CHAPTER IV

MATERIALS AND METHODS

PART I. Preparation of HPV-16 E6 and L1 antigens

1. Plasmid

Plasmid pGEX-4T-2-L1 containing HPV16 L1 fused with GST and pGEX-3X-MT-E6 containing HPV16 E6 fused with GST are kindly provided by Prof. Peter C Angeletti, Nebraska Centre for Virology, School of Biological Sciences, University of Nebraska Lincoln, USA. The plasmids were transformed in *E.col BL-21* by Miss Sarita Yanpinit of the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

2. Plasmid purification

Plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen, Germany) from bacterial cells following manufacturer's instruction. In brief, pelleted bacterial cells were resuspended in 250 µl buffer P1 and were transfered to a 1.5 ml microcentrifuge tube. 250 µl of buffer P2 was added and mixed by inverting and followed by 350 µl buffer N3, mixed and then centrifuged at 13,000 rpm for 10 min. After that supernatant was applied to the QIAprep spin column and centrifuged at 13,000 rpm for 1 min. The QIAprep spin column was washed with 500 µl of wash buffer (PB), centrifuged at 13,000 rpm for 1 min, washed again by 750 µl of wash buffer (PE) and centrifuged at 13,000 rpm for 1 min. The QIAprep spin column then placed in a clean 1.5 ml microcentrifuge tube. To elute plasmid DNA, 50 µl of elution buffer (EB) was added, incubated at room temperature for 1 min and centrifuged at 13,000 rpm for 1 min and kept at -20 ^{0}C until use.

3. Bacterial transformation

All plasmids were transformed into the competent *Escherichia coli DH5α* for increase number of plasmid. Plasmid was transformed using Z-competent *Escherichia coli* transformation kit & buffer set (Zymobroth™) following manufacturer's instruction. In brief, 1 µl of plasmid DNA was added to a tube of thawed Z- competent *Escherichia coli DH5α* cells on ice, mixed gently and spreaded 100 µl of mixture onto a pre-warmed 37 ^{0}C culture plate containing amplicillin100 µg/ml. Then the plate was incubated at 37 ^{0}C 12 hr. The clones containing recombinant plasmid were selected by using the specific antibiotics as recommended. The presence of HPV16 E6 or L1 gene in the transformed bacteria was confirmed by PCR using primers specific to HPV16 E6 and HPV L1(Table 4). After that, the bacteria which containing plasmids were extracted using QIAprep Spin Miniprep kit (Qiagen, Germany). The purified plasmids were transformed into *Escherichia coli BL-21(DE3)* for expression.

4. Induction of expression

The expression condition was optimized by varying concentration of IPTG (0.25, 1 mM), temperature (25 ^{0}C, 37 ^{0}C) and duration of IPTG induction time (4, 6, 8 hr). In short, *Escherichia coli BL-21(DE3)* clones containing pGEX-4T-2-L1 and *Escherichia coli BL-21(DE3)* clones containing pGEX-3X-MT-E6 were grown in 20 ml Luria-Bertani (LB) broth plus 100 µg/ml ampicillin at 37 ^{0}C overnight. The bacterial culture was inoculated into 2 liters of LB broth and continuously grown at 25 ^{0}C or 37 ^{0}C until the O.D value between 0.6 - 0.8 at absorbance wavelength of 600 nm was obtained. The inducity conditions were varied as indicated. After that, bacteria were then harvested by centrifugation, washed with PBS and kept frozen at -80^{0}C until use.

5. Protein extraction

The induced bacteria were thawed, resuspended in PBS containing 2 mM DTT, 1% Triton X-100, lysozyme (1KU/ml), complete proteases inhibitor cocktail (1

tabblet/10ml) (Roche Co., USA) and lysed by sonicator (High intensity ultrasonic processor VC505, SONICS) at amplitude of 35% with pulse on for 30-second and pulse off for 50-second for a total of 6 cycles. Lysates were collected by centrifugation at 17,000 rpm, 4 ^{0}C for 30 min. The supernatant and cell lysate pellet were separately collected and kept at -20^{0}C until further analysis by SDS-PAGE.

