MATERIALS

&

METHODS
1. **PATIENT SELECTION**

The patients with molar pregnancy registering as new cases in the Trophoblastic Clinic of Sree Avittam Thirunal (SAT) Hospital and Regional Cancer Centre (RCC), Thiruvananthapuram, Kerala, India, during the period from 1995-1999 were recruited for the study.

2. **SAMPLE COLLECTION AND PROCESSING**

2.1 *Patient Details*

The details of the patients were collected from medical records maintained in the hospitals and by personal interview in the follow up clinic of the SAT hospital.

2.2 *Tissue*

Samples of molar placentae were collected after suction evacuation and normal placentae of corresponding gestational ages were collected from patients undergoing elective abortions of normal pregnancies at the Medical Termination of Pregnancy Theatre of the SAT Hospital, Thiruvananthapuram. Full-term placentae were collected from patients undergoing normal vaginal delivery in the labour room of the hospital. A part of the tissue was snap frozen in liquid nitrogen until use.
Another part was fixed for 24 hrs in 10% buffered formalin (paraformaldehyde in phosphate-buffered saline) for paraffin embedding.

2.3 Blood

Blood was collected prior to suction evacuation by aseptic venipuncture procedures into plain tubes (10 ml) without the addition of any anticoagulants or preservatives. Serum was separated and stored at -20°C until further use.

3. PATIENT FOLLOW UP

Regression pattern of serum β-hCG was the marker used for follow up. The patients after suction evacuation of the tumour in the hospital were followed up every week for one month, and then biweekly up to 12 months and then followed up once in a month up to 24 months or more, for patients who had elevated serum β-hCG levels.

4. PATIENT CLASSIFICATION

(i) Based on clinical and histopathological features, molar placentae collected were classified as complete, partial, invasive and choriocarcinoma.
(ii) The patients were classified into spontaneously regressing group and persisting disease group (including slow regressing and chemotherapy group) based on serum β-hCG levels and clinical follow up (Balaram et al., 1999; Fig. 2). The SAT hospital considers a patient with serum β-hCG level of < 10 mlU/ml at three consecutive evaluations between 12-16 weeks after evacuation as having attained normal levels and hence this was used as a cut off in the present study. The patients attaining normal serum β-hCG levels (<10 mlU/ml) by 16 weeks following evacuation were included in the spontaneously regressing group (Spont. Regr.). Persisting disease group (PD) consisted of patients in the slow regressing group (Slow Regr.) and chemotherapy group (Chemo Group). Patients in the slow regressing group were those whose serum β-hCG levels did not touch the normal levels of < 10 mlU/ml by 16 weeks of evacuation, but who were not administered chemotherapy till that period. 80% of these patients showed gradual decreasing pattern of β-hCG and attained normal levels with a longer time after evacuation while 20% showed either persistingly higher than normal levels or shooting up of β-hCG levels in the period following 16 weeks of evacuation suggesting persistence of increased trophoblastic proliferation and were administered chemotherapy at a later stage. The chemotherapy group (CT) included the patients who were administered chemotherapy between 2-16 weeks of evacuation due to persistingly very high levels of β-hCG with or without other clinical symptoms of the disease on follow up. This classification was followed for further analysis.
Fig. 2.

BETA HCG REGRESSION PATTERN

Weeks after evacuation

- SPONT. REGR.  - SLOW REGR.  - CHemo
5. LABORATORY INVESTIGATIONS

5.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The ELISA procedure is a comparatively specific, sensitive and reliable serologic procedure first described by Engvall and Perlman (1971,1972). Since then, ELISA test systems have been developed for detection of a wide variety of different antigens and antibodies. The ELISA procedure allows an objective determination of the antibody status to be made on a single dilution of the test specimen and is suitable for screening large number of patient samples. The β-hCG titre of the patient sera collected were quantitated using ELISA.

The patient sera were also screened for previous infection with Human immunodeficiency virus (HIV), Cytomegalo virus (CMV) and Herpes simplex virus (HSV) as described later (Section 5.4).

5.1.1 Quantitation of serum β-hCG by ELISA

Serum β-hCG was quantitated using commercially available Novapath™hCG ELISA kit (Bio-Rad, USA) as per the manufacturer's instructions. The sensitivity of the kit was 5 mIU/ml. The concentration was obtained by taking the optical density of the ELISA plate at 450 nm using an ELISA reader (Bio Rad, USA).
5.2. HISTOPATHOLOGICAL EXAMINATION

5.2.1 Preparation of slides for sections

New slides were washed with chromic acid followed by thorough rinsing with dilute NaOH and distilled water and dried in the hot air oven.

