Elyas K.K. “Isolation and characterisation of tumour associated antigens and their significance in host immune responses” Thesis. Department of Biochemistry, Medical College Thrissur, University of Calicut, 2005
MATERIALS AND METHODS
Chapter III
MATERIALS AND METHODS

3.1. Samples for the study

Patient’s data for the epidemiological studies were collected from histo-pathological laboratory reports, during 1995-1998 periods from Amala Cancer Research Centre, Thrissur, a referral centre for cancer treatment and research in Kerala. The patient’s data with the clinical symptoms and whose tissue samples were histo-pathologically examined were used for the study. The tissue samples were stained at Histo-pathology department of the centre using standard methods (David and James 1999). Tissue samples of all organs and lesions were preserved in neutral formalin; fixed tissues were embedded in paraffin, 5-7 µm thick sections were made and stained with hematoxylin and eosin stains. Special stains were used when needed.

Blood samples for the studies were collected from oral cancer patients and normal controls by venipuncture into clean centrifuge tubes and were allowed to clot. The serum was separated by centrifugation, aliquoted and stored at -20°C until use. Repeated freezing and thawing was avoided as this could cause aggregation of immunoglobulins leading to erroneous results. These samples were used for the isolation, characterisation of CIC and antigens from the serum.

Biopsies and surgery specimen, obtained from histo-pathologically proved oral squamous cell carcinoma cases were used for HPV-DNA hybridization
studies. Age matched control DNA samples were obtained from oral mucosa, from fresh autopsy specimens and minor oral surgical procedures. DNA samples were stored at -20°C until use.

3.2. Detection of HSV-1 and HSV-2 infections in oral cancer patients by ELISA

HSV-1 and HSV-2 infections were determined by the assessment of specific antibodies in serum of patients and controls using commercially available ELISA kits (Human GmbH, Germany) (Bidwell 1977).

3.2.1. Reagents and Chemicals for the ELISA (Human GmbH, Germany)

1. **Dilution buffer** (pH 6.5 ± 0.2)
   - Phosphate buffer - 10 mmol/l
   - Sodium chloride - 8 g/l
   - Albumin - 10 g/l

2. **Substrate buffer** (pH 3.9 ± 0.2)
   - Potassium citrate - 0.2 mol/l
   - Hydrogen peroxide - 6 mmol/l

3. **Washing Solution** (pH 7.2 ± 0.2)
   - Tris Buffer - 10 mmol/l
   - Sodium chloride - 8 g/l

4. **TMB solution**
   - 3,3', 5,5'-tetra methyl-benzidine- 20 mmol/l

5. **Stopping solution**
   - Sulphuric acid - 1.5 mol/l

6. **Anti-IgG conjugate**
   a) Anti-Human IgG (Rabbit), Peroxidase-conjugated
   b) Anti-Human IgM (Rabbit), Peroxidase-conjugated
7. **Microtiter well strips coated with cell culture derived antigen**
   a) HSV-1 antigen for detection of IgG
   b) HSV-2 antigen for detection of IgG
   c) HSV-1 and HSV-2 antigen for detection of IgM

8. **Control serums**
   a) HSV, IgG/IgM-negative-controls (Human)
   b) HSV, IgG/IgM-positive-controls (Human)

3.2.2. Procedure for the ELISA

The presence of anti HSV-1 and anti HSV-2 IgG antibodies and anti HSV IgM antibodies were determined in patients serum samples. Microtitre strip wells as a solid phase were coated with cell culture derived HSV antigens. If corresponding specific antibodies were present in a patient sample or control, they were bound to the specific antigen on the solid phase. After a washing step to remove unbound material, anti immunoglobulin peroxidase conjugate was added, this binds specifically to the specific class of antibodies. The enzyme-linked complexes were detected by incubation with chromogenic solution and the subsequent development of the blue colour, which changed to yellow by stopping the enzymatic reaction with sulphuric acid. The colour developed is directly proportional to the antibodies present in the serum.

Hundred micro-liter of 1:100 diluted serum samples from oral cancer patients and normal healthy individuals were added to the microtitre wells and incubated for 30 minutes at room temperature. The wells were washed 3 times with washing solution to remove the unbound serum materials. The remaining fluid was removed by tapping the wells on a tissue paper. Added 100 μl of rabbit anti-immunoglobulin (anti-IgG or anti-IgM as the case may be) conjugated with
peroxidase enzyme and incubated for 30 minutes at room temperature. Wells were washed four times as described earlier. Added 100 µl TMB solution and incubated at room temperature for 15 min. in the dark and stopped the reaction by using 100 µl of sulphuric acid as the stopping reagent after the development of colour. The absorbance at 450nm (A_{450}) was measured in an ELISA Reader (Merck, Germany).

Negative and positive controls from the kit were also included during the experiment. Substrate blank was also incorporated in the experiment. All the tests were done in duplicates. Calculations were made as follows in accordance to the manufacturer's instruction.

COV = MNC + 0.1 MPC for IgG ELISA

COV = MNC + 0.2 MPC for IgM ELISA

COV: Cut Off Value

MNC: mean negative control absorbance

MPC: Mean Positive control absorbance

Results were interpreted as follows:-

A_{450} patient ≥ COV + 15% anti-HSV IgG/IgM antibody positive.

A_{450} patient < COV + 15% anti-HSV IgG/IgM antibody negative.

Positive patient's specimens were expressed in "Human Units"("HU/ml.")

"HU/ml" = \frac{\text{Absorbance of test sample}}{\text{Mean Absorbance of positive control}} \times 100
3.3. Detection of HPV-16 and HPV-18 DNA from oral cancer tissue samples

For the determination of the HPV association with oral cancer, hybridisation studies were conducted with the DNA isolated from oral cancer tissue samples.

3.3.1. Maintenance and storage of HPV DNA probes

Viral HPV probes were maintained as recombinant plasmid in pBR 322. These recombinant plasmids were used to transform the host *E. coli* by calcium chloride mediated chemical transformation (Sambrook *et. al.* 1989).

3.3.1.1. Reagents and chemicals for Transformation of *E. coli*

1. **Probe DNA**
   a) HPV-16 DNA Cloned at *BamH*1 site of pBR 322.
   b) HPV-18 DNA Cloned at *EcoR*1 site of pBR 322.

2. **Host cell**
   *E. coli*. (HB 101)

3. **Luria Broth Medium (LB)**
   - **NaCl**: 5.0 g (1%)
   - **Yeast extract**: 2.5 g (0.5%)
   - **Bactotryptone**: 5.0 g (1%)
   - Autoclaved and stored at 4°C.

4. **MgCl₂**
   - 100 mM

5. **CaCl₂**
   - 85 mM

6. **Antibiotics**
<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock solution</th>
<th>Working Con.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracycline</td>
<td>12 mg/ml (in 70% Ethanol)</td>
<td>12 µg/ml</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>4 mg/ml (Water)</td>
<td>50 µg/ml</td>
</tr>
</tbody>
</table>
3.3.1.2. Procedure for transformation of pBR 322-HPV recombinant probe plasmids in *E. coli*

HPV-16 and HPV-18 DNA cloned in the plasmid pBR 322 were used for the study. Cloned recombinant HPV-16 and HPV-18 DNA were used to transform and maintain in *E. coli* (HB 101). These clones were used for the preparation of the viral probes. The cloned HPV-16 DNA was at the *BamH*1 site of pBR 322. HPV-18 was cloned at *EcoR*1 site of pBR322.

