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Assessment of human papillomavirus detection using different methods and clinical samples

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ภรจริม นิลยนิมิต : ประเมินการตรวจหาเชื้อไวรัสฮิวแมนเปปิโลมา ด้วยวิธีการที่แตกต่างกันจากสิ่งส่งตรวจทางคลินิกของผู้ป่วย (Assessment of human papillomavirus detection using different methods and clinical samples) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ดร.ยง ภู่วรวรรณ, 121 หน้า.

ไวรัสฮิวแมนเปปิโลมาเป็นสาเหตุของโรคมะเร็งในบริเวณต่างๆ เช่น ปากมดลูก ช่องคลอด อวัยวะเพศชายทวารหนักและ ช่องปาก ในปัจจุบันเทคนิคการตรวจ Pap smear เป็นสิ่งที่แนะนำสำหรับการตรวจคัดกรองมะเร็งปากมดลูก แต่มีเพียงผู้หญิงเพียงส่วนน้อยที่จะเข้ารับการตรวจคัดกรองนี้ งานศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาเทคนิคและชนิดของตัวอย่างในการตรวจหาดีเอ็นเอของไวรัสฮิวแมนเปปิโลมาในคนไทย และเพื่อศึกษาสายพันธุ์ของไวรัสฮิวแมนเปปิโลมาในผู้ป่วยไทยที่มีหูดบริเวณอวัยวะสืบพันธุ์ จากตัวอย่างการป้ายเซลล์จากปากมดลูกและตัวอย่างปัสสาวะจากผู้หญิง 116 ตัวอย่างและผู้ชาย 100 ตัวอย่าง ถูกเก็บรวบรวมก่อนทำการตรวจ Pap smear ตัวอย่างทั้งหมดได้นำไปตรวจสอบทางพยาธิวิทยา ซึ่งสามารถจำแนกได้ 4 กลุ่มคือ เซลล์ปกติ 52 ตัวอย่าง ASCUS 9 ตัวอย่าง LSIL 24 ตัวอย่าง และ HSIL 31 ตัวอย่าง ในชายปกติ 45 รายและชายรักร่วมเพศหรือชายรักชาย 55 ราย นำตัวอย่างทั้งหมดตรวจหาดีเอ็นเอของไวรัสโดยใช้เทคนิคพีซีอาร์ พบไวรัสฮิวแมนเปปิโลมาสายพันธุ์ 16 เป็นส่วนใหญ่ และพบว่าอัตราการตรวจพบไวรัสฮิวแมนเปปิโลมาในตัวอย่างปัสสาวะต่ำกว่าตัวอย่างเซลล์ที่เก็บจากปากมดลูก การเปรียบเทียบเทคนิคระหว่าง DNA chip และพีซีอาร์ในการตรวจหาเชื้อไวรัสฮิวแมนเปปิโลมา ในตัวอย่างเซลล์ที่เก็บจากปากมดลูกพบว่า เทคนิคทั้งสองชนิดพบไวรัสฮิวแมนเปปิโลมาตรงกันร้อยละ 88.8 ในตัวอย่างปัสสาวะเปรียบเทียบกับเซลล์ที่ได้จากการป้ายจากรอยโรคพบว่าตัวอย่างปัสสาวะ กับเซลล์ที่ได้จากการป้ายจากรอยโรคในชายปกติ มีสายพันธุ์ที่ตรงกันมากกว่าเมื่อเทียบกับชายรักร่วมเพศหรือชายรักชาย นอกจากนี้ยังมีตัวอย่างปัสสาวะและเซลล์ที่เก็บจากปากมดลูกจำนวน 164 คู่ ทำการทดสอบด้วยเทคนิค Genoarray ผลการศึกษาพบว่าไวรัสฮิวแมนเปปิโลมา มีความสอดคล้องกันของตัวอย่างทั้งสองเท่ากับ 65.2% นอกจากนี้ยังมีการเปรียบเทียบตัวอย่างเซลล์ช่องคลอดที่เก็บด้วยตนเองและเก็บโดยแพทย์ใน 101 ตัวอย่าง ด้วยเทคนิค DNA chip ผลการทดลองแสดงให้เห็นว่าตัวอย่างเซลล์ช่องคลอดที่เก็บด้วยตนเองและเก็บโดยแพทย์มีความสัมพันธ์ตรงกันอย่างมีนัยสำคัญ และสายพันธุ์ที่พบมากที่สุดคือ ไวรัสฮิวแมนเปปิโลมา สายพันธุ์ 51 นอกจากนี้ยังตรวจหาสายพันธุ์ของไวรัสฮิวแมนเปปิโลมา จำนวน 58 ตัวอย่างโดยเปรียบเทียบ 4 เทคนิคคือ nested-PCR, INNO-LiPA, DNA chip และ NGS ผลการวิจัยพบว่า 7 สายพันธุ์ของไวรัสฮิวแมนเปปิโลมา (16, 18, 31, 33, 45, 56 และ 58) สามารถพบได้ทั้ง 4 เทคนิค แต่มีเพียง 19 สายพันธุ์ที่สามารถพบได้ด้วยเทคนิค NGS การค้นพบนี้แสดงให้เห็นถึงวิธีการอื่นๆในการตรวจหาสายพันธุ์ของไวรัสฮิวแมนเปปิโลมา นอกจากนี้ในการศึกษาการหาสายพันธุ์ของไวรัสฮิวแมนเปปิโลมาของหูดที่อวัยวะเพศ ในประชากรไทย โดยทำการหาสายพันธุ์ไวรัสในตัวอย่างทั้งหมด 206 ตัวอย่างด้วยเทคนิคพีซีอาร์ พบไวรัสฮิวแมนเปปิโลมาในตัวอย่าง 88.3% (182/206) ซึ่งสายพันธุ์ที่พบส่วนใหญ่เป็นสายพันธุ์ที่มีความเสี่ยงต่ำ (สายพันธุ์ 6 และ 11) คิดเป็นร้อยละ 75.2% สรุปผลการศึกษานี้แสดงให้เห็นว่าตัวอย่างปัสสาวะและเซลล์ช่องคลอดที่เก็บด้วยตนเองเป็นตัวอย่างที่เหมาะสมในการนำมาตรวจหาไวรัสฮิวแมนเปปิโลมาและเทคนิค NGS อาจใช้เป็นทางเลือกในการตรวจคัดกรองหาสายพันธุ์ไวรัสฮิวแมนเปปิโลมาในอนาคต นอกจากนี้วัคซีนป้องกันไวรัสฮิวแมนเปปิโลมาชนิดสี่สายพันธุ์อาจจะเหมาะสมในการป้องกันการเกิดหูดที่อวัยวะเพศในคนไทย

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: HUMANPAPILLOMA VIRUS / NEXT GENERATION SEQUENCING / PCR / ELECTROCHEMICAL DNA CHIP / URINE / SELF-SWAB / PAP SMEAR / ANOGENITAL WART

PORNJARIM NILYANIMIT: Assessment of human papillomavirus detection using different methods and clinical samples. ADVISOR: PROF. DR.YONG POOVORAWAN, M.D., 121 pp.

Human papillomavirus (HPV) is the etiological agent implicated cancers include those of the cervix, vulva, vagina, penis, anus, rectum and oropharynx. Currently, the Papanicolaou (Pap) smear is the recommended for cervical cancer screening but only a small percentage of women follow this screening. Overall, this thesis aimed to develop the technique and type of specimen to detect HPV DNA in Thai population and to characterize of the HPV genotypes in Thai patients with anogenital warts. Cervical swab and urine sample of 116 women and 100 men were collected before the Pap smear test. The histological examination of women can classified into 4 group: normal cytology in 52, ASCUS in 9, LSIL in 24, and HSIL in 31. In male patients, there were 45 heterosexual males who visited hospital for health check-up and 55 were documented as homosexuals or MSM. HPV DNA was genotyping by electrochemical DNA chip and PCR sequencing showed HPV16 was the most prevalence in this study. The HPV detection rate was generally lower in urine samples compared with cervical samples. There was good agreement for detection of carcinogenic HPV from female cervical samples between the DNA chip and PCR/sequencing, with 88.8% agreement. In male urine samples, the level of agreement was higher in heterosexuals compared with homosexuals. Moreover, the 164 pair of urine and cervical specimens were tested with Genoarray assay. The result showed overall concordance percentage for HPV detection was 65.2%. In addition, the comparison of 101 self-collected vaginal and physician-collected cervical swabs of known cytology was performed by DNA chip assay. The result showed the concordance was relatively high between self-collected and physician-collected samples. The most common HPV genotype detected was HPV 51. Also, 58 cervicovaginal samples were tested for HPV genotypes using four methods in parallel: nested-PCR followed by conventional sequencing, INNO-LiPA, DNA chip, and NGS. These findings show that 7 HPV genotypes (16, 18, 31, 33, 45, 56, and 58) were identified by all four methods. Nineteen HPV genotypes were detected by NGS, but not by nested-PCR, INNO-LiPA, or DNA chip. This findings further showed the alternative method to detected HPV genotyping. Finally, the study was investigate HPV-associated anogenital warts in the Thai population and whether genotypes found are represented in the vaccine. A total of 206 anogenital swab samples were analyzed by PCR/sequencing. HPV positive was identified in 88.3% (182/206) of the samples. The majority of HPV (75.2%) were low-risk genotypes HPV6 and HPV11. This finding conclusion, this study demonstrated urine and self-collected swab is a promising technique for HPV screening. NGS may serve as an alternative for diagnostic HPV genotyping in certain situations. Moreover, quadrivalent vaccine could potentially prevent the genital warts in the Thai population.

Field of Study: Medical Science Student's Signature .....
Academic Year: 2017 Advisor's Signature .....

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CHAPTER I
Introduction

Human papillomavirus (HPV) infection is the major cause of cervical cancer and genital warts (3) (3). More than 100 genotypes of HPV have been identified and more than 40 genotypes can infect genital areas in human. HPV is transmitted through skin-to-skin contact with the penis, scrotum, vagina, vulva or anus (4). Pap smear is a standard cytology method for screening abnormal cellular changes or premalignant lesions of cervical tissues. This screening method is invasive, uncomfortable for patients and time consuming for healthcare workers (5). HPV DNA detection in urine and self-swab are another alternative, which is more widely accepted because it is easily collected, non-invasive and readily available (6, 7). The widespread detection of HPV DNA can be done by polymerase chain reaction (PCR) on the L1 region. At present, HPV DNA detection have various technique such as electrochemical DNA chip, Genoarray assay. It can detect single and multiple infections of high risk genotypes of HPV with higher sensitivity, specificity, simplicity and speed up when compare with the original methods (8). Moreover, Next-Generation Sequencing (NGS) is an alternative method to detected HPV DNA because of its highly sensitive detection of multiple HPV genotypes. In addition, anogenital warts are caused by HPV genotype6 and 11. These lesion may cause discomfort and affect psychological well-being because they are often untreated (9). The immunization with the HPV vaccine can provide excellent protection against the HPV genotypes comprised in the vaccine (10). This thesis will focus mainly on HPV viruses which are the major cause of cervical cancer and anogenital warts. The HPV patients have a lot of barriers to see the doctor. Therefore, development the technique to detect

HPV DNA is a better way to screening and protecting HPV in Thai population. Additionally, characterization of the HPV genotypes in Thai patients with anogenital warts were determine the genotypes that represented in the vaccine.

Part 1: Detection of human papillomavirus in urine and self-swab by electrochemical DNA chip, PCR sequencing, and GenoArray Diagnostic assay

Research questions

1. What are the incidences of HPV infections in Thai women and men using urine and self-swab?
2. Is testing urine and self-swab samples for HPV comparable to conventional samples?
3. What is the detection sensitivity of self-swab and physician-collected cervical swabs in Thai women?

Objectives

1. To evaluate HPV detection rate in Thai women and men using urine and self-swab sample.
2. To compare HPV genotypes obtained from Pap smear, urine, and self-swab samples.
3. To evaluate the detection of HPV DNA in self-swab collected sample compare with physician-collected cervical swabs.

Part 2: Comparison of Four Human Papillomavirus Genotyping Methods: Next-generation Sequencing, INNO-LiPA, Electrochemical DNA Chip, and Nested-PCR

Research question

1. What is the optimal method to detect the HPV genotyping for the future?
2. Is the Illumina sequencing platform appropriate for the detection of HPV genotyping?

Objectives

1. To compared HPV test results obtained by PCR, INNO-LiPA, electrochemical DNA chip and NGS test on the same specimen.
2. To investigate the use of Illumina sequencing to detect and genotype the HPV types present in a complex multiple infections in a cervical specimen.

Part 3: Human papillomavirus in Thai women and men with anogenital warts

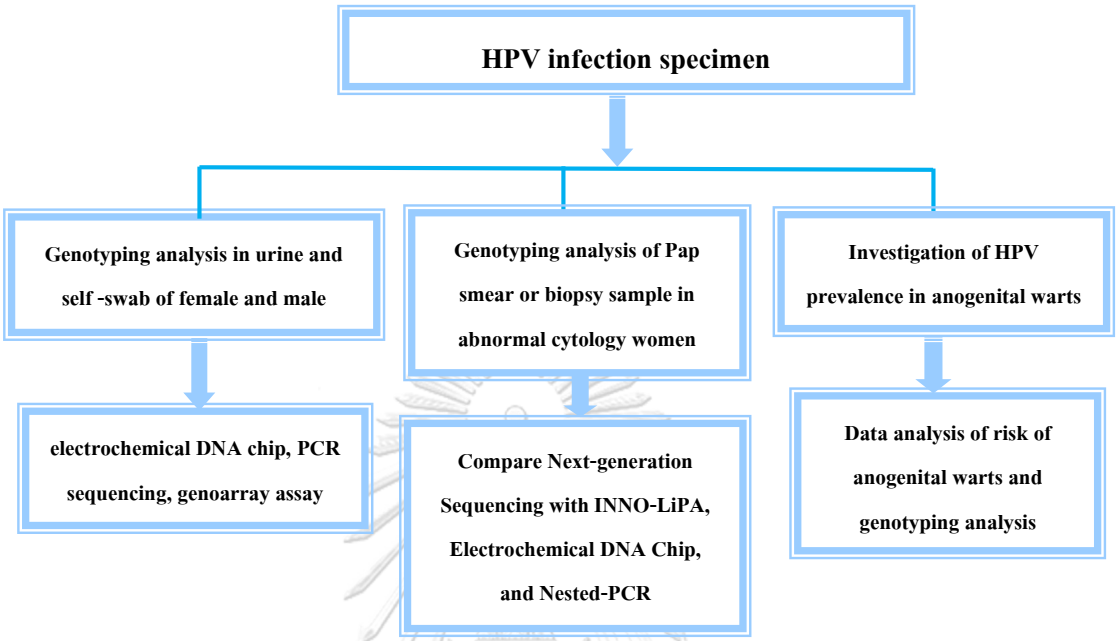
Research questions

1. What is the incidence of HPV infection in anogenital wart among Thais?
2. Are the genotype of anogenital wart in Thais correlate with the vaccine?

Objectives

1. To investigate the prevalence of human papillomavirus infections in anogenital wart among Thai population.
2. To determine the genotypes of anogenital wart in Thais are represented in the vaccine.

Conceptual framework



CHAPTER II
Review of related literature

Human papillomavirus (HPV) is one of the most common causes of sexually transmitted diseases in both men and women around the world, with prevalence rates varying with the studied population and geographical localization (11). The majority of women and men have been infected with HPV at least once during their lifetime (12). Once HPV transmission to the genital tract occurs through sexual contact, the risk factors for the infection and cervical lesions including cervical cancer are the same classic risk factors as for other sexually transmitted diseases. In addition, other indicators of sexual behavior, reproductive activity, heredity, immune and nutritional status, and smoking habit also contribute in some way to the development of cervical cancer (12, 13).

Structure of human papillomavirus

HPV are also classified into five genera designated by letters of the Greek alphabet (alpha, beta, gamma, mu, and nu) according to the identity of the nucleotide sequences of their genomes and the phylogenetic and pathology features (14, 15). The genus Alphapapillomavirus is associated with infections of the anogenital and oral mucosa. This is a group of high risk HPV that causes almost all cases of cervical cancer and a small proportion of cases of other cancers of the genital tract (16). HPV is a small non-enveloped virus that contains a double-stranded, closed circular DNA genome and protected by a capsid with icosahedral symmetry (50-60 nm diameter). The major protein capsid L1 forms a network of intra- and inter-pentameric disulfide interactions, which serve to stabilize the capsid (17). L2 protein is the secondary component of viral capsid, located within the virion. To assemble the viral capsids, the L1 protein join to copies of L2, which occludes the center of each pentavalent capsomere (18, 19).

Viral genome organization

The genome of HPV contains approximately 8000 base pairs and an average of 8 open reading frames, divided into three regions. The first is a long control region (LCR), which has the regulatory function of the transcription of the E6 and E7 viral genes. The second is an early region (E) consisting of six open reading frames (E1, E2 E4, E5, E6, and E7), which encodes non-structural proteins involved in viral replication and oncogenesis. The third is a late region (L), which encodes the L1 and L2 structural proteins (20, 21). The major surface-exposed regions of L1 contain a series of hypervariable amino acid loops that have diverged between different papillomavirus types and in response to the host immune selection pressure. The main functions of the viral proteins was show in Table 1.

