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Effectiveness of testing E6/E7 mRNA and DNA of Human Papilloma Virus in Visual Inspection Acetic acid Positive Women

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Abstract

Background: Several testing tools are used for screening the women for cervical pre-cancer or cancer that are lack in sensitivity, specificity and effectiveness. A range of promising new biomarkers of HPV detection and cancer screening have emerged from the research pipeline, of which HPV DNA and mRNA for oncogenes HPV E6 and E7 are used as molecular evidence of infection and cervical cancer screening. **Objective:** The objective of the study was to evaluate the effectiveness of Human Papillomavirus (HPV) E6/E7 mRNA and HPV DNA test in Cervical Cancer screening of VIA positive patients. Methodology: This cross-sectional analytical study was carried out in the Department of Obstetrics and Gynecology of SSMC and MH, Dhaka in collaboration with the Department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka between July 2014 to June 2016 to evaluate HPV E6/E7 mRNA test as an alternative of HPV DNA test for diagnosis of CC among Visual Inspection Acetic acid (VIA) positive women, with an ultimate goal of improving the effectiveness of cervical cancer prevention and treatment. A total of 50 VIA positive were selected for that purpose and tested for HPV DNA test by Hybrid Capture® 2. Test for HPV E6 and E7 mRNA was performed on same samples with real time PCR tests. **Results:** Among the 50 VIA positive women, 14 (28%) were found positive for HR HPV DNA. On the contrary, 8 (16 %) samples collected from the VIA positive women were positive for HPV E6 or E7 mRNA (any of the two mRNAs. Considering the HPV DNA test as gold standard, the E6/E7 mRNA test showed low sensitivity (57.14 %) but high specificity (100%). On the contrary, in the same way when E6/E7 mRNA was considered as gold standard, the HPV DNA test showed high sensitivity (100 %) and comparatively low specificity (85.71%). In addition, the PPV of HPV mRNA test was 100 % while it was lower for HPV DNA test (75%). On the other hand, the NPV of HPV DNA test was 100 % while 85 % for HPV mRNA test. **Conclusion:** This study reveals that test for HPV E6/E7 mRNA could help more specifically than HPV DNA test in screening for HPV infection of VIA positive women and along with HPV DNA, this test could give better opportunity for diagnosis of CC. *[Journal of National Institute of Neurosciences Bangladesh, January 2022;8(1): 73-78]*

Keywords: E6/E7 mRNA; Real time PCR; HPV DNA; VIA

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Introduction

Human Papilloma virus (HPV) causes cervical cancer (CC), which is the fourth most common cancer in women[1]. A large majority (around 85%) of the global burden of CC occurs in the less developed regions, where it accounts for almost 12% of all female cancers[2]. HPV is one of the most commonly acquired sexually transmitted infections (STIs) which strictly host-specific. Therefore, there is a need of early detection of CC, which could prevent such life-threatening situation of woman.

HPV is recognized as the main causal factor of cervical intraepithelial neoplasia (CIN). In addition, HPVs can also cause benign tumors like papillomas and genital warts as well as asymptomatic infections. HPV genotypes are classified as high-risk (HR) and low-risk (LR)-types according to their clinical behavior. Worldwide, the eight most common HR-HPV types found in CC are all included either in species 7 (HPV18, 45) or species 9 (HPV16, 31, 33, 35, 52, 58)[3]. The majorities of HPV infections in young women are transient and up to 80-90% of these women will clear their HPV infection. The infections that fail to clear spontaneously remain persistent which are considered as the main risk factor and causal link for CIN and CC. These precursors of CC are classified as low-grade lesions (CIN-I) and high-grade lesions (CIN II-III)[4]. Since HPV infection is the requisite common denominator underlying CC, new approaches aimed at prevention have evolved in recent years through improved screening methods and HPV vaccination.

