Molecular testing of human papillomavirus in cervical specimens

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ABSTRACT

Objectives: To improve the diagnosis of cervical neoplasia by early detection of human papillomavirus (HPV) in uterine cervix, by adding molecular testing of HPV using hybrid capture 2 (HC2) and polymerase chain reaction (PCR) tests to Papanicolaou (Pap) test.

Methods: One hundred women were enrolled in this study. The mean age (mean ± SD) was 41.97 ± 8.76 years and the range was 27-65 years. All women had undergone cervical cytological screening with cervical cytology, HPV DNA testing by HC2 and PCR, during the period from January - December 2006, at King Abdul-Aziz University Hospital (KAAUH) and King Fahd Research Centre, Jeddah, Saudi Arabia.

Results: The results we obtained by HC2 for detection of HPV were 5 (5%) high-risk HPV, one low-risk HPV (1%) and 94 (94%) negative cases. The PCR detected only 4 (4%) cases. Using the HC2 test as a reference, the sensitivity, specificity, positive predictive, negative predictive values and accuracy of baseline Pap were 50, 85, 17.7, 96.4, and 83%; of final Pap smear were 100, 96.8, 66.7, 100, 97%, and for PCR were 66.7, 100, 100, 97.9, and 98%. The Pap test was repeated within a year for patients with abnormal Pap or negative Pap test with positive HPV DNA.

Conclusion: Combined screening by cytology and HPV testing using both HC2 and PCR sensitively detects women with existing disease. The absence of HPV DNA provides reassurance that patients are unlikely to develop cancer for several years. We suggest using Pap with HC2 and PCR in screening programs to ensure that women with the double negative result at baseline might safely be screened at longer intervals.

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Cervical carcinoma is a progressive disease that begins with a non-invasive intraepithelial lesion, designated as squamous intraepithelial lesion (SIL) or cervical intraepithelial neoplasia (CIN). The advent of Papanicolaou (Pap) smears, which provide early detection of cervical cancer precursor lesions in women, has led to a significant decrease in deaths due to cervical cancer in countries where there is a good access to health care. In western region of the Kingdom of Saudi Arabia, many studies were performed to evaluate the importance of cervical Pap smears to detect the prevalence of abnormal Pap smear and types of abnormalities by studying retrospectively cervical smears results.[1-7] These studies showed that CIN and invasive cervical carcinoma (ICC) are less common in the Saudi Arabian community compared to the western countries. Studies show that human papillomavirus (HPV) DNA is found in 99.7% of all cervical carcinomas and cells derived from that cancers.[8] The recognition of high-risk HPV as etiological agents of cervical cancer have increased the demands to use testing for HPV for the detection of abnormal cervical smears and for cervical cancer screening.[9] American Cancer Society Guidelines make preliminary recommendations of HPV DNA, and this test may be used with a Pap test for routine screening in women ≥30.[10] A negative HPV test and a normal baseline Pap result give confidence that a woman does not have, and are unlikely to develop, high-grade cervical disease or cancer within the next 3 years as suggested by preliminary recommendations from American Cancer Society.[11] The aim of the present study was to determine the reliability of the detection of HPV DNA by both hybrid capture 2 (HC2) tests, which are a standardized test, approved by the Food and Drug Administration and have been in routine clinical use for more than 5 years in Western countries, and polymerase chain reaction (PCR), in a series of cervical smears. Another aim was to evaluate the significance of multiple Pap tests to screen cervical cellular abnormalities.

Methods. In this study, Digene's HC2, HPV DNA test and PCR were used for HPV infection detection

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as a cancer cervical screening test in conjunction with the Pap test in 100 sexually active women with an age range of 27-65 years visiting the Obstetric and Gynecology clinics at King Abdul-Aziz University Hospital (KAAUH), Jeddah, Saudi Arabia for different gynecological problems. An informed consent was obtained from each patient after an approval of experimental protocol by the Medical Ethics Committee at KAAUH. Three cervical samples were collected. Sample 1 was taken for Pap smear using cytobrushes, and then the specimen was smeared on a glass slide and fixed immediately with alcohol.[12,13] Cytological results were classified according to the 2001 Bethesda system in the Pathology laboratory at KAAUH.