The following method was used to transform *E.coli* cells (Sambrook *et. al.* 1989). Single colony of *E. coli* (HB 101) was inoculated into 1ml of LB medium and incubated at 37°C overnight with shaking at 250 rpm. One ml of the culture was inoculated into 50ml of LB medium and grown for 2-3 hours (until the cell density reached 0.6 OD_{600}). The culture was chilled on ice for 2 minutes and centrifuged at 3000 rpm for 5 minutes at 4°C. The pelleted cells were resuspended in 9 ml of sterile 100mM MgCl$_2$ and subsequently centrifuged at 3000 rpm for 5 minutes at 4°C. The pelleted cells were resuspended in 9 ml of ice-cold 100 mM CaCl$_2$ and incubated on ice for 40 minutes. Subsequently, the cells were pelleted and resuspended in 3.5 ml of a sterile solution containing 85mM CaCl$_2$ and 15% glycerol. The cells were dispensed into 100μl aliquots in pre-chilled microfuge tubes and stored at −70°C. These cells were used for transformation.

About 50-100 ng HPV inserted pBR322 DNA was used to transform 100 μl aliquots of competent *E. coli* cells. DNA sample was added to the cells and incubated on ice for 40 minutes. Then the cells were incubated at 42°C for 90 seconds and subsequently on ice for 5 minutes. One ml of antibiotic free LB
medium was added to the tube and cells were allowed to grow at 37°C for one hour. The cells were plated on LB agar plates containing suitable antibiotics. Successful transformants were further propagated and maintained as continuous culture or glycerol stock.

3.3.2. Isolation of Recombinant pBR322-HPV Plasmids DNA from E. coli

In order to get HPV-16 and HPV-18 for probing genomic DNA, HPV containing tranfomants were grown in appropriate antibiotic media and recombinant plasmids were isolated by alkali lysis method (Birnboim and Doly 1979).

3.3.2.1. Reagents and chemicals for Plasmid isolation

1. Solution I (10ml)                        
   Volume     Final concentration
   Glucose (1M)  0.5ml  50mM
   Tris-Cl (1 M, pH 8.0) 0.25ml  25mM
   EDTA (0.5 M, pH 8.0) 0.2ml  10mM
   Distilled water added to make a final volume of 10ml

2. Solution II (10ml)
   Volume     Final concentration
   NaOH (10N)  0.2ml  0.2N
   SDS (10%)  1ml  1%
   Distilled water added to make a final volume of 10ml

3. Solution III (Sodium Acetate solution) (3M, pH 5.2)
   Sodium acetate - 40.81g
   Distilled water - 80ml
   pH adjusted to 5.2 and distilled water added to make a final volume of 100ml

4. Saturated Phenol
   Distilled phenol - 100ml
   β-Hydroxyquinoline - 80mg (0.8mg/ml)
   β-Merocaptoethanol- 0.2%
   Tris-Cl (1 M, pH 7.5)- 100ml

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Mixed equal amounts of phenol with Tris-Cl (0.1M). It was allowed to settle down and aqueous phase discarded. The extraction was repeated several times with equal volumes of 0.1 M Tris-Cl until the pH of the phenolic phase became 7. The equilibrated mixture was stored under an equal volume of 0.01 M Tris-Cl (pH 7.5) at 4°C in dark glass bottles.

5. **Tris (1M, pH 8.0) stock solution**
   Tris base - 12.1 g  
   Distilled water - 80 ml  
   pH adjusted to 8.0 with concentrated HCl and final volume adjusted to 100ml with distilled water.

6. **EDTA (0.5 M, pH 8.0) stock solution**
   EDTA - 18.6 g  
   Distilled water - 80 ml  
   pH adjusted to 8.0 and distilled water added to make a final volume of 100ml

7. **Tris-EDTA (TE) Working solution**
<table>
<thead>
<tr>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl (1 M, pH 8.0)</td>
<td>1.0ml</td>
</tr>
<tr>
<td>EDTA (0.5 M, pH 8.0)</td>
<td>0.2ml</td>
</tr>
</tbody>
</table>
   Distilled water added to make a final volume of 100ml. Autoclaved and stored at 4°C.

8. **RNAse A (1ml) Solution**
   RNAse A - 10mg  
   Tris-Cl (1 M, pH 7.5) - 10μl (10mM)  
   NaCl - 3μl (15mM)  
   Distilled water - 973μl  
   Heated to 100°C for 15 minutes. Allowed to cool slowly at room temperature, stored at –20°C in aliquots.

3.3.2.2. Procedure for Plasmid Isolation

Single cell colony of the transformant was transferred into 5ml medium containing antibiotics and incubated at 37°C with vigorous shaking (300 rpm) on a rotary shaker for 14 hrs. 1.5 ml of culture was taken in a microfuge tube and centrifuged at 10,000 rpm at 4°C for 10min. The supernatant was discarded and the pellets were re-suspended in 100 μl of ice cold Solution I.
vortexed to mix the contents well and kept on ice for 5 min. 200 μl of freshly prepared Solution II was added, mixed gently by inverting the tubes several times and incubated on ice for 10 min. 150 μl of chilled Solution III was added and gently mixed by inverting the tubes several times and kept on ice for 15 min. Centrifuged at 12,000 rpm for 10 min at 4°C and the supernatant was transferred to a fresh tube. 450 μl phenol-chloroform mixture (1:1) was added, mixed by inverting the tubes several times and kept on ice for 15 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was transferred to a fresh tube and added 2 volumes of chilled 100% alcohol, mixed by inverting the tubes several times and kept on ice for 30 min. Centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was removed carefully and 1 ml of 70% ethanol was added to the pellet and mixed gently by inverting the tube several times. Centrifuged at 10,000 rpm at 4°C for 15 min removed the supernatant carefully and the pellet (plasmid DNA) was dried at 37°C for 1 hour. RNA was removed by treating the plasmid preparation in TE (pH 8) containing DNAase free pancreatic RNAase (20 μg/ml). Purity was checked by agarose gel electrophoresis. The prepared plasmids were kept at −20°C until further use.