5.2.2 Poly-L-Lysine coating

0.2 ml of 0.1% poly-L-Lysine solution (mol wt. 70,000) was spread evenly on each slide and dried well. The slides were stored for 2-3 weeks at room temperature.

5.2.3. Preparation of paraffin sections

1. Fixation and paraffin embedding of tissues were done as for routine histological evaluation taking care not to overfix the tissues.

2. Five micron (μ) sections were cut from the paraffin embedded blocks and were floated on a hot water bath containing distilled water.

3. The flattened sections were collected on clean poly L-lysine coated glass slides and were dried overnight.

4. For optimal adhesion, the slides were placed in 60°C oven for 1 hour.
5.2.4. Haematoxylin and eosin staining

Of the numerous sections cut from formalin fixed paraffin embedded specimens, one was used for routine Haematoxylin and Eosin staining and the others were used for immunohistochemical staining by Avidin - Biotin method.

Haematoxylin and Eosin staining was done on the sections as follows:

1. Dewaxed using xylol and then treated with absolute, 90% and 70% grades of alcohol and then dipped in water.
2. Stained in Ehrlich's Alum Haematoxylin for 30 minutes.
3. Washed well in water until the sections were blue.
4. Differentiated in 0.1% HCl for 5 seconds.
5. Washed well in tap water until the sections were again blue.
6. Stained in 1% eosin for 1 minute.
7. Washed well in running water for 4-5 minutes.
8. Dehydrated in ascending grades of alcohol, cleared in xylol and the sections were mounted in DPX.
9. The sections were evaluated under a microscope.

5.2.5 Immunohistochemistry (IHC)

The term ‘immunohistochemistry’ derives from ‘immuno’ meaning involvement of antigen-antibody reactions, ‘histo’ meaning tissues. As the name
implicates, this technique involves the use of monoclonal or polyclonal antibodies to specific antigens, mostly proteins (antigens) in cells or sections spread on slides. The antigen-antibody reaction in the cells on the slide is developed usually through an indirect procedure using labelled antibodies, substrates and chromogen and visualised under a microscope. Here, we have used the indirect standard avidin-biotin complex (ABC) immunoperoxidase system for staining. The avidin-biotin peroxidase system makes use of the high affinity of egg white avidin for biotin (Vitamin H). One molecule of avidin binds 4 molecules of biotin by a non-covalent interaction which is essentially irreversible. The avidin is labelled with biotinylated enzymes on three of its binding sites leaving one biotin binding site free to bind the biotin molecule of the antibody, giving three enzyme molecules per bound primary antibody. Addition of substrate and chromogen gives rise to the coloured product. We have made use of Horse radish peroxidase (HRP) as the enzyme and Amino ethyl carbazole (AEC) or Diaminobenzidine (DAB) as the chromogen which gives a red or brown coloured reaction product respectively.

5.2.5.1. Materials and Reagents Used

1. Poly L - lysine (Sigma, USA)
2. Antigen-specific monoclonal/polyclonal antibody (Appendix).
3. Phosphate buffered saline (PBS) - Dissolved 8.0 g NaCl and 4.0 g NaH₂PO₄ in 1000 ml distilled water and adjusted the pH to 7.2.
4. Tris buffered saline (TBS) - It was prepared by dissolving 3.05 g Tris Base and 40 g NaCl in 500 ml dist. H₂O and adjusting the pH to 7.2 using 1N HCl.

5. Hydrogen peroxide (0.3%) in methanol.

6. Xylene

7. Alcohol (100%, 90%, 70%, 50%, 30%)

8. Biotinylated second step antibody

9. Blocking reagent BSA (Bovine serum albumin-Fatty acid free, from Sigma, USA)

10. Extra-avidin-biotinylated horse radish peroxidase conjugated tertiary antibody complex (Sigma, USA)

11. Amino ethyl carbazole (AEC) substrate solution - prepared by adding 300 µl of 0.004% AEC in dimethyl sulphoxide to 5 ml of sodium acetate buffer and 3% H₂O₂. Sodium acetate buffer was prepared by mixing 210 ml of 0.1N acetic acid ie., 5.75 ml glacial acetic acid in 1000 ml dist. H₂O and 790 ml of 0.1 M sodium acetate ie., 13.61 g sodium acetate trihydrate in 1000 ml dist. H₂O, pH 5.2. Each day the chromogen was freshly prepared in dark bottles.