Papanicolaou test (Pap smear) smear screening has successfully reduced morbidity and mortality from CC over the past 50 years[5]. However, molecular detection of HPV-DNA provides a different approach for screening and patient management, allowing the identification of HPV infection in patients at risk for disease. As only presence of HPV is not indicative of CC and testing for HPV DNA is expensive which requires specialized laboratory facility, there is an urgent need of new tool to diagnose CC. However, many questions still remain before successful implementation can become feasible.

Despite current updated knowledge about HPV and its interactions with host cells, tissues and immune systems, it cannot be predicted whether a specific infection will regress or persist[6]. Several testing tools including Visual Inspection Acetic acid (VIA), Pap smear and HPV DNA tests are used to screen the women who are suspected for cervical pre-cancer or cancer. These tests are lack of high sensitivity, specificity and effectiveness[7]. A range of promising new biomarkers has emerged from the research pipeline, one of which is mRNA from the HPV E6 and E7 oncogenes, which provides high specificity to distinguish between benign productive infection and those where neoplastic progression has been initiated or already resulted in cancer[8]. Therefore, this study was aims to evaluate HPV E6/E7 mRNA test as an alternative of HPV DNA test for diagnosis of CC among VIA positive women, with an ultimate goal of improving the effectiveness of cervical cancer prevention and treatment.

Methodology

The cross-sectional analytical study was carried out in the Department of Obstetrics & Gynecology of Sir Salimullah Medical College (SSMC) and Mitford Hospital, Dhaka, Bangladesh in collaboration with the Department of Virology at Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh from July 2014 to June 2016. The study subjects were enrolled from Outdoor Patient Department (OPD) and VIA & Colposcopy Room of the Department of Obstetrics & Gynecology SSMC and MH, Dhaka. A total of 50 VIA positive (+) women were included as the study population who were aged between 30-50 years and VIA positive cases without history of prior treatment for cervical pre-cancer and cancer. Before commencing the research work, permission (IRB–SSMC/2014/ 78) was taken from the Institutional Review Board of SSMC, Dhaka. In this study, a purposive sampling technique was followed where a woman who attended the GOPD and VIA & Colposcopy Room of SSMC & MH for VIA examination and found positive was approached with a request to be a participant of the ongoing research. If she agreed, only then she was included in the study for collection of cervical swab samples. Two variables i.e. HPV DNA and HPV E6 and E7 mRNA were studied in this study. For collection of relevant information including different investigations, a questionnaire was developed and all data was collected on it. Cervical samples were collected by Colposcopy on second visit of VIA positive women using a Cervex brush® (Rovers Medical Devices B.V., Holland). The brush was washed in a vial containing PreservCyt solution (Cytyc Corporation, Boxborough, MA) and transferred to the laboratory for HPV analyses to the Department of Virology, BSMMU to perform test for HPV DNA and E6 and E7 mRNA tests. All the cervical swab samples were divided into two parts i.e. one for HC2 HPV DNA Test and another for HPV E6 and E7 mRNA tests. The DNA and mRNA was isolated and subjected to HC2 HPV DNA Test and real time-PCR for detection of HPV DNA and HPV E6 andE7 mRNA respectively. Hybrid Capture® 2 High-Risk HPV DNA Test™ was performed and analyzed as per the manufacturer's instructions using the HR HPV probe cocktail (package insert; Digene Corporation, Gaithersburg, MD). Total RNA was isolated using the Trizol-reagent according to the manufacturer's instructions. The mRNA was reverse transcribed into cDNA using an M-MLV Reverse Transcriptase kit (Solis BioDyne, Tartu, Estonia) according to the manufacturer's recommendations.

Detection of HPV 16 and HPV 18 E6/E7 mRNA in cervical specimens was performed by qualitative reverse transcriptase PCR (RT-qPCR). PCR primers targeting the selected genes were collected from published journal[9]. Statistical analyses was performed using Microsoft excel Version 12.3. Sensitivity, specificity and predictive values and their 95% confidence intervals (CI) for HPV DNA test and E6/E7 mRNA test were calculated using 2 × 2 tables and standard formula. All statistical analysis was performed using an online statistical software- MedCalc[10].

