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Validation of In-House HPV-16 DNA Quantitative Real-Time PCR Testing

Sukjai Pholampaisathit* **Pilailuk Aukapaiboon Okada***
Thanomsiri Soontornthum** **Vorawan Sirchai**** **Veera Suraseraneewong****
Somrat Lertmahalit*** **and Somchai Sangkitporn***

**Department of Medical Sciences, Tiwanond Road, Nonthaburi 11000*

***Bhumibol Adulyadej Hospital, Phahongothin Rood, Bangkok 11220*

****Chulalongkorn University, Phayathai Road, Pathumwan, Bangkok 10330 Thailand.*

ABSTRACT Introduction: HPV is a causative agent of cervical cancer, and is the second leading cancer for Thai women after breast cancer. HPV-16 is the most common subtype associated cancer. Since cytology screening has become a problem that has a large number of borderline results, our purpose is to clinically validate the sensitivity and specificity of the in-house HPV-16 DNA quantitative real-time PCR assay test compared to a reference method, and validate its reliability in order to use it as a combined test for managing abnormal Pap smear samples and to determine if viral load is associated with the virulence of disease. Sixty five out-patients with borderline Pap smear results, from the 'Gynecological Ward' at Bhumipol Adulyadej hospital, Bangkok, Thailand, and 119 normal women presenting annual Pap smear screening were recruited for the study, between 2010 and 2012. All were determined by a colposcopy-directed biopsy as a reference method. The cervical scrape samples were taken to test for HPV DNA using a quantitative real-time PCR primer and specific probe for the L1 region of HPV DNA. The test was validated for clinical sensitivity and specificity, for the detection of HPV2 DNA in precancerous lesions (≥CIN2), and for its reliability. Out of 65 samples with borderline results, 45 cases were HPV-16 DNA positive (69.2%). Only 14/65 cases had precancerous lesions (≥CIN2). For clinical sensitivity, out of 14 of those cases, 12 patients were HPV-16 DNA detected (86%). For the clinical specificity of the test, in normal women; out of 96 normal women who were negative for colposcopy (without CIN2), 76 cases tested negative for HPV-16 DNA (80%). The viral load did not correlate to the virulence of disease. This test might be useful for health care practices as an adjunct to cytology follow-ups for women with borderline results.

Keywords: clinical sensitivity and specificity, HPV-16 DNA, in-house quantitative real-time PCR, Thai women, validation

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Introduction

Cervical cancer is the second most common cancer amongst women worldwide[1]. It is considered to be the second most common cancer amongst Thai women after breast cancer, with an aged standardized rate of 16.7 per 100,000 women per year[2]. HPV has been involved in 99.7% of cervical squamous cell cancers worldwide[3]. About 40 HPV types have been identified and are known to infect the mucosal epithelium. Only 10-15 types are associated with lesions (precancerous and cancerous lesions) that can progress to cancer, and of these HPV-16 is the most common subtype (60%). It was found that HPV-16 and/or - 18 increases the risk of development of precancerous lesions also called cervical intraepithelial neoplasia (CIN), when clearly compared with the non-HPV-16/18 types[4-5]. If a high amount of HPV DNA is present in a cervical scrape then the viral load might be of value in predicting incidents of high-grade cervical intraepithelial neoplasia (CIN3)[6-7].

At present, a standard screening program for cervical cancer has been implemented effectively across the country, using the cytology method or Pap smear. However, its methods show large false positives of up to 40% and false negatives at 5-14%[8-10]. Furthermore, an additional problem is that there are a large number of unclear Pap smear results with borderline results[11-12], causing difficulty in patient management. Such Pap smear results are cytological in findings, thus distinguishing between the classes of: normal, abnormal (low grade or high grade squamous intraepithelial lesion, LSIL or HSIL) and inconclusive or borderline (atypical squamous cells of undetermined significance, ASC-US). They will be tested for histology classification for a conclusion, which usually consist of precancerous lesions (CIN) based upon their severity; this is graded from CIN1 (mild dysplasia), CIN2 (moderate dysplasia) and CIN3 (severe dysplasia). LSIL generally corresponds to CIN1 and HSIL indicates CIN2 or CIN3.

A need to implement new technology is required more and more, as several studies have established testing for the presence of HPV DNA in cervical scrapes to be used for an adjunct or combined Pap smear test for triaging equivocal Pap smears, for the reason that positive HPV test results help distinguish patients who have unclear Pap tests to be closely followed-up[13-15]. Usually HPV testing is established to detect HPV infections that are associated with or develop into ≥CIN2, or have sensitivity against CIN2 or worse (≥CIN2)[13]. Therefore, our purpose is to clinically validate the sensitivity and specificity of the in-house HPV-16 DNA quantitative real-time PCR assay test, compared to a colposcopy-directed biopsy as a reference method, and to validate the reliability of the test and evaluate if viral load can be associated with the virulence of precancerous lesions.