12. Diaminobenzidine dihydrochloride (DAB) solution - It was prepared by dissolving 1mg/ml of DAB in PBS containing 0.02% hydrogen peroxide for 5 minutes.

13. Mayer's Haematoxylin

14. Glycer gel (Dako,USA)/DPX mountant (Glaxo Lab, India)
5.2.5.2 Deparaffinisation

1. The slides were deparaffinised in 2 changes of xylene for 30 minutes each and in 2 changes of xylene : alcohol (1 : 1) for 10 minutes each.

2. The slides were dehydrated as follows:

   Absolute alcohol : 2 changes 5 minutes each
   90% alcohol : 10 mins.
   70% alcohol : 10 mins.
   50% alcohol : 10 mins.
   30% alcohol : 10 mins.

5.2.5.3 Antigen unmasking

Antigen unmasking was done wherever necessary by citrate buffer treatment in pressure cooker as follows:

i) The deparaffinised slides were placed in a rack in boiling citrate buffer in pressure cooker.

ii) The lid was closed and the pressure was allowed to build up for 5 minutes.

iii) The slides were allowed to cool in the buffer for 20 minutes and were then used for the standard staining procedure.
5.2.5.4 Endogenous peroxidase blocking

Endogenous peroxidase activity was blocked by incubating the slides for 30 mins in 0.3% hydrogen peroxide in methanol.

5.2.5.5 Staining


No pretreatment for antigen unmasking was given for sections to be incubated with EGF, TGF-α, EGFR and c-erbB-2 while all the sections to be treated with the other antibodies were given pretreatment for antigen unmasking.

1. The deparaffinised and treated / non treated sections were washed in TBS and the slides were labelled with hydrophobic liner pens (Dako, USA). This was done to prevent the antibodies from flowing off the sections.
2. In order to avoid non-specific binding of antibodies on the cells which could be due to presence of Fc receptors, the sections were incubated with 100 µl of 3% BSA for 30 min at 37°C in a humid chamber.

3. The BSA was drained off, 50-100 µl of diluted primary antibody was applied and incubated (Optimal dilution of the antibody, incubation time and temperature were standardized for different antibodies as shown in Appendix). With each set of staining, positive and negative control slides were included to confirm the specificity of the antibodies and avoid false positivity/negativity.

4. The slides were rinsed three times with TBS for 5 minutes each.

5. Sections were incubated with biotinylated secondary antibody for 30 minutes at 37°C in a humid chamber.

6. The sections were rinsed in 3 changes of TBS, 5 minutes each and the excess TBS was wiped off.

7. 50-100 µl of streptavidin (avidin) - horse radish peroxidase conjugate was applied to each slide and incubated at room temperature for 30 minutes.

8. The slides were rinsed in 3 changes of TBS/PBS

9. 200 µl of the freshly prepared AEC/DAB chromogen solution was applied and the sections were incubated in the dark at room temperature.

10. The chromogen solution was drained off and the slides were rinsed in water for 5 minutes (2 changes).

11. Counterstained the sections in Mayer's haematoxylin solution for approximately 1 minute.
12. The slides were rinsed with tap water for 10 minutes for blueing.

13. The slides stained with AEC were mounted in glycer gel (Dako, USA). In the case of slides stained with DAB, the sections were dehydrated in ascending grades of alcohol, cleared in xylene and finally permanently mounted in D.P.X.

14. In the case of nuclear antigens, DAKO LSAB 2 Kit was used. The technique used in this kit is based on the labelled streptavidin-biotin (LSAB) method. It utilizes a refined avidin-biotin technique in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. When compared to the ABC method, labelled streptavidin avidin-biotin methods have been shown to provide an increase in sensitivity of up to eight times. Endogenous peroxidase activity was quenched by first incubating the specimen for five minutes with 3% hydrogen peroxide. The specimen was then incubated with an appropriately characterized and diluted rabbit or mouse primary antibody, followed by sequential 10 minute incubations with biotinylated link antibody and peroxidase-labelled streptavidin. Staining was completed after a 10 minute incubation with a freshly prepared substrate-chromogen solution.