3.3.3. Excision of HPV probe DNA from recombinant vector

HPV-16 and 18 DNA cloned in PBR 322, were used for the preparation of probes for the study. The vectors containing the HPV inserts were digested with restriction enzymes, BamHI and EcoRI for HPV-16 and HPV-18 respectively (Sambrook et. al. 1989).
3.3.3.1. Reagents and chemicals for restriction digestion

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>10X Buffer System</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Bam H1</em></td>
<td>Tris 100 mM</td>
</tr>
<tr>
<td></td>
<td>NaCl 1 M</td>
</tr>
<tr>
<td></td>
<td>MgCl₂ 100 mM</td>
</tr>
<tr>
<td></td>
<td>DTT 10 mM</td>
</tr>
<tr>
<td></td>
<td>pH: 8</td>
</tr>
<tr>
<td>2. <em>Eco R1</em></td>
<td>Tris HCl 500 mM</td>
</tr>
<tr>
<td></td>
<td>NaCl 1 M</td>
</tr>
<tr>
<td></td>
<td>MgCl₂ 100 mM</td>
</tr>
<tr>
<td></td>
<td>2-mercaptoethanol 50 mM</td>
</tr>
</tbody>
</table>

3.3.3.2. Procedure for restriction digestion

For restriction digestion, in a sterile microfuge tube 3 μl of HPV-plasmid DNA, 5 μl 10x buffer and 5 μl restriction enzyme (10 units/μl) were added and final volume made up to 50μl with distilled water and incubated at 37°C for 3hr. After the digestion, electrophoresis was performed on agarose gel as in section 3.3.4. The HPV-DNA bands were cut out and the DNA was eluted from the gel by electroelution as in section 3.3.5. Eluted DNA was suspended in TE buffer (pH 8) and stored at -20°C until use. These HPV inserts were labelled and used for DNA hybridization studies. Probes were prepared separately for HPV-16 and HPV-18 from the recombinant pBR 322 plasmids.

3.3.4. Agarose gel electrophoresis

This is a method widely used for the separation of DNA molecule based on their size and molecular weight (Slater 1989).
3.3.4.1. Reagents and chemicals for agarose gel electrophoresis of DNA

1. Gel Running buffer  TAE (50 X)
   Tris-base - 24.20 g
   Glacial acetic acid - 5.71 ml
   0.5 M EDTA (pH 8) - 10.00 ml
   50X TAE was diluted to appropriate concentration prior to use with sterile distilled water.

2. Agarose Gel
   1% agarose in TAE buffer

3. Ethidium bromide
   Prepared as 10mg/ml stock solution in deionised water and stored in a dark bottle.

4. DNA loading buffer (6X)
   Glycerol - 30%
   Bromophenol blue - 0.25% (dissolved in sterile deionised water)

3.3.4.2. Procedure for agarose gel electrophoresis

   Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with TAE buffer. The DNA samples were diluted with 1/6 volume of 6X loading buffer and deionised water. Samples were loaded on agarose gel containing 0.5μg/ml ethidium bromide. Electrophoresis was performed at 5 V/cm until the run was complete in a horizontal electrophoresis apparatus (Hoefer USA). After completion of electrophoresis, the gel was visualized on a UV transilluminator (Hoefer USA).
3.3.5. Electoelution of DNA probes from agarose gel

The region of the gel containing the desired HPV DNA fragments was sliced out from the gel using a microscopic cover glass. DNA was eluted by electro elution method (Sambrook et. al. 1989).

3.3.5.1. Reagents and chemicals for electroelution

1) TAE (Tris Acetic acid EDTA) *

2) Saturated Phenol *

*Reagents were prepared as described in section 3.3.4.1

3.3.5.2. Procedure for the elution of DNA fragments from agarose gel

The gel slice containing the band of interest was kept in a dialysis bag, filled with 0.5X TAE buffer and the necks of the bag were sealed. The bag was immersed in an electrophoresis tank containing 0.5X TAE buffer and electric current of 50V was passed for 30 minutes. Polarity of the current was reversed for 30 seconds to release the DNA from the wall of the dialysis bag. The buffer was collected from the bag, extracted with phenol-chloroform (1:1), precipitated with absolute ethanol (100%) and the pellet was washed with 70% ethanol. The pellet was dried, resuspended in TE buffer and stored at -20°C.

3.3.6. Genomic DNA extraction

DNA samples from biopsy and surgery specimens from histopathologically confirmed oral squamous carcinoma cases and from normal oral mucosa sample obtained from fresh autopsy specimens were used in DNA
hybridization studies. Tissue DNA was extracted by the standard phenol: chloroform: isoamyl alcohol method (Sambrook et. al. 1989).

3.3.6.1. Reagents and chemicals for genomic DNA extraction

1. Tris EDTA buffer *
2. Saturated phenol *
3. RNAse A *

*Reagents were prepared as described in section 3.3.2.1

4. Sodium acetate (3M, pH 5.2)
   Sodium acetate - 40.81 g
   Distilled water - 80 ml
   pH adjusted to 5.2 and distilled water added to make a final volume of 100ml .

3.3.6.2. Procedure for genomic DNA extraction from tissue samples

The tissue samples were homogenized in Tris-EDTA buffer (1mg tissue/10ml buffer), 0.5% final concentration of SDS and proteinase-K 100 μg/ml were added to the homogenate and incubated for 3 hour to overnight at 37°C. The digest was then extracted with water-saturated phenol. The aqueous layer was collected and re-extracted with phenol-chloroform–isoamyl alcohol (25:24:1). DNA was precipitated out with 1/10 volume of chilled 3M sodium acetate and 2.5 volumes of absolute ethanol.

The DNA pellet was washed with 70% ethanol, freeze-dried and resuspended in Tris-EDTA buffer. RNase was added to a final concentration of 100μg/ml and incubated at 37°C for one hour. The RNA free DNA was purified by phenol/chloroform extractions, precipitated with ethanol, washed with 70% ethanol,
dried and suspended in TE buffer. Extreme care was taken to avoid cross contamination between samples.

### 3.3.7. Restriction digestion of genomic DNA

The following method was used for the complete digestion of the genomic DNA (Sambrook et. al. 1989)

#### 3.3.7.1. Reagents and chemicals used for digestion of genomic DNA

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>10X Buffer System</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>PstI</em></td>
<td>Tris -100 mM</td>
</tr>
<tr>
<td></td>
<td>NaCl - 1 M</td>
</tr>
<tr>
<td></td>
<td>MgCl$_2$ -100 mM</td>
</tr>
<tr>
<td></td>
<td>DTT - 10 mM</td>
</tr>
<tr>
<td></td>
<td>pH: 8</td>
</tr>
</tbody>
</table>

#### 3.3.7.2. Procedure for Restriction digestion of genomic DNA

In a sterile microfuge tube 8 µl of autoclaved distilled water, 10 µl of chromosomal DNA (approximately 0.2µg DNA) and 2 µl of 10X buffer were added and mixed well. 1 µl of *PstI* restriction enzyme was added, mixed thoroughly and incubated at 37°C overnight. This DNA digest was used for Southern blotting. Digestion of genomic DNA was confirmed by agarose gel electrophoresis (section 3.3.4.) and observation of characteristic pattern (Sambrook et. al. 1989).

### 3.3.8. Capillary blotting of genomic DNA

The DNA was transferred from an agarose gel on to Hybond-N membrane by capillary action as described originally by Southern (Southern 1975). Restriction enzyme digested genomic DNA was electrophoresed in agarose gel
using sample buffer containing 0.25% (w/v) xylene-cyanol and 0.25% (w/v) bromophenol blue. After electrophoresis, placed the agarose gel in 0.25 M HCl until the dyes changed colour, and incubated for an additional 10 minutes. Rinsed gel in distilled water and incubated in denaturation buffer for 30 minutes at room temperature with shaking. Rinsed gel in distilled water and placed in neutralization buffer for 15 minutes at room temperature with shaking and repeated rinsing with neutralisation buffer for three times. The blotting was performed as described by Southern (Southern 1975).