6. Protein purification

The L1 pellet was washed with buffer (50 mM Tris pH 8, 100 mM NaCl, 1mM DTT,1mM EDTA) and solubilized in urea solution (8M urea, 100 mM NaH_2PO_4, 0.1 mM EDTA, 0.1 M DTT, 10 mM Tris pH 8) at room temperature for 2 hr. Debris was removed by centrifugation at 20,000 rpm, for 30 min at the 20 ^{0}C and supernatant containing L1 proteins was collected. Supernatant containing E6 was further purified by GST fusion protein affinity column purification kit (GE healthcare Co., USA). The procedure of E6 purification was followed the recommendation of the company. In brief, the column was equilibrated with 5 ml of binding buffer (140 mM NaCl, 2.7 mM KCL, 10mM Na_2HPO_4, 1.8 mM KH_2HPO_4, pH 7.3), then supernatant E6 was applied using a syringe fitted to luer adaptor onto the column, using a flow rate of 0.2-1.0 ml/min. After that, column was washed with 5 ml of binding buffer or until no material appears in the effluent. To elute E6 protein with 5 ml of elution buffer (50 mM Tris-HCL, 10 mM reduced glutathione, pH 8.0) was added and kept at -20^{0}C until use.

7. Quantitation of proteins

Total proteins amount of L1 and E6 were determined by Quant-iT protein assay kit (Qubit fluorometer; Invitrogen Co., USA). The mixture of the L1 or E6 proteins and Quant-iT buffer were incubated at room temperature 15 min. Concentration of L1 and E6 proteins were measured by Qubit fluorometer. The Qubit fluorometer generates concentration data based on the relationship between the three standards used in calibration.

8. Purity of proteins

Purity of fusion proteins L1 and E6 were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using mouse anti-HPV-16 L1 and E6 monoclonal antibody (Santa cruz), respectively. For SDS-PAGE the solution of proteins to be analyzed were boiled, then 10 µl were run on 10% polyacrylamide gel (40%/0.8/, w/v acrylamide/bisacrylamide, 10% SDS, 1.5 M Tris-HCl pH 8.8, 10% ammonium persulfate, TEMED) in running buffer. Following electrophoresis, the gel was stained with Coomassie blue. After staining, different proteins were appeared as distinct bands within the gel based on molecular weight. The gel after electrophoresis was used for Western blot. The proteins from the gel were transferred to nitrocellulose membrane and were blocked with 3% bovine serum albumin in Tris buffer saline (TBS) incubated at 4^0C overnight. After blocking, primary antibody using mouse anti-HPV-16 L1 and E6 monoclonal antibody (Santa cruz) were incubated with the membrane at room temperature for 1 hr. After that, the membrane was washed three times with TTBS (TBS, 0.1%Tween 20) and incubated with secondary antibody using goat anti-mouse IgG labeled alkaline phosphatase at room temperature for 1 hr. The membrane was washed three times with TTBS and substrate BCIP/NBT was added for detection colored of the proteins.

PART II. Preparation of goldnanoparticles conjugated with antibody

1. Preparation of goldnanoparticles

Golgnanoparticles (AuNPs) were prepared using gold colloidal solution (Sigma-Aldrich, cat.G1527) which have been 10 nm particle size. All unconjugated gold colloids contain approximately 0.01% of $HAuCl_4$ suspended in 0.01% tanic acid with 0.04% trisodium citrate, 0.26 mM potassium carbonate, and 0.02% sodium azide as a preservative.

2. Optimization of goldnanoparticles and antibody conditions

Standard curves, adsorption isotherms, were determined for each antibody in order to determine the minimum amount of antibody and optimum pH conditions required for stabilization of goldnanoparticles. All AuNPs were optimized by varying pH of colloidal gold solution (pH 4, 5, 6, 7, 8, and 9). Antibodies using HPV-16E6 goat polyclonal antibody, HPV-16L1 goat polyclonal antibody and goat normal antibody were optimized by varying concentration of antibody (final dilution 1:200, 1:400, 1:600, 1:800 and 1:1000). One milliliter of AuNPs was adjusted pH with 0.2 M K_2CO_3 for more basis and 1M acetic acid for more acidic. After that 2 µl of the serial dilutions of antibody were added to 100 µl of AuNPs and let the solution stand 10 min at room temperature. Then 10 µl of 10% NaCl were added to each tube of solution, mixed rapidly and allowed to stand for 5 min before determining the optical density at 580 nm. A curve was generated from series of readings and the point where there curve first appears asymptotic with the x axis was taken as the minimum quantity of antibody needed to stabilize the AuNPs. Moreover, If there is not enough antibody to stabilize AuNPs, the solution will chang from red to blue.

