CHAPTER 3

Materials and Methods

3.1 Detection of HPV 16 and 18 DNA in clinical samples

3.1.1. Clinical samples

3.1.1.1. Fresh frozen invasive cervical carcinoma biopsies

Biopsies were obtained from 42 patients who attended the Hospital Kuala Lumpur and the University Hospital, Kuala Lumpur between 1989 and 1992. The tissues were histopathologically confirmed for squamous carcinoma of the uterine cervix (SCC). Upon excision, the biopsies were frozen under dry ice and then stored in liquid nitrogen until use.

3.1.1.2. Cervical scrapes

Papanicolaou (pap) smears were obtained from 30 women with normal cervical cytology attending the Hospital Kuala Lumpur in 1992. Each wooden spatula containing the cervical scrapes were immediately placed in 20 ml of sterile phosphate buffered saline (PBS) pH 7.6 and vigorously vortexed until the exfoliated cells were in suspension. The spatula was then removed. Prior to DNA extraction, the suspension was centrifuged at 3000 rpm for 10 minutes and washed twice with sterile PBS.

3.1.1.3. Noncervical DNA samples

Noncervical DNA used in this study were obtained from leukemia, ovarian cancer and normal oral tissues.

3.1.2. Primers used for the amplification of the β-globin gene

The 268 bp frament of DNA from the β-globin sequences in the human genomic DNA was amplified using the following primers:

Primer 1 (GH 20) N 808-0014 Gene Amplimer (Perkin Elmer Cetus)

Sequence: 5'-GAAGAGCCAAGGACAGGTAC-3'

Primer 2 (PC 04) N 808-0013 Gene Amplimer (Perkin Elmer Cetus)

Sequence: 5'-CAACTTCATCCACGTTCACC-3'

3.1.3. Degenerate primers used for the amplification of HPV sequences

The L1 consensus primer pair were utilized in this study for the amplification of 450 bp HPV DNA. Primers MY 11 and MY 09 for the positive and negative strands, respectively are degenerate in several positions. At the specified positions (indicated in bold) one or more possible nucleotides is inserted during the synthesis of the oligonucleotide, to render them almost completely complementary to each of the five sequenced HPVs. Dashes indicate exact nucleotide matches.

Primer 1 (MY 09) N 808-0011 Gene Amplimer (Perkin Elmer Cetus)

Sequence: 5'-CGTCC**MARR**GGA**W**ACTGATC-3'

HPV 6b -----C-AA---T--------

HPV 11 -----A-GG---A--------

HPV 16 -----**T**-AA---A----------

HPV 18 -----A-GG---T-T------

HPV 31 --A--C-G**T**---A--------

HPV 33 -----C-AA---A---------

Primer 2 (MY 11) N 808-0012 Gene Amplimer (Perkin Elmer Cetus)

Sequence: 5'-GC**M**CAGGG**W**CATAA**Y**AATGG-3'

HPV 6b --C-----A-----C-----

HPV 11 --**T**-----A-----C-----

HPV 16 --A-----**C**--C--T-----

HPV 18 --A-----T-----C-----

HPV 31 --**T**-----A--C--T-----

HPV 33 --A--A--T-----T-----

(Degenarate code: **M** = A or C; **R** = A or G; **W** = A or T; **Y** = C or T)

3.1.4. Plasmids

HPV 16 and 18 DNA inserted separately into a plasmid vector pBR 322 carrying the ampicillin-resistence gene transformed in *Escherichia coli* were kind gifts of Dr. E. M. de Villiers (Germany). 100 µl of the glycerol stock containing the *E. coli* was plated onto a LB agar and incubated at 37°C until colonies grew. Single colonies were innoculated onto another LB agar incorporated with 50 µg/ml of ampicillin and incubation continued at 37°C. A single colony was then innoculated in 2 ml of LB broth containing 50 µg/ml of ampicillin. Incubation was carried out at 37°C for 3 hours in an incubator shaker. Following the period, 2 mls of the culture was innoculated into 98 ml

of LB broth containing 50 μg/ml of ampicillin and incubated at 37°C overnight in an incubator shaker.

3.1.4.1. Plasmids isolation

Plasmids pBR 322 containing inserts of HPV 16 and HPV 18 were isolated using commercial plasmid midi preparations (Qiagenolist, 1990). The method of plasmid isolation was carried out according to the manufacturer's specifications.