3.3.8.1. Reagents and materials for capillary blotting

1. Gel loading Dye (6X) for blotting
   - Bromophenol blue: 0.02g (0.25%)
   - Xylene cyanol: 0.02g (0.25%)
   - Ficoll (MW.400): 1.5g (15%)
   - Distilled water added to make a final volume of 10ml, aliquoted and stored at 4°C
   - Electrophoresis reagents were prepared as described in agarose gel electrophoresis section

2. SSC (20X)
   - NaCl: 17.5g
   - Sodium citrate: 8.8g
   - Distilled water: 80ml
   - pH adjusted to 7 and distilled water added to make a final vol. of 100ml

3. Filter paper 3mm (Whatman, UK)

4. Hybond–N membrane (Amersham, UK)

3.3.8.2. Procedure for capillary blotting

Glass tray filled with blotting buffer (20X SSC) was used for capillary blotting. Made a platform with a glass plate and covered it with a wick made from three sheets of Whatman 3mm filter paper which was saturated with
the blotting buffer from the tray. Placed the gel on the wick and avoided trapping air bubbles beneath it with cling film to prevent the blotting buffer being absorbed directly into the paper towels above. A sheet of Hybond-N membrane cut to the exact size of the gel was placed on the top of the gel. Trapping bubbles beneath the membrane was avoided. Air bubbles that appeared were squeezed out using a glass rod. Placed three sheets of 3mm paper, cut to size and wetted with blotting buffer on the top of the Hybond–N membrane. Placed a stack of absorbent paper towels on top of 3mm paper (approximately 5cm high). Placed a glass plate on top of the paper towels and kept a weight on top. Allowed transfer to proceed over night. After blotting, carefully dismantled the apparatus, marked sides of the membrane with pencil to allow later identification of the tracks. Washed the membrane briefly and carefully in 2X SSC to remove any adhering agarose. Baked the membrane in an oven at $80^\circ$C for 2 hours under vacuum to fix.

3.3.9. HPV DNA probes labelling

Labelling of HPV-16 and HPV-18 DNA were done using Megaprime labelling system (Amersham, UK) (Feinberg and Vogelstein 1983).

3.3.9.1. Reagents and solutions for DNA labelling
(Megaprime labelling system, Amersham, UK.)

1. **Primer solution**
   Random nonamer primers in an aqueous solution

2. **Reaction buffer**
   A concentrated reaction buffer containing Tris HCl, pH 7.5, MgCl$_2$ and 2-mercaptoethanol
3. Non radioactive nucleotide in buffer solutions
Nucleotides in concentrated buffer solutions containing
Tris HCl, pH 8.0, 0.5mM EDTA.
1. dATP
2. dGTP
3. dTTP

4. Radioactive nucleotide
$^{32}$P labelled dCTP (BARC, India)

5. Enzyme solution
1U/µl DNA polymerase I “Klenow” fragment (cloned) in 50mM
Potassium Phosphate pH 6.5, 10mM 2-mercaptoethanol and 50% Glycerol

6. TE buffer (Section 3.3.2.1)

3.3.9.2. Procedure for DNA labelling

Diluted the DNA to a concentration of approximately 5 µg/ml in
10 mM TE buffer. Added 25 ng (5 µl) of template DNA into a clean micro centrifuge
tube and to it added 5 µl of primers. Denatured by heating to 95-100°C for 5
minutes in a boiling water bath. Centrifuged briefly in a micro centrifuge to bring the
contents to the bottom of the tube. Kept the tube at room temperature, added the 4
µl non-radioactive nucleotides and 5 µl 10X reaction buffer, 4 µl radiolabelled
nucleotide, 2 µl DNA polymerase Klenow fragment enzyme and nuclease free
water to make the final volume to 50 µl. Capped the tube and spun for a few
seconds in a microcentrifuge to bring the contents to the bottom of the tube.
Incubated at 37°C for 10 minutes. Stopped the reaction by the addition of 5 µl of
0.2 M EDTA. For use in hybridization, denatured the labelled DNA by heating to
95-100°C for 5 minutes and then chilled on ice.
3.3.10. Hybridization of Genomic DNA with labelled HPV Probes

The probe DNA was hybridized with the membrane transferred patient DNA to detect the presence of HPV (Meinkoth and Wahl 1984)

3.3.10.1. Reagents and chemicals for hybridization

1. Denhardt's solution (50X)
   - Ficoll – 5g
   - Polyvinyl pyrrolidone – 5g
   - BSA (Pantex fraction V) – 5g
   - Distilled water – 500ml
   - Filter sterilized. Stored at -20°C in aliquots.

2. Prehybridization solution
   - Formamide – 50ml
   - SSC 5X – 20ml (Section 3.3.8.1)
   - Denhardt’s solution – 0.02 ml
   - Herring sperm DNA – 5mg
   - Trisodium phosphate 0.5M – 10 ml
   - SDS – 1 mg
   - Added distilled water and made upto 100 ml

3. Hybridization solution
   - Formamide – 50ml
   - SSC 5X – 20 ml (Section 3.3.8.1)
   - Denhardt’s solution – 0.02 ml
   - Herring sperm DNA – 5mg
   - Trisodium phosphate 0.5M – 10 ml
   - SDS – 1 mg
   - Dextran sulphate – 10%
   - Added distilled water and made upto 100 ml

3.3.10.2. Procedure for Probe Hybridization

The blots were prehybridized in prehybridization solution for two hour. Then prehybridized in a shaking water bath at 65°C for 1 hour. For hybridization, denatured labelled HPV probe by heating to 100°C for 5 minutes. Hybridization was carried out for 16-20 hour under stringent condition at 65°C in
the hybridization solution. After hybridization, the membranes were subjected to stringent washes and then autoradiographed.

3.4. Isolation and quantification of circulating immune complexes (CIC) from oral cancer patients

Circulating immune complexes (CIC) are important source of cancer antigen. The antibody part of the CIC is a specific response against the CIC antigen and can be considered as a tumour associated antigen. Polyethylene glycol (PEG) precipitation is the simplest and the most widely used method for the isolation of CIC from other serum components. The isolated complexes are quantified by exploring the biological and physico-chemical properties of it. In the present study, the PEG precipitate was quantified by the ability of CIC to fix serum complement (Harkiss and Brown 1979). CIC fixes the limited amount of externally added complement in the reaction system, in proportion to its amount. The excess complement in the reaction system was quantified using sheep red blood cells (sRBC) and anti-sRBC antibody raised in rabbit as an indicator system. Excess complement acts on the indicator system; lyse the RBC and release haemoglobin. The amount of haemoglobin released depends on the amount of CIC.