Table 1. *The human papillomavirus (HPV) proteins and functions*

Protein	Functions
E1	Binding site in the origin of replication localized in the long control region, a prerequisite for viral DNA replication
E2	Controls viral transcription, DNA replication, and segregation of viral genomes
E4	Favors and supports the HPV genome amplification, besides regulating the expression of late genes, controlling the virus maturation, and facilitating the release of virions
E5	Enhances the transforming activity of E6 and E7; promotes fusion between cells; contributes to immune response evasion
E6	Binds and degrades the tumor-suppressor protein p53, inhibiting apoptosis; interacts with proteins of the innate immune response; activates the expression of telomerase
E7	Binds and degrades the tumor-suppressor protein pRB; increases cdk activity; affects the expression of S-phase genes by directly interacting with E2F factors and with histone deacetylases; contributing to immune response evasion
L1	Major capsid protein; contains the major determinant required for attachment to cell-surface receptors; it is highly immunogenic and has conformational epitopes that induce the production of neutralizing type-specific antibodies against the virus
L2	Minor capsid protein; L2 contributes to the binding of virion in the cell receptor, favoring its uptake, transport to the nucleus, and delivery of viral DNA to replication centers; also, E2 helps the packaging of viral DNA into capsids

Cell cycle entry of human papillomavirus

The HPV cycle initiates when the virus gains access to undifferentiated cells of the basement membrane of the squamous columnar junction epithelium of the ectocervix, after these regions are exposed to mechanical or chemical trauma. The basal cells in the transformation zone retain the ability to differentiate, a property required for virion production. Cervical infection with high-risk HPV typically lasts from 12 to 18 months and in most cases is cleared spontaneously. However, in some women the immune response is insufficient to eliminate the virus, resulting in a persistent, long-term infection that may progress to a malignant lesion (22-24).

Tissue progression to cervical cancer

Cervical cancer can well-defined as pre-malignant phase that can be suspected by cytological test of exfoliated cervical cells and confirmed by histological examination of cervical material. These pre-malignant changes represent a range of histological abnormalities ranging from CIN1 (mild dysplasia) to CIN2 (moderate dysplasia) to CIN3 (severe dysplasia/carcinoma in situ) (25). It is likely that high-risk HPV infection occurs early in life and persists until the influence of some factors promoted cell transformation leading to a gradual progression to more severe disease. Some investigators have correlated HPV type with different grades of CIN and have suggested that CIN1 and CIN2-CIN3 are distinct processes (26). The tissue progression of HPV was initial with basal cells in the cervical epithelium rest on the basement membrane, which is supported by the dermis. After HPV are access to the basal cells through micro-abrasions in the cervical epithelium. Following infection, the early HPV genes E1, E2, E4, E5, E6 and E7 are expressed and the viral DNA replicates from episomal DNA (purple nuclei) (Figure 1). In the upper layers of epithelium (the midzone and superficial zone) the viral genome is replicated and the late genes L1 and L2, and E4 are expressed.

L1 and L2 encapsidate the viral genomes to form progeny virions in the nucleus. The shed virus can then initiate a new infection. Low grade intraepithelial lesions (LSIL) support productive viral replication. An unknown number of high-risk HPV infections progress to high grade intraepithelial lesion (HSIL). The progression of untreated lesions to microinvasive and invasive cancer is associated with the integration of the HPV genome into the host chromosomes (red nuclei), with associated loss or disruption of E2, and subsequent upregulation of E6 and E7 oncogene expression. LCR, long control region (1).

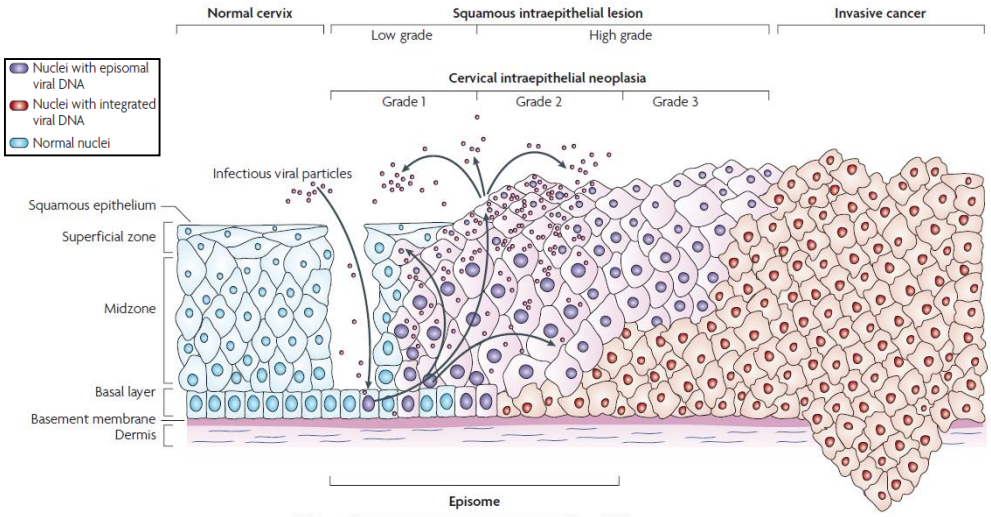


Figure 1. *HPV progression to cervical cancer (1).*

Prevalence of human papillomavirus

The statistics of HPV infection in the cervix uterus ranging from normal cytology (cytologically normal women) to different stages of precancerous lesions (CIN-1, CIN-2, CIN-3/CIS) and invasive cervical cancer. HPV infection is measured by means of HPV DNA detection in cervical cells (fresh tissue, paraffin embedded or exfoliated cells). The prevalence of HPV increases with severity of the lesion. HPV causes virtually 100% of cases of cervical cancer, and an underestimation of HPV prevalence in cervical cancer is most likely due to the limitations of study approaches. Globally, HPV16 and

18 contribute to over 70% of all cervical cancer cases, 41%-67% of high-grade cervical lesions and 16-32% of low-grade cervical lesions. Other HPV16/18, the 6 mostly common HPV types are found in HPV31, 33, 35, 45, 52 and 58 around the world. These 6 types are cause of cervical cancers worldwide for 20% (27). The prevalence of HPV in Thai population have a few database. The prevalence of HPV among women with normal cervical cytology around the world was show in Figure 2.

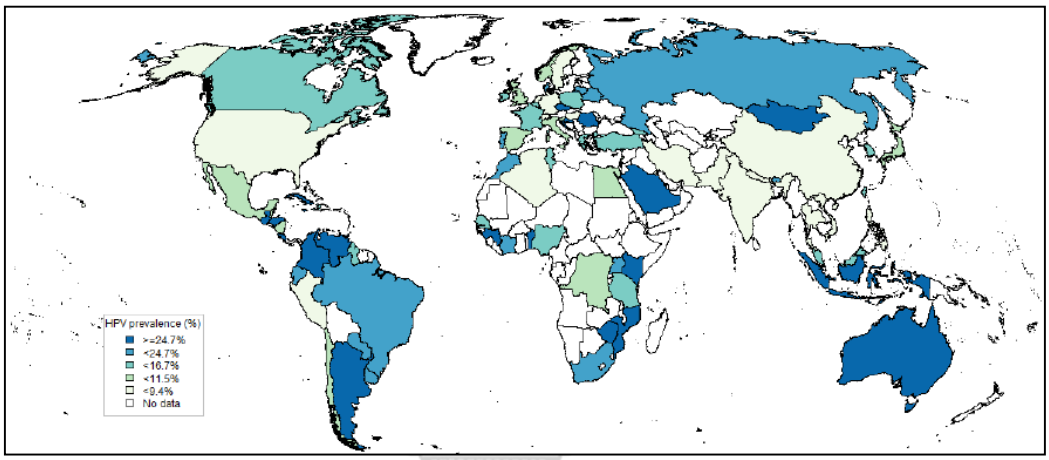


Figure 2. *Prevalence of HPV in women with normal cervical cytology around the world (2).*

Data updated on 15 Dec 2016 (data as of 30 Jun 2015)

Clinical manifestation

HPV infection is associated with a variety of clinical conditions from benign lesions to cervical cancer. The HPV infection may be latent, subclinical, or clinical. In most cases, the infection is transient whereby most individuals healed and eliminated the virus without the presence of any clinical manifestation (28). HPV are classified based on their oncogenic potential as either low-risk or high-risk HPV types (14). The low-risk HPV type causes benign hyperproliferative lesions or genital warts with a very limited tendency for malignant progression. Low-risk HPV infection can effect in

anogenital warts, which present as recurrent papules or cauliflower-like lesions, or flat genital warts. Genital warts turn white with application of a 3-5% acetic acid solution, but this test is nonspecific (29). Meanwhile, the high-risk HPV type is strongly associated with premalignant and malignant cervical lesions (15, 28).

Viral transmission

HPV is transmitted through skin or mucosa contact with infected genital. Genital warts have high viral load and highly infectious; up to 65 percent of sexual contacts develop an infection (30). The usually incubation period for clinical warts is 3-8 weeks (30). Oral infection with genital HPV types may occur but the risk of transmission is low (31). Perinatal transmission also can occur but this is rare case (31). Receptive anal intercourse has been associated with intra- and perianal warts but these warts are not always associated with anal intercourse. Cervical and anal infections often coexist in a persons without a history of anal intercourse (32).

Risk Factors

HPV is a necessary reason of cervical cancer, but it is not a sufficient reason. Other risk factors are necessary for progression from cervical HPV infection to cancer. Tobacco smoking, long-term hormonal contraceptive use, high parity, and co-infection with HIV have been identified as established cofactors. Co-infection with Chlamydia trachomatis and herpes simplex virus type-2, immunosuppression, and certain dietary deficiencies are other probable cofactors. Genetic and immunological host factors and viral factors other than type, such as variants of type, viral load and viral integration, are likely to be important but have not been clearly identified. (33).

Human papillomavirus vaccine

In present, there are 3 vaccines for the prevention of HPV infection, Cervarix (recombinant HPV bivalent vaccine, Types 16 and 18; GlaxoSmithKline), Gardasil (recombinant HPV quadrivalent vaccine, Types 6, 11, 16, and 18; Merck), and Gardasil9 (recombinant HPV nonavalent vaccine, Types 6, 11, 16, 18, 31, 33, 45, 52 and 58; Merck) 9 (34). All three vaccines contain synthetically virus like particles (VLPs) of the L1 epitope. The 3 types of vaccine are intended to be given to females or males before they become sexually active. The CDC's Advisory Committee on Immunization Practices has recommended that the first dose be given to females aged 11 to 12 years and recommended the second and third doses at one to two months and six months after the first dose. The vaccines are prepared for the prevention of HPV infection and do not treat existing HPV infection or disease. Because the development of cervical cancer may take longer than 10 years from the time of HPV infection, but precancerous lesions may be found sooner, many of the endpoints for clinical trials have been rates of CIN or adenocarcinoma in situ (35).

Screening technique of cervical cancer

Papanicolaou (Pap) test is regarded as a simple technique used for screening cervical cancer since the 1940's and has been shown to reduce the incidence of mortality from cervical cancer in countries with an active screening program (36). The abnormal cell changes on the cervix which cause of HPV called cervical dysplasia. The abnormal cell changes can be mildly or seriously abnormal. The degree of abnormal cells of Pap smear will helps the healthcare worker to provide the recommendation for the follow-up. The most common categories of abnormal cell changes on the cervix has divided in 4 stages:

1. ASCUS (Atypical squamous cells of undetermined significance). This means your Pap smear results are borderline, between normal and abnormal.

2. ASC-H (Atypical squamous cells of undetermined significance-cannot exclude HSIL). This means your Pap smear results are borderline but may be more serious.
3. LSIL (Low grade squamous intraepithelial lesion). This means that there are mildly abnormal cell changes on your cervix.
4. HSIL (High grade squamous intraepithelial lesion). This means that the cell changes on your cervix are more serious.

Cervical cancer is such a slowly progressing cancer. Sometimes bacteria or other viruses such as Herpes are identified on Pap smear results (37).

Molecular diagnostic of HPV

HPV DNA testing is an effective screening method for precancerous changes in the cervix, especially for specimens with normal cytology or low-grade squamous intraepithelial lesions (LSIL) (38). Test results help physicians detect precancerous lesions and determine the course of treatment prior to the development of malignancy. HPV genotypes 16 and 18 correlate with an increased risk of developing precancerous lesions and are the most common causes of cervical cancer (39).

Polymerase chain reaction (PCR)

The most gold standard technique is the PCR using the consensus primers MY09 and MY11 (forward and reverse) followed by Sanger sequencing (40). This classical technique involved PCR, which amplifies a 450 bp fragments of L1 region of the HPV genome followed by Sanger sequencing. Recently, another pair of primers (GP5+ / GP6+) that amplifies a fragment of 140 to 150 bp in L1 region superimposed the MY primers (41). Studies comparing these techniques reported that the combination of them increases the accuracy of the test (42, 43).The nested-PCR has a higher sensitivity and consists of two consecutive PCR reactions (in two tubes). The MY primers are used in

the first round, and the GP primers are used in the second round. Then, analysis involved electrophoresis and Sanger sequencing. This technique is very sensitive and has been used in scientific research. However it may fail to detect cumulative infections by multiple HPV genotypes.

INNO-LiPA

INNO-LiPA HPV genotyping is a line probe assay using the SPF10 primer system. Short HPV sequences of about 50 to 65 bp are amplified from the L1 region by multiplex PCR. In addition, a set of primers for amplification of the human HLA-DPB1 gene was added to monitor sample quality and extraction. Probes for the following 28 HPV types are fixed on a membrane strip: 15 high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), 3 probable high-risk (26, 53, 66), 7 low-risk (6, 11, 40, 43, 44, 54, 70), and 3 unclassified (69, 71, and 74) genotypes. The PCR product containing the biotin-labeled primer is hybridized to the strip. Streptavidin-horseradish peroxidase conjugate is linked to biotin and the presence of HPV is detected visually by the addition of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium chloride (NBT) as substrate. Interpretation of the result can be done by direct visualization or using the software LIRAS for LiPA HPV (44).

Electrochemical DNA chip

Electrochemical DNA chip system can detect single or multiple infections of 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. In this system, all processes from reaction through measurement and analysis have been integrated into single compact equipment. It is based on Loop-mediated isothermal amplification (LAMP) and hybridization with a specific DNA probes on a gold electrode with high sensitivity. This method relies on a current-detection technique which measures the

charge released by an electrochemical agent. With this system, HPV can be detected in a short time (45).

Next generation sequencing (NGS)

Next-Generation Sequencing (NGS), or Massive parallel sequencing (MPS). These powerful genotyping techniques possess the advantage of analyzing many samples at the same time. Recently, they have been used for the genotyping of HPV (46-48). A single sequencer may generate NGS reading of millions of DNA sequences in 24 hours, more than hundreds of Sanger type sequencers in the same time. NGS is not yet widely available, and its accuracy for the diagnosis of HPV has yet to be confirmed (49).

The first objective of this thesis is to evaluate HPV detection rate in Thai women and men using urine and self-swab sample and comparative the HPV genotypes obtained from Pap smear, urine, and self-swab samples. The second aim for this study was to compare HPV test results obtained from PCR, INNO-LiPA, electrochemical DNA chip and NGS test on the same specimen and investigate the use of Illumina sequencing to detect and genotype the HPV types present in a complex multiple infections in a cervical specimen. The third purposive was to investigate the prevalence of human papillomavirus infections in anogenital wart among Thai population and determine the genotypes of anogenital wart in Thais are represented in the vaccine. This study helped to evaluate the HPV detection rate in Thai population and develop the technique and type of specimen to detection the HPV. Moreover, characterized the genotypes of HPV detected in Thai patients with anogenital warts can determine the genotypes that represented in the vaccine. This thesis divided into three parts as follows;

CHAPTER III

DETECTION OF HUMAN PAPILLOMAVIRUS IN MALE AND FEMALE URINE BY ELECTROCHEMICAL DNA CHIP AND PCR SEQUENCING

(Part 1.1)

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CHAPTER III

Part 1.1: Detection of human papillomavirus in male and female urine by electrochemical DNA chip and PCR sequencing

Summary

Cervical cancer is the second most common cancer in Thai women after breast cancer. Currently, the Papanicolaou (Pap) smear is the recommended procedure for cervical cancer screening in Thailand, but only a relatively small percentage of women follow this screening program. An alternative method to detect HPV genotypes associated with cervical cancer is self-sampling of urine, which is a more widely accepted method. Our study aimed to evaluate the prevalence of HPV in Thai women using urine and cervical swabs and prevalence of HPV in Thai men using urine samples. Tumorigenic HPV detection was accomplished by electrochemical DNA chip and PCR/direct sequencing. In addition to HPV prevalence, we report the concordance between different methods and sample types. One-hundred and sixteen women and 100 men were recruited. Histological examination revealed normal cytology in 52 women, atypical squamous cells of underdetermined significance (ASCUS) in 9, low-grade squamous intraepithelial lesion (LSIL) in 24, and high-grade squamous intraepithelial lesions (HSIL) in 31. One-hundred men were classified as heterosexuals (n=45) and homosexuals (n=55). The most prevalent HPV genotype in our study was HPV16. The HPV detection rate was generally lower in urine samples compared with cervical samples. Overall, there was good agreement for the detection of carcinogenic HPV from female cervical samples between the DNA chip and PCR/sequencing, with 88.8% total agreement and a kappa value of 0.76. In male urine samples, the level of agreement was higher in heterosexuals compared with homosexuals. Conclusions: Further improvement is required to increase an overall yield of HPV DNA detection in

urine samples before clinical application of a urine-based HPV screening program. The electrochemical DNA chip test is a promising technique for carcinogenic HPV detection.

