Value of Combined use of HPV DNA Analysis and Liquid Based Cytology for Cervical Cancer Screening

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ABSTRACT

Objectives: To evaluate the value of PCR HPV test with Thinprep cytology test for detection of cervical human papillomavirus changes.

Method: Seventy women were studied. All patients were subjected to speculum examination followed by thin-prep cytology test and Multiplex PCR HPV analysis.

Results: ThinPrep cytology result test was NILM in 12 (17.1%) patients, 36 (51.4%) patients had ASC-US, 20 (28.6%) had (LSIL) and 2 (2.9 %) had HSIL. PCR HPV was negative in 26 (37.2%) patients, 24 (34.3%) patients had low risk HPV types, 10 (14.3) patients had HPV 16, 5 (7.1%) patients had HPV 18 infection. ThinPrep cytology result was significantly related to conventional PAP smear result (P =0.030) and PCR HPV result (P=0.000). ThinPrep test was more sensitive but less specific than PCR HPV test.

Conclusion: The combined use of ThinPrep cytology test and PCR HPV test can play a significant role towards accurate diagnosis and screening of cervical cancer.

Keywords
Human papillomavirus (HPV), Cervical cancer screening, Conventional PAP Smear, ThinPrep cytology test, PCR HPV test.

Introduction

Cervical cancer is considered the third most common cancer among women worldwide with 52% mortality incidence ratio [1]. In Saudi Arabia, cervical cancer ranks the twelfth among all females’ cancers and occurs in 2.4% of all new cases [2]. In Middle Egypt with a regional registry in Minia, the incidence of cervical cancer is 1.06% of cancer sites in females [3].

Clinical and epidemiological studies have reported that sexually transmitted infection with human papilloma virus (HPV) is a well-established cause of cervical cancer. Moreover, rates of infection are increasing [4]. In general, HPV is thought to be responsible for more than 90% of cervical cancers. There are more than 200 genotypes of HPV and 40 genotypes were responsible for anogenital infections [5]. According to their oncogenic potential, they are classified into low, high and intermediate risk types. Of these, four are most often associated with precancerous and cancerous lesions of the cervix, type 16, 18, 31, and 45. Among them, HPV 16 and 18 genotypes, are considered the most oncogenic types and are frequently reported in cervical squamous cell carcinoma and adenocarcinoma. Infection by multiple genotypes of HPV has been detected in 10-20% of HPV-positive cases [6]. In Egypt, approximately 78.4% of invasive cervical cancers are due to infection with HPV 16 or 18 [7].

Infection with HPV can be diagnosed by the gross appearance of lesions, cytology, histopathology, and colposcopy. All the aforementioned methods are subjective, with limited sensitivity and are inaccurate. In addition, serology is unreliable especially in discrimination between recent and old infection [8]. On the other hand, immunocytochemistry and molecular methods can help in better screening and diagnosis of cervical cancer especially when
combined with the Pap smear [9].

Liquid-based cytology (LBC) as ThinPrep Pap test is considered more useful than the traditional Pap smear for the diagnosis of cervical squamous intraepithelial lesions (SIL). Moreover, the residual sample in ThinPrep Pap test can be used for further testing for HPV DNA testing [10].

Testing of HPV DNA for cancer-associated HPV DNA is considered valuable in the diagnosis of equivocal cervical cytological findings and also for asymptomatic women without cytological abnormalities [11]. However, the methodology and sensitivity of HPV DNA testing in cervical cancer still requires further studying. Therefore, the aim of this study is to find out the incidence of HPV types and their correlation with cytology findings especially ASCUS samples and to test the validity of the combination between HPV testing and ThinPrep test for early detection and so prevention of cervical cancer.

Materials and Methods
The present retrospective study included 70 patients. A computerized search identified patients with conventional PAP smear results, ThinPrep-cytology results and HPV DNA positive results from January 2017 to January 2018 attending king Faisal hospital, KSA from Gynecology outpatient department of the hospital. The study design was in line with the ethical standards of the committee responsible on human experimentation and with the revised Helsinki Declaration of 2008. The study age range between 23 -53 years and other clinical data were collected. All patients were subjected to speculum examination followed by conventional PAP smear, Thin-cytology test, and Multiplex PCR HPV analysis.

Inclusion Criteria
The present study included patients with a complete medical record; aged more than 22 years; new patients and patients with abnormal previous result either in conventional PAP smear, thin-cytology test or PCR HPV analysis.