Sample 2 was taken for HC2 test using the Digene's cervical sampler. The sample was then placed into the Digene liquid collection medium and transported to the Virology laboratory for testing using Digene HC2, HPV DNA test. The HC2 assay was performed according to the manufacturer instruction (Digene Corporation). The principle of the procedure of HC2, HPV DNA test amplifies the hybridization antibody capture microplate assays that utilize chemiluminescent detection of 18 types of HPV DNA in cervical specimens. The Digene's HPV, HC2 test can differentiate between 2 HPV DNA groups, low-risk HPV types 6, 11, 42, 43, 44, and high/intermediate risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68.[14] Specimens containing the target DNA hybridize with a specific HPV ribonucleic acid (RNA) probe cocktail. The resultant RNA:DNA hybrids are captured onto the surface of a microplate well coated with antibodies specific for RNA:DNA hybrids. Immobilized hybrids are then reacted with alkaline phosphatase conjugated antibodies specific for the RNA: DNA hybrids, and detected with a chemiluminescent substrate. Several alkaline phosphatase molecules are conjugated to each antibody. Multiple conjugated antibodies bind to each captured hybrid resulting in substantial signal amplification. As the substrate is cleaved by the bound alkaline phosphatase, light is emitted which is measured as relative light units (RLUs) on a luminometer. The intensity of the light emitted denotes the presence or absence of target DNA in the specimen. All quality control calculations were performed by the Digene Hybrid Capture System version 2 (DHCS version 2) Software and DML 2000 Instrument.

Sample 3 was collected with a brush and transported into a sterile phosphate buffer saline, then proceeded to test for HPV DNA by PCR in the Biosafety Level 3 laboratories at King Fahd Medical Research Centre, using consensus HPV primers MY09/MY11. In this study, the HC2 test and PCR were used for HPV detection in conjunction with the Pap test as cancer cervical screening. Pregnant women were excluded from this study.

Table 1 - Results of baseline Pap smear of 100 patients in different groups.

Negative for intra-epithelial lesion or malignancy (%)	**Percentage**
Within normal limit*	80
Abnormal cells with benign changes	
Inflammatory smear	4
Normal and atrophic changes	2
Degenerative changes and mild atypical features	1
Intra-epithelial cells abnormalities	
Abnormal with Atypical cells detected	
ASC-US	3
AGC	5
Atypical endocervical cells with reactive changes	1
Abnormal cells with significant changes	
HSIL	2
Koilocytotic changes as (LSIL) suggesting HPV infection	2
Cancer cells	0

*negative for intraepithelial lesion or malignant cells, ASC-US - Atypical squamous cells of undetermined significance, ASC-H - atypical squamous cells cannot rule high-grade dysplasia, AGC - atypical glandular cells, HGSIL - high grade squamous intraepithelial lesion, LSIL - low-grade squamous intraepithelial lesion

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Results. **Table 1** demonstrates that no intra-epithelial lesion or malignant cells were obtained by baseline Pap smear examination in 80 cases (80%). Different abnormalities were showed in the remaining 20 cases (20%). The baseline and the follow-up Pap tests in patients with significant abnormal Pap results or positive HPV DNA in 21 patients were described in **Table 2**. The final cytological changes in the 100 study cases after second and third Pap smears are distributed in different groups and described in **Table 3**.