The bacterial culture from section 3.1.4 was harvested by centrifugation at 6000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet washed and resuspended in 4 ml of buffer P1 (100 μg/ml RNAse, 50 mM Tris-HCL, 10 mM EDTA, pH 8.0). Four milliliters of buffer P2 (200 mM NaOH, 1% SDS) was then added, mixed gently and incubated at room temperature for 5 minutes. Four milliliters of buffer P3 was then added to the mix, mixed gently and centrifuged at 20000 rpm at 4°C for 30 minutes. The supernatant was transferred to a fresh tube and centrifuged again for 10 minutes to obtain a particle-free, clear lysate.

The Qiagen column was equilibrated with 3 ml of buffer QBT (750 mM NaCL, 50 mM MOPS, 15% ethanol. 0.15% Triton-X-100, pH 7.0) and allowed to empty by gravity flow. Following this, the clear lysate was carefully pipetted into the column and allowed to enter the resin by gravity flow. As the lysate flowed through, the anion-exchange resin contained in the Qiagen column selectively filtered the plasmids, thus separating them from other nucleic acids present in the sample which drained out. Five milliliters of buffer QC (1.0 mM NaCL, 50 mM MOPS, 15% ethanol, pH.7.0) was then applied to the column twice to wash out contaminating impurities. Plasmid DNA was subsequently eluted out with 5 ml of buffer QF (1.25 M NaCL, 50 mM MOPS, 15% ethanol, pH 8.2) and collected in a sterile tube. DNA was precipitated with 0.7 volumes of isopropanol

and spun down at 4°C for 30 minutes. The DNA pellet was washed with 70% ethanol, dried, redissolved in sterile deionized water and stored at 4°C.

3.1.5 Cell lines

Cell lines use in this study were cervical cancer-derived lines:

(1) HPV 16-containing SiHa and CaSki cell lines

(2) HPV 18-containing HeLa cell line

SiHa and CaSki cell lines were kind gifts of Dr. Hans Ulrich Bernard (Singapore).

3.1.5.1 Reviving and culturing of cells

The cell lines cryopreserved in liquid nitrogen were revived and cultured as follows. The cryovial containing the frozen cells were removed from liquid nitrogen into a beaker of ice and then straight into a 37°C waterbath for rapid thawing. The cells were subsequently transferred into a fresh tube and washed with RPMI base media. The cells were recovered after centrifugation at 800 rpm for 5 minutes. The supernatant was decanted. The pellet was resuspended in suplemented RPMI 1640 containing 20% fetal calf serum (FCS) and incubated in a tissue culture flask at 37°C in a CO_2 incubator.

The cells were routinely checked under an inverted microscope for bacterial or fungal contamination. The culture media was replaced with fresh media when a colour change was observed and then further incubated until the cells were confluent. Following this, trypsin digestion with 0.25% trypsin was carried out to dislodge the monolayer. Fresh culture media or supplemented RPMI 1640 containing 10% FCS was added and the suspension was split into two or three culture flasks depending on the density of the cells. The flasks were further incubated.

3.1.5.2. Cryopreservation of cells

Cell lines were cryopreserved for future reference and use. Six milliliters of exponentially growing cells were pelleted in a sterile tube by centrifugation at 1000 rpm for 5 minutes. The pellet was then washed with RPMI culture media and resuspended in 3 ml of freezing solution. Aliquots of about 1 ml of cell suspension were dispensed into cryovials and stored on ice in a styrene box. The styrene box was then sealed and placed in a -70°C freezer overnight. The freezing rate was about 1°C per minute. The following day, the cryovials were removed and stored in liquid nitrogen.

3.1.6. DNA extraction

Total DNA was extracted from clinical samples and cultured cells according to the Cooper Fast Dounce DNA extraction method (personal communication by Professor M. Yadav)). The tumour biopsy was cut into small pieces with a scalpel and then homogenized gently in a dounce (Bellco, USA) containing 8 ml of 1X SSC and 0.5 ml of 20% SDS. For extraction of DNA fom cultured cells, confluent cultures were trypsinized and harvested by washing twice with sterile phosphate buffered saline (PBS). Cells were resuspended in a similar solution mentioned above.

The tissue homogenate or cells were placed in a tube and digested with 250 µl of 20 mg/ml of proteinase K. An equal amount of phenol saturated with 1X SSC was added to the lysate and mixed thoroughly but gently for about 5 to 10 minutes until emulsification was observed. Phenol removes the bulk of proteinaceous fraction of the lysate and selectively removes denatured DNA. Two volumes of chloroform : isomyl alcohol (24:1, v/v) was then added and mixed as before. The two organic phases allow better removal of proteins from the lysate. The mixture was centrifuged at 3000 rpm at room temperature for 5 minutes to separate the phases. The organic phase was

subsequently discarded and 5 ml of 1X SSC was added, mixed and centrifuged as before.