Cytological Screening
For liquid based cytology; all samples were prepared using a ThinPrep 2000 processor (Hologic Inc., Marlborough, MA 01752). The ThinPrep system for Pap specimens is FDA approved. PreservCyt specimens were collected by inserting a cytobrush into the endocervical canal. The cytobrush was immediately placed in a vial of PreservCyt transport medium (ThinPrep Pap Test; Cytyc Corporation, Boxborough, Mass.). PreservCyt vial is then capped, labeled, and sent to a laboratory equipped with a ThinPrep processor. These specimens were stored at 15 to 20°C and transported to the laboratory within 24 hours of collection. The ThinPrep processor homogenizes the sample by spinning the vial (T3000), creating shear forces in the fluid that are strong enough to disaggregate randomly joined material, break up blood and mucus while keeping cell clusters intact. The cells are then collected onto the membrane of the TransCyt filter and transferred onto a glass slide to create a monolayer deposit of cells, ~20 mm in diameter. The slide is then ejected automatically into a fixative bath of 95% ethanol and then stained with Papanicolaou stain. With regards to specimen adequacy; cellularity is assessed easily in a liquid-based preparation, either by comparison with reference images or by counting well-preserved squamous cells in a specific number of fields at high power (x400 power field) (Number of cells per x40 high-power field 3.8 cells per high-power field = 5000 cells with ThinPrep). Cytological reports were formulated according to the Bethesda System, including evaluation of correct sampling of the transformation zone (presence of metaplastic and/or columnar cells). PreservCyt specimens were stored at 15 to 20°C for as long as 6 weeks, in case the sample had to be retested.

PCR HPV Analysis
PreservCyt specimens were tested by PCR within 1 week of collection. The PreservCyt transport medium containing residual endocervical cells was vortexed vigorously. DNA sequence files for HPV types 6, 11, 16, 18, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66 and 68 were obtained from Genbank (http://www.ncbi.ni.gov/genbank/). Primers were designed for each HPV type with confirmed specificity by BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) and appositive control of a primer pair specific for β-globin was included.

The QIAGEN Multiplex PCR kit (Qiagen, Germany) was used in all Multiplex PCR reactions, according to manufacturer instructions. Each PCR was done in a DNA thermal cycler (MaxyGene Gradient Thermal Cycler, Axygen Scientific, USA) with the following conditions: initial denaturing step at 95°C for 15 min, 10 cycles of 30 s at 94°C, 90 s at 65°C, and 90 s at 72°C, followed by 30 cycles of 30 s at 94°C, 90 s at 63°C, and 90 s at 72°C, with a final extension at 72°C for 10 min. PCR products were tested by electrophoresis on a 2% agarose gel stained with ethidium bromide. The band sizes were detected by comparison with a 100 bp molecular weight marker (GeneRuler 100bp DNA Ladder, Fermentas International, Canada), and gels were photographed in a UV transilluminator (UVP, USA) with a Canon PowerShot A60 digital camera (Canon, USA). HPV type was resulted based on the amplification pattern. Some PCR amplified fragments were selected to be cloned into a pGem-T vector (Promega, USA). Then each cloned product was sequenced with universal primers (forward and reverse) to confirm fragment identity. After that, the selected amplified fragments were prone to digestion with restriction enzymes Alul, HaeIII, RsaI, or Mspl (New England Biolabs, USA). The digestion patterns were seen in a 2% agarose gel to also confirm fragment identity. As mentioned previously, HPV consensus PCR was tested using special primers; PGMY09/PGMY11, that were constructed to amplify a fragment of the HPV L1 gene of nearly 450bp [12]. HPV genotype was detected by sequencing the amplified fragments using primers PGMY11.

Aagarose gel electrophoresis of 5-plex PCR amplification products was used for identification of HPV-types (Lane M shows ferments 100 bp DNA molecular marker, Lane 1 is positive sample for HPV 33, Lane 2 is positive sample for HPV 16, Lane 3 is positive sample for HPV 18, Lane 4 is positive sample for HPV 31, Lane

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5 is positive sample for HPV 45, Lane 6 is positive sample for all five types of HPV, Lane 7 is positive sample for HPV 18 and 45, Lane 8 is positive sample for HPV 18, 31 and 45, and Lane 9 is negative control contain HPV 54). HPV 6, 11,42, 43 and 44 are considered low risk types, HPV 16 and 18 are high risk types and HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 are among other types.

Interpretation of Cytological Results
The slides were screened and interpreted by cytopathologist and classified according to the 2014 Bethesda System for reporting [13]. The Bethesda system included the following terms: negative for intraepithelial lesion or malignancy, ASCUS (atypical squamous cells of undetermined significance), ASC-H (atypical squamous cells, cannot exclude high grade squamous intraepithelial lesion), LSIL (low grade squamous intraepithelial lesion), and HSIL (high grade squamous intraepithelial lesion), or squamous cell carcinoma. All the screening and diagnosing of the smears were done without the data of HPV results.

