 | 1.3 | 1.3 |
| 18. | 0.0412 | 0.0301 | 1.3 | 2.0 |
| 19. | 0.0394 | 0.0265 | 1.4 | 1.9 |
| 20. | 0.0478 | 0.0363 | 1.3 | 2.3 |
| 21. | 0.0399 | 0.0278 | 1.4 | 1.9 |
| 22. | 0.0387 | 0.0278 | 1.3 | 1.9 |
| 23. | 0.0422 | 0.0311 | 1.3 | 2.1 |
| 24. | 0.0386 | 0.0276 | 1.3 | 1.9 |
| 25. | 0.0543 | 0.0392 | 1.3 | 2.7 |

Table 7.2 Showed the purity ratio and the yield of DNA obtained.

These results showed that buccal cell samples collected by mouthwash rinses and processed under optimal conditions can be used for genome-wide association studies with results comparable to those obtained from DNA extracted from other alternatives. Mean average of DNA obtained was 2µg and the individual amount is represented in table 7.2. The mean value for 260/280 ratio was 1.3.

Although buccal cells gave a smaller amount of DNA than blood, recently developed methods of genotyping use very small amounts of DNA (2-10ng per assay) and thus allow the use of buccal cells as a source of DNA. We did not evaluate the potential presence of bacterial DNA in these buccal samples.

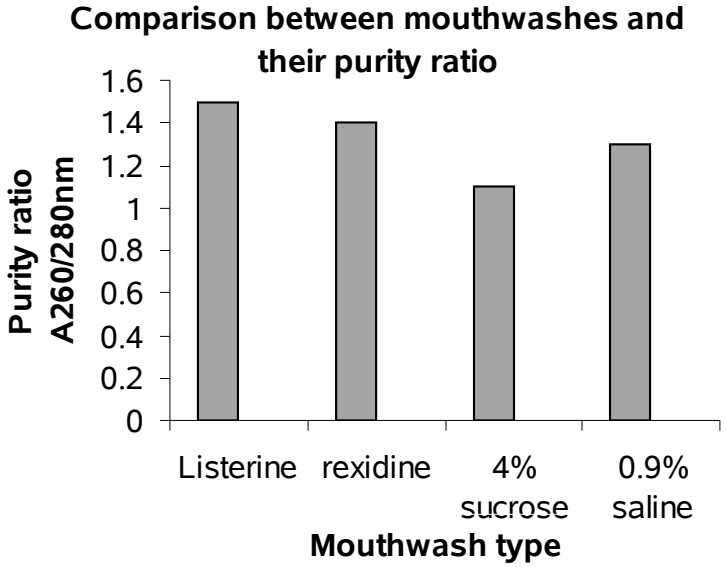


Fig. 7.9. Graphical representation of various mouthwashes used and their purity ratio at A260/280nm.

From the above figure it can be said that Listerine a commercial mouthwash which contains thymol, menthol, eucalyptus oil and alcohol 26%v/v, yielded the pure DNA with good yield and alcohol present in it reduced the microbial DNA presence.

Next went Rexidine which was also a commercial mouthwash containing chlorhexidine gluconate. Even though, it yielded a moderate amount of DNA the dye was present in the supernatant in the last extraction which hindered the DNA isolation process and its purity. 0.9% saline was given to cancer patients and the yield and purity was average but bacterial contamination should be addressed. The average purity obtained with this solution was around 1.3 to 1.4. To the last went the 4% sucrose solution which gave purity around 1.1 and 1.2. The purity ratio of DNA at A260/280nm should be around 1.8-2.0. Below this ratio the DNA is contaminated with protein and again should be extracted with phenol and chloroform. As phenol and chloroform extraction method was used to isolate uncontaminated genomic DNA, without yeast sporule or bacteria, that could be stored for many years and can be used for DNA analysis.