The DNA was precipitated with at least two volumes of ice-cold 100% ethanol and recovered by spooling with a heat-sealed sterile glass pipette. The pipette tip laden with DNA was air-dried and resuspended in 4 ml of 0.1X SSC and 0.5 ml of 10X SSC. 125 µl of 10 mg/ml of RNAse was added to the mixture and incubated for 30 minutes at 37°C. Following this, 62.5 µl of 20 mg/ml of proteinase K was added. The mixture was incubated again for 30 minutes at 37°C. Extraction with phenol:chloroform and isoamyl alcohol was carried out as before. The DNA was then extracted repeatedly with chloroform : isoamyl alcohol to remove traces of phenol and until the interphase between the aqueous and organic layer was clean. DNA precipitation with ice-cold 100% ethanol was performed, following which, the spooled DNA was submerged in 70°C ethanol for the duration of several hours to overnight at 4°C. The DNA was dried, suspended in sterile deionized water and stored at 4°C.

3.1.6.1. Quantification of DNA samples

DNA samples were quantified spectrophotometrically according to the procedure described by Davis et al. (1986). A dilution of 1:250 of DNA was made with sterile deionized water. The optical density (OD) of each sample was recorded at 260 nm and 280 nm on 0.5 mm light slit, 1 ml quartz cuvettes (Hellma 280 QJ).

The total amount of double-stranded DNA in each sample was calculated from the absorbance value at 260 nm using the formula given below. At the specified dilution an absorbance reading of 1 correspond to a DNA concentration of 50 µg/ml.

Concentration of DNA (µg/ml) = dilution factor x OD at 260 nm x 50

The purity of each DNA sample was assessed from the ratio of OD at 260 nm to that at 280 nm. The resulting value ranging from 1.8 to 2.0 indicates a fairly pure DNA sample. Samples with the ratio of below 1.6 were subjected to further purification with phenol : chloroform.

DNA stock solutions of 10 ng/ul were prepared for use as template in PCR.

3.1.7. Polymerase chain reaction

The PCR was carried out with the Gene Amp TM DNA Amplification Reagent kit, N 801-0043 (Perkin Elmer Cetus) according to the procedure described by Saiki et al. (1985) and Mullis and Faloona (1987).

Aliquoting of reagents were done in a laminar flow hood (Gelaire, Australia). Reagents were added into a 0.5 ml sterile, siliconized Gene-Amp microcentrifuge tube using positive displacement pipettes (Eppendorf). A 50 µl reaction mixture contained sterile distilled water; 1X reaction buffer (10mM Tris-HCL pH 8.3, 50 mM KCL, 1.5 mM MgCL, 0.01% w/v gelatin); 200 µM each deoxynucleotide triphosphate (dNTP); up to100 ng of template DNA; 20 pmole of each primer; 2.5 U Ampli Taq DNA polymerase. The mixture was then overlaid with 40 µl of light mineral oil (Sigma Co.) to prevent evaporation during PCR. After a brief centrifugation, the tubes were placed in the DNA thermal cycler (Perkin Elmer Cetus Instruments).

3.1.7.1. PCR amplification of the β-globin gene

The reaction mix for the amplification of β-globin sequences in the human genome with primers PC 04 and GH 20 is as shown in Table 3.1.

Table 3.1 Reaction mix for the amplification of β-globin gene sequences in the DNA obtained from fresh frozen tissues and pap smears

Components	Volume / μl
Sterile dH_2O	29.2
10X reaction buffer	5
d NTPs : dATP	1
dCTP	1
dGTP	1
dTTP	1
DNA template (10 ng/μl)	10
Primer 1 (PC 04)	0.5
Primer 2 (GH 20)	0.5
Amplitaq DNA polymerase	0.5
Total	50.0

3.1.7.2. PCR Amplification of the HPV DNA

The reaction mix for the amplification of HPV DNA using primers MY 09 and MY 11 is as shown in Table 3.2.