3.4.1. Antibody to sheep red blood cells raised in rabbits.

In order to produce the above mentioned indicator system, anti-sheep RBC antibodies were raised in rabbits (Catty and Ray 1988).
3.4.1.1. Reagents and materials for production of anti-sRBC serum

1. **Anticoagulant Dextrose Citrate (ADC) 100 ml**
   
   - Citric acid (anhydrous) – 0.8 g
   - Sodium citrate (dihydrate) – 2.5 g
   - Dextrose (anhydrous) – 12.0 g
   
   Sterilised by autoclaving

2. **sRBC** were collected from slaughterhouse, in sterile ACD solution.

3. **Alserver’s solution 100 ml**
   
   - Dextrose – 2.05 g
   - NaCl – 0.42 g
   - Trisodium citrate – 0.30 g
   - Citric acid – 0.05 g
   
   Sterilised by filtration.

4. **Phosphate Buffered Saline (5X)**
   
   - NaCl – 32.43 g
   - KH₂PO₄ – 6.10 g
   - Na₂HPO₄ – 24.72 g
   
   Distilled water – 1 litre
   
   PH – 7.2
   
   Diluted 1:5 in water prior to use

3.4.1.2. Procedure for production of anti-sRBC antibodies

Sheep red blood cells (sRBC) were harvested under sterile condition into anti-coagulant dextrose citrate (ADC) solution. Aliquot of whole blood (20 ml) were centrifuged at 1500 rpm for 20 min in sterile centrifuge tubes and the plasma and buffy coat were removed. The cells were then washed four times in 20 ml Alserver’s solution and resuspended in same volume. If necessary, they were stored for weeks at 4°C in this condition. For immunization, the cells are washed twice in PBS and was made up to 10% (v/v) suspension in PBS that may contain approximately 10⁹ cells/ml. For the good antibody response against the sRBC, 10 intravenous injections of 1 ml cells/kg body weight was given in to the
lateral ear veins for the first four days and then on alternate days. Third day, after the final immunization, the rabbits were bleeded for harvesting serum. Blood was collected from the lateral ear veins and allowed to clot for 1h at 37°C. The clots were freed from the walls of the container and left at 4°C overnight for the clot to retract. The serum was spun at 1500 rpm to remove red cells and stored at −20°C without any preservatives. Anti-sRBC antibodies were checked by haemagglutination test.

3.4.2. Standardisation of number of sheep red blood cells (sRBC) and its sensitization

The number of erythrocytes used in complement consumption assay was standardised in order to reduce the day-to-day variation in the release of haemoglobin.

3.4.2.1 Reagents and chemicals for standardisation of number of sRBC

1) Borate buffer
   Boric acid  – 6.18gm
   Disodium tetraborate  – 4.38gm
   NaN₃  – 1.00gm
   Distilled water  – 1 litre
   PH 8.4

2) Phosphate buffered saline (Section 3.4.1.1.)

3) Sodium carbonate solution (1%)
   1 g Na₂CO₃ in 100 ml water

3.4.2.2. Procedure for Standardisation of number of sRBC

For Standardisation of number of sRBC 10 ml of 50% sheep blood in ACD solution was centrifuged at 3000 rpm for 5min, removed the supernatant
and buffy coat. Washed the Sheep RBC (sRBC) three times with PBS solution, centrifuged at 3000 rpm for five minutes and 1ml of packed cells were suspended in 19 ml of PBS solution. After thorough mixing, 200μl of cell suspension was mixed with 2.2 ml of 1% Na₂CO₃ solution. After haemolysis, the cell debris was removed by centrifugation at 3000 rpm for 5 minutes and absorbance of the supernatant was measured at 545 nm in 1cm cuvette.

Following equation was used for the adjustment of the optical density (OD) of the supernatant to a final value of 1.50.

\[ V_t = V_i \cdot \left( \frac{OD}{1.50} \right) \]

\( V_i \) = initial volume of PBS solution (20ml)
\( V_t \) = final volume of PBS in which the sRBC is to be suspended in order to get an OD of 1.5.

The suspended sRBC was sensitised by incubating with rabbit antiRBC antibody at a two fold dilution of its tire, in a shaking water bath at 37°C for 30 minutes.

3.4.3. PEG precipitation and compliment consumption assay

3.4.3.1. Reagents and chemicals for the assay

1) Borate buffer (Section 3.4.3.1)

2) PEG-borate solution - 2.5%
   2.1 g PEG in 100 ml of borate buffer

3) PEG-borate solution - 12.5%
   125 g PEG in 1 liter of borate buffer

3.4.3.2. Procedure for PEG precipitation and compliment consumption assay

This assay was done as reported by Harkiss and Brown with modifications (Harkiss and Brown 1979). It is based on the isolation of CIC by PEG
precipitation and quantitation of the precipitate by their ability to activate the complement cascade. To 300μl of serum, 50μl of borate buffer (pH 8.4) and 50μl of 0.2M EDTA were added and mixed gently. An aliquot of 100μl of 12.5% PEG was added and the tubes were vortexed and then kept at 4°C for 90 minutes. They were centrifuged at 1700 x g for 10 minutes at 4°C; the supernatants discarded and the pellets were washed with 1 ml of 2.5% PEG at 1700Xg for 15 minutes at 4°C. Once again, the supernatant was discarded and the pellet was dissolved in 30μl of warm borate buffer by vortexing. To this is added 15μl of pooled normal human serum (fresh) as a source of complement and incubated at 37°C for 30 minutes and then kept on ice. Each sample was then made up to 750μl with warm borate buffer and then 250μl of warm 0.2% sensitized sheep RBC was added to each tube and further incubated at 37°C for 15 minutes. The optical density of each sample was then noted at 545nm. A 100% haemolysis control, containing 30μl complement fixing diluent plus 15μl of pooled normal human serum and 0% haemolysis control, containing 45μl complement fixing diluent and no normal human serum was also included in the study. The results were expressed as the percentage of complement consumption (Raghunath et. al. 1987)

3.5. Determination of Immunoglobulin Classes in Circulating Immune Complex

A quantitative comparison of the CIC immunoglobulin classes present in various age groups of oral cancer patients and normal healthy individuals were studied.
3.5.1. Quantification of Immunoglobulin classes by ELISA

A specific, sensitive and reproducible solid-phase enzyme immunoassay was developed to perform the detection of total and CIC immunoglobulin classes in serum samples.

3.5.1.1. Reagents and chemicals for Quantification of Immunoglobulin classes by ELISA

1. Phosphate buffered saline - 0.1M (Section 3.4.1.1)

2. Coating buffer (Carbonate/bicarbonate buffer)
   Prepared (I) 0.2 M solution of anhydrous Na₂CO₃ (21.2g in 1000ml) and (II) 0.2 M solution of Na₂HCO₃ (16.8 g in 1000ml), used 16 ml of solution I and 34 ml of solution II and diluted to a total of 200 ml will yield the approximate pH of 9.6.

3. Washing buffer
   PBS with 0.05% Tween20

4. Substrate buffer
   Citric acid - 7.3 g
   Na₂HPO₄·2H₂O - 11.8 g
   Dissolved in 1000 ml distilled water and adjust the pH to 5.0 with acid at the same molarity.