Table 4 showed the relation of Pap tests with significant cellular changes or atypical cells and negativity by HC2 and PCR tests. The 13 cases had no HPV DNA detected by both HC2 and PCR. Meanwhile, some of these 13 cases had serious cytological changes as atypical squamous cells of undetermined significance (ASC-US) and atypical glandular cells (AGC). Ten out of these 13 cases turned to be within normal limit (WNL) within almost a year. The PCR amplified products of positive specimens are visualized by ultraviolet (UV) illumination following ethidium bromide staining (**Figure 1**). The relation between HC2, PCR, and Pap test of the 6 positive HPV specimens are described in **Table 5**. Quantitative results of HC2 test in high-risk HPV positive patients were illustrated in **Table 6**, while that in low-risk HPV positive patients were illustrated in **Table 7**. A cross tabulation between grouped cases of Pap smear, HC2, and PCR results for detection of HPV in our study was described in **Table 8**.

Table 2 - Follow-up of Pap test in patients with significant abnormal Pap results or positive human papillomavirus (HPV) DNA, (hybrid capture 2 [HC2]) or polymerase chain reaction (PCR) (n=21).

Serial no.	Patient no.	Pap 1	Pap 2	Pap 3
1	14*	Inflammatory smear	Marked inflammation with reactive cellular changes	Marked inflammation with reactive cellular changes
2	18†	WNL	Marked inflammation with reactive cellular changes	Marked inflammation with reactive cellular changes
3	19	AGC	WNL	WNL
4	20	AGC	WNL	WNL
5	24	ASC-US (with previous treated cancer breast)	Few atypical squamous cells. ASC-H Candidate hyphae seen	ASC-US
6	31	LSIL suggesting HPV infection	WNL	WNL
7	46	Squamous cell carcinoma, stage II: Treated with radiotherapy (WNL)	WNL	WNL
8	47	HGSIL	WNL + biopsy: normal	WNL
9	52	Degenerative changes and mild atypical features	Mild reactive cellular changes WNL	WNL
10	53†	WNL	ASC-US	ASC-US
11	58	ASC-US	WNL. Satisfactory smear with excess blood and inflammation exudates	Mild inflammation. Atypical squamous cells
12	65	Atypical cells	WNL	WNL
13	66	AGC	WNL	WNL
14	71	Treatment by total hysterectomy and radiation (WNL)	WNL	WNL
15	87	ASC-US cannot rule out high-grade dysplasia (ASC-H)	ASC-H	ASC-US
16	88†	WNL	ASC-US	ASC-US
17	89	AGC	WNL	WNL
18	92†	Atypical endocervical cells	Actinomyces present Atypical endocervical cells	Atypical endocervical cells
19	95	HGSIL	WNL	WNL
20	96	AGC	WNL	WNL
21	100*	Koilocytotic changes suggesting HPV infection	Koilocytotic changes suggesting HPV infection	Koilocytotic changes suggesting HPV infection

*HPV DNA was only detected by HC2 and not by PCR (Patients number 14 and 100). †Patient having HPV DNA detected by both HC2 and PCR Tests.
LSIL - Low-grade squamous intraepithelial lesion, WNL - within normal limits, negative for intraepithelial lesion or malignant cells
ASC-US - Atypical squamous cells of undetermined significance, ASC-H - Atypical squamous cells cannot rule high-grade dysplasia
AGC - Atypical glandular cells, HGSIL - High Grade Squamous Intraepithelial Lesion

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Table 3 - Results of second or third Pap smear of 100 patients in different groups.

Negative for intraepithelial lesion or malignancy (%)	Percentage
Within normal limit*	90
Abnormal cells with benign changes	
Abnormal cells with benign changes	0
Intraepithelial cells abnormalities	
Abnormal with atypical cells detected	
ASC-US	4
AGC	2
Atypical endocervical cells with reactive changes	1
Abnormal cells with significant changes	
Atypical squamous cells	1
Koilocytotic changes as LSIL suggesting HPV infection	1
Cancer cells	0

*negative for intraepithelial lesion or malignant cells, ASC-US - Atypical squamous cells of undetermined significance, ASC-H - atypical squamous cells cannot rule high-grade dysplasia, AGC - atypical glandular cells, HGSIL - high grade squamous intraepithelial lesion, LSIL - low-grade squamous intraepithelial lesion

Table 4 - Relation of Pap tests of abnormal cells with significant changes or atypical cells and negative by hybrid capture 2 and polymerase chain reaction tests (n=13).