Statistical Analysis
Statistical analysis was performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) statistical software. Chi-square test was used to determine the significance of any differences between the ThinPrep-cytology test results and HPV DNA test results and clinical data. A P-value <0.05 was considered statistically significant.

Results
Regarding patients' clinical data; the mean age (SD) is 34.6 + 0.923, the median age (SD) is 32.5 + 7.7. The age range is between 23 years and 53 years. Age is grouped according to its median value into 2 groups one below 32 years (includes 36 patients) and the other is equal or more than 32 years (includes 34 patients). Regarding marital status; 22 patients are single and 48 patients are married. Among married patients 37 are multiparous and 10 patients use contraceptives.

ThinPrep cytology result test was negative for intraepithelial lesion or malignancy in 12 (17.1%) patients, 36 (51.4%) patients had atypical squamous cells of undetermined significance (ASC-US), 20 (28.6%) had a low grade squamous intraepithelial lesion (LSIL) and 2 (2.9 %) had a high grade squamous intraepithelial lesion (Figure 1). Regarding PCR HPV result, it was negative in 26 (37.2%) patients, 24 (34.3%) patients had low risk HPV types, 10 (14.3%) patients had HPV 16, 5 (7.1%) patients had HPV 18 infection and other types were identified in 5 (7.1%) patients. Overall, 63 (90%) patients manifest HPV infection by a single genotype and 7 (10%) by dual HPV genotypes. Among women with dual genotypes there were 5 patients with combined HPV16 and other types; stratified as 2 with HPV16 and either low-risk types and other types, one with HPV18 infection. The remaining 2 showed combined infection with HPV18 and low-risk types.

There was no statistically significant relation between ThinPrep cytology result and any of the clinical data regarding age, marital status, gravidity or contraception. On the other hand, ThinPrep cytology result was significantly related to PAP smear result (P =0.030).

HPV infection was significantly related to the age (P=0.008). The prevalence of HPV 16 was more in age ≥ 32 years, whereas HPV 18 was more in age younger than 32 years. But, the overall prevalence of high-risk HPV types was significantly higher in women aged <32 years than ≥ 32 years age (P=0.008). HPV infection was not related to conventional PAP smear results (P=0.456). A highly significant relation between ThinPrep cytology result and PCR HPV result was found (P=0.000). All HSIL patients have HPV16 and 18 infections (Table 1).

The percentage of patients who have atypical squamous cells of undetermined origin ASC-US on conventional PAP smear was 15.7% and it was upgraded by ThinPrep test up to 51.4%. Twenty-four from thirty-six (66.7%) patients that were diagnosed as ASC-US by ThinPrep test show negative results for HPV infection by PCR HPV test. Whereas, all patients diagnosed as HSIL in
ThinPrep test showed HPV 16,18 infection and 6/20 patients diagnosed as LSIL showed HPV 16,18 infection (Table 2). The sensitivity of the ThinPrep test was 75% and that of PCR HPV test was 56.9%. But the specificity of PCR HPV test was more. Therefore, the combined use of ThinPrep cytology test and PCR HPV test can play a significant role towards accurate diagnosis especially for patients diagnosed as ASCUS.

Regarding cases in the present study, full data about subsequent histological follow up for all patients is not available.

**Discussion**

Human papillomavirus (HPV) is a well-established cause of most cervical cancers. So that, lots of interest is directed towards detection of HPV in cervical cancer prevention programs in combination with cytological screening especially for cervical dysplasia. Several trials towards more sensitive and specific cytological tests for HPV detection have been made. Therefore, the evolution of liquid-based gynecologic specimen has resulted. Liquid-based preparations become superior to conventional smears because of improved fixation; standardizations of cell transfer and decreased obscuring factors. Another advantage of liquid-based cytology is that the remaining specimen could be stored and used for immunostaining and HPV DNA testing [14].

The percentage of unsatisfactory smears are high with conventional PAP smears as up to 90% of the scraped materials from the cervix may be discarded. On the other hand, liquid-based cytology could reduce unsatisfactory specimens to 2.6% [15].

HPV DNA has gained lots of attention due to its higher sensitivity and detection of “high-risk” HPV types that most commonly affect the cervix [16]. HPV DNA test is considered an effective way to identify high-risk HPV types. HPV test has been approved by the FDA to follow-up women with equivocal cytology results and for the screening of woman more than 30 years of age [17].