5. Bovine Serum Albumin- 1% solution in PBS

6. Chromogen: O-Phenylenediamine dihydrochloride 4mg in 10ml substrate buffer containing 2μl H₂O₂ solution.

7. Antibody diluting buffer
   PBS pH 7.4 with 1% BSA and 0.05% Tween20.

8. Antibody conjugate (Sigma USA)
   Antihuman IgG-peroxidase
   Antihuman IgM-peroxidase
   Antihuman IgA-peroxidase

9. Standard Immunoglobulin (Sigma USA)
   human IgG
   human IgM
   human IgA
3.5.1.2. Procedure for Quantification of Immunoglobulin classes by ELISA

Microplate wells were added separately with 100 µl of class specific anti-human Immunoglobulin antibodies (anti-IgG/ anti-IgM/ anti-IgA antibodies), 10µg/ml, diluted in coating buffer and incubated for 18-20h at 4°C. After washing three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), the wells were incubated in PBS containing 2% BSA at 4°C for 18-20 h for blocking unwanted sites on the plate. Washed again three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). 100µl serum CIC in antibody diluting buffer were added and left for incubation for 1h at 37°C. After washing three times with PBS-T wells were incubated with peroxidase conjugated class specific anti-human immunoglobulin antibodies diluted in PBS-T containing 2% BSA at 37°C for 1h.

The plates were again washed three times with PBS-T and then 100µl of the orthophenaline diamine (OPD) substrate solution was added and left for 15-30 min at 37°C till the colour developed. Adding 100µl of 2M H₂SO₄ stopped the colour reaction and the absorbance values were read on an ELISA reader at 495 nm (A₄₉₅). Each sample was tested in duplicate and their average Optical density was used for data analysis. The ELISA experiments were carried out on the same ELISA plates under identical conditions and appropriate blanks were also included in the test. Serially diluted immunoglobulins in PBS-T BSA were used as a standard.
3.6 Purification and Characterisation of Oral Cancer Antigen

3.6.1. Affinity purification of CIC using Protein-A column

Protein-A, synthesized from *Staphylococcus aureus* is a group specific ligand that binds to the Fc region of immunoglobulin from many species (Lindmark *et. al.* 1983, Hermanson *et. al.* 1992). Protein-A agarose column (Genei, Bangalore, India) with 5mg/ml binding capacity was used for the present study.

3.6.1.1. Reagents and Chemicals for Protein A Column

1. **Tris Buffer**
   - 0.1M Tris
   - 0.10 M
   - NaCl
   - 0.15 M
   - pH
   - 7.5

2. **Glycine HCl Buffer (elution buffer)**
   - Glycine
   - 0.1 M

3. **Neutralising solution**
   - KCl
   - 1 M

3.6.1.2. Procedure for purification of CIC

The column was equilibrated with Tris buffer. The immune complexes with a protein concentration of 5mg/ml were applied on the column. The column was then washed with Tris buffer until the absorbance at 280nm was less than 0.02. The bound IgG-Ligand complex was eluted with dissociating buffer and the eluted immune complex was neutralized immediately with 1M KCl. The absorbance of the eluted fractions was measured at 280 nm and the protein peak was calculated. Then, the sample with the highest peak was dialyzed against phosphate buffered saline and was stored at 4°C.
3.6.2. Sephadex G-200 gel filtration of CIC

Sephadex G-200 (Pharmacia) filtration was performed according to the method of Andrews (Andrews 1965).

3.6.2.1. Reagents and materials required for the G-200 gel filtration.

1. Sephadex – G 200 (Pharmacia)
2. Phosphate buffered saline (Section 3.10.1.1.)

3.6.2.2. Procedure for Sephadex G-200 gel filtration

The gel material was swelled in distilled water at 90°C in a water bath, for 5 hrs. After cooling, the gel was packed into a 90 x 1.6 cm glass column to get a bed height of 70 cm. The column was equilibrated for a flow rate of 12 ml/hr with 9M urea containing phosphate buffered saline and calibrated using standard molecular weight markers. A graph relating the molecular weight and the elution volumes were plotted (Andrews 1965)

Three ml of Protein-A purified CIC (1 mg/ml) was carefully applied on to the column and the fractions were collected in 5 ml volumes. The fractions were read at 280 nm in a UV spectrophotometer to monitor protein content. The protein containing fractions were pooled, dialysed, concentrated and stored at −20°C.
3.6.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

(SDS PAGE)

Separation of various component proteins from CIC and determination of its molecular weight was done by SDS-PAGE both in presence and absence of β-mercaptoethanol, following modified Lammelli method (Lammelli 1970). Gel electrophoresis in presence of the detergent sodium dodecyl sulphate (SDS-PAGE) is widely used to determine the molecular weight of polypeptides. It is unmatched in its ability to resolve proteins and polypeptide in a complex protein mixture. The detergent SDS breaks hydrogen bonds and causes a considerable degradation of tertiary structure, but does not affect disulphide bonds. Addition of β-mercaptoethanol reduces disulphide bonds, completing the denaturation process.

3.6.3.1 Reagents and chemicals for SDS-PAGE

Stock solutions for SDS PAGE

1. Sample treatment buffer
   - Tris \(1.21\) g/l
   - EDTA \(0.29\) g/l
   - SDS \(10.00\) g/l
   - Bromophenol blue (0.01%) \(10\mu l\)

2. Stacking gel buffer
   - Tris \(1.5\) M
   - SDS \(4g/l\)
   - pH was adjusted to 6.8

3. Separating gel buffer
   - Tris \(1.5\) M
   - SDS \(4g/l\)
   - pH adjusted to 8.8
4. Acrylamide solution
   Acrylamide – 300g/l
   Bis acrylamide – 8g/l
   Filtered and stored in amber coloured bottle.

5. Electrophoresis buffer
   Tris – 6.0 g/l
   Glycine – 28.8 g/l
   SDS – 1.0 g/l
   pH adjusted to 8.3.

Working solution for SDS PAGE

6. Preparation of separating gel 10% (35ml)
   Distilled water – 14.5 ml
   Separating gel buffer – 8.75 ml
   TEMED – 35 μl
   Acrylamide stock – 11.4ml
   APS (10%) – 350 μl

7. Preparation of separating gel 15% (35ml)
   Distilled water – 8.8 ml
   Separating gel buffer – 8.75 ml
   TEMED – 35 μl
   Acrylamide stock – 17.1 ml
   APS (10%) – 350 μl

8. Preparation of stacking gel 7% (10ml)
   Distilled water – 5.3 ml
   Stacking gel buffer – 2.5ml
   TEMED – 10 μl
   Acrylamide stock – 2.1ml
   APS (10%) – 100 μl

9. Gel fixative solution
   Acetic acid – 50ml
   Methanol – 100ml
   Mixed and made upto 500ml

10. Coomassie blue stain solution
    Solution A – 0.2% Coomassie blue in 95% ethanol
    Solution B – 20% acetic acid in water
3.6.3.2. Procedure for SDS PAGE

A discontinuous buffer system with two gel components was used; where the 'stacking gel' concentrates and the 'separating gel' separates the protein sample. A vertical slab gel electrophoretic apparatus was used (Broviga, USA). Marker proteins (proteins of known molecular weight) were also run parallel. The vertical slab gel units were assembled in the casting mode using 1.5 mm thick spacers. Separating gel solution was taken leaving out APS and TEMED. Vacuum was applied for few minutes in a vacuum desiccator. TEMED and APS were added to the solution after de-aeration and mixed with swirling. Then, the solution was pipetted into the sandwich to a level of about 4.0 cm from the top. The gel was overlaid immediately with 1-2 ml butanol. When the gel was polymerized, butanol was poured off and stacking gel was casted over the separating gel layer. De-aeration of the stacking gel solution was also done as described above and mixed with APS and TEMED. The portion above the separating gel was filled with the stacking gel solution and the comb was inserted into it and allowed to polymerize for one to two hours at room temperature.