Introduction

Human papillomavirus (HPV) is a non – enveloped, DNA virus that is transmitted through skin-to-skin contact with the penis, scrotum, vagina, vulva or anus of an infected person. More than 100 types of HPV have been identified, of which more than 40 can infect genital areas in human (50). HPV infection is quite common among sexually active people. It can cause both benign diseases and malignant neoplasms of the genital tract (51). An individual can be infected with multiple types of HPV, and HPV infection in males can be found in many parts of genital organs such as penis, scrotum and anus (52). HPV prevalence in Thai women is 7.8%, with HPV16 most frequently detected (17.9%) (53), whereas prevalence of anal HPV infection in homosexuals or men who have sex with men (MSM) was 85% in HIV-positive and 58.5% in HIV-negative individuals (54). Hadzisejdic et al. has suggested that HPV DNA can be detected in more than 90% of cervical lesions in women (55).

Cervical cancer is the second most common cancer in Thai women, and several studies have shown that certain types of HPV are the etiological cause of this disease (56). HPV16 and HPV18 are the two most prevalent carcinogenic HPVs found in normal female cytological samples, and they are the genotypes correlated with the highest increased risk of developing precancerous lesions (39). The high anal HPV prevalence in MSM is also associated with anal cancer incidence, which is estimated to be 44 times higher in MSM than among the general population (52). Similar to women, HPV16 was found to be the most common high-risk HPV type in MSM (54).

The American Cancer Society recommends that HPV testing should be used as an adjunct to cervical cytology for cancer screening in women more than 30 years of age

(57). Pap smear is a method for cancer screening based on detecting abnormal cellular changes or premalignant lesions of cervical tissues. However, HPV testing is a better method for screening high-risk HPV infection in samples with normal cytology or low-grade squamous intraepithelial lesions (LSIL) (38). While Pap smear cytology screening has proved to be effective in reducing the incidence and mortality from cervical cancer (58), this screening method requires a pelvic exam, which is invasive and uncomfortable for patients and time consuming for health-care workers (5). Cervical cancer screening in Thailand is still currently based on the Pap smear, but only a small percentage of women follow this screening program (59). HPV detection in urine sample is another alternative, which is more widely accepted because it is easily collected, non-invasive and readily available (6). However, urine samples have some limitations as it doesn't directly come from the original sites of the disease. In addition, some reports (60, 61) have demonstrated a lower sensitivity of HPV detection in urine compared with cervical swab samples, although other reports (62, 63) have shown comparable sensitivity.

The detection of HPV DNA usually focuses on the L1 region of the major capsid-forming gene (64), and various techniques can be used to detect it such as polymerase chain reaction (PCR) with specific primers, Hybrid capture2 test (HCII) and linear array (65). Recently, a new technique for HPV DNA detection using an electrochemical DNA chip system combined with loop-mediated isothermal amplification (LAMP) was introduced. This technique can detect 13 high-risk (HR) genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) by using a single compact piece of equipment. The electrochemical DNA chip can detect single and multiple infections of high risk genotypes of HPV with higher sensitivity, specificity, rapidity and simplicity when compared with other methods such as direct sequencing (8). Other studies have also demonstrated good agreement in electrochemical DNA chip testing when compared to direct sequencing (45, 66).

The aim of this study was to evaluate HPV detection rate in Thai women using urine and cervical samples. We also evaluate the prevalence of HPV in Thai men, both homosexuals and heterosexuals, using urine samples. In order to evaluate the performance of electrochemical DNA chip, the results of HPV genotypes detected were compared with those obtained from PCR/sequencing methods, which is the current gold standard.

Materials and methods

Ethical consideration

The research protocol was approved by the Institutional Review Board (IRB number 389/2555 in female and IRB number 411/2555 in male) of the Faculty of Medicine, Chulalongkorn University. The objective of the study was explained to the patients and written consent was obtained for all participants. All specimens were sent and kept at –20°C until tested. The coding of all samples was anonymous.

Population study

Female subjects were recruited from King Chulalongkorn Memorial Hospital and the National Cancer Institute between June 2012 and February 2013. The collected samples were divided into 2 groups. The first group was from healthy women who came for a routine Pap smear checkup with normal cervical cytology confirmed by a cytotechnologist and pathologist. The second group consisted of female patients older than 15 who came for a follow-up visit at the colposcopy clinic. All of the patients in the second group had abnormal cytology at various stages and were classified into 3 groups; atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL).

Male urine samples were also collected from 2 groups of people. The first group was recruited from the Thai Red Cross AIDS Research Centre in Bangkok, Thailand. These subjects consisted of 18 and older Thai MSM. The second group was recruited from healthy Thai subjects who attended a routine health checkup at the Bangpakok 9 International Hospital, Bangkok

Sample collection and preparation

Cervical swabs

Cervical swabs were collected from the Department of Obstetrics and Gynecology, Chulalongkorn hospital, Bangkok and Department of Gynecology, National Cancer Institute, Bangkok, Thailand. The cervical swab was collected before the gynecologist performed Pap smear, and all specimens were kept in phosphate buffered saline (PBS) and stored at -20 °C until tested. Cytological results were assessed by a specialized cytotechnologist and pathologist. The specimens were sent as anonymous with the coding number and patient's age to the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Urine

Female urine samples were collected before the Pap smear test was performed. Male urine was collected before seeing the doctor during clinical visits. All of the urine specimens were collected in sterile 50 ml tubes (Invitrogen co., Carlsbad, CA) and stored at 4 °C for not more than 1 day. The urine was processed by centrifugation at 13,000 rpm for 20 min, the supernatant was discarded, and then the pellet was re-suspended with 1 ml of PBS buffer and stored at -20 °C until tested.

Pathological classification of cytological findings

Cytological determinations were characterized according to the 3 types of the Bethesda System, which is the international standard for reporting Pap smear results. The 3 types are; ASCUS, LSIL and HSIL (67). Each type was diagnosed by a specialized cytotechnologist and confirmed by a pathologist.

Laboratory method

DNA extraction

DNA extraction and purification was performed using the Qiamp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. After extraction, all of specimens were tested for the β-globin gene to serve as an internal control. The DNA samples were stored at -20 °C until tested.

HPV detection and genotyping

Polymerase Chain Reaction (PCR)/Sequencing

The DNA was amplified by polymerase chain reaction (PCR) using a nested MY/GP primer set to amplify the L1 gene. The MY/GP primer set can detect 39 different HPV genotypes which overlap the 13 carcinogenic HPV genotypes detected by the electrochemical DNA chip (68). The PCR reaction mixture contained 2 μL DNA template, 0.5 μL of 10 mmol of each primer, 15 μL of 2.5× PerfectTaq Plus MasterMix (5 PRIME Inc., Hamburg, Germany) and sterile distilled water to a final volume of 25 μL. The nested PCR reactions were performed by using Eppendorf Thermocycler (Eppendorf, Hamburg, Germany). The PCR conditions were previously reported [26] and are the followings: the first PCR amplification was initially denatured at 94 °C for

5 min, followed by 40 cycles at 94 °C for 30 s (denaturation), at 55 °C for 45 s (annealing), at 72 °C for 1.30 s (extension) and a final extension step at 72 °C for 7 min. The second PCR amplification used 50 °C for 45 s (annealing). All amplified products were separated by electrophoresis in a 2% agarose gel (FMC Bioproducts Rockland, ME) stained with ethidium bromide and visualized with UV transillumination (Gel Doc1000, BIO-RAD, Hercules, CA). The PCR products were purified from agarose gel with the PCRExtract&GelExtract Mini Kit (5 PRIME, Hamburg, Germany). The product of GP primer was subjected to direct sequencing by First BASE Laboratories Sdn Bhd (Selangor Darul Ehsan, Malaysia). Nucleotide sequences were edited and assembled using SEQMAN (LASERGENE program package, DNASTAR) and consequently subjected to BLAST program on the GenBank Database (www.ncbi.nlm.gov/BLAST) for genotype diagnosis.

Electrochemical DNA chip

The electrochemical DNA chip package consists of six Loop mediated isothermal amplification (LAMP) reagents, an intercalation reagent and an electrochemical DNA chip, which has L1 specific DNA probes for 13 carcinogenic high risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The conditions of the LAMP procedure are the following; denaturation at 95 °C for 5 min, followed by 65°C for 90 min and 80 °C for 5 min. The automated hybridization of probe and primer and the subsequent quantification of the resulting electrochemical signals was done on the GLH-2C601 GenelyzerTM (Toshiba, Tokyo, Japan). A previous study done in our lab using the same electrochemical DNA chip reported no cross hybridization (45).

Statistical analysis

The PCR/sequencing technique was compared with the electrochemical DNA chip by using analyses of agreement (Kappa value and percent total agreement). The Kappa values ranging from 0.0 to 0.20 were considered as poor agreement, from 0.21 to 0.40 as fair agreement, from 0.41 to 0.60 as moderate agreement, from 0.61 to 0.80 as good agreement and from 0.81 to 1.00 as excellence agreement. We also compared HPV detection rate in urine and cervical samples. Sensitivity, which is the probability of an HPV-positive urine sample given an HPV-positive cervical sample, and specificity, which is the probability of an HPV-negative urine sample given an HPV-negative cervical sample were reported. Concordance was measured as the percentage of paired urine and cervical samples that yielded the same results. Type-specific concordance was calculated as the percentage of paired urine and cervical samples that were positive for the same HPV genotypes. Two-sided P-value was calculated with values less than 0.05 being considered statistically significant. All of these analyses were carried out using SPSS Software version 17.0 (IBM Corporation, Somers, NY).

Results

Patient's characteristics

There were a total of 116 women recruited for this study. Histological examination revealed normal cytology in 52, ASCUS in 9, LSIL in 24, and HSIL in 31 women. The average age of women with normal cytology was 43.1 years (range, 27-61 years), while in women with abnormal cytology the average age was 40.0 years (range, 15-63 years). In male patients, there were a total of 100 men recruited, of which 45 were heterosexual males who visited hospitals for health check-up and 55 were documented

as homosexuals or MSM. Among 55 MSM, 21 (38.1%) were HIV-positive. The average age of MSM was 28.1 years (range, 18-46 years).

HPV concordance in paired urine and cervical sample by PCR/sequencing technique

Table 2 showed that the highest prevalence of any-type HPV detected by PCR was found in women with HSIL; 61.3% in urine samples and 83.9% in cervical samples. The most prevalent HR-HPV types detected by PCR in cervical samples with precancerous lesions were HPV16 (26.56%), HPV31 (6.25 %) and HPV58 (6.25%). Among 116 women, there were 14 samples (12.07%) that were high-risk HPV (HR-HPV) positive in the cervical samples but negative in urine samples. In contrast, there were 7 samples (6.0%) that were HPV positive in urine but negative in cervical samples. The sensitivity of HPV detection in urine samples by PCR ranged between 44.4-66.7%, while specificity ranged between 60-100% and concordance ranged between 64.5-82.7%.

Table 2. *HPV concordance in paired urine and cervical samples by PCR/sequencing and the electrochemical DNA chip.*

Population	Technique	HPV type	HPV positivity		Sensitivity	Specificity	Concordance	Type-specific Concordance (%)
			Urine sample n (%)	Cervical sample n (%)				
Female with normal cytology (n=52)								
	PCR	Any type HPV	8 (15.4)	9 (17.3)	44.4	90.7	82.7	23.1
	DNA chip	HR-HPV	4 (7.7)	7 (13.4)	57.1	100	94.2	42.8
Female with abnormal cytology								
Total (n=64)	PCR	Any type HPV	33 (51.6)	46 (71.9)	65.2	83.3	70.3	57.1
	DNA chip	HR-HPV	27 (42.2)	42 (65.6)	64.3	100	75	62.8
HSIL (n=31)	PCR	Any type HPV	19 (61.3)	26 (83.9)	65.4	60	64.5	57.1
	DNA chip	HR-HPV	16 (51.6)	27 (87.1)	59.3	100	61.3	57.1
LSIL (n=24)	PCR	Any type HPV	9 (37.5)	14 (58.3)	64.3	100	79.2	50
	DNA chip	HR-HPV	9 (37.5)	11 (45.8)	81.8	100	91.7	72.7
ASCUS (n=9)	PCR	Any type HPV	5 (55.5)	6 (66.6)	66.7	66.7	66.7	42.8
	DNA chip	HR-HPV	2 (22.2)	4 (44.4)	50	100	77.8	50

HPV concordance in paired urine and cervical samples by the electrochemical DNA chip

Prevalence of HR-HPV infection detected by the electrochemical DNA chip was also reported highest in HSIL group; 51.6% in urine samples and 87.1% in cervical samples. These are comparable numbers to the PCR technique. The most prevalent HR-HPV types in women with precancerous lesions detected in cervical samples by the electrochemical DNA chip were HPV16 (31.25%), followed by HPV58 (10.94 %) and HPV52 (9.38%). Among 116 women, dual or multiple HPV infections were detected in 5 of 116 (4.3 %) samples extracted from urine and in 9 of 116 (7.8 %) cervical samples. There were 18 samples (15.5%) that were HR-HPV positive in the cervix but negative in urine, however, none of the samples were positive in urine but negative in cervical samples. Sensitivity of HPV detection in urine samples by the electrochemical DNA chip ranged between 50-81.8%, while specificity was 100% and concordance ranged between 61.3-94.2%.

Agreement in HR-HPV detection by the electrochemical DNA chip in cervical and urine samples

Among 116 women, the overall agreement for the detection of HR-HPV genotypes by electrochemical DNA chip in urine and cervical samples was 84.48% with a kappa value of 0.67. There was good agreement in women with normal cytology (k=0.70) and moderate agreement in women with abnormal cytology (k=0.55). The highest level of agreement was found in women with LSIL, which reported 91.67% total agreement with a kappa value of 0.83 as shown in Table 3.

Table 3. *Comparison of HPV detection in cervical and urine samples detected by the electrochemical DNA chip.*

	Cervical swab –		Cervical swab +		%agreement	Kappa	95%CI	p value
	Urine-	Urine+	Urine-	Urine+				
All(n=116)	67	0	18	31	84.48	0.67	0.52-0.81	<0.001
Normal cytology group (n=52)	45	0	3	4	94.23	0.7	0.37-1	<0.001
Abnormal cytology group (n=64)	22	0	15	27	76.56	0.55	0.36-0.75	<0.001
HSIL(n=31)	4	0	11	16	64.52	0.27	0.00-0.62	0.01
LSIL(n=24)	13	0	2	9	91.67	0.83	0.60-1	<0.001
ASCUS(n=9)	5	0	2	2	77.78	0.53	0.00-1	0.04

HPV positivity in male urine sample

Among heterosexual males (n=45), HPV DNA was detected in 3 urine samples (6.7%) by PCR and 2 urine samples (4.4%) by electrochemical DNA chip. Among HIV-positive MSM (n=21), HPV DNA was detected in 2 urine samples (9.52 %) by PCR and 4 urine samples (19.05 %) by electrochemical DNA chip. When considering only HIV negative MSM (n=34), HPV DNA was detected in 5 urine samples (14.7 %) by PCR and 2 (5.88 %) by electrochemical DNA chip. The genotypic distribution of HPV is shown in Table 4.

Table 4. *HPV prevalence in male urine samples by PCR/sequencing and the electrochemical DNA chip.*

Population	**Technique**	**HPV type**	**HPV positivity in urine sample n (%) and HPV genotypes**
Heterosexuals(n=45)	PCR	LR-HPV	2 (4.4); HPV6, 70
	PCR	HR-HPV	1 (2.2); HPV18
	DNA chip	HR-HPV	2 (4.4); HPV35+52, 18
Non-HIV MSM (n=34)	PCR	LR-HPV	4 (11.8);HPV6, 11, 84, 87
	PCR	HR-HPV	1 (2.9); HPV82
	DNA chip	HR-HPV	2 (5.9); HPV31+68, 45
HIV-positive MSM (n=21)	PCR	LR-HPV	1 (4.8); HPV81
	PCR	HR-HPV	1 (4.8); HPV45
	DNA chip	HR-HPV	4 (19); HPV35, 35+68, 35+52+68, 45

Agreement in HR-HPV detection in PCR/sequencing and DNA chip in female cervical and urine samples

In the PCR/sequencing technique, HPV positive results were excluded if the specimens showed negative results from the internal control (β-globin gene). All positive PCR products were sequenced in order to identify the HPV genotypes. The DNA chip used primer sets for each HPV and amplified only one or multiple matching types among 13 carcinogenic types. When we compared HPV detection between the electrochemical DNA chip and PCR/sequencing technique in female urine and cervical samples, the PCR/sequencing technique was considered positive only when they were positive in one of the 13 carcinogenic HPV types detectable by the DNA chip. Table 5 shows that among 116 women, overall agreement for the detection of HR-HPV genotypes by electrochemical DNA chip and PCR/sequencing was 88.79% and 86.21% in cervical samples and urine samples, respectively. In women with normal cytology, carcinogenic HPV detection rate showed fair agreement with a kappa value (k-value) of 0.41 in cervical samples and 0.73 in urine samples. In women with HSIL, there was good agreement in HPV detection rate from cervical samples; 90.32% agreement and a k-value of 0.67. However, in urine samples the agreement dropped down to 83.87% with a k-value of 0.67. In women with LSIL and ASCUS, the level of agreement was also higher in cervical samples than in urine samples as shown in table 4. In summary, the overall percentage of agreement in HR-HPV detection by the two methods in female samples ranged from 70.83 – 96.15% with the kappa value ranging from 0.22-0.77.