**Restriction fragment length polymorphism results**

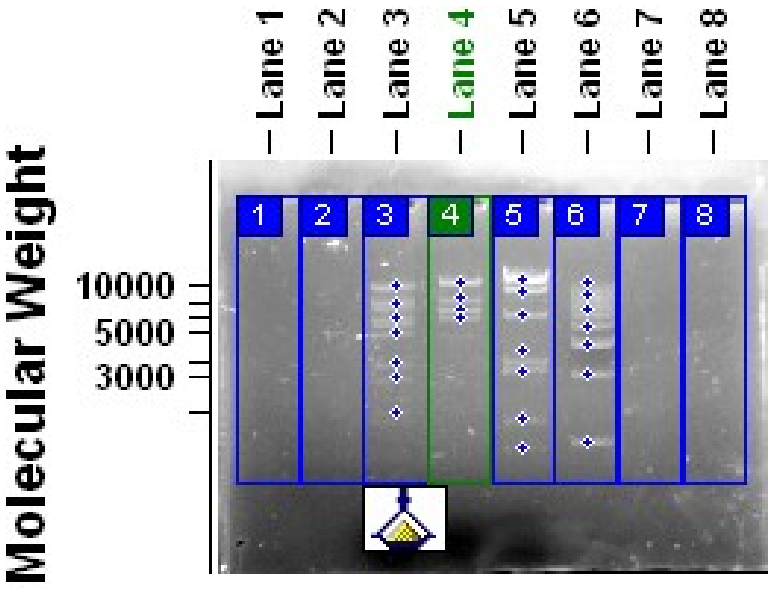


FIG. 7.9: Agarose gel photo of RFLP.

This agarose gel photo showed the fragments generated by restriction endoculeases Eco RI and Hind III with samples 6 of a healthy volunteer (Listerine lane no. 4), sample 12 of a tobacco chewer (Listerine lane no. 5) and sample 18 of a cancer patient (0.9% saline lane no. 6). Lane 4 was loaded with DNA molecular Weight marker.

If the DNA of two individuals were cut with the same enzyme, EcoR V for example, two patterns of DNA fragments are produced, making it possible to distinguish them on the basis of the variation in the length of the fragments because each pattern of fragments is unique to each individual. The occurrence of many patterns of fragments with different lengths is called RFLP The relative position of bands, like a bar code, reveals the fragment sizes. The pattern of bands can then be used reliably to identify the individual source of the DNA.

In the above figure in Lane no.4 the band of normal patient when compared with a tobacco chewer in Lane no.5 there might have some mutation undergone in third fragment i.e., in approximately 6000 bp region. 1kb Marker was loaded to the Lane 3.

Lane no.5 also showed a difference at 8000 bp when compared with Lane no.4. Lane No.6 shows some gap in the 2000bp region. It was assumed that some variation i.e., polymorphism is present between the genes of normal, tobacco chewer and cancer patient. Mutation may be a reason for such polymorphism.

RFLP was developed by Alec Jeffreys in England, in the beginning of the 1980s, this technique is based on the distance between restriction sites in the DNA.

Evaluation of exfoliated cells like buccal cells or bladder epithelium is an innovative tool for genotoxicity studies which is very promising due to the easy obtainment and handling[24]. The results indicated that epithelial cells, could be a good biomarkers of early effects, and can be utilized for human monitoring, since in some cases, this kind of cell is the first to interact with xenobiotics.