Standard marker proteins were used to compare the molecular weight of CIC components. The protein markers used were mixed at a final concentration of 2mg/ml, with sample treatment buffer. CIC was mixed in 1:1 proportion with sample treatment buffer containing bromophenol blue. The combs were carefully removed from the gels without disturbing the well dividers. The gel was placed in a vertical position in the electrophoresis apparatus and the upper and lower buffer chambers were filled with electrophoresis buffer up to the marked
level. Electrodes were connected to power pack and pre-electrophoresed the gel at 200-300 V for about 30 minutes. The power cables were disconnected after pre-electrophoresis and then the sample and protein marker mixture were applied in the wells in a volume of about 10-40 μl. Power supply was set to a constant voltage of 800 watts. When the dye reached to the bottom of the gel, indicating the completion of electrophoresis run, the power supply was turned off. The gel was removed from the apparatus and kept immersed in fixative for about three hours. The gel was put into the stain solution for 4-8 hours with gentle shaking and then transferred to destaining solution.

3.6.3.3. SDS-PAGE under reducing condition

The electrophoresis was repeated incorporating 5% β-mercapto-ethanol in protein sample treatment buffer. Pre heating of the CIC sample with the buffer for 10 minutes, in boiling water bath breaks the disulphide linkages in the protein molecule. Molecular weight of the polypeptides separated by the SDS-PAGE was calculated by comparing their mobility with that of marker proteins.

3.7. Detection of oral cancer antibody from patients' sera by ELISA

The detection and quantification of the oral cancer antibody from patient's sera were determined by the sandwich ELISA technique using purified oral cancer antigen (Engvall and Perlmann 1971).
3.7.1. Reagents for ELISA

1. Phosphate buffered saline - 0.1M (PBS)
   - NaCl - 8 g
   - KCl - 0.2 g
   - KH2PO4 - 0.2 g
   - Na2HPO4.2H2O - 1.48 g
   - Distilled water - 1 L
   - PH - 7.4

2. Antigen coating buffer (Carbonate/bicarbonate buffer)
   (I) Na2CO3 - 0.2 M
   (II) Na2HCO3 - 0.2 M
   16 ml of solution (I) and 34 ml of solution (II) diluted to a total of 200 ml will yield the approximate pH of 9.6.

3. Washing buffer (PBST)
   - PBS - 0.1M (pH 7.4)
   - Tween20 - 0.05%

4. Diluting buffer PBST (Incubation buffer)
   - PBS - 0.1M (pH 7.4)
   - Tween20 - 0.05%
   - Bovine Serum Albumin - 1%

5. Substrate buffer (100 ml)
   - Citric acid - 7.3 g
   - Na2HPO4.2H2O - 11.8 g
   - pH - 5.0

6. Chromogenic substrate
   - O-Phenyldiamine (OPD) - 4mg
   - Substrate buffer - 10ml
   - H2O2 - 2μl

7. Antibody conjugate
   - Antihuman antibody conjugated to HRPO (Sigma USA)

3.7.2. Procedure for detection of oral cancer Antibody from patient’s sera by ELISA

Hundred micro-liter purified antigen (1mg/ml) in coating buffer was added to a microtitre plate and incubated overnight at 4°C. The wells were washed three times with PBS-Tween20 and blocked with 250μl BSA (1%) in antigen
coating buffer and incubated overnight at $4^\circ$C. Washed three times, with PBS-Tween20. Oral cancer patient’s serum or normal healthy control serum was diluted ten times in antibody diluting buffer and added 100μl into the microtitre wells. Incubated microtitre plates for 1h at $37^\circ$C and again washed 3 times. 100 μl of secondary antibody conjugate in diluting buffer was added to the wells. Incubated for 1h at $37^\circ$C and washed 3 times with PBS-Tween20 and added 100μl substrate solution (OPD/H$_2$O$_2$) for 15 min until a blue colour was developed. The reaction was stopped by adding 100μl of 2N H$_2$SO$_4$ and read the absorbance at 450nm in an ELISA reader. Appropriate antigen and antibody controls were incorporated in the test.

3.8. Production of Polyclonal antibody against the Oral Cancer antigen in rabbit.

3.8.1. Preparation of pure antigen from PAGE

Electrophoretically purified antigens were cut out and eluted from the reducing SDS PAGE and used for the immunization of rabbits.

3.8.1.1. Reagents and chemicals for antigen elution

1) PAGE reservoir buffer (Section 3.6.3.1)

2) PBS (Section 3.4.1.1)

3.8.1.2. Procedure for antigen elution from the PAGE

For pure antigen preparation, gel elution of oral cancer antigen was performed. Protein-A purified CIC fraction was electrophoresed and a portion of
the gel was cut and stained in Coomassie blue. This was matched with the remaining unstained portion of the gel for oral cancer antigen and cut out from the unstained gel. The pieces were chopped and transferred to dialysis tube. Three ml of reservoir buffer for Native PAGE was added and dialysis tube was closed with closure clips without any air bubbles. The protein was allowed to elute for overnight in a horizontal electrophoretic tank filled with native reservoir buffer at 80 V. After elution, the current was reversed for 30 seconds to allow the detachment of protein bound to dialysis membrane. The elutant was dialysed in phosphate buffer saline (PBS, pH 7, 0.1 M) and lyophilised. The preparation was used as purified oral cancer antigen.

3.8.2. Immunization of rabbit with oral cancer antigen

Purified antigens from PAGE were used for generating polyclonal antibody in rabbits (Stills 1994).

3.8.2.1. Reagents and Chemicals for immunization

1) Freund’s complete adjuvant (GENEI Bangalore)

2) Freund’s incomplete adjuvant (GENEI Bangalore)

3.8.2.2. Procedure for immunization of rabbit

Antibodies were raised against electrophoretically pure oral cancer antigen in New Zealand white rabbits by Intramuscular injection of 100μg of protein emulsified with an equal volume of Freund’s complete adjuvant. Three booster injections were given at an interval of 15 days with 200 μg of protein emulsified
with an equal volume of Freund's incomplete adjuvant. After the 3rd booster injection, blood was collected from the lateral ear vein of the rabbit and allowed to clot at room temperature. Serum was separated by centrifugation at 1700 x g for 15 minutes, aliquoted and stored.