Results

The mean (±SD) age and weight of women of the study was 41.2±4.9 years and 51.3 4.7 kg respectively. The mean age of attaining menarche and getting married was 13.8±1.48 and 19.41±3.91 respectively. The mean duration of marriage of the participants was 15.4±4.7. Among the participants 31(62.0%) had 2 number of pregnancy whereas 19(38.0%) had more than 2 pregnancies. A total of 78.0% (39/50) women were pre-menopausal and 22.0% (11/50) were post-menopausal. Forty six percent (23/50) of the study participants had primary education while 14.0% (7/50) studied up to high school and above and 24.0% (12/50) had no formal education. All the study subjects were divided into 4 age groups with 5 years of interval. HPV DNA and HPV mRNA was detected in all the groups with increase trend of detection in higher age group (Table 1).

The highest rate of HPV DNA and HPV mRNA were 46.15% (6/13) and 30.76% (4/13) in 46-50 year group respectively whereas lowest rate like 8.3% (1/12) for both type of tests was detected in 30 to 35 years of age group. In addition, 8.3% (1/12) HPV E6/E7 mRNA detection rate was also observed in age group 36 to 40 years, although detection of HPV DNA was more (25.0%) in this group. Among the study participants, 62.5% (5/8) were positive for E6 gene of HPV-16 and 37.5% (3/8) were positive for E6 gene of HPV-18 and of those 8 E6 HPV mRNA positive samples, 7 were positive for E7 HPV mRNA (Table 2).

Table 2: Distribution of HPV E6 and E7 genes among HPV DNA positive women

Type	E6 mRNA	E7 mRNA
HPV-16	5(62.5%)	4(57.1%)
HPV-18	3(37.5%)	3(42.9%)
Total	**8(100.0%)**	**7(100.0%)**

* E7 gene was not detected in 1 sample.

Moreover, of those E7 gene positive samples, 4 (57.14%) were for HPV-16 whereas 3 (42.85 %) were for HPV-18. Among the 50 VIA positive women, 8 were both positive for HPV DNA and mRNA, while 6 samples were negative for HPV mRNA but were positive for HPV DNA (Table 3).

Table 3: Test Results of HPV DNA and HPV mRNA Test among the VIA Positive Women

HPV DNA	HPV RNA		Total
	Positive	Negative	
Positive	8	6	14
Negative	0	36	36
Total	**8**	**42**	**50**

None of the HPV DNA negative samples was positive for HPV mRNA. A total of 36 VIA positive woman were negative for both HPV DNA and mRNA. The test characteristics of HPV mRNA among the VIA positive women in terms of sensitivity, specificity and positive and negative predictive values (PPV, NPV) were considering HPV DNA test result as gold standard. The sensitivities of HPV mRNA to detect HPV infection were 57 % whereas the NPV of 85.0% (Table 4).

TTable 4: Test characteristics of HPV mRNA tests considering HPV DNA test as Gold Standard Test in Detecting HPV Infection among the VIA Positive Women

Variables	Value	95.0% CI
Sensitivity	57.14%	28.86% to 82.34%
Specificity	100.0%	90.26% to 100.0%
PPV	100.0%	63.06% to 100.00%
NPV	85.7%	71.46% to 94.57%

PPV=Positive Predictive Value; NPV=Negative Predictive Value

Table 1: Age-wise Distribution of VIA Positive Women with their HPV DNA and mRNA Result