Utmost care was taken to avoid drying of the slides at any point of time during the entire staining procedure.
5.2. 5.6 Microscopic Evaluation

a) Evaluation of cytoplasmic and membraneous staining

The staining intensity of the tissue sections were evaluated under a microscope (Leica, Germany) and were scored in the absence of any clinical data. All the sections were evaluated by another observer also and the mean of the two observations were recorded in each case. The sections were examined for the presence of a red/brown coloured end product at the site of the target antigen. The absence of the specific staining in the negative control specimens was checked to confirm the specificity of the primary antibody. The intensity of the staining was graded as:

-ve (negative), + (mild positive), ++ (moderate positive), +++ (intense positive).

Score 1 was given for negative, 2 for mild, 3 for moderate and 4 for intense staining pattern. Sections in which >80% of cells displayed mild positivity were taken as mildly positive; moderate and intense staining were scored in the same manner. The sections were blindly evaluated without knowing the histopathological diagnosis. The villous and extravillous trophoblasts were graded separately and the total of the staining by cytotrophoblasts and syncytiotrophoblasts were used to denote staining by villous trophoblasts. These scores were used to analyse the behaviour of the lesions with respect to regression pattern of the tumour based on the clinical outcome.
b) Evaluation of nuclear staining

Nuclei showing a clear red colour were taken as positive. Sections were examined at high power and 20 fields per section were chosen at random. The spatial distribution of positive staining in the sections was assessed. A quantitative estimate of nuclear labelling index immunoreactivity was made by scoring positive nuclei per total number of counted nuclei in a minimum of 1000 trophoblast cells in each case. In normal placentae and molar tissue, both cytotrophoblasts and syncytiotrophoblasts were counted. The nuclei were scored as positive or negative as follows:

Scores 1-4 were given: 1 - negative; 2 - 1-20% +ve cells; 3 - 21-40% +ve cells; 4 - 41-60% +ve cells; 5 - >60% +ve cells. The scores were entered in the computer and used for analysis.

5.3 SCREENING FOR VIRAL ANTIBODIES USING ELISA

5.3.1 HIV Test

The micro-ELISA kits for the detection of IgG antibodies to Human immunodeficiency virus (HIV) were obtained from Ranbaxy Diagnostics, New Place, New Delhi (India) under the commercial name ‘Eliaids 1/-2’.

The principle of the technique was ‘sandwich method’ using HIV 1/2 peptides bound to solid phase and anti IgG-Alkaline Phosphatase Conjugate
resulting in the formation of HIV - 1/2 peptides Human IgG anti IgG-Alkaline Phosphatase complex.

The procedure employed for ELISA was the same as described in the manual supplied by the manufacturer along with the kits. In brief, the procedure followed is given below:

HIV specific antibodies were detected by adding 200 µl of test samples in the wells precoated with HIV - 1/2 peptides in micro-ELISA plates and incubating at 37°C for 30 min. After washing the wells 4 times, 200 µl of conjugate was added into each well and incubated at 37°C for 30 min. Again the wells were washed and 200 µl of chromogenic substrate (paranitro phenyl phosphate) solution was added into each well and incubated in dark at room temperature for 30 min. 50 µl of stopping solution (NaOH) was then added to all the wells and the reading was taken in an ELISA reader at an absorbance of 405 nm. Positive and negative control sera were included along with each batch of testing.

5.3.2 CMV Test

The CMV IgG kit of Sigma Diagnostics, USA, an enzyme-linked immunosorbent assay (ELISA) designed for the qualitative determination of IgG antibodies to cytomegalo virus (CMV) in human serum was used.
The assay was carried out as per the manufacturer's instructions. The procedure employed is given below in brief:

Prepared a 1:21 dilution of the Negative control, Low Positive Standard, High Positive Control and each patient's serum as follows:

Added 10 μl of each sample and 200 μl of sample diluent to a separate well of the dilution plate.