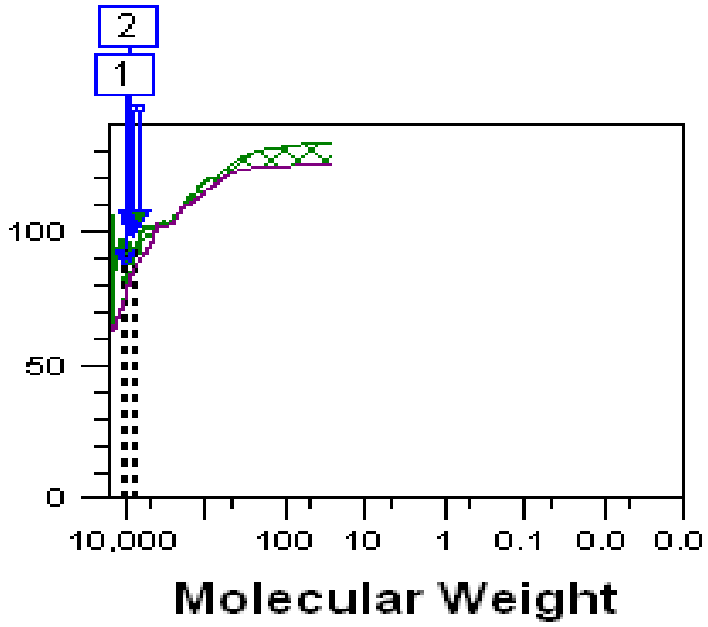


Fig.No.7.9a. A graphical representation. Results are shown below.

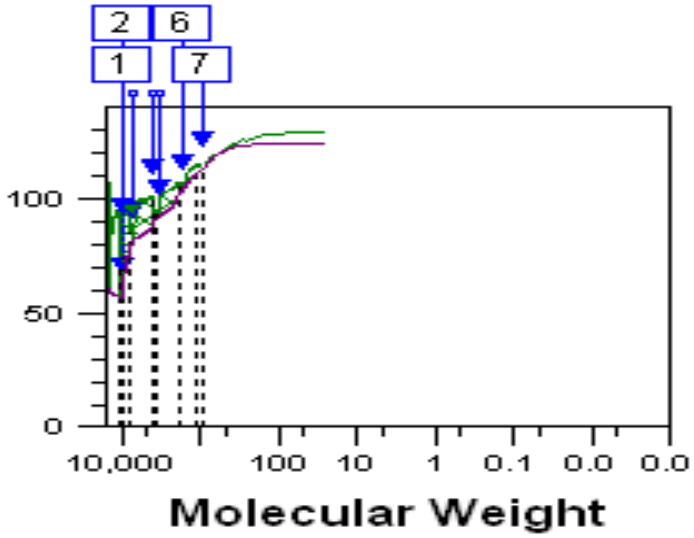


Fig.No.. 7.9b. Graphical representation and results are shown below.

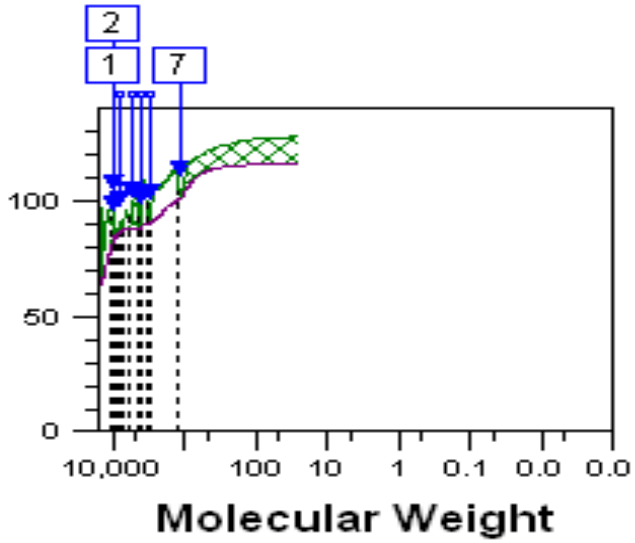


Fig.No.7.9c. Graphical representation, results shown below.

**Sample 1**

| | Band Posn | Volume | Vol+BkGnd | Calib | Vol(ng) | AreaLane% | MW | Rf |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 99 | 5254.95 | 47943.00 | 576.00 | 2.33 | 10263.16 | 0.30 |
| 2 | 2 | 118 | 11337.34 | 99370.00 | 1080.00 | 5.03 | 9334.74 | 0.36 |
| 3 | 3 | 131 | 646.81 | 18860.00 | 216.00 | 0.29 | 8291.61 | 0.40 |
| 4 | 4 | 142 | 6006.46 | 1512862.00 | 3168.00 | 15.98 | 7000.00 | 0.43 |