3. Conjugation of goldnanoparticles and antibody

The AuNPs conjugated with anti-HPV16 E6 polyclonal antibody or anti-HPV16 L1 polyclonal antibody or goat a normal antibody were prepared according to the method of Yokota, S., Fujimori, O. 1992 (69). Briefly, AuNPs solution was adjusted to pH 8.0 with 0.2 M K_2CO_3, 10 µl of antibody solution, at the optimum concentration of 0.5 µg/ml, was incubated with 90 µl of the AuNPs solution for 30 min at room temperature. After the addition of 10 µl of 10% bovine serum albumin in 20 mM sodium borate, pH 8, the mixture was incubated at room temperature for another 10 min. And the conjugated AuNPs were washed by centrifugation 25000 rcf at 10^0C for 30 min with washing buffer (20 mmol/L sodium borate, pH 8, 1% BSA and 0.1% sodium azide). The precipitate was resuspended in the washing buffer and stored at 4^0C until use.

PART III. Immunogoldagglutination assay

1. Immunogoldagglutination assay with HPV antigens

HPV antigens, i.e., E6 and L1 proteins were obtained from *E.coli* expression as described in Part I. The immunogoldagglutination assay was performed by mixing 20 µl of purified E6 proteins with 50 µl of AuNPs conjugated HPV-16E6 polyclonal antibody. While the purified L1 proteins were reacted with AuNPs conjugated HPV-16L1 polyclonal antibody. The rate of agglutination was measured as a function of temperature, duration of process and antigen concentration. The temperature-dependent experiments were conducted at various temperatures 4^0C, room temperature (22-25^0C) and 37^0C. Different concentrations of E6 or L1 proteins (0.625, 2.5, 10, and 40 µg/ml) were used to determine the optimal concentration for immunogoldagglutination assay. All experiments were carried out in 2 independent measurements using spectrophotometer (Smart™ Spec, BIO-RAD) at 520, 580 and 620 nm for 0, 30, and 60 min after mixing the reaction.

2. Immunogoldagglutination with control cell line

2.1 Control cell lines and culture

The CaSki cell line, a human cervical carcinoma cell line with about 600 copies of HPV type 16 was kindly provided by Associate Professor Dr. Parvapan Bhattarakosol of the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. The CaSki cells were grown at 37^0C in 5% CO_2 air atmosphere as monolayers in Eagle's Minimum Essential Medium (GIBCO™ , Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (PAA laboratories GmbH, Austria). The CaSki cell line was used as a control cell line for detection HPV-16 L1 and E6 antigens with immunogoldagglutination assay.

2.2 Cell lysis

Cell grown in monolayer (CaSki cell line) can detached from the culture flask by trypsinization and was washed with phosphate buffer saline (PBS), centrifuged for 5 min at 3000 rpm. Then the pellet of approximately 10^7 cells were resuspended with 200 µl of lysis buffer (150 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 and protease inhibitor 1 tabblet/10 ml lysis buffer), mixed by vortex for 5 min or until cell complete lyses and kept at -20^{0}C until use.

2.3 Immunogoldagglutination

Immunogoldagglutination between control cell line and AuNPs conjugated HPV16 E6/L1 polyclonal antibody were performed. Twenty microlitres of CaSki cell lysate (approximately. 1,000,000 cells) were reacted with 50 µl of AuNPs conjugated either HPV16 E6 or HPV16L1 polyclonal antibody. The agglutination assay was monitored for 0.5, 1, 2 and 24 hr at room temperature.

3. Sensitivity of immunogoldagglutination assay

3.1 HPV16E6 or HPV16L1 fusion protein

The minimum amount of each antigen necessary to detection by immunogoldagglutination assay was determined an absorption change at 620 nm using either HPV16E6 or HPV16L1 fusion protein at concentrations 0.625, 2.5, 10 and 40 µg reacted with either AuNPs conjugated HPV16 E6 or HPV16L1 polyclonal antibody was detected.

3.2 Cell lysate containing HPV16E6 or L1

The minimum amount of number of HPV-16 positive cells was determined by agglutination of various number cells of CaSki cells lysate which containing HPV16E6

and L1 proteins (10^4, $5x10^4$, 10^5, $5x10^5$, 10^6, and $2x10^6$) and either AuNPs conjugated HPV16 E6 or HPV16L1 polyclonal antibody.

4. Control experiment of immunogoldagglutination assay with HPV16 fusion proteins

Several control experiments were carried out to prove that nonspecific agglutination does not occur under experimental conditions. Control experiment using HPV16 E6 or L1 monoclonal antibody or normal goat antibody conjugated with AuNPs and unconjugated AuNPs for detection E6 or L1 fusion proteins were done. All experiments were carried out in 2 dependent measurements using spectrophotometer (Smart™ Spec, BIO-RAD) at 620 nm for 0, 30, and 60 min after mixing the reaction.