Table 5. *Comparison between PCR/sequencing and the electrochemical DNA chip in female and male samples.**

			DNA chip- PCR/ Sequencing -	DNA chip- PCR/ Sequencing +	DNA chip+ PCR/ Sequencing -	DNA chip+ PCR/ Sequencing +	%agreement	Kappa	95%CI	p value
Female all (n=116)		Cervical swab	66	1	12	37	88.79	0.76	0.64-0.88	<0.001
		Urine	80	6	10	20	86.21	0.62	0.45-0.80	<0.001
Normal cytology (n=52)		Cervical swab	45	0	5	2	90.39	0.41	0.00-0.90	<0.001
		Urine	47	1	1	3	96.15	0.73	0.36-1.00	<0.001
Patients (n=64)	HSIL (n=31)	Cervical swab	4	0	3	24	90.32	0.67	0.34-1.00	<0.001
		Urine	12	3	2	14	83.87	0.67	0.42-0.93	<0.001
	LSIL (n=24)	Cervical swab	12	1	3	8	83.33	0.66	0.36-0.96	<0.001
		Urine	15	1	6	2	70.83	0.22	0.00-0.59	0.09
	ACUS (n=9)	Cervical swab	5	0	1	3	88.89	0.77	0.34-1.00	0.009
		Urine	6	1	1	1	77.78	0.36	0.00-1.00	0.14
Male	All (n=100)	Urine	92	0	6	2	94	0.38	0.00-0.76	<0.001
	Heterosexuals (n=45)	Urine	43	0	1	1	97.78	0.66	0.03-1.00	<0.001
	MSM (n=55)	Urine	49	0	5	1	90.91	0.26	0.00-0.68	0.002

* PCR/sequencing technique was considered positive only when they were positive in one of the 13 carcinogenic HPV types detectable by the DNA chip.

Agreement in HR-HPV detection in PCR/sequencing and DNA chip in male urine samples

Among healthy males (n=100), there was moderate agreement of HR-HPV detection in urine samples by the 2 methods, with 94.00% agreement, and a k-value of 0.38. As shown in Table 5, the level of agreement and k-value was higher in heterosexual male urine samples than in MSM urine samples.

Discussion

The present study evaluated the detection rate of HPV genotypes by comparing PCR/sequencing with an electrochemical DNA chip, which is a new automated DNA test that detects 13 carcinogenic HPV genotypes (66). Both assays used the same DNA sample obtained from the same patient specimen. Moreover, we also compared HPV detection in cervical and urine samples in women, as urine is considered to be a preferable biological specimen for HPV DNA detection (6).

In this study, HPV prevalence was determined to be 17.3 % in cervical samples with normal cytology by PCR, which is higher than what a previous survey found in Thai women (7.6%) (53), but slightly lower than a study of Japanese women (22.5%) (69). In women with abnormal cytology, we found that HPV prevalence detected by PCR was higher than in the normal group with a prevalence of 83.9%, 58.3% and 66.6 % for HSIL, LSIL and ASCUS, respectively. These results are consistent with another report (69), which demonstrated a higher prevalence of HPV in precancerous lesions. The 3 most common types of HPV detected by PCR in our study were HPV 16 (26.6%), HPV 31 (6.3%) and HPV 58 (6.3%). This was different from a previous survey in Thai women which reported HPV16 as the most common genotype identified (17.9%), followed by HPV 90 (16.6%) and HPV 71 (10.3%) (53). Regardless of differences in collection methods and PCR techniques, HPV 16 is still the most prevalent HPV genotype in Thailand as well as in many other countries (60, 69, 70).

Several other studies have compared HPV sampling rates between urine and cervical samples in order to gauge the ability to detect HPV prevalence independently of cervical cytology (60, 63, 70-72). Daponte et al. suggested that sensitivity of urine testing for HPV 16 and 18 was higher in cervical cancer (88.8%) than in high grade and low grade lesions (76.5 and 45.5%, respectively) (63). A US study reported that the prevalence of any HPV genotype in HIV-infected women was lower in urine samples (58% for the cervical swab specimens and 48% for the urine specimens) but not significantly different (72). Our study demonstrated that the HPV detection rate was generally lower in urine samples compared with cervical samples, with sensitivity ranging between 44.4-81.8% and specificity 60-100% by PCR and electrochemical DNA chip. The sensitivity was also higher in the abnormal cytology group compared to the normal cytology group. Variation of sensitivity in urine samples is likely due to the relatively small amount of HPV DNA in urine or due to some PCR inhibitors in urine such as nitrites, which can inhibit amplification (73). The urine HPV DNA yield could

be improved by using first-voided, epithelial cell-rich morning urine or collecting larger urine volumes. There were 7 samples (6.0%) that were HPV positive in urine but negative in cervical samples. This was probably due to HPV contamination in urine or improper cervical swab technique, which may have resulted in a low yield of HPV DNA. Concordance of HPV detection in urine and cervical samples in our study ranged from 64.5-94.2%. The use of urine samples for HPV testing has some advantages as it is easy to collect and considerably less invasive, however, testing urine samples yields lower sensitivities than self-collected vaginal or vulva samples (71). Previous studies concluded that further improvement in the PCR process was required in order to increase an overall yield before clinical application of a urine-based HPV screening program for precancerous lesions and cervical cancer (63, 73). Our study showed that urine HPV testing was helpful for the detection of HPV DNA in high grade cervical lesions, which need immediate treatment. HR-HPV detection in urine samples of women with normal cytology could help identify those who are at risk of developing precancerous lesions and may need frequent follow up. However, urine HPV testing cannot currently substitute for cytologic evaluation for cervical cancer screening.

The sensitivity of the electrochemical DNA chip for HPV detection has been found to be 102 copies/µl (66), while it has been shown that PCR L1 gene detection requires at least 103 copies/µl (74). When comparing the two techniques for HPV detection, our results showed good agreement for the detection of HR-HPV genotypes in cervical samples from 116 women (k = 0.76). In women with normal cytology, carcinogenic HPV detection rate showed moderate agreement (k-value = 0.41) compared to women with HSIL (k value = 0.67), LSIL (0.66) and ASCUS (0.77). A previous study (66) showed better agreement between these two techniques with kappa values of 0.77, 0.94, 1.00 and 1.00 in women with normal cytology, cervical intraepithelial neoplasia grade I (CIN I), CIN II and CIN III, respectively. The difference in kappa values between our study and the previous study (66) were probably due to a

small sample size and an unequal distribution of samples between groups. Moreover, the electrochemical DNA chip can detect multiple HR-HPV genotypes while PCR with a consensus primer can detect only one predominant type due to possible amplification bias (75). Thus, in women infected with multiple types of HPV, the electrochemical DNA chip seems to be a better choice for rapid diagnosis and identification. However, the electrochemical DNA chip also has some limitations as it can currently only detect only 13 of the 15 HR-HPV genotypes so far identified (76). This was apparent in our study as we found HPV82 in 2 of our samples by PCR/sequencing technique, which the electrochemical DNA chip is not designed to find.

We also demonstrated that prevalence of anytype of HPV detected in MSM urine samples by the PCR technique was higher than in heterosexual males; 12.7% and 6.7%, respectively. The overall prevalence of HR-HPV detected by electrochemical DNA chip in HIV MSM (19%) was higher than in non-HIV MSM (5.9%). A previous report showed higher prevalence of HPV infection from anal samples in HIV-positive MSM (85%) than in HIV-negative MSM (58.5%) (54). These results indicate that anal samples are superior to urine samples for the purpose of HPV detection. The low prevalence of HPV DNA in male urine samples can be explained by failure to extract small amounts of DNA from urine or the presence of PCR inhibitors (77). The 2 most prevalent types of HPV detected in male in our study were HPV 35 (4%) and HPV 45 (3%). These results were different from a previous survey in Thailand which reported HPV16 as the most prevalent (54). A survey in Italy demonstrated that the two most prevalent HPV genotypes from male anogenital sites samples were HPV 6 (13%) and 16 (7%) (78). Our study showed that the electrochemical DNA chip has a higher detection rate of HR-HPV in male urine samples compared to PCR techniques. However, the differences in genotypes detected by these two techniques were most likely due to different reagents for amplification or PCR conditions. Overall, there was fair agreement in HR-HPV detection by the 2 methods (kappa value of 0.38). The level of agreement and the kappa

value were higher in heterosexual male urine samples compared with MSM; 97.78% overall agreement with k-value of 0.66 in heterosexual group and 90.91% overall agreement and a k-value of 0.26 in MSM.

A limitation of our study was a small study population. Moreover, urine samples have limitations such as low cellular load and they were not taken directly from the HPV infection site. PCR inhibitors can affect these results as well. Thus, the results obtained from urine samples might not reflect the real prevalence of HPV infection.

In conclusion, there was good overall agreement between the electrochemical DNA chip and PCR/sequencing methods for HR-HPV detection in female cervical samples, while there was fair agreement in male urine samples. The detection rates of HR-HPV were generally higher in samples from cervical lesions than in samples extracted from urine. Further improvement of PCR process and specimen collection is required to increase an overall yield of HPV detection in urine samples before the technique can be used in a clinical setting. The electrochemical DNA chip test is a promising technique for carcinogenic HPV detection.

CHAPTER III

COMPARISON OF HUMAN PAPILLOMAVIRUS DETECTION IN URINE AND CERVICAL SAMPLES USING THE HPV GENOARRAY DIAGNOSTIC ASSAY

(Part 1.2)

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CHAPTER III

Part 1.2: Comparison of human papillomavirus detection in urine and cervical swab samples using the HPV GenoArray Diagnostic assay

Summary

Human papillomavirus (HPV) is the leading cause of cervical cancer. Urine-based HPV testing would be a simple and non-invasive method. It may have the advantage of increasing acceptance of screening. A total of 164 pairs of cervical swab and urine samples from Thai women who underwent cancer screening were used for HPV testing with HPV GenoArray Diagnostic Kits. The overall concordance percentage for HPV detection in the cervical swab and urine samples was 65.2%. The HPV genotypes most commonly detected were HPV16 and HPV18. An analysis of the urine samples and a second analysis of the cervical swab samples showed that the differences in the overall HPV detection rate between women with normal and abnormal cytology were not significant ($p > 0.05$). Urine samples processed with the HPV GenoArray assay may be useful for the clinical management of HPV infection and the technique could be an accurate, noninvasive method for monitoring HPV infections in women.

Introduction

Human papillomavirus (HPV) is the main etiological agent for the development of cervical cancer (51). Nowadays, 170 genotypes have been identified (15), and about 40 genotypes infect the human anogenital tract (79). The genital HPVs are classified into high-risk and low-risk genotypes depending on their association with uterine cervical cancer (76). The high-risk genotypes most commonly detected in uterine cervical cancer are HPV16, 18, 31, 33, 35, 45, 52, 58, 39, 51, 56, and 59 (80).

The Papanicolaou (Pap) test is a general technique used for screening for cervical cancer. The test results help physicians to detect precancerous lesions and determine the course of treatment. The use of this technique has been shown to reduce the incidence of mortality from cervical cancer (81). However, this test is primary for detecting invasive cervical cancer and it is insufficient for screening (36, 82). Nevertheless, encouraging results have been reported for HPV DNA assays used in combination with the traditional Pap smear test (83).

HPV DNA detection in urine samples could be a feasible alternative to HPV DNA detection in cervical specimens. Urine collection could provide an especially simple, non-invasive method for screening (84). The benefits of using urine for HPV DNA detection have been evaluated in disease surveillance surveys and screening for cervical cancers involving specific genotypes. HPV DNA urine testing is an alternative way to monitor HPV vaccination in adolescent girls and young women who do not wish to have a vaginal examination (85, 86). Some studies have reported a high HPV detection sensitivity for urine-based assays (87-89), while the others have reported a low HPV detection sensitivity for urine-based assays (90, 91).

There are many available molecular methods for HPV testing, such as PCR/sequencing (41), the INNO-LiPA HPV Assay (92), and the Hybrid Capture 2 test (HCII) assay (93). In addition, the HPV GenoArray Diagnostic Kit (Hybribio Ltd.,

Sheung Wan, Hong Kong) is a recently developed PCR-based HPV genotyping assay. This test uses L1 consensus primers to amplify 21 HPV genotypes, which is followed by flow-through hybridization with immobilized genotype-specific probes. The test is currently used in several hospitals in China (94). A previous study showed that the sensitivity of the HPV GenoArray assay was 97.8% and the specificity was 100% (95).

The aim of this study was to evaluate the use of a urine-based assay as a non-invasive method for HPV detection and to genotype the samples using the HPV GenoArray assay.

Material and methods

Ethical consideration

The research protocol was approved by the Ethics Committee of the National Cancer Institute, Bangkok, Thailand (number EC COA 037/2012), and the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (number 389/2555). The objective of the study was explained to the patients, and written consent was obtained from all participants. Each specimen was sent to be tested in an anonymized way, with a participant-specific numerical code and the participant's age.

Clinical specimens

The 164 pairs of biospecimens (a Pap smear sample from the cervix and a first-void urine sample) were divided into three groups: 95 samples indicating normal cytology, 50 samples indicating low-grade squamous intraepithelial lesions (LSIL), and 19 samples indicating high-grade squamous intraepithelial lesions (HSIL). The ages of the patients enrolled in this study were between 19 and 69 years.

Sample preparation

Each Pap smear sample (which are the standard samples for HPV genotyping) was evaluated by a specialized cytotechnologist and the results were confirmed by a pathologist. All the Pap smear samples were kept in liquid-based cytology (LBC) buffer (ThinPrep®, Hologic, Marlborough, MA, USA).

The participants were asked to collect first-void urine (FVU) samples of 30–50 mL in a sterile Cell PrepPlus (Biodyne, Gyeonggi-do, Korea) urine bottle. The urine samples were stored at 4 °C and processed within 3 days. Approximately 15 mL of each FVU sample was centrifuged at 3000 rpm for 5 min, and then the supernatant was removed. Subsequently, 800 μL of the sample was added to a 1.5-mL tube to wash the pellet, the sample was centrifuged at 8000 rpm for 5 min, and the supernatant was removed. The DNA was extracted from the cervical swabs and urine pellets using a DNA Prep Kit (Chaozhou Hybribio Biochemistry Ltd., Guangdong, China) and stored at -20°C until testing.

HPV GenoArray Diagnostic Assay

The extracted DNA from the cervical swab and urine samples was subjected to an HPV genotyping assay using HPV GenoArray Diagnostic Kits (Hybribio Ltd., Sheung Wan, Hong Kong). These kits make use of both DNA amplification and a proprietary flow-through hybridization technique to identify 21 HPV genotypes. The kits can detect 13 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), two probable high-risk types (HPV 53 and 66), and six low-risk or unknown risk types (HPV 6, 11, 42, 43, 44, and CP8304 [HPV-81]). The HPV GenoArray assay is based on an L1 consensus primer-based PCR assay (this is not the same as that used in the Linear Array HPV Genotyping Test, which uses MY09 and MY11 primers) (94).

Subsequently, the assay procedure involves using a flow-through hybridization technique on a nylon membrane covered in immobilized HPV genotype-specific oligonucleotides probes. After hybridization, the presence of a positive result for the internal control and the biotin dots within the membrane indicated that the isolated DNA was of good quality, the enzyme conjugate was valid, and the hybridization process was proper. The results were manually interpreted using the manufacturer's guidelines. The normal detection limit of the kit (as reported by the manufacturer) is around 500 copies per μL of target HPV DNA. There is no reported cross-reactivity regarding the amplification/detection of the 21 HPV genotypes.

Statistical methods

A statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Pearson's chi-square test for matched pairs was used to compare the performance of the two types of samples regarding the detection of HPV genotypes. Statistical significance was defined as $p<0.05$.

RESULTS

The mean age of the 164 participants was 45.8 years. Among the women with normal cytology, the mean age was 50.5 years, while among those with abnormal cytology (LSIL or HSIL), the mean age was 41.1 years. The pairs of cervical swab and urine samples were suitable for analysis for this study because the biotin and internal controls were positive, which means that PCR amplification would be effective.

According to the cervical swab samples, 65 of the 164 samples (39.6%) were HPV DNA positive (Table 6). In total, 18 (11.0%) contained multiple HPV genotypes. The most common HPV genotypes detected were HPV16 (12 samples) and HPV18 (8 samples). Thus, 20 of the 164 samples (12.2%) contained either HPV16 or HPV18. In

the normal cytology group, 11 of the 95 samples (11.6%) were HPV DNA positive. In contrast, the LSIL and HSIL groups had 35 (10.0%) and 19 (100.0%) HPV DNA-positive samples, respectively.

Table 6. *Detection of HPV genotypes and concordance between cervical swab and urine samples.*

Cytology	**Specimen (% positive)**				**Concordance (percentage)**
	Any HPV positive		**HPV16&18**		
	Cervical swab	**Urine**	**Cervical swab**	**Urine**	
Normal (N=95)	11 (11.6)	10 (10.5)	1 (1.1)	4 (4.2)	68 (71.6)
LSIL (N=50)	35 (10.0)	35 (10.0)	11 (22.0)	13 (26.0)	31 (62.0)
HSIL (N=19)	19 (100.0)	8 (42.1)	8 (42.1)	3 (15.8)	8 (70.5)
Total (N=164)	65 (39.6)	53 (32.3)	20 (12.2)	20 (12.2)	107 (65.2)

Regarding the urine samples, 53 of the 164 samples (32.3%) were HPV DNA positive (Table 6). Of all the urine samples, 13 (7.9%) had multiple HPV genotypes. The most commonly detected HPV genotypes were HPV18 (17 samples) and HPV16 (four samples). In total, 20 of the 164 samples contained HPV16 or HPV18 (12.2%). In the normal cytology group, 10 of the 95 samples (10.5%) were HPV DNA positive. In contrast, the LSIL and HSIL groups had 35 (10.0%) and 8 (42.1%) HPV DNA-positive samples, respectively.