**Sample 2**

| | Band posn | Volume | Vol+BkGnd | Calib | Vol(ng) | Area Lane% | MW | Rf |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 96 | 4100.34 | 32667.00 | 504.00 | 1.25 | 10421.05 | 0.29 |
| 2 | 2 | 112 | 38951.12 | 174933.00 | 1944.00 | 11.92 | 9652.57 | 0.34 |
| 3 | 3 | 139 | 35872.99 | 277395.00 | 2880.00 | 10.98 | 7385.21 | 0.42 |
| 4 | 4 | 180 | 11961.17 | 120600.00 | 1224.00 | 3.66 | 4236.70 | 0.54 |
| 5 | 5 | 202 | 26501.42 | 419195.00 | 4104.00 | 8.11 | 3508.58 | 0.61 |
| 6 | 6 | 259 | 8136.84 | 259528.00 | 2376.00 | 2.49 | 1775.00 | 0.78 |
| 7 | 7 | 291 | 1005.74 | 49184.00 | 432.00 | 0.31 | 975.00 | 0.88 |

**Sample 3**

| | Band Posn | Volume | Vol+BkGnd | Calib | Vol(ng) | Area Lane% | MW | Rf |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 99 | 11569.50 | 112099.00 | 1224.00 | 4.34 | 10263.16 | 0.30 |
| 2 | 2 | 115 | 599.00 | 42817.00 | 504.00 | 0.22 | 9502.86 | 0.35 |
| 3 | 3 | 131 | 1521.00 | 57546.00 | 648.00 | 0.57 | 8291.61 | 0.40 |
| 4 | 4 | 153 | 13467.00 | 133091.00 | 1368.00 | 5.05 | 5536.02 | 0.46 |
| 5 | 5 | 172 | 1853.65 | 45931.00 | 504.00 | 0.69 | 4322.30 | 0.52 |
| 6 | 6 | 207 | 2005.20 | 40770.00 | 432.00 | 0.75 | 3183.76 | 0.63 |
| 7 | 7 | 286 | 28890.96 | 430202.00 | 3672.00 | 10.83 | 1100.00 | 0.86 |

# 8. SUMMARY AND CONCLUSION

The basic sequence of the human genome has been completed and in the coming years a majority of human genes will be identified. The next step is to elucidate the differences among people in sequences, genes, and gene expression patterns to explain what role these differences play in disease, and in some cases to develop genetic tests for these variants.

We found that total DNA yield was moderate and enough for PCR amplifications and RFLP. Whole blood cells and buccal cells are commonly collected to obtain a DNA sample. Processing of buccal cells from mouthwash samples is an attractive, non-invasive method for obtaining relatively large amounts of DNA. Once DNA is collected, the genomic DNA must be isolated from other cellular material. Next, a specific region or unknown region must be identified or amplified, performed via PCR (RAPD). Gel electrophoresis is often performed after PCR to verify that PCR was successful and that the amplified target sequence is the correct size. Numerous methods are available to determine a person's genotype and differ based on allele discrimination and detection. PCR coupled with restriction fragment length polymorphism (RFLP) analysis, a conventional genotyping method, does not rely on automated technology and is practical for laboratories that genotype a limited number of samples.

The mouthwash method for obtaining DNA is simple, cost-effective and does not require elaborate instrumentation. In addition, as repeated sampling is possible, there ought to be no paucity for using this DNA for various molecular biology tests, a

problem commonly encountered when working with peripheral blood samples. Genomic DNA in mouthwash is stable for prolonged periods at room temperature, and the quantity of DNA recovered from this method is more than sufficient for pharmacogenetic studies.

As 4%sucrose, 0.9% saline and branded mouthwashes were used to collect the exfoliated cells, there should be no problem to the donor.

The RFLP performed would be useful as a diagnosing tool when gone for Southern hybridization by using a probe. The polymorphism was seen in the gel run and by sequencing the gene which has underwent mutation, can be screened out when sequenced or by using other advanced molecular techniques. In humans, RFLPs were first identified in the vicinity of the globing gene and have been used for diagnosing sickle cell anemia. It can be also used for mapping genes and hence for characterizing genetic defects even if the gene in question is completely unknown[28].