Table 3.2 Reaction mix for the amplification of HPV DNA sequences

Components	Volume / μl
Sterile dH_2O	29.5
10X reaction buffer	5
d NTPs : dATP	1
dCTP	1
dGTP	1
dTTP	1
Primer (MY 09)	0.5
Primer (MY 11)	0.5
DNA template (10 ng/μl)	10
Amplitaq DNA polymerase	0.5
Total	50.0

The cycling parameters for the PCR is as follows:

Thirty amplification cycles were carried out with each cycle consisting of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and chain

extension at 72°C for 1 minute. In the last cycle, the extension step was extended to 5 minutes.

For each PCR run, 50 ng each of DNA template from cervical cancer-derived, HPV-containing cell lines SiHa and HeLa were used as positive controls while sterile deionized water was used in place of DNA template in the negative control. Following amplification, the samples were kept at 4°C until analysis with electrophoresis and Southern blot hybridization.

3.1.8. Agarose gel electrophoresis

The amplified DNA samples were subjected to electrophoresis in 1.5% agarose submerged in 0.5X tris borate EDTA buffer (TBE). The correct amount of powdered agarose (Sigma Co.) was added to 0.5X TBE buffer. The slurry was then heated until the agarose was dissolved. The homogeneous molten solution was subsequently cooled to approximately 50°C before casting and allowed to set for 30 minutes around a well comb. Air bubbles, if any, were removed with a pasteur pipette. After the gel has set and the comb removed, the gel slab was submerged into 0.5X TBE in the electrophoresis unit.

Eight microliters of each amplified DNA sample mixed with 2 μl of 6X loading buffer was carefully loaded into appropriate wells with a micropipette. Electrophoresis was carried out from cathode to anode at a constant voltage of 80V for 3 hours. Upon completion of the run, the gel was stained in 50 μg/ml of ethidium bromide solution for 20 minutes with gentle rocking and then destained with distilled water for 5 minutes.

The amplified DNA bands intercalated with ethidium bromide in the agarose gel were visualized on a long wavelength ultraviolet-transilluminator (Model TM-30, UV products, Inc., USA) and photographed with a MP-4 Land camera (Polaroid

Corporation, USA) using the Polaroid Land 665 black and white films. Following this, Southern transfer and hybridization was carried out.

3.1.9. Non-radioactive HPV DNA probe preparation and labelling

Probes for HPV 16 and 18 were prepared by PCR from plasmids PBR 322 containing inserts of the HPV 16 and HPV 18 sequences. During PCR the amplicons were labelled with digoxigenin (DIG) using the Non-Radioactive DNA labelling and Detection Kit (Boehringer Mannheim). The HPV L1 concensus primer pair MY 09 and MY 11 were utilized for this purpose. Amplification and labelling were carried out as specified in Table 3.3.

Table 3.3 Reaction mix for DNA probe labelling

Components	Volume / µl
Sterile dH_2O	36.5
10X reaction buffer (Perkin Elmer)	5
Dig DNA labelling mixture (Boehringer, Mannheim)	2
DNA Template	5
Primer (MY 09)	0.5
Primer (MY 11)	0.5
Amplitaq DNA polymerase	0.5
Total	50.0

The 10X DIG DNA labelling mixture contained 2 mM dATP, 2 mM of dCTP, 2 mM of dGTP, 1.3 mM of dTTP and 700 µM of Dig-11-dUTP. Cycling parameters were employed as described in section 3.1.7.2. The two resulting probes were the 450 bp digoxigenin-labelled L1 DNA region of HPVs 16 and 18. Labelled probes were stored at 4°C.

3.1.10. Southern transfer of PCR amplified products

Transfer of DNA from agarose gel to nylon membrane, Hybond N+ (Amersham) was performed according to specifications described by Southern (1975). The gel was first trimmed by cutting away unused areas of the gel with a scalpel. DNA denaturation was then carried out by soaking the gel in several volumes of 1.5 M NaCL and 0.5 M NaOH for I hour at room temperature. Following this, the gel was neutralized in 1M Tris.CL (pH 8.0) and 1.5 M NaCL for 1 hour at room temperature. Both denaturation and neutralization steps were carried out with constant shaking.

A platform slightly larger than the size of the gel was wrapped in a piece of Whatman 3 mm filter paper and placed inside a large baking dish (reservoir). The dish was filled with 10 X SSC almost to the top of the platform. The gel was then placed on the platform with its underside up. Air bubbles trapped between the damp 3 mm paper and gel were smoothed out with a pasteur pipette. A piece of nylon membrane which was cut about 1 to 2 mm larger than the size of the gel on all sides was wet in 2 X SSC for 2 to 3 minutes and then placed on the gel. The membrane was overlaid by two pieces of pre-wet Whatman 3 mm papers cut exactly to the same size as the gel. Trapped air bubbles were removed as before. A stack of paper towels with the size smaller than that of the Whatman paper was placed on top and weighed down with a 500 g weight. The entire set-up was designed so that the DNA fragments were eluted out with the flow of the buffer from the reservoir through the gel and deposited onto the membrane.