Materials and Methods

Materials

Cervical scrape samples were collected from 65 out-patients aged 35-60 years with borderline Pap test results, from the Gynecological Ward at Bhumibol Adulyadej hospital, Bangkok, Thailand, and also collected from 119 normal women aged 35-60 years who presented an annual Pap smear screening between 2010 and 2012. The study's proposal was approved by

the Institute Ethic Committee at the Department of Medical Sciences, Ministry of Public Health, Thailand. All women were informed, and a written consent form was taken from all participants before participating in the study.

Methods

Study Design

All cervical samples were taken using quantitative HPV-16 DNA real-time PCR. Patients who had borderline Pap smear results has cervical scrapes collected for HPV- 16 DNA testing, and were examined at that time for colposcopy-directed biopsy. Normal women had cervical samples collected for Pap smear and HPV testing, and were examined at that time for colposcopy.

Preparation of Clinical Specimens for HPV Detection and Colposcopy Performance

Before applying acetic acid into the grey zone area for colposcopy examination, the cervical scrape samples were collected from patients using cotton swabs. The specimens were kept in a tube containing 1 ml of transport medium with swab sticks, and stored at -70 ˚C until use. The patients were then examined via a colposcopy-directed biopsy as the reference method, and a biopsy was conducted in cases where the colposcopy results were positive. The clinical results were separated into three grades of precancerous lesion: CIN1, CIN2 and CIN3. The specimens were then extracted (Invisorb®, Hoffmann, USA) to obtain their viral DNA.

HPV DNA Quantitative Real-time PCR Testing

The HPV DNA quantitative real-time PCR amplification reaction was developed by Mark van Duin et al.7, using a primer set designed for the L1 region of HPV DNA, and this was performed along with specific TaqMan probing. A real-time PCR reaction was set up by adding 5 ml of a sample DNA template in a 25 ml of reaction mix of: Primers, Probe, TaqMan Universal PCR Master mix, with UNG and distilled water. The amplification was then carried out (Applied Biosystem, Foster city, CA, USA).

A standard curve was set up for quantitating the viral load, by plotting the serial diluting known amounts of HPV-16 pBR 322 Plasmid DNA (NIBSC, WHO), which were 5, 50, 500, 5,000 and 50,000 duplicate copies (Fig.1); and this was performed in every run of detecting HPV DNA in the specimen, showing a slope = -3.189 Y = 40.621, R^2 =0.992.

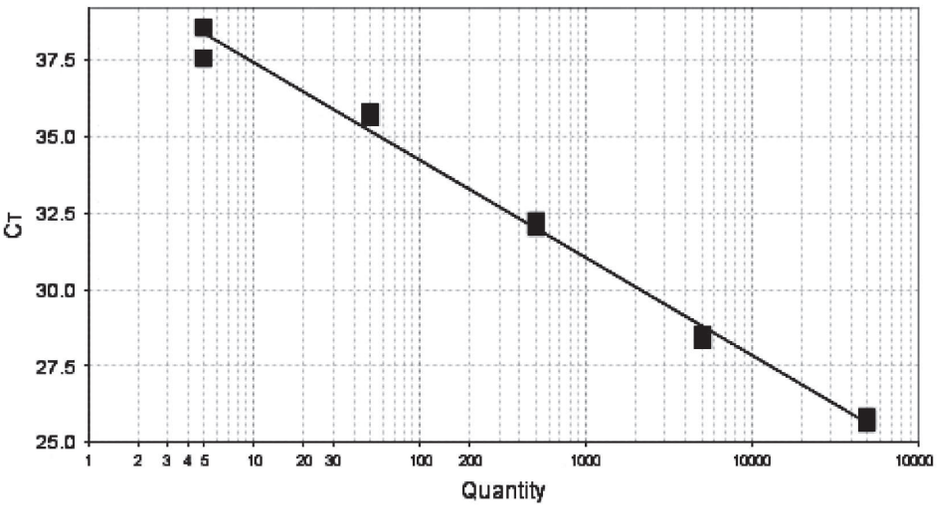

Figure 1 Standard curve plotting by the serial dilution of HPV 16 - plasmid DNA in every run for the quantitative viral load.

To control the DNA sample adequacy, the human β-globin gene was also amplified in a separate HPV reaction, by adding 5 µl of a sample DNA template into a 25 µl of reaction mix of β-globin primer7 and probe and Master mix, in the same fashion as the HPV DNA reaction mix was. A standard curve for quantitating the globin gene was also set up using human placental DNA ranging from 2, 20 and 200 fg.