Serial no.	Patient no.	Nationality	Age	PAP Result	HC2	PCR
1	19	Philippine	50	Baseline Pap: AGC Final Pap: WNL	ND	ND
2	20	Philippine	49	Baseline Pap: AGC Final Pap: WNL	ND	ND
3	24	KSA	50	Baseline Pap: ASC-US Final Pap: ASC-US	ND	ND
4	31	KSA	45	Baseline Pap: LSIL with HPV infection Follow-up Pap: WNL	ND	ND
5	47	Philippine	55	Baseline Pap: HGSIL Final Pap: WNL	ND	ND
6	52	Screening	44	Baseline Pap: Degenerative changes and mild atypical features (Mild reactive cellular changes) Final Pap: WNL	ND	ND
7	58	KSA	42	Baseline Pap: ASC-US favouring changes Final Pap: Atypical squamous cells	ND	ND
8	65	KSA	43	Baseline Pap: Atypical cells Final Pap: WNL	ND	ND
9	66	KSA	38	Baseline Pap: AGC Follow-up Pap: WNL	ND	ND
10	87	KSA	50	Baseline Pap: ASC-H Follow-up Pap: ASC-H	ND	ND
11	89	KSA	50	Baseline Pap: AGC Follow-up Pap: WNL	ND	ND
12	95	KSA	28	Baseline Pap. HGSIL Follow-up Pap: WNL	ND	ND
13	96	KSA	51	Baseline Pap: AGC Follow-up Pap: WNL	ND	ND

WNL - within normal limits = Negative for intraepithelial lesion or malignant cells, ASC-US - Atypical squamous cells of undetermined significance, ASC-H - Atypical squamous cells cannot rule high-grade dysplasia, AGC - Atypical glandular cells, HGSIL - High Grade Squamous Intraepithelial Lesion, ND - Not detected, KSA - Kingdom of Saudi Arabia

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In our study, using the HC2 test as a reference (**Table 9**), the sensitivity, specificity, positive predictive, negative predictive values, and accuracy of baseline pap were calculated.

Discussion. Cervical cancer is one of the few highly preventable cancers. The early detection and removal of precancerous cervical lesions effectively abolish the development of invasive cervical cancer. Finding abnormalities as cervical dysplasia earlier is a benefit while intervention is easier. This study presents the results from a trial that compares conventionally the single and multiple Pap test with the use of HPV testing by both HC2 and PCR in 100 patients (n=100), where the age range of the positive cases is 28-52 years. The results obtained and described in **Tables 1-3** show that a single Pap smear is not a conclusive result; thus, multiple consecutive Pap smears are needed (**Table 3**). This may be affected by the quality of the specimen gathered, preservation of the specimen, or the presence of certain cells that are not typical of cancer but that still appear unusual. An inconclusive Pap smear does not necessarily mean a cervical dysplasia is present. Some other Pap smear had an irregular result, which means abnormalities in the cells, which are not always linked to cancer. Once the irritant is removed, the cells return to normal. Certain changes in the tissue may stem from inflammation caused by infections or objects present in the vagina (tampons, diaphragms, and so forth). If an irregular cytology reading is determined, case management may encompass additional clinical visits for further evaluation. Higher sensitivity levels of re-collected HPV DNA specimens increase the likelihood of identifying high-risk type HPV infections notoriously linked with cervical cancer and concurrently deter potentially unnecessary higher cost diagnostic procedures. The positive HC2 and PCR tests with the irregularity of Pap test results were demonstrated in this study among 21 patients in **Table 2**, where the follow-up of pap tests in patients with abnormal results of the baseline pap test, or HPV DNA was described. Abnormal pap tests (**Table 2**) are reported. Out of 18 abnormal pap tests, the 12 patients showed WNL pap results in the follow-up Pap tests. Three out of 6 specimens reported in baseline Pap test as WNL and having positive HPV DNA with HC2 or PCR tests (5 high-risk and 1 low-risk), showed cellular changes in the follow-up Pap tests. Most of results obtained after multiple Pap smear examination (**Table 3**) were WNL (91%) of cases, while other 9 (9%) specimens