Using a multichannel pipett, transferred 100 μl of each diluted sample and control from the dilution plate to the test plate. Withdrew and expelled the samples several times before the final transfer to ensure proper mixing of the samples. Covered the wells with a plate sealer and incubated the plate at room temperature for 20 minutes. Washed the multi-well strips three times with wash buffer. Added 100 μl of properly diluted peroxidase conjugate solution to each well at the same rate and order as the specimens were added. Covered the plates with the plate sealer and incubated at room temperature for 20 min. Washed the strips three times. Added 100 μl of TMB substrate. Incubated the plates at room temperature for 10 minutes. Hydrolysis of the substrate by peroxidase produces a colour change. Added 50 μl of stop solution (1N sulphuric acid) and the plate was gently tapped several times to ensure thorough mixing of the samples. Wiped the bottom of the plate to make it free of any residual liquid and/or fingerprints which could alter the optical density. The optical density of each well was measured against
the reagent blank in a Multi-well ELISA reader at 450 nm. The colour intensity of
the solution directly correlated with the antibody concentration in the test sample.

5.3.3 HSV Test

Anti-HSV type 2 IgG/IgM/IgA test kits for the determination of antibodies
against herpes simplex virus (HSV) were obtained under the trade name VIR-ELISA
for Anti-HSV-2 from Rashmi diagnostics Pvt. Ltd., Bangalore.

The detection of antibodies was based on the principle of sandwich-ELISA
as above. The procedure employed in brief is given below:

The purified, homogeneous antigen is fixed to each well of the micro-plate.
Any specific antibodies present in the patient's serum was bound during the first
incubation. After removing unbound material by washing, the presence of specific
antibodies was detected using anti-Human IgG, IgM or IgA, peroxidase linked
conjugate during the second incubation. Excess conjugate was then removed and
TMB substrate (solution containing 3,3',5,5'-tetramethyl benzidine in dimethyl
sulfoxide) was added, resulting in the development of a blue colour. The enzyme
reaction was terminated by the addition of a stop solution (1N sulphuric acid). The
intensity of the yellow colour thus developed was proportional to the concentration
of the antibodies in the sample. The optical density and concentration was read
at 450 nm using an ELISA reader. Positive, negative and cut off controls were
included with each batch of testing.
5.4 VDRL Test

Veneral Disease Research Laboratory test kits were obtained from Lupin Laboratories Ltd., Biotechnology Division, Kalina, Santacruz (E), Bombay (India) under the trade name ‘Treposcreen-rapid Plasma Reagin (RPR) Card Test for diagnosis of Syphilis’.

The principle of the technique is based on flocculation and it helps to diagnose syphilis by detecting nontreponemal antibodies which react with cardiolipin resulting in clumping of carbon particles.

The procedure employed for VDRL test was the same as described in the manual supplied by the manufacturer along with the test kits. In brief, the procedure for detecting VDRL positivity is given below: 50 $\mu l$ of the serum sample was added on the slide. To this was added 20 $\mu l$ of RPR Antigen Reagent and thoroughly mixed. The slides were observed after 8 minutes under bright light for clumps of charcoal particles and graded as positive or negative. Positive and negative control sera were included along with each batch of testing.

6. STATISTICAL ANALYSIS

Staining intensity (score) was expressed as mean score and standard error. The mean staining score was calculated using Student's 't' test. Statistical analysis
to compare the staining intensities of the various antibodies in the molar placentae and normal placentae was done using Chi Square test and non parametric Mann-Whitney U-Wilcoxon Rank Sum test. Comparison of numerical variables was done by the Student's 't' test. P value <0.05 was considered significant. Comparisons were also made on the basis of gestational age, prognosis and invasiveness of the disease.

The parameters that showed significance in univariate analysis were then analysed by multivariate analysis (Logistic Regression) to look into their potential as independent prognostic variables.

The different parameters were analysed by Bivariate Correlation using Spearman Rank Correlation method in order to find out whether there was any significant correlation between the variables. P value <0.005 was considered significant.

7. MOLECULAR ANALYSIS

7.1. DNA Extraction

Samples that were freshly collected and snap frozen in liquid nitrogen were used for DNA extraction. High molecular weight DNA was isolated by digestion of the cells with proteinase K in the presence of a detergent such as sodium dodecyl sulphate (SDS) followed by extraction with phenol. The details of the protocol followed is given below.
7.1.1 Solutions and Reagents

(1) Extraction buffer

10 mM Tris HCl, 10 mM NaCl, 0.1% SDS, 10g/ml proteinase K

(2) Saturated Phenol (equilibrated with 0.5M Tris HCl, pH 8.0) (Bangalore Genei, India)

(3) Phenol: Chloroform mixture (1:1)

(4) Phenol: Chloroform : Isoamylalcohol (25:24:1)

(5) TE Buffer pH 8.0 (10 mM Tris - HCl, 1 mM EDTA)

7.1.2 Protocol

1. All glasswares, plastic pipette tips, centrifuge tubes, solutions and buffers were autoclaved to avoid DNase contamination. All chemicals used were of the molecular biology grade. Gloves were worn during isolation procedures and vigorous shaking was avoided to prevent DNA shearing.