Pap smear samples have been used as standard samples for detecting HPV. The aim of this study was to evaluate the efficacy of detection using urine compared with detection using the standard sample. Comparing the cervical swab and urine specimen results, the overall concordance was 65.2% (107/164). There was a concordance of

71.6% (68/95) in the normal cytology group and a concordance of 56.5% (39/69) in the abnormal cytology group (Table 6). The sensitivity and specificity of the urine-based assay (using HPV GenoArray Detection Kits), using the Pap smear results as the reference, were 56.5% and 70.6%, respectively. The sensitivity and specificity from this study was lower than other studies (Table 7). The positive and negative predictive values were 53.8% (95% CI = 41.9 to 65.4) and 72.7% (95% CI = 63.2 to 80.5), respectively.

Table 7. *Studies of human papillomavirus DNA detected in paired urine and cervical samples from females of all ages.*

Author	Country	HPV detection assay	Age, years range	Total sample size	Lesion/HPV types	Sensitivity (%)	Specificity (%)	Concordance (%)
Strsuss et al. (1999)	UK	PCR with MY and GP primers	16–57	144	All/any type	76.4	73.3	75.7
Daponte et al. (2006)	Greece	In house type-specific primers and commercial	N/A	77	All/HPV16/18	70.3	100.0	85.7
Gupta et al. (2006)	India	In house L1 consensus primers	N/A	30	All/any type	100.0	100.0	100.0
Cuschieri et al. (2011)	UK	HPV INNO–LiPA	16–25	90	All/any type	90.5	67.6	59.8
Nilyanimit et al. (2013)	Thailand	Electrochemical DNA chip	27–61	116	All/HR-HPV	64.3	100.0	75
Bernal et al. (2014)	Spain	Cobas 4800HPV test	21–65	125	All/any type	90.5	85	88
Hagihara et al. (2016)	Japan	Anyplex II HPV28	19–58	240	All/any type	68.4	99.9	98.4
This study	Thailand	Hybribio GenoArray	19–69	164	All/any type	56.5	70.6	65.2

N/A, not applicable.

Regarding multiple HPV infections, the cervical swab-based assays were able to detect more HPV genotypes in each sample. However, in the normal cytology group, for each pair of biospecimens, the most common number of genotypes per sample was one. Similarly, in the abnormal cytology group, 36 of the 69 cervical swab samples (52.2%) and 31 of the 69 urine samples (44.9%) had a single genotype (Table 8).

Table 8. *Number of HPV genotypes detected using the HPV GenoArray assay.*

No. of HPV genotypes detected	Normal (N=95)		Abnormal (N=69)	
	Cervical swab	Urine	Cervical swab	Urine
0[a]	84 (88.4)	85 (89.5)	15 (21.7)	26 (37.7)
1	11 (11.5)	9 (9.4)	36 (52.2)	31 (44.9)
2	-	1 (1.1)	14 (20.3)	7 (10.1)
≥3	-	-	4 (5.8)	5 (7.3)

An analysis of the urine samples and a second analysis of the cervical swab samples showed that the differences in the overall HPV detection rate between the normal and abnormal cytology groups were not significant ($p > 0.05$).

DISCUSSION

As a result of the use of the Pap test, screening attendance remains low (96), while the estimated incidence of invasive cervical cancer remains high (82). In Thailand, 25–38% of women aged 30–65 years have had only one Pap test (97). When cervical testing for HPV is required, these results suggest that urine sample collection could be an alternative non-invasive sampling method for monitoring HPV infection in women. In a previous study, the overall percentage agreement between HPV detection in urine and cervical samples was 88% (89) and, in this study, the percentage was 65.2%. However, the results must be interpreted with caution owing to variation between the studies in terms of the participant characteristics, surrogate nature of using cervical HPV detection to screen for cervical disease, and lack of standardized urine testing methods.

Urine sample assays cannot be used to detect all of the genital HPV infections, but these assays provide an alternative for use in epidemiological surveys in which

invasive sampling is difficult to perform; in these cases, testing urine for HPV DNA could be considered (84). Previous studies have compared HPV detection rates between cervical and urine samples in order to evaluate the ability of urine-based assays to detect the prevalence of HPV independently of cervical cytology assays (63, 70). Research indicates that the sensitivity of urine testing for HPV 16 and 18 was higher for participants with cervical cancer (88.8%) than for those with high- and low-grade lesions (63). These data showed that HPV DNA detection in urine samples from most groups of patients (HSIL, Normal) was lower than detection in cervical swabs. The reasons for this could be low-efficiency amplification (due to the presence of inhibitory substances in the urine), HPV DNA loss during urine processing, or the urine samples being truly HPV DNA negative (73).

The HPV DNA analysis of urine samples needs to be developed further before a urine-based assay can replace the Pap smear test. It is possible that a greater amount of urethral cells in the urine samples could increase the sensitivity of the test. An analysis of the urine samples and a second analysis of the cervical swab samples showed that the differences in the overall HPV detection rate between women with normal and abnormal cytology were not significant ($p > 0.05$). This result suggests that urine could be used as a substitute for cervical swabs. However, the urine samples should be optimized by preventing DNA degradation during extraction and storage, recovering cell-free HPV DNA in addition to cell-associated DNA, processing a sufficient volume of urine, and collecting the first portion of the urine stream in the morning (85).

Using traditional cytological analysis, it is difficult to determine accurate screening results for HPV-associated anogenital tumors. Therefore, HPV genotyping is an alternative screening method to be used in combination with traditional cytology for identifying patients at high risk of developing squamous cell carcinoma (98, 99). Nowadays, there are many HPV genotyping techniques for detecting HPV DNA, such as PCR, real-time PCR, restriction fragment length polymorphism (RFLP), Hybrid

Capture, and Linear Array (83, 100, 101). However, PCR and real-time PCR need specific expensive equipment (such as a thermal cycler), and these methods have not yet become common procedures in hospital laboratories (8). This study used the HPV GenoArray Diagnostic Kit for HPV genotyping, which is a commercial kit that has recently been started to be used, especially in China (94). The results from the HPV GenoArray assay used in this study were a percentage-point (39.6%) higher compared to the results from a previous survey of Thai women (7.6%) (53) and one of Japanese women (22.5%) (69). The higher percentage may be due to the small number of participants in our study sample.

The Linear Array HPV Genotyping Test has been widely used as a standard reference method for evaluating new methods. However, the HPV GenoArray Diagnostic Kit is an alternative technique for studies conducted in resource-limited laboratories because the cost of the HPV GenoArray Diagnostic Kit is lower than that of the Linear Array HPV Genotyping Test and the hybridization time is also lower (94, 102). Moreover, the HPV GenoArray assay can distinguish and identify HPV 52, which is one of the most common high-risk HPV genotypes in women in eastern and southeastern Asia (103, 104).

In conclusion, the HPV GenoArray assay was shown to possess reliable clinical performance for HPV genotyping both cervical swab and urine samples. Urine assays could be a useful noninvasive method for monitoring HPV infection in women and the use of this method may increase the number of women who undergo cervical cancer screening, as some women avoid this critical preventive screening because of the embarrassment and discomfort associated with the traditional Pap smear method. Additional research based on a larger sample from a general screening population is required.

CHAPTER III

COMPARISON OF DETECTION SENSITIVITY FOR HUMAN PAPILLOMAVIRUS BETWEEN SELF-COLLECTED VAGINAL SWABS AND PHYSICIAN-COLLECTED CERVICAL SWABS BY ELECTROCHEMICAL DNA CHIP

(Part 1.3)

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CHAPTER III

Part 1.3: Comparison of detection sensitivity for human papillomavirus between Self-Collected Vaginal Swabs and Physician-Collected Cervical Swabs by Electrochemical DNA chip

Summary

Human papillomavirus (HPV) DNA testing is an effective method to screen for precancerous changes in the cervix. Samples from self-collection rather than Pap smear can potentially be used to test for HPV as they are more acceptable and preferred for use in certain settings. The objective of this study was to compare HPV DNA testing from self-collected vaginal swabs and physician-collected cervical swabs. A total of 101 self-collected vaginal and physician-collected cervical swabs of known cytology from Thai women were tested by electrochemical DNA chip assay. The specimens were divided into 4 groups: 29 with normal cytology, 14 with atypical squamous cells of undetermined significance (ASCUS), 48 with low-grade squamous intraepithelial lesion (LSIL), and 10 with high-grade squamous intraepithelial lesion (HSIL). Positive detection rates of HPV from self-collected swabs were similar to those from physician-collected swabs. Among specimens with abnormal cytology, HPV was found in 50% of self-collected swabs and 47.2% of physician-collected swabs. In specimens with normal cytology, 17.2% of self-collected swabs and 24.1% of physician-collected swabs were positive for HPV. Concordance was relatively high between results from self-collected and physician-collected samples. The most common HPV genotype detected was HPV51. HPV DNA testing by using self-collected swab is a feasible alternative to encourage and increase screening for cervical cancer in a population who might otherwise avoid this important preventive examination due to embarrassment, discomfort, and anxiety.

Introduction

Human papillomavirus (HPV) causes benign and malignant neoplasms of the genital tract including cervical cancer (51). HPV genotypes 16 and 18 correlate with an increased risk of developing precancerous lesions and are the most common causes of cervical cancer (39). HPV DNA testing is an effective screening method for precancerous changes in the cervix, especially for specimens with normal cytology or low-grade squamous intraepithelial lesions (LSIL) (38). Papanicolaou (Pap) test is a simple technique used for screening cervical cancer since the 1940's and has been shown to reduce the incidence of mortality from cervical cancer in countries with an active screening program (36). Test results help physicians detect precancerous lesions and determine the course of treatment prior to the development of malignancy.

In developing countries with few or no screening for cervical cancer by Pap test, the incidence of cervical cancer is high (105). Women generally do not visit their doctor for gynecologic examination when the disease is asymptomatic, which creates a barrier to HPV screening. Other barriers include the embarrassment, discomfort and fear of the results. Moreover, the cost of testing may discourage patients from choosing to screen for HPV (106). To improve screening coverage, self-administered sample collection could be an alternative to Pap smear test (107). Previous studies examined the sensitivity and predictive value of HPV detection by comparing self-collected and physician-collected samples for HPV screening. They found that HPV self-collection was an acceptable and feasible method to confirm cytology results in cervical cancer screening (36, 108). In Thailand, most screening of cervical cancer is done by Pap smear, but few women follow this screening program (59). Patients who avoid HPV testing may do so because of cultural and behavioral factors specific to certain countries.

The universal detection of HPV DNA usually focuses on the L1 region of the major capsid gene (64). Detection of the HPV DNA can be done by various techniques

such as polymerase chain reaction (PCR) with specific primers, hybrid capture test, and linear array (65). Recently, a new technique to detect HPV DNA uses electrochemical DNA chip system combined with loop-mediated isothermal amplification (LAMP). This technique can detect 13 high-risk (HR) HPV genotypes. It can also detect single and multiple infections of high risk genotypes of HPV with higher sensitivity, specificity, simplicity, and speed when compared with other methods (8).

Due to the many advantages of using self-collected samples for HPV testing, this study aimed to compare the HPV DNA test results between self-collected and physician-collected cervical swabs in Thai women.

Materials and methods

Ethical consideration

All self-collected and physician-collected specimens were obtained from the King Chulalongkorn Memorial Hospital and Bangpakok 9 International Hospital in Bangkok, Thailand, between October 2013 and March 2014. The Pap smears were evaluated by a specialized cytotechnologist and confirmed by a pathologist. The research protocol was approved by the Institutional Review Board (IRB number 519/56) of the Faculty of Medicine, Chulalongkorn University. The objective of the study was informed to participants and written consents were obtained. The specimens were sent as anonymous.

Population study

Self-collected vaginal swabs and physician-collected cervical swabs were obtained from 101 females between ages 20-70 years. The specimens were separate into five groups: normal (n = 29), atypical squamous cells of undetermined significance (ASCUS) (n = 14), low-grade squamous intraepithelial lesions (LSIL) (n = 48) and high-

grade squamous intraepithelial lesions (HSIL) (n = 10). Participations were voluntary and were solicited during colposcopy clinic and routine clinic. Both methods to collect the specimen were performed during the same visit for all participants.

Specimen collection and preparation

Physician-collected cervical swabs

Physician-collected cervical swabs were collected before the gynecologist performed Pap smear. The doctor inserted the Flexible minitip flocked swab (Copan Diagnostics, Murrieta, CA) into the cervix and twirled it for 3 seconds, after which the swab was placed in a collection tube and sent to the Center of Excellence in Clinical Virology Laboratory within 6 hours. After transportation to the laboratory, 1 ml of phosphate buffered saline (PBS) was added to the samples and vortexed. Then, the specimens were transferred to 1.5 ml tube and stored at -20 °C until used.

Self-Collected Vaginal Swabs

Self-collected specimens were collected before Pap smear was performed. For self-collected vaginal swabs, patients were instructed to insert the Flexible minitip flocked swab (Copan Diagnostics, Murrieta, CA) into the vagina and twirl it 2-3 times. Self-collection was conducted in private room. All specimens were sent to the laboratory and the collected samples were treated by the same method as physician-collected cervical swabs.

Pathological classification

All specimens in this study were subjected to cytological evaluation to characterize the pathology. Cervical smears for cytology analysis were reported in accordance with the Bethesda System, which is the international standard for reporting

Pap smear results. This system classifies histological morphology into 3 types; ASCUS, LSIL and HSIL (Solomon et al., 2002).

Laboratory method

DNA isolation

DNA was extracted from gynecological specimens using the Qiamp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. After extraction, the DNA samples were stored at -20 °C until tested.

Electrochemical DNA chip

The electrochemical DNA chip consists of six loop-mediated isothermal amplification (LAMP) reagents, an intercalation reagent and an electrochemical DNA chip, which has L1 specific DNA probes for 13 carcinogenic high risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). The reaction conditions and detection were performed using Electrochemical DNA chip (Toshiba, Tokyo, Japan) according to manufacturer's instructions. Genotyping for HPV was accomplished by automated hybridization of probe and primer and the subsequent quantification of the resulting electrochemical signals was done on the GLH-2C601 Genelyzer™ (Toshiba, Tokyo, Japan). The specific primers amplified only respective target. Cross-hybridization of the electrochemical DNA chip was not observed.

Statistical analyses

The self-collected vaginal swabs were compared with physician-collected cervical swabs by using analyses of agreement (Kappa value and percent total agreement). The Kappa values ranging from 0.0 to 0.20 were considered poor agreement, from 0.21 to 0.40 as fair agreement, from 0.41 to 0.60 as moderate

agreement, from 0.61 to 0.80 as good agreement and from 0.81 to 1.00 as excellent agreement. Concordance was measured by the percentage of paired self-collected vaginal swabs and physician-collected cervical swabs that yielded the same results. Type-specific concordance was calculated as the percentage of paired self-collected vaginal swabs and physician-collected cervical swabs samples that were positive for the same HPV genotypes. All statistical analyses used SPSS Software version 17.0 (IBM Corporation, Somers, NY).

Results

Among the 101 women participants in this study, the age ranges were between 20 to 70 years and the majority of the women were less than 50 years old. Among those with normal cytology, the average age was 58.3, while in women with abnormal cytology the average age was 41.8 years. In both the physician-collected and self-collected specimens, all 13 genotypes in which the DNA chip can detect were identified in the samples. As an internal control, β-globin gene was detected in all samples, indicating adequate DNA sampling. Among the samples with abnormal cytology, the DNA chip identified HR-HPV in 50% of self-collected samples and 47.2% of physician-collected samples (Table 9).

Table 9. *Analysis of the cytology results and HPV detected in paired samples (self-collected versus physician-collected) using electrochemical DNA chip.*

	Number of HPV positive samples/total (%)		Concordance (%)
	Self-collected	**Physician-collected**	
Total	41/101 (40.6)	41/101 (40.6)	91
Abnormal cytology	36/72 (50.0)	34/72 (47.2)	94
HSIL	5/10 (50.0)	4/10 (40.0)	90
LSIL	24/48 (50.0)	22/48 (45.8)	96
ASCUS	7/14 (50.0)	8/14 (57.1)	93
Normal cytology	5/29 (17.2)	7/29 (24.1)	83

From samples with normal cytology, HPV was identified in 17.2% of self-collected samples and 24.1% of physician-collected samples. The most common HR-HPV genotype found in both types of samples was genotype 51. Overall, HPV was detected in 40.6% of the samples in the self-collected and physician-collected specimens. The overall concordance between the results for the two collection methods was 91%. There was a 94% concordance in the abnormal cytology group and 83% in the normal cytology group.

The level of agreement was high between self-collected and physician-collected samples (Table 10). Among specimens with abnormal cytology, there was an excellent agreement in the HPV detection rate as measured by the kappa value (k-value). The k-value of HSIL was 0.80, LSIL was 0.92 and ASCUS was 0.86. For specimens with abnormal cytology, there was a fair agreement in HPV detection rate (k-value of 0.58). The sensitivity and specificity of HPV detection in self-collected and physician-collected ranged between 80-100% (Table 10).