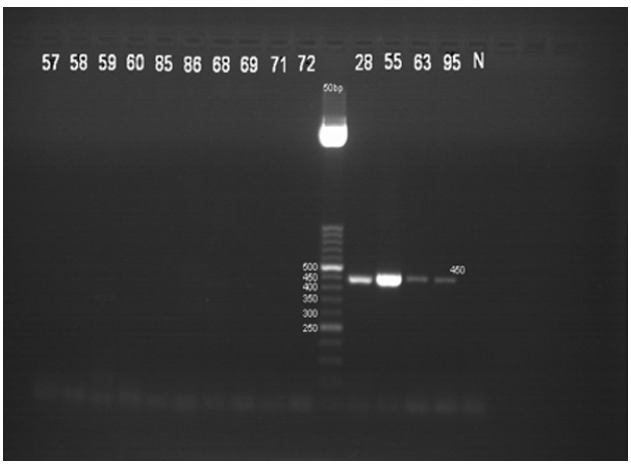

Figure 1 - Agarose gel electrophoresis of polymerase chain reaction (PCR)-amplified products of 14 different samples of the 100 study specimens. The specimens 57, 58, 59, 60, 85, 86, 68, 69, 71, and 72 were identified by PCR test as negative. These 10 negative specimens were previously identified as negative by hybrid capture 2 (HC2) test. The specimens 28, 55, 63, and 95 showed 4 bands and were identified as positive human papillomavirus. These positive 4 specimens (28, 55, 63, and 95) were also identified as positive by HC2 test.

Table 5 - Relation between hybrid capture 2 (HC2), polymerase chain reaction (PCR) results of positive human papillomavirus (HPV) samples and Pap smear.

Nationality	HC2	PCR	AGE	HC2	PCR	PAP RESULTS	
						Baseline Pap	Final Pap
KSA	14	94	27	High-risk	Not Detected	Inflammatory smear	Inflammatory smear
Philippine	18	95	47	High-risk	Detected	WNL	Marked inflammation with marked cellular changes
KSA	53	28	52	High-risk	Detected	WNL	ASC-US
KSA	88	63	30	High-risk	Detected	WNL	ASC-US
KSA	92	55	48	High-risk	Detected	Atypical endocervical cells	Atypical endocervical cells
Philippine	100	86	34	Low-risk	Not Detected	Koilocytotic cell changes suggesting HPV infection	Koilocytotic cell changes suggesting HPV infection

WNL - within normal limits, negative for intraepithelial lesion or malignant cells
ASC-US - Atypical squamous cells of undetermined significance, KSA - Kingdom of Saudi Arabia