For internal control, a positive control was set up using the known value of SiHA cell line containing 1-2 copies per cell, integrated with HPV-16 DNA dilution of 1:100 (approximately 7,500 copies), which served for accurate quantification of the HPV load, and which was included in every run of amplification.

Validation of the HPV DNA Quantitative Real-time PCR Testing

The clinical sensitivity and specificity of the test were determined for the detection of the confirmed pathological sign (≥CIN2), as the disease assessment by reference method for validity of the test[16].

The HPV DNA test was verified using HPV-16 pBR322 plasmid DNA as a DNA template to measure the detection limit, the linearity, cross reactivity, reproducibility and repeatability. The detection limit was performed by making ten times dilution of the known amount of plasmid DNA from 50,000 - 5 copies. The reproducibility was performed by testing the plasmid DNA 1000 fg and 10 fg 5 times between runs, while the repeatability was completed intra-run by repeating those concentrations 5 samples at a time. The linearity of the test was determined by its regression oefficient of the standard curve included in every test run. For other microorganisms that colonize the genital tract, such as Chlamydia trachomatis, Herpes simplex virus 1-2 were tested for cross-reaction or analytical specifications.

Statistical Analysis

The clinical sensitivity and specificity were calculated using the 'Table 2 by 2 Method', and the 'Mann-Whitney' test was used to test the differences in viral load.

Results

Out of 65 cases of borderline results, 45 cases were HPV-16 DNA positive (69.2%). Only 14/65 cases had precancerous lesions (≥CIN2), or were positive for a colposcopy-directed biopsy (Table 1), whereas CIN1 had 11 cases whose symptoms were usually mild and disappeared without treatment.

Table 1 The amount of precancerous lesion and no lesion with HPV infection

HPV testing	No precancerous lesion	Precancerous lesion	
		CIN1	≥CIN2
Neg	15	3	2
Pos	25	8	12
total	40	11	14

For clinical sensitivity, out of the 14 ≥CIN2 using the reference method, HPV-16 DNA was detected in 12 cases (86%) using in-house quantitative real-time PCR. For the specificity, out of 96 normal women with CIN2 negative by the reference method, 76 cases were negative (80%) for HPV-16 DNA (Table 2).

Table 2 Clinical sensitivity and specificity of the in-house real-time PCR for detecting HPV DNA 16

In-house HPV DNA testing	Reference test *		Total
	≥CIN 2	Normal	
positive	12	20	32
negative	2	76	78
total	14	96	110

*Reference test: colposcopy - directed biopsy

The verification of tests was examined. The detection limit was tested by calculating the genome equivalent to original viral load of 50,000 copies, and making a serial ten times dilution; the final dilution was detected at 5 copies/reaction. For reproducibility, the testing of five replicates of 10 and 1000 fg of HPV-16 pBR322 during the day (in 5-day testing) showed 5.21% CV and 11.89% CV, respectively. Repeatability of HPV-16 pBR322 detection of five replicates with 10 and 1000 fg intra-run (in a same day) showed 13.40% CV and 7.20% CV, respectively. Linearity was determined by the regression coefficient = 0.991-0.999. There was no cross-reactivity of Chlamydia trachomatis and Herpes simplex 1-2 in this method.

The specimens in this study were detected in ranges from 5 copies to more than 50,000 copies. There was no statistical significant difference (p = .433) in viral load between CIN 1 and ≥CIN2 using the Mann-Whitney test (data not shown).

Discussion

This study showed clinical validation of the HPV-16 DNA real-time PCR testing in cervical scrapes in women aged 35-60, in order to help diagnose borderline Pap smear results. The sensitivity and specificity of this in-house testing was the first study in Thailand that was set up with patients with abnormal Pap smears and normal women who were confirmed with precancerous lesions using a colposcopy-directed biopsy as the reference method. According to the validation guidelines[13], the HPV-16 PCR test results were compared to the end stage of disease (≥CIN2) by reference method, and showed a sensitivity of 86% in detecting disease, which correlates with a study of Tsiodras et al.[17], whose sensitivity showed 80% detection of CIN2 and 100% of CIN3, which would imply 86% detection of ≥CIN2. Furthermore, in seven other studies which conducted meta-analyses, their pooled sensitivity of the PCR system for CIN2+ was 80.9[15] which was lower than our study. Additionally, another system of detection using 'Hybrid Capture II' showed a sensitivity of ~ 90%.

However, for specificity our 80% result was quite low, considering others showed more than 90%. This could be a result of the different primers and the test performance used in the detection system. We also found that the HPV positive rate of normal colposcopy-confirmed women was 21% while another study[18] showed 20% in normal cytology samples. This showed that the prevalence of HPV infection of the normal population was quite the same in different areas.