Table 10. *Concordance comparison of HPV DNA detection between self-collected and physician-collected swab.*

	Self-collected + Physician-collected +	Self-collected + Physician-collected -	Self-collected - Physician-collected +	Self-collected - Physician-collected -	% agreement	Sensitivity	Specificity	Kappa	95% CI	*P*-value
Abnormal cytology (n=72)										
HSIL (n=10)	4	1	0	5	90.0	80.0	100	0.800	0.044-0.052	0.048
LSIL (n=48)	22	2	0	24	95.8	91.7	100	0.917	0.000	0.000
ASCUS (n=14)	7	0	1	6	92.9	100	85.7	0.857	0.004-0.007	0.006
Normal cytology (n=29)	4	1	3	21	86.2	80.0	87.5	0.583	0.005-0.008	0.006

Discussion

In this study, we assessed the concordance of HPV DNA detection between specimens from self-collected swabs and physician-collected swabs in a cohort of Thai women. We found that the agreement rate between self-collected and physician-collected specimens for HPV DNA detection was high. These findings were similar to a previous study (109). Among specimens with normal cytology, 6.9% of these samples later tested positive for HPV. This is comparable to the rate of 7.6% in a previous study of Thai women (53). Among samples with abnormal cytology, 33.7% of the specimens were positive for HPV, which was higher than in the normal group. Previous report showed a higher prevalence of HPV in precancerous lesions (69). The detection of HPV DNA also depends on the grade of anogenital disease and position of sampling (110). Although our study population was small, our results showed high levels of agreement in the detection of HR-HPV among samples with abnormal cytology (k-value= 0.80-0.92) while there was a fair agreement (k-value=0.58) among the specimens with normal cytology. High agreement of HPV DNA detection between self-collected and physician-collected (k-value=0.75) was also observed in a study of women in Uganda (7). Study size, the technique used to collect samples, and methods used to detect HPV DNA can contribute to the differences in HPV detection.

Previous study in Thailand found that 25-38% of Thai women have had Pap smear test. In this group, women ages 30-65 have had only one Pap smear test done (97). Reasons women avoid Pap test include embarrassment associated with gynecologic exam and the fear of pain from speculum, therefore self-collected swabs could be an alternative way to facilitate increased screening for HPV (107). Self-collection was well-accepted by the women in this study, although some women expressed doubt in their confidence in performing the collection correctly. Even if a simplified collection method is standardized, another barrier to the increased screening of HPV DNA in Thailand is cost (106). Although the acceptability of urine sampling for HPV detection has been reported, sensitivity of this sampling method was not well-established (71).

The most common type of HPV detected in our study was HPV51. It was different from a previous survey in Thai women, which reported HPV16 as the most common genotype identified (17.9%) (53). HPV 16 remained the most prevalent HPV genotype in Thailand as well as in many other countries (60, 69, 70).

Self-collected vaginal swab for HPV DNA testing is a viable alternative for screening the HPV genotyping. The self-collected testing may be the alternative approach to clinician-collected specimens because it is less costly, less-invasive, and relatively practical in low-resource setting and in remote population (111). In addition, self-collected is overwhelmingly preferred over Pap smear test because it can be done in relative privacy and less invasively. In addition to requiring less resource on the healthcare system, this sampling method will help increase the number of women who choose to pursue HPV screening in the future.

Testing for HPV using self-collected sampling is a feasible alternative to encourage and increase screening for cervical cancer in the population who might otherwise avoid this crucial preventive examination due to embarrassment, discomfort and anxiety.

CHAPTER IV

COMPARISON OF FOUR HUMAN PAPILLOMAVIRUS GENOTYPING METHODS: NEXT-GENERATIONSEQUENCING, INNO-LIPA, ELECTROCHEMICAL DNA CHIP, AND NESTED PCR

(Part 2)

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CHAPTER IV

Part 2: Comparison of Four Human Papillomavirus Genotyping Methods: Next-generation Sequencing, INNO-LiPA, Electrochemical DNA Chip, and Nested-PCR

Summary

Human papillomavirus (HPV) infection causes cervical cancer, thus necessitating early detection by screening. Rapid and accurate HPV genotyping is crucial both for the assessment of patients with HPV infection and for surveillance studies. Fifty-eight cervicovaginal samples were tested for HPV genotypes using four methods in parallel: nested-PCR followed by conventional sequencing, INNO-LiPA, electrochemical DNA chip, and next-generation sequencing (NGS). Seven HPV genotypes (16, 18, 31, 33, 45, 56, and 58) were identified by all four methods. Nineteen HPV genotypes were detected by NGS, but not by nested-PCR, INNO-LiPA, or electrochemical DNA chip. Although NGS is relatively expensive and complex, it may serve as a sensitive HPV genotyping method. Because of its highly sensitive detection of multiple HPV genotypes, NGS may serve as an alternative for diagnostic HPV genotyping in certain situations.

Introduction

Human papillomavirus (HPV) infection is one of the most common causes of sexually transmitted disease (112). Young women are more likely than men to become infected with HPV and often contract multiple strains of the virus (113). To date, over 120 different types of HPV have been identified, approximately 40 of which can infect the cervicovaginal mucosa. High-risk HPV (HR-HPV) types are strongly associated with premalignant and malignant cervical lesions, while low-risk HPV (LR-HPV) types are primarily linked to benign anogenital warts (15, 28).

HPV has a circular double-stranded DNA genome of approximately 8,000 bp. The genome is divided into early (E), late (L), and noncoding (upstream regulatory) regions. Variations among HPV genotypes occur primarily in the L1 region, where nucleotide sequence variation may be greater than 10% (15).

There are several molecular diagnostic tests for detecting HPV DNA including direct sequencing, hybridization with genotype-specific probes, or restriction fragment length polymorphism analysis (65, 114). Although a combination of several techniques may be used, identification of HPV DNA has traditionally relied on PCR amplification of the major capsid gene, L1, using the degenerate primers, MY09/11 (115). Alternatively, primers GP5+/GP6+ (extended versions of the MY primers) have been used to amplify a 140 – 150 bp fragment of the L1 region, resulting in higher detection sensitivity (41). This combination of primers gives rise to increased PCR accuracy, although the assay may not be reliable for detecting multiple HPV genotypes (42).

The INNO-LiPA HPV Assay (Innogenetics, Gent, Belgium) is a commercially available HPV genotyping test based on reverse hybridization of amplified HPV products onto a membrane strip containing multiple probes immobilized as parallel lines, which can detect 28 different HPV genotypes (92, 116). Alternatively, the

electrochemical DNA chip system can detect single or multiple infections caused by 13 HR-HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68); all processes of this system, from reaction to measurement and analysis, are integrated into a single compact piece of equipment, which can determine HPV genotype relatively quickly (45).

The emergence of next-generation sequencing (NGS) technologies provides an opportunity to directly examine viral diversity in clinical samples without previous sequence information (117). This system can yield data outputs ranging from 300 kilobases to 1 terabase in a single run and uses primers tagged with initial sequences of specific nucleotides to identify each sample (118). This results in a massive number of parallel sequencing reactions and DNA fragments, all of which possess initial sequence tags specific to the original individual samples (barcodes), which are analyzed molecule by molecule. This method is not widely used for HPV diagnosis, and its accuracy is yet to be confirmed (49). The objective of this study was to compare the diagnostic performance of four techniques for HPV detection and genotyping: nested-PCR followed by conventional sequencing, INNO-LiPA, electrochemical DNA chip, and NGS.

Materials and methods

Ethical consideration

This study was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University (IRB number 603/2558). The IRB waived the need for consent because the clinical samples were de-identified and anonymous. All experiments in this research involved conveniently archived samples derived from an earlier study (Chinchai et al., 2011), which examined the prevalence of cervicovaginal HPV infection.

Population study and specimen collection

Gynecological samples were selected from the samples obtained during routine Pap smear checkups, investigations, or treatment of patients. All cervical cytology was confirmed by a cytotechnologist and a pathologist. Samples were categorized into four groups based on cytology: normal, low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), and cervical cancer. The coding of all samples was anonymous. All samples were kept in LBC buffer (ThinPrep; Hologic, West Sussex, UK) or phosphate buffered saline (PBS). Approximately 15 mL of each LBC was centrifuged at 3000 rpm for 5 min, and the supernatant was removed. Then, 800 μL of the sample was added to a 1.5 mL tube to wash the pellet, the sample was centrifuged at 8000 rpm for 5 min, and the supernatant was removed.

Laboratory method

DNA isolation

DNA was extracted from cervicovaginal samples in LBC using the QIAamp DNA mini kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's protocol. The DNA samples were stored at -20°C until tested. All samples were tested in parallel for the β-globin gene as an internal control.

Nested-PCR and sequencing

DNA was amplified using the nested MY/GP primer set targeting the L1 gene, as previously described (Ermel et al., 2010). Sanger sequencing was performed by FirstBASE Laboratories SDN BHD (Selangor Darul Ehsan, Malaysia). Nucleotide sequences were analyzed using BLAST via the NCBI website for comparison with HPV sequences in the GenBank database.

The INNO-LiPA method

HPV genotyping was performed using the INNO-LiPA HPV genotyping Extra test (Innogenetics N.V., Ghent, Belgium), following the manufacturer's instructions. This assay can identify 28 different HPV genotypes, including all known HR-HPV genotypes and probable HR-HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82), as well as several LR-HPV genotypes (6, 11, 40, 43, 44, 54, and 70) and a number of additional types (69, 71, and 74), based on nested-PCR amplification of a fragment of the L1 region of the HPV genome. Amplified products were denatured under alkaline conditions and immediately incubated with the test strip in hybridization buffer. The results were visually interpreted by two independent investigators by comparing them with a template provided with the assay.

Electrochemical DNA chip

The electrochemical DNA chip is comprised of specific DNA probes targeting the L1 region of 13 carcinogenic HR-HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The assay consists of six loop-mediated isothermal amplification (LAMP) reagents, an intercalation reagent, and an electrochemical DNA chip. The conditions for the LAMP procedure are denaturation at 95 °C for 5 min, followed by 65 °C for 90 min and 80 °C for 5 min. The automated hybridization of probe and primer generates electrochemical signals quantifiable with a GLH-2C601 Genelyzer (Toshiba, Tokyo, Japan). A previous study using the same electrochemical DNA chip demonstrated that there was no cross-hybridization (45).

NGS using the MiSeq platform

Fifty-eight cervicovaginal samples (seven normal, 18 LSIL, 12 HSIL, and 21 cervical cancer samples) previously genotyped by nested-PCR, INNO-LiPA, and

electrochemical DNA chip, were selected for HPV genotyping by NGS using the MY/GP primer set for amplification. These nested-PCR primers amplify a 180-bp fragment of the L1 region (74). Amplicons were purified using Expin Gel SV (GeneAll Biotechnology Co., LTD., Seoul, Korea), according to the manufacturer's instructions. Purified DNA concentration was determined using a Qubit fluorometer (Life Technologies Corporation, Carlsbad, CA, USA). DNA libraries were prepared using the NEBNext Ultra DNA Library Prep Kit for Illumina, following the manufacturer's recommendations (New England BioLabs, Herts, UK). Briefly, purified amplicons were end-repaired, adaptor-ligated, and cleaned up. Subsequently, DNA libraries obtained from each sample were amplified by nested-PCR with different index primers and then purified using AmPure XP beads (Beckman Coulter, Porterville, CA, USA). DNA libraries with different index primers were pooled in equal amounts to generate a 2-nM master DNA library, which was denatured with NaOH and then diluted using HT1 buffer to yield a 15 pM DNA library. Paired-end (150 × 2) deep sequencing was performed using the MiSeq v2 reagent kit (Illumina, Inc., San Diego, CA, USA) on the MiSeq platform (Illumina, Inc.) using a standard protocol.

NGS Data analysis

FASTQ data were processed and analyzed using CLC genomic workbench version 8 (http://www.clcbio.com/). Low-quality reads (Q-score < 30) and adaptor sequences were excluded, and low-quality sequence regions were trimmed. The pass-filter reads (Q-score $\geq$ 30) were aligned with sequences of multiple HPV genotypes obtained from the NCBI and Papillomavirus Episteme (PaVE; http://pave.niaid.nih.gov) databases. HPV genotypes were identified and quantified based on the number of reads matched to each genotype.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) software version 17.0 (IBM, Somers, NY, USA) was used for statistical analysis. The Pearson's chi-square test was calculated to compare the detection of HPV genotyping between the four techniques. *P* values < 0.05 were considered statistically significant.

RESULTS

HPV detection results varied substantially between the four methods tested using the same 58 samples. For NGS, DNA samples were combined into three pools of 24, 24, and 10, yielding total sequence outputs of 2.38, 2.44, and 1.64 Gb, respectively. Short sequence reads were trimmed, and a cut-off quality score of 30 was applied. The average number of pass-filter reads per sample was > 2×10^4 (Table 11). Paired-end reads were assembled to generate L1 sequences of approximately 180 bp. These reads were used for HPV genotyping based on sequence comparison with HPV reference sequences.

Table 11. *Summary of next-generation sequencing data generated from each run.*

Run	Raw data	% > Q30	Data with > Q30	Total reads	Passed filter reads	Reads identified	No. of samples	Average reads / sample
1	2.38 Gbp	83.82	1.99 Gbp	80,60,047	77,89,497	49,76,709	24	2,07,362
2	2.44 Gbp	80.11	1.95 Gbp	86,13,601	80,15,471	52,95,020	24	2,20,625
3	1.64 Gbp	84.88	1.39 Gbp	55,27,404	53,81,851	27,63,042	10	2,76,304

NGS identified a variety of HPV genotypes in each sample; generally, more genotypes were detected using NGS than with the other methods (Table 12). HPV16 was the predominant genotype among the samples analyzed by NGS, consistent with the results of the other methods. Of the sequence reads, > 50% matched at least one major HPV genotype. The minor HPV genotypes in each sample (detailed in Table 12) are indicated in red ($\geq$ 30%), orange (20% – 29.9%), yellow (10% – 19.9%), green (1% – 9.9%), and blue (< 1%).

Table 12. *Summary of four HPV genotyping methods (HPV major type and minor types detected by next-generation sequencing (NGS) for all samples/percentage of total reads obtained in the sequencing)*

Sample	Cytology	PCR	INNO-LiPA	DNA CHIP	NGS									
					HPV major type (>50%)	NGS HPV minor type (<50%, top10)								
B511C	Normal	16	16, 51	16, 51	16(95.89%)	109/0.36	164/0.27	50/0.26	168/0.25	119/0.16	59/0.15	87/0.14	98/0.14	24/0.13	11/0.13
B198C	Normal	70	74	Negative	70(82.66%)	109/1.55	164/1.38	168/1.1	82/0.95	50/0.94	87/0.91	119/0.73	59/0.68	98/0.65	153/0.6
B135C	Normal	16	52	16, 31, 39, 51, 52	16(93.95%)	109/0.59	164/0.46	168/0.41	50/0.34	31/0.28	119/0.26	59/0.23	98/0.22	153/0.22	172/0.21
B12C	Normal	18	18, 51, 52	16, 18, 39, 51, 58	18(93.72%)	16/0.77	107/0.45	87/0.45	164/0.31	168/0.3	45/0.28	33/0.27	50/0.27	119/0.18	24/0.17
B603C	Normal	16	16, 51, 52, 74	16, 58	16(97.54%)	87/0.3	11/0.2	109/0.14	168/0.11	97/0.1	50/0.1	164/0.1	119/0.08	59/0.06	167/0.06
B807C	Normal	6	39, 52	39, 51, 58, 59, 68	6(94.03%)	97/0.5	109/0.43	168/0.33	50/0.32	164/0.3	119/0.23	59/0.21	87/0.18	11/0.18	35/0.17
S131C	Normal	66	44, 56, 58, 66	58	66(89.34%)	109/1.07	50/0.76	164/0.75	168/0.67	119/0.48	24/0.45	59/0.43	172/0.42	57/0.39	163/0.38
S113C	LSIL	56	33	33, 56, 58	56(92.78%)	109/0.65	50/0.4	164/0.39	168/0.38	119/0.31	16/0.31	87/0.3	45/0.29	59/0.29	24/0.27
S30C	LSIL	16	16, 18, 56	16, 18	16(67.49%)	18/23.86	109/0.75	168/0.51	164/0.48	50/0.46	167/0.39	59/0.37	87/0.34	42/0.33	12/0.33
S32C	LSIL	16	51	16, 51	16(64.19%)	70/27.94	109/0.7	168/0.49	87/0.48	164/0.44	50/0.41	59/0.33	163/0.28	119/0.27	98/0.27
S215C	LSIL	16	16, 44, 56, 58, 66	16, 58	16(88.64%)	58/4.83	109/0.47	168/0.39	66/0.36	164/0.32	87/0.32	59/0.3	50/0.28	119/0.24	98/0.23
S212C	LSIL	56	16, 45, 53, 54, 56, 58, 73	16, 45, 56, 68	81(71.94%)	16/21.29	56/3.15	87/0.3	109/0.28	164/0.22	50/0.21	168/0.2	62/0.16	119/0.13	59/0.12
S25C	LSIL	66	31, 39, 51, 66, 82	31, 39, 51, 58	66(79.16%)	31/12.85	58/1.79	6/0.58	109/0.51	164/0.41	50/0.36	168/0.36	87/0.35	62/0.23	119/0.22
S11C	LSIL	81	16, 39	16, 39	81(63.23%)	16/29.08	109/0.64	87/0.58	168/0.53	164/0.46	50/0.37	167/0.3	59/0.27	119/0.27	24/0.25
S13C	LSIL	66	52, 56, 66	52, 56	66(57.15%)	62/17.04	56/11.11	87/2.35	109/1.33	168/0.87	164/0.83	50/0.74	59/0.63	98/0.63	167/0.47
S27C	LSIL	66	33, 66	33, 59	33(51.82%)	66/34.51	109/1.37	168/0.93	164/0.89	50/0.77	167/0.67	59/0.61	98/0.52	119/0.51	163/0.49
S34C	LSIL	31	31, 33, 44, 51, 82	31, 51	31(92.4%)	109/0.79	50/0.49	164/0.46	168/0.4	59/0.33	24/0.31	163/0.29	119/0.28	98/0.26	12/0.25
S38C	LSIL	58	16, 39, 58	39, 58	58(91.33%)	109/0.84	164/0.59	168/0.59	50/0.52	59/0.37	119/0.3	98/0.3	12/0.28	68/0.28	35/0.28
S272C	LSIL	16	52	16, 39, 45, 52	16(96.14%)	11/0.33	87/0.31	109/0.31	168/0.2	50/0.18	164/0.17	58/0.13	119/0.11	167/0.11	59/0.11
S312C	LSIL	58	58, 70	52, 58	58(96.21%)	35/0.29	109/0.26	168/0.22	62/0.18	70/0.16	33/0.16	164/0.16	87/0.15	50/0.14	59/0.12