5. Specificity of immunogoldagglutination assay

Immunogoldagglutination assay was developed for detection of HPV16 from clinical specimens such as cervical swab which contaminates with many organisms. The organisms which are commonly found in vagina such as bacteria, virus and fungus. To prove this assay that nonspecific agglutination and no cross-reactivity of other antigens were occurred. Antigens from various bacteria were used including several types of cervical cell line (Table 7, 8). Solution containing AuNPs conjugated either HPV16E6 or L1 polyclonal antibody were reacted to each bacterial cells lysate (10^7 CFU/ml) or each standard cells lysate (10^7 cells). All bacteria cells were provided by Dr. Tanitha Chatsuwan of the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. All standard cell lines were purchased from American Type Culture Collection (ATCC, USA). The positive control solution contained AuNPs conjugated HPV16 E6 or L1 polyclonal antibody and E6 or L1 purified proteins or 10^7 CaSki cells lysate. The agglutination assays were monitored for 24 hr. at room temperature.

Table7. Bacteria cells used in control experiment

Bacteria cells
Staphylococcus aureus
Streptococcus viridans
Enterococcus spp.
Escherichia coli
Pseudomonas aeruginosa

Table8. Standard cell lines used in control experiment

Cell lines	Type of cell line	HPV DNA
CaSki	Human cervical carcinoma	~ 600 copies HPV-16 DNA
SiHa	Human cervical carcinoma	1-2 copies HPV-16 DNA
Hela	Human cervical carcinoma	HPV-18 DNA
ME-180	Human cervical carcinoma	HPV-39 DNA, HPV-18 DNA
MS751	Human cervical carcinoma	HPV-18 DNA, HPV-45 DNA
C-33A	Human cervical carcinoma	Negative for HPV DNA
HEp-2	Human laryngeal carcinoma	Negative for HPV DNA

PART IV. HPV detection in clinical samples

1. Samples

1.1 Standard HPV DNA

Standard HPV DNA used as positive control in this study, was prepared from CaSki and SiHa cell lines, purchased from American Type Culture Collection (ATCC, USA). The cells were grown at 37^0C in 5% CO_2 air atmosphere as monolayers in Eagle's Minimum Essential Medium (GIBCO™ , Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (PAA laboratories GmbH, Austria).

1.2 Clinical specimen

Forty patients were enrolled from women attending the Gynecology Clinic at King Chulalongkorn Memorial Hospital, Bangkok, Thailand from January 2009 to January 2010. Ten patients were diagnosed normal cytology. Twenty patients were diagnosed as having CIN: 10 patients with CIN I, 4 patients with CIN II and 6 patients with CIN III. Ten patients were diagnosed as having cervical carcinoma. Cervical swab of these patients were collected for detection HPV-16 DNA by PCR and immunogoldagglutination assay.

2. Extraction of DNA

DNA either from cervical swab or cell culture was extracted using QIAamp DNA Mini Kit (Qiagen, Germany) following manufacturer's instruction. In brief, Either one microliter of cervical swab solution was placed in 1.5 ml microcentrifuge tube and centrifuged 3000 rpm for 5 min or 10^7 cells grown in monolayer were trypsinized and centrifuged at 300 rcf for 5 min. The cells pellet was resuspended in 200 µl of PBS, then was added with 200 µl of lysis buffer (AL) and 20 µl of proteinase K. The suspensions were then incubated at 56^oC for 10 min. After that, 200 µl of absolute ethanol was added. The suspension was applied to QIAamp spin column and centrifuged at 8,000 rpm for 1 min. The QIAamp spin column was washed with 500 µl of wash buffer (AW1),

centrifuged at 8,000 rpm for 1 min, washed again by adding 500 µl of wash buffer (AW2) and centrifuged at 14,000 rpm for 3 min. The QIAamp spin column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 100 µl of elution buffer (AE) was added onto the spin column, incubated at room temperature for 5 min and centrifuged at 8,000 rpm for 1 min. The eluate was kept at -20 °C until use.

3. Quantitative analysis of DNA extraction

The DNA was quantitated by Quant-iT protein assay kit (Qubit fluorometer; Invitrogen Co.). The mixture of the DNA and Quant-iT buffer was incubated at room temperature for 2 min. Concentration of DNA was measured by Qubit fluorometer. The Qubit fluorometer generates concentration data based on the relationship between the two standards used in calibration.