Age Group	VIA	DNA	mRNA	HPV-16		HPV-18	
	Positive	Positive	Positive	E6 mRNA	E7 mRNA	E6 mRNA	E7 mRNA
30 to 35 Years	12	1(8.33%)	1 (8.3%)	1(8.33%)	0(0.0%)	-	-
36 to 40 Years	12	3(25.0%)	1 (8.3%)	0(0.0%)	0(0.0%)	1(8.3%)	**1(8.3%)**
41 to 45 Years	13	4(30.8%)	2(15.4%)	1(8.3%)	1(8.3%)	1(8.3%)	**1(8.3%)**
46 to 50 Years	13	6(46.2%)	4(30.8%)	3(25.0%)	3(25.0%)	1(8.3%)	**1(8.3%)**
Total	**50**	**14(28.0%)**	**8(16.0%)**	**5(41.7%)**	**4(33.3%)**	**3(25.0%)**	**3(25.0)**

Discussion

Even though currently there is an effective prophylactic HPV vaccine for prevention of CC, the only way to reduce the mortality associated with CC among women already infected with HPV is through effective and sustainable cancer screening program and management of screen positives. Cervical cancer incidence and mortality have declined significantly in those places that have effectively implemented Pap test-based screening[11]. Yet CC remains the second most common female cancer and third most common cause of female cancer-related mortality globally[12]. This seeming contradiction is explained by the fact that CC incidence and mortality are approximately 10-fold greater in low- and middle-income countries (LMIC), where Pap programs have failed to be established because of the technical and financial barriers to implementation[11].

Currently, the combined or individual use of cervical cytology, VIA and HPV DNA testing is the mainstay of CC screening programs in Bangladesh. A cross-sectional study from Bangladesh involving some 3600 women concurrently tested with VIA and cytology, reported 2.0% sensitivity and 98.0% specificity for cytology to detect high-grade disease as opposed to 79.0% sensitivity and 57.0% specificity for VIA[12]. Hence for better performance and low cost, VIA is regarded as primary HPV screening test in Bangladesh. To increase the efficiency of screening, presently molecular testing for HPV DNA in Bangladesh has started. This HPV test is more sensitive than Pap testing[13,14]. Studies comparing the performance of cervical cytology with HR-HPV DNA detection for identification of CC lesions have shown that the Pap smear lacks the sensitivity to detect pre-cervical cancer or cervical cancer lesions in all women, despite the fact that the specificity of the Pap smear is greater than 90.0%[13]. A key attribute of HPV DNA testing related to its high sensitivity is its excellent negative predictive value, providing near complete reassurance following a negative test that the woman does not have cancer or precancer[15,16]. On the contrary, although the sensitivity of high risk HPV DNA is superior to that of cytology for the detection of pre-cervical cancer or cervical cancer, the low specificity of these assays leads to false-positive results[17] which challenges of using HPV DNA testing in management of screen-positive women. It was observed that most women with a positive screening test (80% to 90%) will not have concurrent disease (i.e., cervical precancer or cancer[18]. A mid of such puzzling situation related to diagnosis of HPV infection, there is urgent need of introduction of newer tools for better diagnosis. Presently a new molecular test for detection of cancer markers like E6 and E7 mRNA of HPV are presently in the way of development and implementation. The rationale behind targeting these viral mRNAs is to observe whether actual oncogenic process is initiated by persistent high-risk HPV infection which is mediated by the upregulation of the E6/ E7 oncoproteins[19]. Continuous expression of the HR-HPV E6 and E7 oncoproteins is necessary for transformation of normal cells to dysplastic cells[20]. This is directly related with an increased risk of lesion progression[21]. On this basis, it would stand to reason that the detection of E6/E7 oncogene activity should be more specific and should be a better predictor of CC risk than HPV DNA detection methods[22].

This current study was undertaken to compare the effectiveness of HPV E6/E7 mRNA testing of HR-HPV over HPV DNA in VIA positive women. For that purpose 50 VIA positive women were selected and tested for HPV DNA test by Hybrid Capture® 2 and test for HPV E6 and E7 mRNA was performed on same samples with real time PCR. Among the 50 VIA positive women, 14(28%) were found positive for HR HPV DNA. On the contrary, 8(16.0%) samples collected from the VIA positive women were positive for HPV E6 or E7 mRNA (any of the two mRNAs). It indicates that among the study subjects only 28.0% were infected with HR-HPV and among them oncogenic activities of E6 or E7 gene was going on only in 16.0% VIA positive women. The HPV infection rate and expression of both the oncogenes increased with age reaching highest at 45 to 50 years age group.