> 30% 20-29.9% 10-19.9% 1-9.9% <1%

Table 12. *(continued)*

Sample	Cytology	Nested-PCR	INNO-LiPA	DNA CHIP	NGS									
					HPV major type (>50%)	NGS HPV minor type (<50%, top 10)								
S322C	LSIL	11	39, 52	39, 52	11 (89.14%)	81/3.2	87/0.74	109/0.65	50/0.65	168/0.44	164/0.41	119/0.32	24/0.28	172/0.26	16/0.25
S28C	LSIL	33	33, 58	16, 33, 58	33 (97.66%)	109/0.18	58/0.16	16/0.16	35/0.14	164/0.12	50/0.11	168/0.1	18/0.09	97/0.08	59/0.07
S96C	LSIL	33	33	33, 58	33 (98.8%)	35/0.12	97/0.09	18/0.07	109/0.06	50/0.06	164/0.06	58/0.05	87/0.05	168/0.04	16/0.03
S9C	LSIL	16	31	16, 31, 52, 68	16 (90.29%)	31/0.95	109/0.92	168/0.65	164/0.59	50/0.47	59/0.39	119/0.36	167/0.33	98/0.29	24/0.28
S137C	LSIL	56	31, 33, 44, 66	16, 31, 56	66 (97.03%)	31/1.33	87/0.41	97/0.09	16/0.08	109/0.08	56/0.06	81/0.06	168/0.06	33/0.06	50/0.06
S820C	HSIL, ASC-H	33	16, 33, 39	33, 39	33 (90.32%)	109/0.97	168/0.77	164/0.59	50/0.44	119/0.44	42/0.42	110/0.41	167/0.38	68/0.35	98/0.34
S853C	HSIL, CIN II	45	16, 39, 45, 51	39, 45, 51	45 (96.73%)	109/0.27	164/0.2	50/0.19	168/0.18	87/0.18	59/0.11	119/0.11	98/0.11	24/0.09	172/0.09
S835C	HSIL, CIN II	16	16	16, 58	16 (97.03%)	87/0.31	11/0.22	109/0.21	168/0.17	50/0.15	33/0.14	164/0.13	97/0.1	59/0.07	12/0.07
S841C	HSIL, CIN II	16	16, 52	16, 45, 52	16 (95.79%)	168/0.35	109/0.29	87/0.28	50/0.28	164/0.22	11/0.21	110/0.15	59/0.13	42/0.13	163/0.13
S842C	HSIL, CIN II	58	31 , 44, 58	31, 58	58 (95.13%)	109/0.38	35/0.28	168/0.27	164/0.22	50/0.19	59/0.18	62/0.18	167/0.18	98/0.17	87/0.17
S878C	HSIL, CIN II	16	16	16, 39	16 (96.42%)	109/0.34	168/0.26	50/0.22	164/0.18	59/0.15	87/0.15	119/0.15	98/0.15	11/0.11	12/0.11
S864C	HSIL, CIN III	31	31, 33, 44, 52, 54	31	31 (94.19%)	109/0.55	164/0.43	50/0.41	168/0.37	59/0.23	119/0.23	98/0.2	24/0.2	57/0.19	163/0.19
S819C	HSIL, CIN III	33	33, 52, 54	33, 51	33 (97.16%)	35/0.24	109/0.2	18/0.17	168/0.15	87/0.14	164/0.13	50/0.13	97/0.11	52/0.08	58/0.08
S839C	HSIL, CIN III	45	16, 45	16, 45	45 (72.41%)	16/23.26	109/0.36	87/0.3	164/0.25	168/0.22	50/0.18	97/0.17	33/0.16	18/0.16	54/0.16
S859C	HSIL, CIN III	16	16	16, 33, 68	16 (95.67%)	109/0.36	87/0.33	164/0.22	11/0.21	168/0.21	50/0.17	59/0.16	119/0.14	167/0.14	98/0.14
S871C	HSIL, CIN III	18	18	18, 31	18 (86.82%)	109/1.49	164/1.17	168/1.05	50/0.98	109/1.50	164/1.18	172/0.52	59/0.51	167/0.49	24/0.48
S836C	HSIL, CIN III	18	18, 52	18, 52	18 (95.96%)	87/0.38	109/0.26	50/0.23	16/0.22	87/0.39	109/0.27	164/0.19	168/0.15	59/0.13	97/0.12
T100C	CA	33	16, 33, 58, 68	16, 33, 58, 59, 68	16 (85.38%)	109/1.43	164/1.13	50/1.06	168/1.05	119/0.62	33/0.61	59/0.61	98/0.54	24/0.52	163/0.51
T36C	CA	16	16, 44, 52	16	16 (89.92%)	109/1.05	164/0.78	50/0.73	168/0.7	59/0.44	119/0.43	98/0.39	172/0.37	57/0.35	167/0.34
T26C	CA	67	Negative	16, 56	56 (89.4%)	109/0.99	16/0.87	164/0.74	50/0.69	168/0.64	119/0.43	59/0.41	98/0.38	163/0.36	24/0.36

> 30% | 20-29.9% | 10-19.9% | 1-9.9% | <1%

Table 12. *(continued)*

Sample	Cytology	PCR	INNO-LiPA	DNA CHIP	NGS									
					HPV major type (>50%)	NGS HPV minor type (≤50%, top 10)								
T32C	CA	33	33	33, 39	33 (87.98%)	81/6.44	109/0.64	50/0.49	164/0.43	168/0.3	59/0.24	172/0.21	12/0.2	24/0.2	119/0.2
T37C	CA	45	45, 52	31, 45, 51, 52	45 (98%)	87/0.15	109/0.15	97/0.13	33/0.11	50/0.11	168/0.1	164/0.1	83/0.08	172/0.05	119/0.05
T44C	CA	16	16, 33	16, 33	16 (92.73%)	109/0.71	50/0.65	33/0.57	164/0.57	168/0.44	59/0.29	124/0.27	12/0.27	119/0.26	24/0.25
T60C	CA	18	18	18, 58	18 (66.46%)	109/3.53	168/2.79	164/2.52	50/2.36	59/1.6	98/1.46	119/1.41	163/1.33	24/1.31	167/1.27
T69C	CA	67	Negative	Negative	67 (96.06%)	109/0.34	50/0.25	168/0.24	97/0.23	164/0.22	31/0.16	24/0.14	119/0.14	57/0.13	172/0.13
T85C	CA	16	16	16, 31, 39	16 (88.68%)	109/1.34	50/0.89	164/0.85	168/0.8	24/0.47	119/0.46	59/0.44	12/0.4	163/0.4	57/0.38
T94C	CA	16	16	16, 68	16 (96.7%)	109/0.33	50/0.27	168/0.21	164/0.21	87/0.14	124/0.14	11/0.13	59/0.12	24/0.11	119/0.11
T95C	CA	18	18	16, 18	18 (98.62%)	16/0.18	87/0.18	45/0.15	33/0.11	97/0.08	109/0.07	168/0.05	50/0.04	164/0.03	172/0.03
T129C	CA	18	18, 51	18, 51	18 (70.56%)	109/3.27	168/2.69	164/2.22	50/2.21	110/1.31	119/1.22	59/1.2	98/1.1	167/1.09	163/1.06
T134C	CA	58	58	39, 52, 58	58 (97.85%)	18/0.22	16/0.16	35/0.15	50/0.15	109/0.15	168/0.11	164/0.1	33/0.09	124/0.08	45/0.07
T144C	CA	45	51	45, 51	45 (98.5%)	16/0.18	87/0.15	97/0.13	33/0.11	109/0.08	83/0.06	164/0.06	50/0.05	44/0.04	168/0.04
T151C	CA	16	16, 58	16, 31, 58	16 (97.42%)	109/0.22	87/0.15	164/0.15	11/0.14	168/0.14	50/0.13	59/0.09	6/0.08	119/0.08	24/0.07
T153C	CA	16	16, 52	16, 52	16 (98.4%)	11/0.14	87/0.12	109/0.12	50/0.09	164/0.09	168/0.08	97/0.06	33/0.05	119/0.05	59/0.04
T180C	CA	16	16	16, 58	16 (80.09%)	109/2.52	168/1.53	164/1.53	50/1.32	59/1.02	119/0.89	12/0.85	167/0.84	98/0.79	172/0.79
T29C	CA	45	45	16, 45	45 (96%)	109/0.43	168/0.3	164/0.25	50/0.23	16/0.15	68/0.15	87/0.15	59/0.14	163/0.13	119/0.13
T28C	CA	16	16	16, 18, 33, 52	16 (80.88%)	109/2.29	50/1.53	164/1.45	168/1.4	59/0.9	119/0.85	24/0.77	172/0.75	12/0.73	163/0.72
T19C	CA	16	16	16, 33	16 (96.73%)	109/0.31	168/0.22	164/0.22	50/0.21	87/0.15	11/0.14	59/0.12	119/0.12	98/0.11	68/0.11
T23C	CA	16	16, 52	16, 52	16 (83.06%)	109/1.92	164/1.38	168/1.29	50/1.24	59/0.85	119/0.74	98/0.68	24/0.65	172/0.65	163/0.65

> 30% | 20-29.9% | 10-19.9% | 1-9.9% | <1%

Only a single genotype was identified per sample using the nested-PCR; the most frequent genotype was HPV16 (36.2%). Typing by INNO-LiPA revealed multiple infections in 45 of the 58 samples, with only one sample producing a negative result. The maximum number of genotypes/sample detected by INNO-LiPA was 10. The electrochemical DNA chip analysis findings indicated that 55 samples were from individuals with multiple infections, with a maximum of five genotypes/sample. Multiple infections were identified in all 58 samples by NGS analysis. The Pearson's chi-square test showed a significant difference between NGS and electrochemical DNA chip (P = 0.004). In contrast, there was no significant difference between NGS and INNO-LiPA for HPV genotyping. The concordance rate of the four methods (using sequences derived from nested-PCR as gold standard) was 100% for NGS, 77.6% for INNO-LiPA, and 82.8% for electrochemical DNA chip.

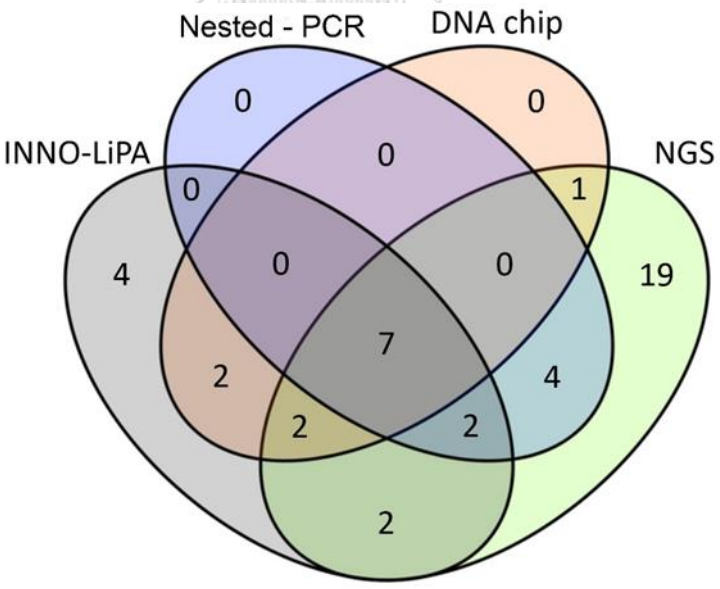

Figure 3. *Venn diagram of HPV genotypes identified by four different techniques.*

A comparison of the four methods is presented in Figure 3 as a Venn diagram. Seven HPV genotypes (16, 18, 31, 33, 45, 56, and 58) were identified using all four techniques. All of these were HR-HPV genotypes and were found primarily in samples from malignant or pre-malignant lesions. Interestingly, four genotypes (44, 53, 73, and 74) were detected by INNO-LiPA, but not by the other methods. Nineteen HPV genotypes (12, 24, 42, 50, 57, 62, 87, 97, 98, 109, 110, 119, 124, 153, 163, 164, 167, 168, and 172) identified by NGS were not detected by nested-PCR, INNO-LiPA, or the electrochemical DNA chip method.

DISCUSSION

HPV genotyping is important for epidemiological studies, potential improvement of the current HPV vaccine, and more effective cervical cancer screening. Currently, several methods are used for HPV genotyping, which differ according to sample preparation, analysis methods, and (for nested-PCR -based assays) primers used. In addition, each method varies in both sensitivity and specificity (119). Our NGS results were in agreement with those of the other three techniques and were in good agreement with a previous finding, which also showed that NGS had high sensitivity and was suitable for detecting multiple HPV infections (46).

Massively parallel sequencing has transformed genetic research (120), enabling increased characterization of genomes, transcriptomes, epigenomes, and microbiomes (121). This study presents proof-of-principle that NGS can be used for the characterization of HPV genotypes and suggests its potential advantages over existing hybridization-based genotyping systems. In this study, NGS HPV genotyping was performed using the Illumina MiSeq platform and the genotyping results were compared with those using the other three methods. Comparison of the results demonstrated that 19 HPV genotypes were detected by NGS, but not using the other approaches. These 19 genotypes are not usually identified during HPV screening. HPV genotypes 42, 50, 57,

62, 87, and 97 are associated with LSIL, which causes common skin warts and cutaneous lesions (15, 122-125), while the remaining 13 genotypes detected solely by NGS are largely uncharacterized. While none of these LSIL genotypes confer any measurable increased risk of developing cervical intraepithelial neoplasia, the seven HPV genotypes identified by all four techniques were high-risk genotypes that commonly cause cervical cancer (126).

The results of a previous study using 454 NGS technology and HPV-specific primers to amplify the L1 region demonstrated that the NGS results correlated well with those obtained using the INNO-LiPA HPV Genotyping Extra assay (46). In this study, the Illumina MiSeq platform HPV typing results were consistent with those generated using the other techniques, indicating that HPV typing using the Illumina MiSeq method is accurate. In contrast, four HPV genotypes were detected by INNO-LiPA, but not robustly identified by NGS. HPV44 was also identified by NGS; however, the frequency of detection was < 0.5%. The three other genotypes detected solely by INNO-LiPA, HPV73 (HR-HPV), HPV53 (probably high-risk) (33), and HPV74 (additional genotype), may represent occasional false-positive results generated by this hybridization-based assay. INNO-LiPA utilizes reverse hybridization with multiple probes specific for different genotypes, and interpretation of the results can be quite difficult, depending on the number and density of the bands present on the strip. A number of genotypes, which are not commonly distributed worldwide, may be problematic to interpret.

One limitation of the NGS assay is the amplification of the HPV genome L1 region using the MY/GP primers; these primers yield a 180 bp HPV DNA amplicon, while the INNO-LiPA assay amplifies a similar region using the SPF10 primers, yielding a product of only 65 bp (127). A previous study demonstrated that the SPF10 primer set is more sensitive than the MY09/11 primers (128). Moreover, nested-PCR amplicon size can influence assay sensitivity, with smaller amplicons associated with

greater sensitivity (129). Hence, the lower sensitivity of the NGS method for some HPV genotypes (relative to INNO-LiPA) may be due to the decreased sensitivity of the MY/GP-based primers.

Several HPV detection methods primarily involve genotyping based on nested-PCR amplification using consensus primers located in the L1 gene followed by HPV type identification by oligonucleotide probe hybridization or direct sequencing. Sanger sequencing methods have the potential to identify a broad range of HPV types, although distinguishing multiple infections may be problematic. Hybridization-based methods can discriminate HPV types in multiple infections, but can only identify HPV types represented by the probes (130). The advantage of the NGS method over hybridization-based methods is high specificity genotyping, because the method is based on massively parallel sequencing and the results are less susceptible to misinterpretation. The other advantage of NGS is that it can detect multiple infections. Multiple infections are common, but have no additive or synergistic effects on the development of high-risk cervical cancer. In fact, reduced high-risk cervical cancer rates have been correlated with multiple infection-, rather than single-genotype infection profiles, suggesting possible intergenotypic competition or more effective immune responses triggered by multiple infections (131).

A single NGS run can generate millions of DNA sequence reads in 24 h, which is more than can be achieved with hundreds of Sanger type sequencers over the same time period (129). However, compared with conventional HPV typing methods, NGS has a longer turnaround time per sample and higher costs, mainly associated with the instrument (Table 12). The NGS technique will improve with automation and standardization of protocols. Moreover, NGS costs decrease with increasing data throughput. With the development of less expensive instruments, NGS may soon become a cost-effective platform for molecular diagnosis of HPV.

A limitation of this study was the small study population. Additionally, depending on the method used and the purity of DNA, different internal controls were used. Therefore, the quantity of amplified DNA differed. Moreover, the NGS method used in this study had an intrinsic drawback because the primers used were based on the MY/GP primers, which have low sensitivity for some HPV genotypes (64). However, our results show that NGS is a promising method for HPV genotyping because of its high sensitivity for multiple infections and its ability to detect a wide range of HPV genotypes.

Detection of HPV DNA is a useful tool in the early diagnosis of cervical pre-cancer when used in conjunction with cytology. NGS is an alternative technique for carcinogenic HPV detection. This technique has high sensitivity for multiple infections and offers the potential to detect a broad spectrum of HPV genotypes.