Short circuiting of buffer between paper towels and the 3 mm paper under the gel was prevented by placing the a watertight border of saran wrap on the sides of the gel. Transfer was allowed to proceed overnight. Prior to removing the membrane, the positions of the wells were marked on the membrane with a soft pencil. Before being

discarded the gel was restained with ethidium bromide to ensure that all the DNA has been transferred. The membrane was soaked in 6X SSC at room temprature for 5 minutes, left to dry on a piece of 3 mm paper and subsequently UV-irradiated for 3 minutes to bind the DNA fragments onto the membrane. The membrane was then dried between two sheets of 3 mm paper at room temperature until being used in hybridization.

3.1.11. Non-radioactive hybridization

The method of DNA hybridization was carried out using the method described by Boehringer Mannheim (1989). One hundred picograms of the digoxigenin-labelled HPV DNA probe was denatured by heating to 95°C for 10 minutes and chilled on ice.

The membrane was first placed between two cloth meshes, wet in 2 X SCC, rolled and placed in a hybridization bottle (Hybaid). 20 ml prehybridization solution which was prepared fresh 1 hour before, was added to the bottle and prehybridization was carried out for 1 hour at 68°C. At the end of the period, the prehybridization solution was replaced with hybridization solution. Denatured digoxigenin-labelled HPV DNA probe was added to 2.5 ml of prehybridization solution to make up the hybridization solution. Hybridization proceeded overnight at 68°C. Prehybridization and hybridization steps were carried out with constant rotation in a hybridization oven (Hybaid).

On the following day, the membrane was removed. It was then subjected to two low strigency washes with wash solution 1 (2 X SSC, 0.1% (w/v) SDS) for 15 minutes each at room temperature and two high stringency washes with wash solution 2 (0.1 X SSC, 0.1% (w/v) SDS) for 30 minutes each at 68°C. Both high stringency and low stringency washes were performed with constant shaking. The membrane was then

used directly for the detection of hybridized DNA or air-dried and stored for later detection.

3.1.12. Detection of hybrids on nylon membranes

The colour detection of the digoxigenin-labelled probes was carried out according to the specified instructions by Boehringer Mannheim (1989). All incubations steps were performed with constant shaking at room temperature. The volumes of the solutions stated below were calculated for a membrane size of 100 cm^2.

After equilibration with buffer 1 (100 mM Tris-HCL, 150 mM NaCL, pH 7.5) for I minute, the membrane was incubated with 100 ml of buffer 1 containing 0.05% blocking reagent for 30 minutes. Thereafter, the membrane was washed again briefly with buffer 1 and incubated for 20 minutes with 20 ml of antibody-conjugated solution diluted 1:5000 in buffer 1. At the end of the period, two washes of 15 minutes each was carried out with 100 ml of buffer 1 to remove any unbound antibody conjugate. The membrane was equilibrated for 2 minutes with 20 ml of buffer 3 (100 mM Tris-HCL, 10 mM NaCL, 50 mM $MgCL_2$, pH 9.5) and then incubated in the dark in a sealed plastic bag with freshly prepared colour solution (0.45% nitroblue tetrazolium salt, 0.35% 5-bromo-4-chloro-3-indoyl-phosphate in buffer 3). Colour reaction was carried out without shaking. When the desired bands were detected, the reaction was stopped by washing the membrane with buffer 4 (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) for 5 minutes. Results were recorded by photography and the membrane air-dried and stored at room temperature.

3.2 . Detection of HPV 16 and 18 DNA by *in situ*hybridization

3.2.1. Paraffin embedded cervical biopsies

Seventy four sections of formalin-fixed paraffin-embedded biopsies of various cervical lesions were obtained from Melaka General Hospital, Sultanah Aminah Hospital, Johor Bharu and Subang Jaya Medical Centre, Selangor. Thirty of the 74 biopsies had histologic features of SCC, five were adenocarcinomas (ADC) and 39 cervical intraepithelial neoplasia (CIN)/carcinoma *in situ*(CIS) lesions comprising of 20 cases of CIN 3/CIS, 17 cases of CIN 2 and four cases of CIN 1. The paraffin-embedded tissues were cut into 5 μm sections, mounted on prewashed and pretreated slides. Adjacent sections were stained with hematoxylin eosin for histologic assessment and confirmation. The slides with sections were stored in a box at room temperature until use.