It can be concluded that mouthwash technique for obtaining DNA is a feasible and cost-effective method for large scale epidemiologic studies. The DNA obtained from buccal cells could be utilized for genotyping, DNA finger printing and other genotyping assays. RFLP study based upon comparison of band profile generated after cutting the DNA with Restriction enzymes is a useful tool in identifying polymorphism in individuals. The study showed difference in DNA fragments of Healthy, Tobacco users and Cancer patient generated after RFLP. RAPD-PCR was successful with IFN primer sequence which was of human origin. Hence it can be concluded that tobacco use in any form is injurious to health and might lead to cancer.

Therefore Prevention is better than cure.

# 9. BIBILIOGRAPHY


1. Bernard, R., Glick, Jack., and Pasternak. J., Molecular biotechnology, Principles and Applications of Recombinant DNA, 2nd Edn., ASM Press, (1998) 19-96.

2. http://basepair.library.umc.edu.

3. http://www.emc.maricopa.edu/faculty/farabee/BIOBK/BioBookDNAMOLGEN.html#The structure of DNA

4. Sambrook and Russel, *Molecular Cloning- A lab manual*, CSHL Press,3rd Edn.,1,(2001) A5.4-5.14.

5. Hartwell, Hood, Goldberg, Reynolds and Silver Veres., *Genetics*, Mc Graw Hill, (2000) 150.

6. De Robertis D.P., and De Robertis, M.F., Jr, Cell and Molecular Biology, 8th Edn., Published by B.I. Waverly Pvt. Ltd.,(1995) 35.

7. Karen Steinberg, *et al*., DNA Banking for Epidemiologic Studies: A Review of Current Practices, *Epidemiology*,13(2002) 246-254.

8. Dominique quinque, *et al*., Evaluation of saliva as a source of Human DNA for population and associated studies, *Analytical Biochemistry,* 2(353), (2006) 272-277.

9. Heather Spencer Feigelson, *et.al*., Genome Wide Scan in Paired Blood and Buccal samples, *Cancer Epidemiology Biomarkers and Prevention,* 16 (2007) 1023-1025.

10. www.bioserve.com/downloads/presentations//fullscreen.htm.

11. Montserrat Garcia-Closas., *et al,* Collection of Genomic DNA from Adults in Epidemiology studies by Buccal Cytobrush and Mouthwash, *Cancer Epidemiology Biomarkers and Prevention*, 10(2001) 687-696.

12. Michael C.R. Alavanja, *et.al*., *The Agricultural Health Study,* (2002).

13. (http;//www.exatest.com/whyuseBuccalCells.htm)

14. Jan Kieleczawa, DNA Sequencing II: Optimizing Preparation and clean up, *Jones and Bartlett publishers*, (2006) 151.

15. Jonathan S.Olshaker, *et.al.,* Forensic Emergency Medicine, *Wolters Kluwer Health*, (2006); 237.

16. Priya Koppikar and Rita Mulherkar, A simple method for extraction of high molelcular weight genomic DNA from buccal cells in mouthwash, *Indian Journal of Biotechnology,* 5(2006) 477-481.

17. Lea C. Harty, *et al*., Self Collection of Oral Epithelial Cell DNA under instruction form Epidemiologic interviewers*, American journal of Epidemiology*, 151(2000) 199-205.

18. Lea C. Harty, *et al.,* Collection of Buccal cell DNA Using Treated Cards, *Cancer Epidemiology Biomarkers and Prevention,* 9(2000); 501-506.

19. Ellen M. Heath, *et al.,* Use of Buccal Cells Collected in Mouthwash as a source of DNA for Clinical Testing, *Arch. Pathol Lab Med*, 125(2001).

20. Neuhas T., *et al.,* Reliability of non-invasively acquired human genomic DNA as a substrate for real-time PCR-assisted analysis of genetic polymorphisms, *On line publications*. (2004).