CHAPTER V

HUMAN PAPILLOMAVIRUS IN THAI WOMEN AND MEN WITH ANOGENITAL WARTS

(Part 3)

(Submitted)

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CHAPTER V

Part 3: Human papillomavirus in Thai women and men with anogenital warts

Summary

Anogenital warts are caused by human papillomavirus (HPV). HPV genotype 6 and 11 are most often associated with anogenital warts. The diversity of HPV genotypes found in Thai patients with genital warts is not well-characterized. The objective of this study was to investigate HPV-associated anogenital warts in the Thai population and whether genotypes found are represented in the vaccine. A total of 206 anogenital swab samples were analyzed for HPV DNA by PCR and sequencing. HPV positive was identified in 88.3% (182/206) of the samples. The majority of HPV (75.2%) were low-risk genotypes HPV6 and HPV11. Thus, HPV6 and HPV11 were most common infection in genital wart. We conclude that the quadrivalent vaccine could potentially prevent 84.5% of the genital warts found in the Thai population.

Introduction

Human papillomavirus (HPV) is the most common pathogen transmitted through sexual contact. The virus has more than 100 genotypes, and at least 40 genotypes can cause genital warts (50), which are the most common benign tumor in the anogenital region (132). Typical anogenital warts appear as flesh-colored, exophytic lesions on the external genitalia, including the penis, scrotum, vulva, perineum, and perianal skin (4). The highest occurrence of genital warts has been reported in individuals between 20 and 24 years of age (133). Globally, genital warts are estimated to affect approximately 1% of the human population (134), although the incidence in sexually transmitted disease clinics is approximately 13% (51). Although genital warts are benign, they may cause discomfort and affect psychological well-being because they are often untreated (9, 135, 136).

Risk factors for genital warts include multiple sex partners, anal intercourse, lower immunity, tobacco use, ultraviolet radiation exposure, and pregnancy (50, 137). As many as 50% of men who have sex with men (MSM) presented with squamous cell carcinoma of the anus have a history of anorectal warts, while only 20% of women and heterosexual men have this reported history (137). The treatment of genital wart is often painful, expensive, and unsuccessful (138).

HPV genotype 6 and 11 often infect the mucosa and skin of the anorectum and genitalia are considered low-risk for cancer development due to their low oncogenic potential and infections often result in genital warts or low-grade precancerous lesions (LSIL) (139). HPV genotypes 31, 33, 35, 39, 40, 43, 45, 51-56, and 58 are considered to have a moderate oncogenic potential, whereas HPV genotypes 16 and 18 are high-risk HPV genotypes associated with the development of high-grade intraepithelial neoplasia (HSIL), carcinoma of the anus, vulva, vagina, cervix, penis, and head and neck cancer (140).

HPV-related anogenital warts are assessed clinically by anoscopy, skin biopsy, and by HPV DNA detection (50, 141, 142). Dacron swab is a simple method of collecting anogenital wart samples (143). Immunization with a bivalent vaccine (containing HPV16 and 18) (Cervarix, GlaxoSmithKline Biologicals, Rixensart, Belgium) or the quadrivalent vaccine (Gardasil, Merck Sharp and Dohme Corp., NJ, USA) (containing HPV16, 18, 6 and 11) can provide excellent protection against the HPV types comprised in the vaccine). The quadrivalent vaccine has also demonstrated significant cross-protection to other oncogenic types in both naive and previously HPV-infected individuals (10). A recently approved nonavalent vaccine (Gardasil9, Merck Sharp and Dohme Corp., NJ, USA) containing HPV16, 18, 6, 11, 31, 33, 45, 52 and 58 in the U.S. has been recommended for previously unvaccinated MSM and immunocompromised patients through age 26 (144). Here, we characterized the genotypes of HPV detected in Thai patients with anogenital warts to determine whether these genotypes are represented in the vaccine.

Materials and methods

Ethical consideration

This cross-sectional analysis used data from enrollment visits of cohort study at King Chulalongkorn Memorial Hospital, Bang Rak Sexually Transmitted Infections Center and Chiang Mai University Hospital. This study was approved by the Institutional Review Board (IRB number 509/57) of the Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from all participants. The specimens were ananomyzed by number codes.

Population study

Individuals older than 18 years of age who came for a follow-up visit at the hospitals were recruited between December 2014 and October 2015. A total of 206 specimens were catagorized into three groups: females (n=69), males (n=91), and MSM (men who self-reported to have sex with other men or who were bisexual) (n=46). All of the patients had anogenital warts. Patient demographics, clinical and behavioral data were extracted from medical records.

Sample collection and preparation

All anogenital wart swab samples were collected by using Flexible mini tip flocked swab (Copan Diagnostics, Murrieta, CA). Swabbed specimens were added to phosphate buffered saline (PBS), vortexed, and stored at -20 °C until tested.

Viral nucleic acid preparation

The total viral nucleic acid was extracted from 100 µl of clinical specimens using the Qiamp DNA mini kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. After extraction, all of the specimens were tested for the β-globin gene to serve as an internal control. The DNA samples were stored at -20°C until tested.

Detection of HPV and genotyping

The polymerase chain reaction (PCR) of HPV DNA was amplified using MY/GP primer sets to amplify the L1 gene (41). The PCR reaction mixture contained 2 µL DNA template, 0.5 µL of 10 mmol of each primer, 15 µL of 2.5× PerfectTaq Plus MasterMix (5 PRIME Inc., Hamburg, Germany) and sterile distilled water to a final volume of 25 µL (74). For the first PCR amplification, cycling parameters were initial denaturation at 94 °C for 5 min followed by 40 cycles at 94 °C for 30 s (denaturation), 55 °C for 45 s (annealing), 72 °C for 1.30 s (extension), and a final extension at 72 °C for 7 min. The second PCR amplification was identical except annealing was at 50 °C for 45 s. The β-

globin gene served as an internal control and was successfully amplified from all samples. All amplified HPV PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide and purified from the agarose gel using Expin Gel SV kit (GeneAll, Seoul, Korea). HPV was genotyped by Sanger sequencing. Sequences were analyzed using BLAST program on the GenBank Database (www.ncbi.nlm.gov/BLAST).

Statistical analysis

Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Chi-squared tests were used to assess the statistical significance of any differences in prevalence. Statistical significance was defined as $p<0.05$. Quantitative data are expressed as mean (SD).

Results

Demographic data such as age, alcohol consumption status, smoking status and the number of sex partner were summarized in Table 13.

Table 13. *Characteristics of patients with anogenital warts.*

	Mean age (years)	HPV positive	Alcohol consumption	*P*-value	Smoking	*P*-value	Sex partner >1	*P*-value
Female (n=69)	28.7	61 (88.4%)	31 (44.9%)	<0.001	8 (11.6%)	<0.001	46 (66.7%)	<0.001
Male (n=91)	30.9	77 (84.6%)	38 (41.8%)	<0.001	20 (22.0%)	<0.001	9 (9.9%)	<0.001
MSM (n=46)	27.2	44 (95.7%)	20 (43.5%)	<0.001	4 (8.7%)	<0.001	9 (19.6%)	<0.001

The average age of female patients in this study was 28.7 (range, 15-49 years). The average age of male and MSM patients was 30.9 (range, 16-58 years) and 27.2 (range, 18-54 years), respectively. HPV DNA was detected in 88.3% (182/206) of the

samples. HPV6 and HPV11 were the most frequent genotype (Figure 4) and this finding is consistent with published reports from other countries (Table 14).

Table 14. *Studies of human papillomavirus DNA detected in anogenital warts.*

country	sample type	Total sample size (M/F)	Age X/range (years)	HPV positive	mostly found genotype (%)			others genotype HR* LR	HPV detection assay	collection method	reference
					6	11	16				
France	CA	516 (260/256)	30.0/18-72	99.0%	69.0	16.0	9.0	**51**,**52**,66,53,**31**,**18**	INNO-LiPA assay	cytobrush	Aubin F et al (2008)
Sweden	CA	621 (376/245)	28.4/15-69	96.3%	61.7	10.3	12.9	42,66,90,**51**,**18**,**31**, **59**,53,67,91,40,**56**,**39**,43, **33**,**52**,81,**58**,73	Luminex assay	cytobrush	Shuresärd E et al (2013)
UK	GW	31 (ND)	ND	100.0%	90.0	32.0	ND	42,**45**,54,55,**59**,62,67, 69,84	Linear Array	biopsy	Ball SL et al (2011)
Spain	GW	184 (184/0)	ND/17-72	75.0%	51.6	17.7	42.3	**52**,**59**,84,54,70,53,66, 73,61	Linear Array	Dacron swab	Arroyo LS et al (2016)
Korea	GW	80 (80/0)	35.4/ND	57.3% LR, 26.3% HR	39.7	13.8	3.4	**18**,40,42,43,44,**51**,**68**, **58**,53,**33**,**35**,66	Anyplex II HPV28	biopsy or cytobrush	Kwon T et al (2016)
Czech Republic	CA	64 (ND)	ND/15-69	90.6%	71.9	20.3	1.6	73,26,**33**,42,**45**,**51**, 84,81	PCR and reverse line blot hybridization	cervical scrape, biopsy	Tachezy R et al (2011)
Denmark	GW; AW	201 (124/58)	27.0/18-85	74.0% (HPV6,11)	67.6 GW 59.9 AW	7.1 GW 9.5 AW	16.5 GW 20.2 AW	42,90,66,**52**,**51**,**56**,**31**, **18**,43,89,40,**59**,**39**,70,81, **68**,53,**45**,**58**,67,30	PCR (modified MGP primer)and Luminex-based HPV genotyping	cytobrush	Kofoed K et al (2014)
Iran	GW	100 (0/100)	26.1	73.0%	49.0	67.0	ND	ND	PCR GP5/GP6 and Type-specific PCR,HPV6,11	biopsy	Jamshidi M et al (2012)
USA	CA	39 (27/12)	29.1 M/ND 26.8 F/ND	100.0%	94.0	8.0	ND	54,**58**,**16**,**18**,26,40,42,54, **56**,57,59	PCR MY09/11	Dacron swab	Greer CE et al (1995)
China	GW	1,005 (449/556)	32.0/17–86	88.7%	41.3	37.6	10.4	**58**,7,8,**59**,**45**,61,66,67	PCR MY09/11,GP5+/6+	biopsy	Chang L et al (2013)
Argentina	GW	160 (0/160)	ND/15-45	93.3%	80.0	12.7	2.0	73	PCR MY09/11,GP5+/6+	biopsy	Elaider LA et al (2016)
Thailand	GW; AW	206 (137/69)	29.2/15-58	88.30%	37.9	37.4	6.8	**18**,**39**,54,**59**,62,66,81,83	PCR MY09/11,GP5+/6+	flocked swab	Present study

CA: Condyloma acuminata, GW: Genital wart, AW: Anal wart, M: male, F: female, ND: no data

*Bold indicates high-risk genotype

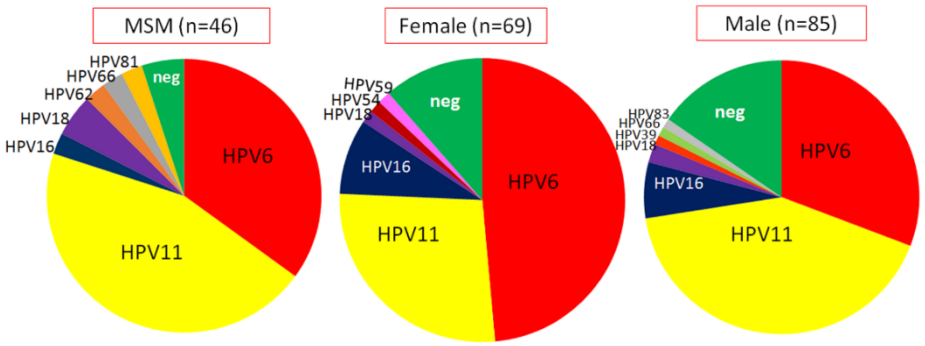

Figure 4. *HPV genotype distribution in anogenital wart.*

The most frequently encountered HPV genotypes among 206 samples were (in decreasing order of frequency) HPV6 (76/206, 36.9%), HPV11 (75/206, 36.4%), HPV16 (13/206, 6.3%), HPV18 (5/206, 2.4%) and HPV66 (2/206, 1.0%). In this study, the relationship between alcohol consumption, smoking status, number of sex partners and HPV positivity was statistically significant (P-value < 0.001). The percentage of HPV positive samples compared to each type of available vaccines coverage are shown in Table 15.

Table 15. *The percentage of HPV positive coverage compared with each type of vaccine.*

	Bivalent vaccine n (%)	**Quadrivalent vaccine n (%)**	**Nonavalent vaccine n (%)**
Female (n=69)	7 (10.1)	59 (85.5)	59 (85.5)
Male (n=91)	8 (8.8)	74 (81.3)	74 (81.3)
MSM (n=46)	4 (8.7)	41 (89.1)	41 (89.1)
Total (n=206)	19 (9.2)	174 (84.5)	174 (84.5)

Discussion

HPV genotyping distribution studies of genital warts in the Thai population and within Southeast Asian region are limited. A previous study on the genital warts in Thailand found that around 40% of women presenting with external anogenital warts who were not immunocompromised had recurrent warts within 6 months after completing the treatment (145). Benign lesions found in 90% of genital warts are caused by low-risk genotypes HPV6 and HPV11 (1). However, our study found low-risk genotypes HPV6 and HPV11 in 155/206 (75.24%) of the samples, and 23 of these samples were positive from high-risk HPV genotype in anogenital warts tissue. A screening of HPV in patients in genital wart found 88.7% in China, 75.0% in Spanish men and 93.3% in Argentinian women (143, 146, 147). Although there is no reliable prevalence data of HPV infection in men with genital warts, low-risk genotype such as HPV6 in men has been reported (148). In this study, the HPV6 and HPV11 genotypes were most commonly detected similar to studies from the U.S., which found between 74%-100% of HPV6 or HPV11 in genital wart samples (149, 150). Variation in reported prevalence is likely due to variances in sampling techniques, the populations studied, collection sites sampled and the sensitivity of HPV DNA detection methods used (151). Moreover, HPV prevalence is higher when samples are collected from a greater number of anatomic sites (152).

The risk of anogenital warts becoming cancerous may be related to the oncogenic nature of HPV. Although smoking, alcohol consumption, and behavior (e.g. male homosexuality, higher sexual activity or multiple sex partners) are also known to be involved in oncogenic risk factors, they may be confounders (153-155). In this study, we showed that smoking, alcohol consumption and the number of sex partners was significant associated with HPV positive status in the genital wart samples consistent with a previous study (156). High intake of alcohol is significantly associated with an increased risk for HPV infections among men (155). Additionally, alcohol is a potent

modulator of immune function which can lead to immune deficiency and increased susceptibility to various chronic and infectious diseases (157, 158). Smoking has been shown to weaken the immune response against viral infections, and more smokers are HPV-positive than non-smokers (159). Furthermore, multiple lifetime sexual partners increase the likelihood of oral-genital HPV co-infection (154).

Laryngeal papillomas can be found in infants and children who may have contracted HPV from their mothers during childbirth, although the possible mechanisms of vertical transmission are not well understood. The prenatal transmission of HPV is also supported by the presence of HPV lesions on some infants at the time of birth (160). HPV transmission from women without clinical evidence of HPV to the neonate is estimated to be between 1-18% (161, 162). In women who had detectable HPV during pregnancy, transmission rate ranged from 5-72% (161, 163). The laryngeal papilloma is not immediately apparent and may be quite difficult to diagnose until they become symptomatic. The HPV genotypes most often found in laryngeal papilloma were HPV6 and 11 (45, 164, 165). Thus, preventing HPV6 and HPV11 infection can reduce the incidence of genital warts.

The HPV16, 18, and 45 are among the most prevalent types of cervical cancer worldwide (166). The quadrivalent vaccine, which includes HPV6 and 11, may prevent infection in 84.5% of our patient population. The bivalent vaccine also contains the high-risk HPV16 and 18, genotypes found in the genital warts specimens in this study, and could theoretically prevent infection in an additional 9.2% of our patient population. Thus, Gardasil9 or Gardasil vaccination may prevent infection in 84.5%.

This study has several limitations. Our samples were collected from only 3 hospitals and therefore may not be generalizable to all populations. We were only able to identify genital warts from adolescents and young adults, individuals most likely to experience infection and seek medical care. We do not have information regarding the anatomical

locations of the genital warts from which the samples were derived due to privacy and different coding practices on the medical records from different hospitals. Also, the description of the anatomic location of genital warts was affected by bias; often times, physicians only performed limited clinical examination on patients.

HPV 6 and 11 were the most detected HPV genotypes identified in anogenital warts in this study. Since they are components in the quadrivalent HPV vaccine, appropriate immunization could potentially prevent a significant number of infection in Thailand

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APPENDIX

VITA

The author of this thesis was born on 12 November 1987 in Chonburi, Thailand. She received her Bachelor degree of Science Major in Biochemistry in 2009 from Faculty of Science, Chulalongkorn University. In 2011, she received her Master degree of Science Major in Biomedical Science from Faculty of Medicine, Chulalongkorn University. In 2012, she enrolled in the Dutsadi Piphat Scholarship in graduate program for degree of Philosophy Program in Faculty of Medicine, Chulalongkorn University under supervision of Prof. Yong Poovorawan. Her research focused on the development of technique and sample for detection human papillomavirus and study the prevalence of anogenital wart in Thai population. She also did the characterization of bacteria invasion in cell culture and in host cell. She did the involvement of host receptor and the microbiome. This work was performed at the University of Texas Medical Branch (Galveston, TX) under supervision of Dr.Grant L. Hughes and Dr.Shivanand Hegde.

Publications:

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