3.2.2. Pretreatment of slides

Slides used for *in situ*hybridization and immunohistochemistry were cleaned and activated to facilitate and enhance tissue adherence. Using pretreated slides, loss of tissue sections and cells during harsh experimental conditions were greatly minimized. Pretreatment procedures were carried out as described by Boehringer Mannheim (1992).

Slides were boiled in 0.1 M HCL for 20 minutes. They were then washed twice in distilled water for 5 minutes each at room temperature and in 100% ethanol for 30 minutes. The slides were air-dried.

The sterilized slides were incubated in 1X Denhardt's solution for a minimum of 9 hours to overnight at room temperature. Fixation in alcohol : glacial acetic acid (3:1)

was carried out for 20 minutes at room temperature. This was followed by hydration in decreasing concentrations of ethanol - 100%, 70%, 50% and 30% for 5 minutes each at room temperature. The slides were then incubated in 1% (v/v) organosilane solution (gamma-amino-propyl-trithoxysilane) pH 3.45 at 70°C overnight. The following day, the slides were washed five times in distilled water for 5 minutes each at room temperature and dried overnight at 100°C. At this point if not directly activated, the slides could be stored for up to six months at room temperature.

The slides were activated with 10% (v/v) glutardialdehyde in PBS for 30 minutes, washed in distilled water for 5 minutes and stabilized in 0.1 M sodium-m-periodate for 15 minutes at room temperature. The slides were then washed three times in PBS (pH 7.2) for 5 minutes each at room temperature and finally dried at 42°C. When dried, white crystalline precipitate formed on the surface of the slides. Unused slides could then be stored for up to eight weeks at room temperature.

3.2.3. Culturing cell lines on slides

CaSki and HeLa which served as positive controls for HPV 16 and 18, respectively were either fixed or cultured on UV-irradiated, pretreated slides. Method of culturing the cells on slides were carried out as described below.

Confluent cells were trypsinized and transferred to a sterile tube. Trypsin was removed by washing twice with sterile culture media containing 10% FCS. The cells were then resuspended in 5 ml of fresh culture media. One drop of cell suspension and one drop of culture media were carefully placed onto each well on the slides. The slides were then carefully deposited into petri dishes and left to incubate overnight in the CO_2 incubator. When the cells were confluent, the slides were removed and ashed

twice in PBS (pH 7.2). The cells were fixed to the glass surface with 100% ethanol for 5 minutes and then used immediately.

3.2.4 *In situ*hybridization

The technique of *in situ*hybridization for the detection of HPV 16 and 18 sequences on sections of formalin-fixed, paraffin-embedded cervical tissues and cell lines were formed according to the protocol described by Boehringer Mannheim (1992) with some modifications.

Sections were deparafinized at 56°C for 20 minutes and dewaxed in xylene for 3 minutes. From this point onwards slides with CaSki and HeLa were routinely included as positive controls. Negative controls were tissues untreated with the probe only. The slides were rehydrated in decreasing concentrations of ethanol - 100%, 95%, 90%, 80% at two minutes each and in distilled water for 5 minutes. Treatment with 0.2 N HCL was carried out for 20 minutes, followed by washings in distilled water and PBS (pH 7.2) at 5 minutes each. The tissues and cells were then subjected to digestion with 100 mg/ml Proteinase K (Sigma Co.) at 37°C for 20 minutes. The enzymatic activity was subsequently inhibited by incubation with 0.2% glycine for 10 minutes. The specimens were washed with PBS for 5 minutes. Tissue and cells were fixed with 4% formaldehyde for 20 minutes, washed with PBS and 1X SSC for 10 minutes each and dehydrated in increasing concentrations of ethanol - 80%, 90%, 95% and 100% at 2 minutes each.

Prehybridization was carried out with 50 µl of prehybridization solution for 1 hour at room temperature, after which the slides were washed briefly with 2X SSC. Subsequently, silicone gum was applied as a ring surrounding each well, followed immediately by the addition of 50 µl of hybridization solution (1 X Denhardt's solution,

5% dextran sulfate, 50% formamide, 4 X SSC, 0.2 mg/ml sonicated salmon sperm DNA and 500 ng digoxigenin-labelled probe). Coverslips were then fixed on top of the silicone ring. Care was taken to prevent the hybridization solution from coming in contact with the coverslip. The probe and target DNA was denatured at 95°C for 6 minutes and chilled on ice for 1 minute. Hybridization was then allowed to proceed in a humid chamber at 42°C overnight.