21. Stephan Richards, Damaging your DNA: New interpretations of Mortality differentials. (2005) 1-3, http://www.learmonth.co.uk/dna.pdf.

22. Wulf Crueger and Anneliese Crueger, A text book of industrial microbiology. Biotechnology. 2nd Edn., Panima Publidhing Corporation, New Delhi/Bangalore,(2003) 11-15.

23. http://en.wikipedia.org/wiki/DNA_repair.

24. Rojas E.,,*et al.,* DNA damage in exfoliated buccal cells of smokers assessed by the SCGE assay, *Mutation Research,* 370(1996)115-120.

25. Satia-Abouta, *et al.,* Buccal cell DNA Yield, Quality and Collection Costs: Comparison of methods for Large-Scale studies, *Cancer Epidemiology, Biomarkers and Prevention,* 11(2002) 1130-1133.

26. Audrey F Saftlas, *et al.,* Optimizing Buccal cell DNA yields in Mothers and Infants for Human Leukocyte Antigen Genotyping, *American journal of Epidemiology,* 160(2004) 77-84.

27. David N. Cooper., Human gene evolution. Academic Press.(1999) 11.

28. Ernst Winnacker, *Introduction to Gene technology*, Genes to Clones. 23-26.

29. Robert F. Mueller., Ian D. Young., Ewery's elements of Medical Genetics, 11th Edn.,(2001) 65.

30. P. Nagarajan and N. Senthil Kumar. Molecular biology, Principles and methods, a practical approach. 29-32.

31. http:www.agencourt.com/technical.

32. Mac Pherson M.J., and Hames B.D., Science PCR 2: A practical approach. (1995) 221.

33. Walsh, DJ, *et al*. Isolation of DNA from Saliva and Forensic Sciences, *JFSCA,* 37(1992) 387-395.

34. Lum A and Le Marchand, A simple mouthwash method for obtaining genomic DNA in molecular epidemiologic studies, *Cancer Epidemiology, Biomarkers and Prev,* 7(1998) 719-724.

35. Mina J. Bissell. Lawrence Bereley National laboratory, Purity Measurement and Quantification of DNA.

36. Ingrid Meulenbelt, *et al*., High Yield Non Invasive Human Genomic DNA isolation mehod for Genetic studies in Geographically Dispersed Families and Populations, *American Journal of Human Genetics*, 57(1995) 1252-1254.

37. Amy H. Walker, *et al.,* Collection of Genomic DNA by Buccal Swabs for PCR reaction based Biomarker Assays, *Environmental Health Prospectives*, 107(1999) 517-520.

38. Loie Le Marchand., *et al.,* Feasibility of Collecting Buccal Cell DNA by Mail in a Cohort Study, *Cancer Epidemiology, Biomarkers and Prevention,* 10(2001) 701-703.

39. Heather Spencer Feigelson, *et al.,* Determinants of DNA Yield and Quality from Buccal Cell samples collected with Mouthwash, *Cancer Epidemiology Biomarkers and Prevention*, 10(2001) 1005-1008.

40. Schichun Zheng, *et al.,* Whole genome Amplification increases the efficiency and validity of Buccal cell Genotyping in Pediatric populations, *Cancer Epidemiology, Biomarkers and Prevention,* 10(2001) 697-700.

41. Stephanie J.London., *et al.,* Collection of Buccal Cell DNA in Seventh Grade Children using water and a Toothbrush, *Cancer Epidemiology*, *Biomarkers and Prevention,* 19(2001) 1227-1230.

42. Tara Engeman Andrisin, *et al.,* Collection of Genomic DNA by the Non-invasive mouthwash method for use in Pharmocogenetic studies, *Pharmocotherapy*, 22(2002) 954-960

43. Philip E. Castle., *et al.,* Effects of Electron-beam irradiation on Buccal Cell DNA, *American journal of Human Genetics,* 73(2003) 646-651.

44. Claire Mulot, *et al.,* Collection of Human Genomic DNA from buccal cells for Genetic Studies Comparison between Cytobrush, Mouthwash and Treated Card, *Journal of Biomedicine and Biotechnology,* 3(2005) 291-296.