4. HPV DNA detection

HPV detection in clinical samples were performed by PCR amplification of HPV L1 region. The system used L1C1-L1C2 primer set (L1C1/C2) (75)and produced approximately 250 bp amplified product (Table9). As a control for DNA amplification, human ß-globin gene was amplified by GH20 and PC04 primers and produced 268 bp amplified product (48) (Table 9). The 50 µl of PCR mixture contained 5 µl of DNA sample, 1X PCR buffer (100 mM KCl, 20mM Tris pH 8.0), 1.5 mM $MgCl_2$, 200 µM dNTPs, 25 pmole of each L1C1/C2 primer, 2 unit of Taq polymerase (Fermentas, EU). The PCR was started at 95 °C for 10 min, one cycle and followed by denaturing at 95 °C for 1.30 min, annealing at 40 °C for 1.30 min and extension at 72 °C for 2 min. The step was repeated for 40 cycles, followed by an extension at 72 °C for 10 min. In parallel, the human ß-globin gene was amplified by GH20 and PC04 primers. Size of the amplified product was determined by gel electrophoresis. Ten microlitres of amplified product was run on a 1.5% agarose gel in 0.5X Tris borate EDTA (TBE) buffer. The DNA bands were stained with ethidium bromide and photographed under ultraviolet (UV) transillumination.

5. HPV type 16 DNA detection

Amplification of HPV type 16 using type specific Pr1/Pr2 primer set was performed as previously described by Frank et al (27). The 50 µl of PCR mixture contained 5 µl of DNA sample, 1X PCR buffer (100 mM KCl, 20mM Tris pH 8.0), 1.5 mM $MgCl_2$, 200 µM dNTPs, 20 pmole of each Pr1/Pr2 primer, 1unit of Taq polymerase (Fermentas, EU). The PCR was started at 94 °C for 5 min, one cycle and followed by denaturing at 94 °C for 30 sec, annealing at 57 °C for 30 sec and extension at 72 °C for 1 min. The step was repeated for 35 cycles, followed by an extension at 72 °C for 10 min. In parallel, the human ß-globin gene was amplified by GH20 and PC04 primers. Size of the amplified product was determined by gel electrophoresis. Ten microlitres of amplified product was run on a 1.5% agarose gel in 0.5X Tris borate EDTA (TBE) buffer. The DNA bands were stained with ethidium bromide and photographed under ultraviolet (UV) transillumination. The amplified HPV-16 E6 (Pr1/Pr2) product was approximately 119 bp (Table9).

Table9. Sequence of oligonucleotides used as primers for PCR amplification

Primers	Sequence (5'-3')	Target	PCR product (bp)	Reference
L1C1	CGTAAACGTTTTCCCTATTTTTTT	L1	250	131
L1C2	TACCCTAAATACTCTGTATTG			
GH20	GAAGAGCCAAGGACAGGTAC	ß-globin	268	17
PC04	CAACTTCATCCACGTTCACC			
Pr1	TCAAAAGCCACTGTGTCCTGA	E6	119	27
Pr2	CGTGTTCTTGATGATCTGCAA			

PART VI. Immunogoldagglutination in clinical specimen

1. Cell lysis

Cervical epithelium cells from cervical swab solution were collected by centrifuged at 3000 rpm for 5 min. The cells pellet was washed with PBS and resuspended in 200 µl of lysis buffer (150 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100 and protease inhibitor 1 tabblet/10 ml lysis buffer), mixed by vortex for 5 min or until cells complete lyse and kept at -20^{0}C until use.

2. Quantitation of proteins from cell lysate

Total proteins of cell lysate were determined by Quant-iT protein assay kit (Qubit fluorometer; Invitrogen Co.). The mixture of the cell lysate and Quant-iT buffer were incubated at room temperature 15 min. Concentration of L1 and E6 proteins were measured by Qubit fluorometer. The Qubit fluorometer generates concentration data based on the relationship between the three standards used in calibration.

3. Immunogoldagglutination assay

Immunogoldagglutination between cells lysate and AuNPs conjugated either HPV16 E6 or HPV16L1 polyclonal antibody. Twenty microlitres of cell lysate were reacted with 50 µl of AuNPs conjugated with either HPV16 E6 or L1 polyclonal antibody. The agglutination assay was monitored for 0.5, 1, 2, 4, 8, 16 and 24 hr. at room temperature.