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were of different diagnosis. We found that 100% of those who tested positive for high-risk types of HPV and reported as WNL by Pap test in the baseline test started to develop clinically significant cervical cellular abnormalities within a year. Our results showing follow-up of Pap tests of HPV DNA positive specimens (**Table 5**) correlate approximately with Manos et al[15] study on women with a history of previous normal Pap smears, where it was found that approximately 80% (100% in our study) of those who tested positive for high-risk types of HPV developed clinically significant cervical lesions within 4 years. Women with ASC-US who had a negative result for high-risk HPV DNA were recommended to have a repeat cytological testing at 12 months follow-up[16] and make a report on any cellular changes. An abnormal Pap smear results could indicate the presence of the HPV or cervical dysplasia. Several methods are used to classify severity of non-typical cellular anomalies. However, the interpretation may vary from one pathologist to another. Every year approximately 3.5 million women receive a diagnosis of abnormal cervical cytology requiring further evaluation or follow-up.[16] Meanwhile ignoring this low-grade abnormal cervical cytology is clearly dangerous; and yet performing immediate colposcopy for large number of women, which are definitely unnecessary. The Pap test is a screening test and, like any other test, it is not 100-percent accurate. False positive and false negative results as shown in **Table 2** can cause anxiety and can affect a woman's health. If the sample is not normal giving a false positive result, the patient does not have to panic. Although it can be frightening to hear that her Pap smear has been judged abnormal, she is unlikely to have cancer. An abnormal result usually means that there's a minor problem with the cervix, one that may not even need treatment. She will probably be asked to go back for either a second Pap smear or further examinations. In any event, an abnormal Pap-smear does not have to be bad news. British researchers analyzed the medical records of almost 350,000 women over 20 years and found that at least 80% of those with abnormal cervical cells of high grade dysplasia in the cervix never went or progress on to develop cancer. In the rare cases in which invasive cancer is found, there are a variety of treatment

Table 6 - Quantitative results in high-risk human papillomavirus positive patients and controls.

Patient number	Relative light units (RLU)
14	1174
18	7686
53	4138
88	1590
92	21288
Negative control - 136 RLU, Positive cut-off - 916 RLU	

Table 7 - Quantitative results in low-risk human papillomavirus positive patients.

Sub-heading	Sub-heading
Patient number 100	1188
Negative Control	102
Positive Cut-off	569.33

Table 8 - Cross tabulation between grouped cases of Pap smear, hybrid capture 2 (HC2) and polymerase chain reaction (PCR) results for detection of human papillomavirus (HPV).

Final Pap smear results	Number	HC2 results			PCR results	
		Negative (n=94)	Positive (n=6)		Not detected negative (n= 96) n (%)	Detected positive (n=4) n (%)
		n (% to group)	High-risk (n=5) n (%)	Low-risk (n=1) n (%)		
Within normal limits	91	91 (100)	-	-	91 (100)	-
Atypical cells of unknown significance	4	2 (50)	2 (50)	-	2 (50)	2 (50)
Marked inflammation with reactive cellular changes	2	-	2 (100)	-	1 (50)	1 (50)
Atypical endocervical cells	1	-	1 (100)	-	-	1 (100)
Koilocytotic changes suggesting HPV infection	1	-	-	1 (100)	1 (100)	-
Atypical squamous cells (1%)	1	1 (100)	-	-	1 (100)	-
Cancer cells seen	-	-	-	-	-	-
Total	**100**	**94 (94)**	**5 (5)**	**1 (1)**	**96 (96)**	**4 (4)**

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options, including radiation.[17] Experts agrees that the conventional single Pap test has a certain irreducible error rate.[15] It means that even the most conscientious laboratories, the laboratory technologist may accurately not analyze the cell sample giving false negative or false positive results, as they sometimes classifies a normal smear as suspicious or fail to detect abnormal cells.[15] Factors that also affect the accuracy of any Pap test include whether the healthcare provider performing the screening are collecting cells correctly, collecting an adequate cell sample or preparing the microscope slide properly.

A false negative Pap test may delay the diagnosis and treatment of a precancerous condition. However, regular screening helps to compensate for the false negative result. If abnormal cells are missed immediately, chances are good that the cells will be detected the next time. From 5-25% of cervical cytology specimens reported as normally will have an abnormality when reviewed for a second time;[15] this is called the false-negative rate for a single smear. In the present study 3 cases showed false negativity in the baseline Pap test gave significant clinical changes in the following test. Sometimes, a tissue sample obtained during the test may show abnormalities in the cells that are not truly related to cancer or other vaginal problems. In the present study, 12 cases showed false-positive results in the baseline Pap then became WNL in the following test (**Table 2**). Changes in the cells may be caused by inflammation of the vagina that could be due to the use of spermicidal, tampons, and vaginal creams and jellies. Intercourse 24 hours before the procedure may also lead to minor changes in the cells. A few cells may look abnormal in the presence of semen and other liquids that get into female organs during ejaculation. Bacterial and viral infections as well as sexually transmitted diseases may make the cells look inflamed. Molecular HPV DNA detection by PCR or HC2 enables clinicians to classify equivocal Pap smears as either normal or abnormal.[15] The HPV testing improves the accuracy of screening for cervical cancer, reduces the need for costly and invasive follow-up procedures, and provides healthcare cost savings.[15]