45. http://www.salaambombay.org/index.htm

46. http://avery.rutgers.edu/WSSP/StudentScholars/project/archives/onions/rapd.html

47. Christina L. Aquilante *et al.,* Common laboratory methods in pharmoacogenomic studies, *American journal of Health System Pharmacy,* 63(2006) 2101-2110.

48. de Vries, *et al*., Analyzing DNA from buccal cells is reliable method for the exclusion of cystic fibrosis, Results of a pilot study, *Genetics in Medicine*, 8(2006) 175-177.

49. Miles D. Thompson, *et al.,* Whole genome amplification of buccal cell DNA; genotyping concordance before and after multiple displacement amplification. (2005).

50. Glei M., Habermann N., *et .al.,* Assessment of DNA damage and its modulation by dietary and genetic factors in smokers using the Comet Assay; a biomarker model, *Biomarkers,* 10(2005) 203-217.

51. Jeffrey A.M and Williams G.M., Risk assessment of DNA-reactive carcinogens in food, *Toxicology and Applied Pharmacology*, 207(2005) S628-S635.

52. Timothy L. Lash, *et al*., A case only analysis of the interaction between N-acetyltransferase 2 haplotype in breast cancer, *Etiology, BiomedCentral Ltd*, 7(2005) 1-11.

53. Jon J. Nordby and Stuart H. James. Forensic Science: An Introduction to scientific and investigation techniques, *CRC Press*, (2005) 289.

54. V.S. Dhillon, *et al.,* Comparison of DNA damage and repair following radiation challenge in buccal cells and lymphocytes using single-cell gel electrophoresis, *International journal of Radiation Biology*, 80(2004) 517-528.

55. Chiouo FS, *et al.,*. Extraction of human DNA for PCR from chewed residues of betel quid using a novel "PVP/CTAB" method, *Journal of Forensic Sciences,* 46(2001).

56. John M. Butler, Forensic DNA typing Biology and Technology behind STR markers, Academic *Press publisher, Elsevier.* (2001)189.

57. Judith M, Rumsey and Monique Emst., Functional Neuroimaging in Child Psychiatry, *Cambridge University Press,* (2000) 324.

58. Marja L. Laine, *et al.,* The Mouthwash: A Non_invasive sampling method to study cytokine gene polymorphisms, *Journal of Periondontology*, 71(2000) 1315-1318.

59. Barbara Seligea, *et al.,* Mass spectrometry of Biological Methods, Screening of DNA polymorphism by MALDI/MS, CRC Press, (1998) 422.

60. Judith Scheppler, *et al.,* Biotechnology Explorations, Applying the fundamentals. 91-95. 141-155.

61. R.G.H. Cotton, E. Edkins, S. Forest., Mutation Detection, A practical approach. (2004) 7.

62. Henry A. Erlich. PCR technology, Principles and Applications for DNA amplifications, Oxford University Press, (1992) 3-5.

63. Benjamin Lewis, *Genes VII,* Oxford University Press.(1999) 38.

64. Sumitra Sen, Dipak Kumar Kar, Cytology and Genetics, Narosa Publishing house, (2005) 37.

65. Free Patents Online. Methods for simultaneously detecting multiple mutations in a DNA samples.

66. Jac A. Nickloff and Merl. F. Hoekstra., DNA repair in higher eukaryotes, Contemporary Cancer Research. DNA damage and repair, Humana Press, 542.

67. National research council, Evaluating Human Genetic Diversity. 36.

68. V. Kumaresan. Biotechnology, Saras Publications (2000) 706-723.

69. Gardner, Simmons, Snustad, Principles of Genetics, 8th Ed., 18,(2006) 97-100.

70. Primrose S.B., R.M. Twyman,. Old. *Principles of gene manipulation*, 6th Ed., 9,22.

71. http://www.genome.gov/Pages/Hyperion/DIR/VIP/Glossary/Illustration/Images/chromosome.gif

72. http://www.accessexcellence.org/RC/VL/GG/nucleotide2.html.