2. About 5 mg of tissue sample was chilled with liquid nitrogen and powdered in chilled mortar and pestle, in the frozen state.

3. The liquid nitrogen was allowed to evaporate and the powder was spread over 1 ml of extraction buffer in a microcentrifuge tube and shaken well to submerge the material.

4. The samples were left overnight in the lysis buffer at 37°C for protein digestion.
5. After 14-16 hrs of incubation the suspension of lysed cells was boiled for 3 min in a water bath to denature the proteinase K.

6. The solution was cooled to room temperature and an equal volume of phenol equilibrated with 0.5 M Tris HCl (pH 8.0) was added and the two phases were gently mixed by slowly turning the tube end over end for 10 minutes.

7. The two phases were separated by centrifugation at 12,000 g for 7 min at 4°C in a IEC Centra MP4R (International Equipment Company, USA) refrigerated high speed centrifuge.

8. With a wide bore pipette (0.3 cm diameter orifice) the viscous aqueous phase was transferred to a clean centrifuge tube without disturbing the interphase layer of proteins.

9. An equal volume of phenol:chloroform (1:1) was added, gently mixed and centrifuged at 12,000 g for 7 min at 4°C.

10. Supernatant was transferred to fresh tubes and an equal volume of phenol: chloroform : isoamyl alcohol (25:24:1) was added, gently mixed and centrifuged at 12,000 g for 7 min at 4°C. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamyl alcohol reduces foaming during extraction.

11. Supernatant was transferred to fresh tubes and an equal volume of chloroform: isoamyl alcohol (1:1) was added and centrifuged for 7 min at 4°C.
12. The supernatant was transferred to fresh tubes and 1/10 volume of chilled sodium acetate and 2 volumes of ice cold absolute ethanol were added and left overnight at -20°C.

13. The precipitated DNA was pelletized by centrifugation at 12,000 g for 7 min at 4°C.

### 7.2 Quantitation of DNA

The DNA was checked for purity by measuring the absorption at 260 nm and 280 nm in a uv-visible spectrophotometer (Spectronic® Genesys® Spectrophotometer, Spectronic Instruments, NY) after appropriate dilution of the sample in TE buffer. The $A_{260}/A_{280}$ ratio of the samples were around 1.8. The absorbance value at 260 nm was used to calculate the concentration of DNA (Sambrook et al., 1989) using the following formula:-

$$\text{DNA (μg/ml)} = A_{260} \times 50 \text{ μg/ml} \times \text{dilution factor}$$

### 7.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) has been employed extensively in the medical and biological sciences since it was formally introduced at the Cold Spring Harbor 51st Symposium on Quantitative Biology (Mullis et al., 1986). PCR produces many copies of a particular template DNA sequence in vitro. This method takes advantage of thermally stable DNA polymerase, which is not
Denatured by temperatures high enough to melt apart duplex DNA, allowing repeated cycles of thermal template denaturation, primer annealing and DNA synthesis.

The reaction mix contains template DNA, polymerase, deoxynucleoside triphosphate, Mg2+, buffer and 2 primer oligonucleotides. One primer complements a site upstream from the sequence being amplified, the other primer complements a region on the opposite strand, downstream from the sequence. Primers are usually 18 to 30 bases long.

The exponential in vitro amplification of genomic sequences by PCR (Saiki et al., 1985; Mullis and Faloona, 1987) has facilitated enormously the detection of oncogenic genetic alterations and the analysis of their role in tumourigenesis. The PCR has found applications in the detection of chromosomal translocations, gene amplification, integration of viral sequences, and intragenic deletions and insertions (McCormick, 1989).