The relation of Pap tests with significant cellular changes or atypical cells and negativity by HC2 and PCR tests described in **Table 5** showed that 13 cases had no HPV DNA detected by both HC2 and PCR. Meanwhile, some of these 13 cases had significant cytological diagnosis as ASC-US and AGC. Ten out of these 13 cases turned to be WNL within almost a year. The negativity of HC2 and to certain limit, the PCR had a significant effect on the decision in treating the patients. By having negative HPV DNA, no colposcopy, or further manipulation is needed.

The HC2 test detects HPV types regarding the high/intermediate and low risk factors involved in the development of cervical neoplasia.[14] Women with persistent HPV infection should be identified. Most HPV infections are transient. Women infected with persistent, cancer-associated HPV types are at greater risk of progressing to HSIL then to cancer. Patients with a positive result for high-risk HPV types with persistent infection will undergo colposcopy, biopsy, and surgery, when necessary. In our study, the low distribution of cancer cervix in our population is in the agreement with previously local published series,[1-7] if we consider that the 6 persistent positive HPV patients will certainly be exposed to cervical cellular transformation. Results obtained by HC2 for detection of HPV (**Table 5**), 5 high-risk HPV (5%), one low–risk HPV (1%), and 94 negative cases (94%). By using PCR test (**Table 5**), 4 cases (4%) were detected, one (1%) marked inflammation with marked cellular changes, 2 ASC-US (2%), and one (1%) atypical endocervical cells. The HC2 detected those 4 plus 2 extra one (1%) as marked inflammation and 1 (1%) as koilocytotic changes with HPV infection. The HC2 detecting more HPV cases is not unexpected, as reported by other investigators.[18] In Dominguez-Gil et al study in 2005,[18] it was concluded that the PCR identification technique lack to detect a significant number of HPV positive samples. Our agreement with the interesting study of Dominguez-Gil et al that it is of great importance to represent an effort to asses better tests to identify HPV and its clinical correlation. Patients with positive HPV DNA result, on routine screening, but with a negative Pap result had repeat Pap tests within a year.

Table 9 - Descriptive statistical analysis of the results of the 100 patients. Polymerase chain reaction (PCR) and human papillomavirus (HPV).

Hybrid Capture 2 (HC2)	Baseline Pap	Final Pap	PCR
True positive (n)	3	6	4
True negative (n)	80	91	94
False positive (n)	14	3	0
False negative (n)	3	0	2
Sensitivity (%)	50	100	66.7
Specificity (%)	85	96.8	100
Positive predictive value (%)	17.7	66.7	100
Negative predictive value (%)	96.4	100	97.9
Accuracy (%)	83	97	98

True positive means that final Pap and PCR found HPV detected as HC2. False positive means results found detected by Pap 2 and PCR but negative by HC2. True negative means results found negative by Pap 2 and PCR and confirmed negative by HC2. False negative means results found HPV not detected by PCR and proven to be detected by HC2.

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Two cases (patients 14 & 100) were detected by HC2 and had no HPV DNA detected by PCR (**Table 5**), giving certainty accuracy of HC2 test. This might be due to the use of MY09-MY11 which might miss these cases deleted in L1. Complementary studies should be carried out later with other pairs of consensus primers, such as Cp primers in E1, may be able to help to identify almost all cases of HPV.[19] Virtually our results were agreeing with those obtained by Clavel et al in 1998[14] who argued that HC2 assay is a more convenient and easier test than the PCR assay for routine use.