3.8.3. Purification of IgG by DEAE-Cellulose Chromatography

Partial purification of antibody was done by Diethylaminoethyl-cellulose (DEAE-cellulose) ion-exchange chromatographic method (Johnstone and Thrope 1996). IgG was purified from serum by ion-exchange chromatography. IgG has a higher or more basic isoelectric point than most serum proteins. Therefore, at pH below the isoelectric point, the immunoglobulins do not bind to an anion exchanger. This principle was used for partial purification of antibody from majority of other serum proteins that binds to the column matrix.

3.8.3.1. Reagents and Chemicals for the ion exchange chromatography

1) DEAE-Cellulose (Pharmacia, Uppsala, Sweden)
2) Sodium Phosphate buffer

3.8.3.2. Procedure for the ion exchange chromatography

The anion-exchange reactive group, DEAE covalently linked to cellulose was used for this purpose. Initially, globulin fraction was separated from the immune serum. Double dilution of the serum was made with phosphate buffer and added ammonium sulphate to get 0-30% saturation. The precipitate was centrifuged at 10,000 rpm for 10min, dissolved in minimum amount of phosphate buffer.
buffer, pH 6.3 and dialysed exhaustively against the same buffer for 24 hrs with 4 changes. Dialysed sample was applied to DEAE-Cellulose column (30 x 1 cm) which was pre equilibrated with the same buffer. After whole sample enters the column, it was washed with 2 column volumes of the same buffer and immediately fractions were collected till the absorbance at 280 nm falls to the baseline. The fraction was pooled, concentrated and stored in refrigerator; and used as purified polyclonal anti-oral cancer antibody.

3.8.4. Gel double diffusion (Ouchterlony method)

The production of polyclonal antibody was checked for its immunoreactivity by immunodiffusion of the serum samples from immunised rabbits (Ouchterlony 1958).

3.8.4.1. Reagents and Chemicals for gel double diffusion

Agarose 1% in PBS

3.8.4.2. Procedure for double diffusion

The method relies on passive diffusion of antibody and antigen solutions within the gel. Antigen and antibody solutions are placed in opposing wells cut into a horizontal agarose gel of approximately 1.5 mm depth. Diffusion occurs radially from the wells and precipitation line developed within the gel between opposing wells. Gels were prepared by adding 1 g of agarose powder to 100 ml of hot PBS in a conical flask and stirred on a hot plate magnetic stirrer. Poured enough molten agarose onto a clean and grease free glass slide with the help of pipette without disturbing the eddies, so as to get a uniform layer of gel on
the surface.Allowed the gels to set, and made wells using a gel punch. The plugs were removed by suction or gentle lifting. Filled the wells with antigen and antibodies using micropipettes. Placed the slide in a petridish on a dampened filter paper. Incubated the slides at 4°C overnight to get the precipitation reaction. The slides were washed many times in PBS and dried in an incubator at 37°C by applying water-dampened filter paper onto the gel surface. If necessary, stained the slides in Comassie blue stain and observed bands.

3.8.5. Antibody coupling to Horseradish peroxidase

The enzyme peroxidase was conjugated to the DEAE-cellulose purified IgG antibodies by periodate method (Nakane and Kawaoi 1974).

3.8.5.1. Reagents and Chemicals for enzyme coupling

1) HRPO Horse Radish Peroxidase (GENEI Bangalore)

2) Sodium periodate 0.1 M in 10 mM sodium phosphate

3) Sodium acetate buffer 1 mM (pH 4)
   Acetic acid       – 1 mM
   Sodium acetate   – 1 mM
   82 ml acetic acid and 18 ml sodium acetate to make 100 ml

4) Carbonate buffer
   (I) Na₂CO₃   – 0.2 M
   (II) Na₂HCO₃ – 0.2 M
   16 ml of solution (I) and 34 ml of solution (II) diluted to a total of 200 ml will yield the approximate pH of 9.6.

3.8.5.2. Procedure for enzyme coupling

The polyclonal antibody raised in rabbits were conjugated to horseradish peroxidase by periodate coupling method. 5mg of Horseradish
peroxidase (HRPO) was dissolved in 1.2ml distilled water. 30 µl of freshly prepared 0.1 M sodium periodate in 10mM sodium phosphate (pH 7.0) was added to the above solution and incubated at room temperature for 20 min. The mixture was dialysed against 1 mM sodium acetate buffer (pH 4.0) at 4°C with several changes of buffer. 5 mg antibody in 20mM carbonate buffer (pH 9.6) was added to the mixture and incubated at room temperature for 2h. The Schiff's bases thus formed had been reduced by adding 100 µl of 0.4% sodium borohydrate in water and incubated at 4°C for 2h. It was dialysed against PBS and used directly with appropriate dilutions.

3.9. Detection of Oral cancer antibody in patients' serum by Immuno Blotting Technique

Identification of proteins separated by gel electrophoresis is compounded by the small pore size of the gel, which limits penetration by macromolecular probes. Overcoming this problem can be achieved by blotting the proteins on to a nitrocellulose membrane (Towbin et. al. 1979; Johnstone and Thorpe 1996)

3.9.1. Reagents and Chemicals for Immuno Blotting

1. Blotting buffer
   Tris      - 14.4 gm
   Glycine   - 3.0 gm
   Methanol  - 200 ml
   Water to make 1 liter

2. Blocking solution (in PBS pH 7.2)
   Tween 20 - 0.3%
   BSA       - 1%
3. Antibody dilution buffer
   Tween 20 - 0.05% in PBS

4. Washing buffer
   Tween 20 - 0.05% in PBS

5. Localising Chromogen (GENEI Bangalore)
   TMB (tetramethyl benzidine) with H₂O₂

3.9.2. Procedure for Immunoblotting

The transfer apparatus was assembled and the tank was filled with transfer buffer. Two pieces of filter paper was cut to the size of the cassette clamp, soaked in transfer buffer and placed one on the cathodal side of the cassette on top of a wetted sponge pad. The specific oral cancer antigen was electrophoretically separated on a Polyacrylamide slab gel in presence of SDS.

Then the gel was placed on the filter paper covering the cathodal side of the cassette. The gel was kept wet all times with transfer buffer, soaked the nitrocellulose sheet (cut to the same size as the gel) in transfer buffer, and placed it on the gel. Trapping of air bubbles throughout the process was avoided. Filter paper was placed over the nitrocellulose and expelled all air bubbles between the nitrocellulose and gel. Finally, a wetted sponge pad was placed on top of the filter paper and clamped securely in the cassette in a tight fit manner. Then cassette was placed in the tank and the lid was fitted. Electrophoresis was done overnight at 0.5A. The nitrocellulose was processed.

The nitrocellulose paper was incubated for three hours in blocking solution. Then, washed thoroughly with washing solution and flooded with the
primary antibody solution followed by incubation at 37°C for three hours. Washed with PBS Tween 20 solution, flooded the paper with Enzyme conjugate system and incubated for 45 minutes. The substrate solution was added and incubated till the blue colour developed on the paper. The excess colour formation was stopped by washing the membrane with distilled water. The protein bands were observed as bluish bands on the nitrocellulose paper.