Following hybridization, the coverslips and silicone gum were removed. Two high stringency washes were performed with 2X SSC and 1X SSC for 1 hour each at room temperature and two low stringency washes with 0.1X SSC at 30 minutes each, one at 68°C and one at room temperature.

3.2.5 Detection of hybrids on slides

The detection of digoxigenin-labelled probes were detected according to the specified instructions of Boehringer Mannheim (1989). All incubations were carried out at room temperature.

The tissues were equilibrated with buffer 1 for I minute and followed with a wash for 30 minutes with buffer 1 containing 0.5% of blocking solution. Thereafter the slides were rinsed with buffer 1 and incubated with 50 µl/well of the diluted antibody-conjugated solution (anti-digoxigenin-AP) for 30 minutes, after which the slides were were rinsed twice for 5 minutes each with buffer 1 and equilibrated for 2 minutes with buffer 3. The slides were incubated overnight in a humidified chamber in 100 µl of freshly prepared susbtrate solution. Colour development was stopped with 50 µl/well of buffer 4. The slides were then rinsed briefly in distilled water, counterstained with Mayers hematoxylin for 1 minute, rinsed again in distilled water and immersed in ammonia solution for 10 seconds. After the final rinse with distilled water the slides

were mounted with glycergel (Dako) prewarmed to 53°C. The slides were then left to dry in the dark before being analysed under light microscope (Olympus, Japan).

3.2.6 Statistical analysis

The data obtained by PCR, Southern blot hybridization and *in situ*hybridization were analysed statistically using the chi-square or Fisher's exact test as appropriate.

3.3. Detection of HPV proteins

3.3.1. Monoclonal antibodies

The monoclonal antibodies used in this study were directed against:

(1) the E6 protein of HPV 18 (Dako) and

(2) the L1 protein of HPV 16. This monoclonal antibody was a gift from Dr Margaret Stanley, University of Cambridge, United Kingdom.

3.3.2. Immunohistochemistry

The technique of immunohistochemical staining were carried out using the Labelled Streptavidin Biotin (LSAB) Peroxidase Kit and the AEC Substrate System (Dako) according to the specifications described by the manufacturer with some modifications. All washing steps required constant shaking and incubations with reagents were carried out in a humidified chamber.

Sections were deparafinized at 56 °C for 20 minutes and dewaxed in xylene for 3 minutes. From this point onwards slides with HeLa were included in the runs as positive control for HPV 18 E6 protein. Negative controls were tissues untreated with the HPV-specific monoclonal antibodies. The tissues and cells were rehydrated in

decreasing concentrations of ethanol - 100%, 95%, 90% and 80% at 2 minutes each and washed in PBS for 5 minutes.

Incubation with a few drops/ well of 3% hydrogen peroxide was carried out for 10 minutes to remove endogenous peroxidase activity. The slides were then rinsed with PBS after which areas surrounding the specimens were blot dry. Fifty microlitres of anti-HPV 16 L1 monoclonal antibody (diluted 1 : 50 in sterile PBS) and of anti-HPV 18 E6 monoclonal antibody (diluted 1 : 80 in sterile PBS) were added to appropriate wells. Incubation with the antibodies were allowed to proceed for I hour at room temperature. Following this the slides were washed twice with PBS for 15 minutes each, incubated with 50 µl/ well of biotinylated anti-mouse immunoglobulin for 10 minutes at 37°C and washed again with PBS. The slides were then incubated with 50 µl/ well of streptavidin horseradish peroxidase conjugate for 10 minutes and washed with PBS.

Fifty microliters of substrate consisting of 3-amino-9-etholcarbazole (AEC) and hydrogen peroxide in 0.1 M acetate buffer pH 5.2 (freshly prepared according to manufacturer's specifications) was applied to each well and left to incubate at 37 °C. The colour reaction was monitored until the desired colour intensity developed. The slides were rinsed with distilled water, counterstained with Mayers hematoxylin for 2 minutes, rinsed again and immersed into ammonia solution for 10 seconds. After the final rinse in distilled water, the slides were mount with glycergel (Dako) prewarmed to 53 °C. The slides were then left to dry in the dark before being analysed under the light microscope (Olympus, Japan).