The main aim of the present study is to assess the accuracy of HPV DNA testing in conjunction with Thinprep cytology test as a screening tool for detection of HPV infection. The present study revealed that HPV infection was significantly related to the age. HPV infection was higher in age <32 years (23/36; 63.9%) than in age ≥32 (8/34; 23.5%) especially for HPV 18. It was found that the peak of HPV infection was observed in 25-35 years old women.
where the prevalence rate was significantly higher than in older women (60.7% vs 45.4%) [18]. This finding reflects a higher risk in young sexually active women who usually have a transient and asymptomatic infection, with a spontaneous HPV clearance in 70-90% of cases within 12-13 months [19].

In the present study, there is a trend towards the presence of cellular abnormalities in younger age as 58.3% patients (21/36) had ASCUS and all patients with HSIL were younger than less than 32 years. On the contrary, it was reported that cytological abnormalities did not change with age, especially among women over 30 years [20]. While others suggested an age-stratified approach for the use of

HPV testing in the triage of women 45 to 50 years and older with low-grade abnormalities [21]. There was a significant association between age and HPV infection in the current study (p=0.008). Similarly, another study found that age is significantly associated with an increased risk of HPV infection. They reported that young sexually active women show considerable fluctuation in their HPV status and HPV type overtime and women <35 years of age are likely to acquire genital infections with oncogenic HPV with a risk of developing cancer [22].

Overall, 90% of patients of the current study manifest HPV infection by a single genotype and 10% by dual HPV genotypes and HPV16 was more prevalent than HPV18. A highly significant relation between ThinPrep cytology result and PCR HPV result was found (P=0.000). All HSIL patients have HPV16 and 18 infections. A previous study reported that the mean prevalence of HPV infection in 8610 women with normal cervical cytology was 12.6%, with HPV16 being the most frequent HPV type. The overall HPV DNA prevalence in women with high-grade cervical lesions was 78.1%. HPV DNA was found in 86.6% of cervical cancers and the combined prevalence of HPV16/18 among HPV positive cases was 87.5% [23].

It was reported that HPV-16 was predominant among women affected by H-SIL, confirming that HPV-16 is the most frequent HPV type associated with high-grade lesions [24]. Many studies found that HPV-52 was the most common genotype being present in all HPV DNA positive women [25,26]. Moreover, Worldwide HPV-52, together with HPV-16, HPV-18, HPV-31, HPV-58 is considered one of the five most common types contributing to the 50% of all HPV infections [5].

The current study revealed that the percentage of patients who have atypical squamous cells of undetermined origin ASC-US on conventional PAP smear was 15.7% and it was upgraded by ThinPrep test up to 51.4% and 33.3% of them have HPV infection by PCR HPV test. Whereas, all patients diagnosed as HSIL in ThinPrep test showed HPV infection. This indicated that ThinPrep test is more sensitive than conventional PAP smear. The ThinPrep test was more sensitive but less specific than PCR HPV test. So, the combined use of both tests together can be helpful for accurate diagnosis especially for patients diagnosed as ASCUS.

Approximately 50% of ASCUS specimens demonstrate HR HPV infections [27]. Another study reported that in a study designed to evaluate the sensitivity and accuracy of the HPV DNA test in conjunction with Thinprep cytology test as a screening method of human papillomavirus (HPV) infection. This study has confirmed that the sensitivity and accuracy of detecting CIN and cancer are raised when HPV DNA test is done in conjunction with thin prep cytology test [28]. A previous study compared between HPV testing and cytologic testing as screening tests in women age more than 30 years; HPV DNA testing was 94.6% sensitive in detecting CIN 2 or CIN 3, compared with 55.4% for cytology [29].

Regarding comparing the sensitivity of Multiplex PCR and pap smear in the detection of HPV type. It was detected that more sensitivity of Multiplex PCR as compared with pap smear results [30]. It was reported that concerning the association between HPV and cytological results, overall 46.9% of women were HPV infected and 93.7% of them harbored HR-HPV genotypes [21]. According to current studies, the presence of HR-HPV was significantly higher in women affected by L-SIL and HSIL than in those affected by ASCUS, strengthening the role of persistent infection sustained by HR genotypes in cervical lesions. It was found that HPV testing is more sensitive than cytology, while cytology is more specific [31]. Several studies have reported slightly higher sensitivity and a lower specificity of LBC for detecting any degree of CIN [28,32].

**Conclusion**

The combined use of HPV DNA test and Thinprep cytology test are more effective in diagnosing patients with ASCUS and SIL and screening of cervical cancer which can be later confirmed by cervical biopsy. Further studies are needed on a larger number of patients.

**References**