7.3.1 Reagents and solutions

1. PCR super mix (Gibco BRL) (1.1X concentration).
   100mM Tris-HCl (pH 8), 55 mM KCl, 1.65 mM MgCl$_2$, 220 $\mu$M dGTP, 220 $\mu$M dATP, 220 $\mu$M dTTP, 220 $\mu$M dCTP, 22 U recombinant Taq DNA Polymerase/ml, stabilizers.
2. Mineral oil (Bangalore Genei, India)
3. Genomic DNA (100 ng/µl)
4. Primers

Custom oligonucleotide primers purchased from Gibco BRL (USA) were used. The primers were reconstituted in sterile TE to a final concentration of 1 nM/µl. The following oligonucleotide sequences were used as primers in this study. By convention, the sense primers at the 5' end of interest is called the forward primer (F) and the antisense primer at the 3' end is called the reverse primer (R).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Amplimer size (approx.bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin</td>
<td>BGKM29 5'GGTTGGCCAAATCTACTCCAGG</td>
<td>530</td>
</tr>
<tr>
<td></td>
<td>BGRS42 GCTCACTCAGTGCGGCAAG</td>
<td></td>
</tr>
<tr>
<td>HPV</td>
<td>MY09 5'GCACAGGGACATAAATGG</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>MY11 5'CGTCCAAAGGGAAAAGGTC</td>
<td></td>
</tr>
</tbody>
</table>

7.3.2 PCR Assembly for reaction

1. Set up 0.2 ml thin walled PCR tubes on ice.
2. Added the following components to each reaction tube.
   (a) 45 µl PCR super mix.
(b) Primer solution (200 mM final concentration of the required forward and reverse primers).

(c) 10μl of template DNA solution.

3. The contents of the tubes were mixed and the reaction was overlaid with 2 drops of sterile mineral oil.

4. Each sample DNA was also analysed for integrity using β-globin primers.

5. Genomic DNA from genital warts containing HPV was used as positive control.

6. Each time a negative control reaction was included with no added template DNA to check the purity for contaminating DNA.

7. The tubes were loaded in a PTC-100 thermal cycler (MJ Research, Massachusetts, USA) and the program file was cycled as given below:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cycle</td>
<td>95° C for 3.5 min</td>
<td>50° C for 1.5 min</td>
<td>72° C for 2 min</td>
</tr>
<tr>
<td>Subsequent 39 cycles</td>
<td>95° C for 1 min</td>
<td>50° C for 1.5 min</td>
<td>72° C for 1 min</td>
</tr>
<tr>
<td>Last cycle</td>
<td>95° C for 1 min</td>
<td>50° C for 1.5 min</td>
<td>72° C for 10 min</td>
</tr>
</tbody>
</table>

The yield of PCR product (”,amplicon”) was examined by agarose gel electrophoresis.
7.4 Agarose Gel Electrophoresis

7.4.1. Reagents and Solutions

1. Agarose (Sigma, USA)
2. Tris borate EDTA (TBE) buffer
3. Loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol and 40% sucrose).

7.4.2. Procedure

Agarose gel electrophoresis was carried out as follows:-

(1) 1% agarose solution was made in 1X TBE electrophoresis buffer by heating agarose in the buffer in a microwave oven until agarose dissolved to give a transparent solution.

(2) The solution was cooled to about 60°C and then gently poured into the mould of the electrophoresis apparatus (Bangalore Genei Pvt. Ltd., Bangalore) with the comb in place. The gel thickness was about 4 to 5 mm.

(3) After the gel was completely set, the comb was removed and the gel was mounted with bottom plate in the electrophoresis tank, and electrophoresis buffer was added into the tank to cover the gel to a depth of about 1mm.

(4) 10 μl of each PCR product was drawn off from under the oil with a pipetman and placed in fresh tubes and mixed with 5 μl of gel loading buffer.
(5) The solutions were loaded into the slots of the submerged gel using micropipette with disposable tips.

(6) HaeIII digested pBR-322 DNA molecular weight marker was also added into a slot of the gel.

(7) Voltage was applied (about 2-3V/cm) and the gel was run for 3-4 hrs till the bromophenol blue has migrated 2-3 cm from the other end of the gel.

(8) After the run was completed the gel was stained with a solution of ethidium bromide for 45 min.

(9) The gel was examined by viewing under ultraviolet light on a uv-visible transilluminator (UV-25, Hoefer Pharmacia Biotech Inc., USA).