In Kulmala et al[9] study, they compared the performance of the HC2 assay with that of PCR. Their results showed that the agreement between the HC2 assay and PCR was substantial. The sensitivities of both HC2 assay and PCR for the detection of high grade squamous intraepithelial lesion (HGSIL) were 85.2% and 74%, and the specificities were 67.2% and 64.1%, concluding the performance of the HC2 assay for the detection of HGSILs was excellent (p<0.0001). Their results of PCR and the HC2 assay was concordant for 85% of samples, resulting in substantial reproducibility. Both tests had low positive predictive value (PPV), equal specificities, and equal negative predictive value (NPV) for the detection of HGSILs; but the sensitivity of the HC2 assay was slightly better.

There was a highly significant difference in the viral loads measured by the RLUs between those women who acquired high-risk HPV infection as shown in **Tables 6 & 7**. By comparing the RLUs values of controls and positive specimens shown in these tables, we found that the signal generated from positive specimens was proportional and concordant to the amount signal originated from the DNA present in the positive controls. Both the presence of high-risk HPV DNA at the baseline HC2 and a high viral load is strong risk factors for incident abnormal Pap smear results during monitoring. Thus, assessment of the viral load might help in identifying women at risk for high-grade CIN as reported by other investigators.[20,21] Schlecht et al[20] established the clear-cut association between HPV DNA copy number and the development of incident squamous intraepithelial lesion in the Pap smear. The patient number 92 had 21288 RLUs while the positive cut-off was only 916. This patient has to have closed follow-up as her Pap showed atypical endocervical cells in the second Pap as shown in **Table 5**. Meanwhile, the other 5 patients are showing the significant amount of RLUs as shown in **Tables 6 and 7.** The HPV screening should be well considered in our country as the western world started to recommend HPV vaccines to their young population.[22] There is an agreement that HPV prevalence is much lower in our country than the western, but advanced technologies should be introduced in our system to protect the minor percent of HPV positive patients from acquiring HPV and cancer cervix. The diagnosis of HPV infections in patients at risk of disease in a clinical setting requires a different approach from that used for epidemiological studies, vaccination trials, and natural history studies.[22,23] However, diagnostic test results should be interpreted with caution and required careful laboratory validation.[24] The implications of HPV DNA detection for patient management purposes need to be addressed properly. Using the HC2 test as a reference, the sensitivity, specificity, PPV, negative predictive value (NPP), and accuracy of baseline Pap were 50, 85, 17.7, 96.4, and 83%; for final Pap smear were 100, 96.8, 66.7, 100, 97%, and for PCR were 66.7, 100, 100, 97.9, and 98%, as shown in **Table 9**. This analysis clarify that single Pap is inconclusive as showing sensitivity only 50% while multiple Pap shows 100%. Single Pap also shows poor PPV accuracy comparing to multiple Pap test and molecular techniques. The results of our study regarding the HC2 assay and PCR were concordant for 86% of samples, resulting in substantial reproducibility. The sensitivity and the specificity were slightly better for the HC2 assay. The negativity of HC2 and to certain limit PCR had significant effect on the decision taken in the treatment of the study patients. By having negative HPV DNA, no colposcopy, or further manipulation was carried out. Finally, the benefit of molecular technology is now well appreciated and has to take place in combination with the single routine Pap test.

In conclusion, the HC2 test detects HPV types regarding high/intermediate and low risk, involved in the development of cervical neoplasia. The HC2 method and MY09-MY11-PCR identified nearly equivalent prevalence of HPV in cervical smears specimens. The combination of Pap smear and HPV DNA testing or PCR is more effective in detecting invasive cancers and high-grade lesions than either test alone, by detecting women with existing cervical neoplasia and in identifying those at risk of future disease. Patients who are HPV DNA-positive and Pap smear negative are the group at highest risk of subsequently developing an abnormal Pap smear.

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