In our samples with borderline results, the HPV-16 positive rate was 69%, while the other showed 50%; hence, this was quite different from ours even though the detection included 11 types of HPV probe. This may be associated with the differences of abnormal smear samples studied.

The reproducibility and repeatability showed the data with no more than 15% CV considering the reliability of the assay. The viral load of the in-house real-time PCR test did not differentiate CIN1 from CIN2/3, which was quite similar to the results from the study of Schmitt et al.[19] and Zerbini's study 20, which showed that mean viral loads in LSIL and HSIL were not significantly different.

Conclusion

HPV infection was found in the majority of precancerous lesions population, and was associated with CIN2/3 and invasive cervical cancer. The clinical sensitivity and specificity of the tests against the disease displayed at 86% and 80%, respectively. This HPV-16 DNA real-time PCR test might be useful for health care practices as an adjunct to cytology follow-ups for women with borderline results.

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ประเมินวิธีการตรวจหา HPV-16 DNA ด้วยวิธี Quantitative Real-Time PCR

สุขใจ ผลอำไพสถิตย์* พิไลลักษณ์ อัคคไพบูลย์ โอกาดะ* ถนอมศิริ สุนทรธรรม วรวรรณ ศิริชัย** วีระ สุรเศรณีวงศ์** สมรัตน์ เลิศมหาฤทธิ์*** และสมชาย แสงกิจพร***

**กรมวิทยาศาสตร์การแพทย์ ถนนติวานนท์ นนทบุรี 11000*

*** โรงพยาบาลภูมิพลอดุลยเดช ถนนพหลโยธิน กรุงเทพฯ 10220*

****จุฬาลงกรณ์มหาวิทยาลัย พญาไท ปทุมวัน กรุงเทพฯ 10330*

บทคัดย่อ ไวรัส HPV เป็นสาเหตุการเกิดโรคมะเร็งปากมดลูก จัดเป็นอันดับสองของโรคมะเร็งในหญิงไทย HPV-16 เป็นชนิดที่มีความเสี่ยงต่อการเกิดมะเร็ง ตรวจพบมากในคนไข้มะเร็งปากมดลูก การตรวจคัดกรองมะเร็งปากมดลูก มักพบการรายงานผล Pap smear ผิดปกติ ผลก้ำกึ่ง เป็นจำนวนมาก การศึกษานี้เป็นการประเมินวิธีหาความไว ความจำเพาะของวิธี เปรียบเทียบกับวิธีอ้างอิงมาตรฐาน เพื่อใช้เป็นวิธีตรวจเพิ่มเติมในคนไข้ที่มีรายงานผลไม่ชัดเจน และปริมาณของไวรัสบอกความรุนแรงของโรคหรือไม่ ทำการเก็บตัวอย่างจากคนไข้ที่มีผลก้ำกึ่งด้วยวิธี Pap smear 65 ราย จากหน่วยนรีเวชกรรม โรงพยาบาลภูมิพลอดุลยเดช และหญิงปกติที่มารับการตรวจ Pap smear ประจำปี จำนวน 119 คน เข้าร่วมในการศึกษาระหว่างปี 2553 - 2555 ทุกคนจะได้รับการตรวจยืนยันด้วยวิธีอ้างอิงมาตรฐาน เก็บตัวอย่างเยื่อบุปากมดลูก ตรวจหา HPV-16 DNA ด้วยวิธี real-time PCR ใช้ primer ขยายปริมาณ DNA ใน L1 region และตรวจจับด้วย specific probe ประเมินหาความไว ความจำเพาะของวิธี ในการตรวจหา HPV-16 DNA ในรอยโรคก่อนมะเร็งระยะที่ 2 ($\geq$CIN2) จากผลการศึกษาคนไข้ที่มีผลแบบก้ำกึ่ง 65 ราย มีผลบวกต่อ HPV-16 จำนวน 45 ราย (69.2%) พบ 14 ราย เป็นรอยโรคก่อนมะเร็งระยะที่ 2 ($\geq$CIN2) แต่มีเพียง 12 ราย ที่มีผลบวกต่อ HPV-16 DNA ความไวของวิธี เป็น 86% (12/14) ในจำนวน 96/119 ราย ไม่พบเป็น CIN2 ด้วยวิธีอ้างอิงมาตรฐานและไม่พบการติดเชื้อ HPV ใน 76/96 ราย ความจำเพาะของวิธีเป็น 80% พบว่าปริมาณของไวรัสไม่สัมพันธ์กับความรุนแรงโรค วิธีนี้มีประโยชน์ใช้ตรวจเพิ่มเติมเมื่อพบผล Pap smear ผิดปกติ เพื่อติดตามคนไข้