3.3.3. Statistical analysis

The data obtained in immunohistochemistry were analysed statistically using the chi-square and Fisher's exact test as appropriate. The expression of the E6 and the L1 protein were correlated with the presence of HPV 16 and 18 DNA, respectively.

$$R = \frac{\text{L1 or E6 protein positivities}}{\text{presence of HPV 16 or 18 DNA}}$$

3.4. Serology to HPV

3.4.1. Peptides

Synthetic peptides used in this study were kindly provided by Dr. Joakim Dillner (Sweden). They are as described in Table 3.4.

Table 3.4 Synthetic peptides of HPV 16 and HPV 18

Peptide number	ORF	HPV type
13	L1	16
30	L1	16
49	L2	16
5	E7	16
245	E2	16
245	E2	18

3.4.2. Blood samples

Blood samples were collected from three groups of women:

1. Eighty patients diagnosed of having invasive carcinoma of the uterine cervix seen at the University Hospital, Kuala Lumpur and the Kuala Lumpur Hospital,

2. Thirty-two healthy pregnant women attending the ante-natal clinic at the Kuala Lumpur Hospital and

3. Thirty female students attending the University clinic at the University of Malaya, Kuala Lumpur for reasons other than CIN and cervical carcinoma.

About 10 ml of blood were collected and centrifuged at 2000 to 3000 rpm for 10 minutes. The sera collected were dispensed into aliquots of 0.5 ml and stored at -70 C. Prior to use in the ELISA, the serum samples were heat-inactivated in a 56°C water bath for 30 minutes and kept on ice.

3.4.3. Enzyme-Linked Immunosorbent Assay

ELISA techniques were carried out as communicated by Dr Joakim Dillner. Peptides were first diluted in coating buffer (10 mM carbonate buffer, pH 9.6) to a concentration of 20 µg/ml. Fifty microlitres of the diluted peptide was added to each of the 30 wells on half-area of the 96-well microtitre plates (Costar), while leaving the edge wells empty.The second half of the plate, also comprising of 30 wells were filled with 50 µl of coating buffer. The plate was sealed and incubated overnight at room temperature. On the following day, the plate was emptied and washed once with 150 µl of PBS containing 0.05% Tween 20 (PBS-T) for 2 minutes after which the PBS-T was discarded. The plate was tapped thoroughly against an absorbent paper until no residual buffer was visible in the wells. Following this, 150 µl of 10% lamb serum (heat-inactivated and filtered (Gibco)) in PBS (LS-PBS) was added to each well. The blocking step was carried out for 1 hour to block the remaining protein attachment sites in the wells. The plates were then washed four times at 2 minutes each with PBS-T. Fifty microlitres of human sera diluted 1:30 in LS-PBS were added to each coated well. The same sera were also added to uncoated wells. Reaction was allowed to proceed at 37°C for 2 hours. Following that, the sera were discarded and the plates were subjected to five washes with PBS-T.

Detection of IgA

Monoclonal anti-human IgA (alpha-chain) -horseradish peroxidase conjugate (Dako) was diluted in LS-PBS to a working dilution of 1:1000. Fifty microliters of the diluted antibody was added to each well and incubated for 2 hours at 37°C. The plate was washed five times with PBS-T and developed with 50 µl / well of peroxidase substrate diluted 1:50. A_{415} values were recorded after 60 minutes.

Detection of IgG

Fifty microliters of monoclonal anti-human IgG (gamma chain)-alkaline phosphatase conjugate (Dakopatts) diluted 1:750 in LS-PBS was added to each well and left to react for 2 hours at 37°C. The plate was washed five times with PBS-T and once with 150 µl/well of diethanolamine buffer. Fifty microliters of phosphatase substrate was then applied to each well and the plate was read at 405 nm after 90 minutes.

Detection of IgM

The plate was incubated with 50 µl/well of monoclonal anti-human IgM (mu-chain)-glucose oxidase conjugate (Seralab) diluted to 1:800 in LS-PBS for 2 hours at 37°C. After five washes with PBS-T, 50 µl of glucose oxidase substrate was added to each well and left to develop for 60 minutes. Absorbance values were recorded at 415 nm.

3.4.4. Statistical analysis

Difference between proportions of positive sera in the different groups were evaluated with the use of chi-square or Fisher's exact test as appropriate. The absorbance values for IgA, IgG and IgM tests for each of the peptides were compared for statistically significant differences between the different groups using the 2-sided (Mann Whitney test), the 3-way nonparametric continuous tests (Kruskall Wallis test) or the nonparametric multiple comparison test as appropriate. A p value of below 0.05 was considered to indicate a significant difference.