Considering the HPV DNA test as gold standard, the E6/E7 mRNA test showed low sensitivity (57.14%) but high specificity (100%). On the contrary, in the same way when E6/E7 mRNA was considered as gold standard, the HPV DNA test showed high sensitivity (100.0%) and comparatively low specificity (85.71 %). In addition, the PPV of HPV mRNA test was 100.0% while it was lower for HPV DNA test (75%). On the other hand, the NPV of HPV DNA test was 100.0% while 85.0% for HPV mRNA test. In earlier report, it was observed that HPV DNA testing is far more sensitive than cytology and able to detect small numbers of HPV genomes. The biggest advantage of HPV DNA testing is the it's negative predictive value (~99%)[23-24]. A woman who tests negative for HR HPV will probably not need CC screening for the next six years (range 3-10 years)[14]. Even though the use of assays to detect only HPV DNA is undesirable for

clinical use, because it would produce unacceptably high levels of positive results among women who would have cleared their infections without intervention[25]. This study demonstrates that HPV DNA testing with HC2 is a more sensitive method for detecting HPV infected VIA positive women than test for detecting mRNA but it detects 8.0% VIA positive women who were not expressing any of the oncogenes. Unfortunately, this excellent analytical sensitivity of HPV DNA testing makes it much less clinically specific. Because of this, it can lead to unnecessary colposcopy and biopsy examinations in women who are positive for HR- HPV DNA[26]. This could happen because HPV DNA testing will identify those women who are infected with HPV, but do not have severe dysplasia and thus have an 80% chance to clear the infection without treatment. This positive result is caused due to positive signal generated from infected cells that are destined to be cleared without symptoms, to be cleared after mild dysplasia or to develop into cancer[23].

Presently several commercial assays have been designed to detect mRNA of the E6/E7 oncogenes of HR HPV. Expression of E6/E7 oncogenes increases with the severity of the lesion. In high-grade squamous interepithelial lesions (HSIL) and CC, high-level expression of E6/E7 mRNA is present due to the associated integration of E6 and E7 genes into the host's cellular DNA. Expression of these viral genes in low-grade squamous interepithelial lesions (LSIL) is usually low. In some studies, HPV mRNA assays have shown approximately the same sensitivity as HPV DNA assays, with a higher specificity and PPV for high-grade lesions[23,4,27]. In subjects with a high expected prevalence of disease (e.g. groups at risk, symptomatic patients, and patients with persistent cytological abnormalities after negative colposcopy results), HPV RNA assays will provide better risk predictions than HPV DNA tests[28]. HPV mRNA assays may also predict which women with LSIL or ASCUS (atypical squamous cells of undetermined significant lesions have the potential to progress to CC. Reductions in the number of cases referred for colposcopy, improved patient wellbeing, and significant reductions in costs have been suggested as possible benefits of introduction of HPV mRNA test[28].

In HPV infection, only 20% of HR-HPV infections cause morphologic changes in the epithelium of the cervix without intervention[29]. However, progression of premalignant lesions is preceded by clearance of HPV. It is suggested that the cases that are HPV positive but have negative cytological test should be follow up more frequently[30-31]. Nevertheless, the women who are HPV negative as well as cytologically negative and have absence of inflammation, might be screened at longer interval. On the contrary, as expression of E6/E7 mRNA indicates chances of possible malignant transformation, it would help physician to follow up and treat the patient more judiciously with great attention and accuracy.

The results of this study demonstrate that comparing with each other HPV DNA test has higher sensitivity and high negative predictive value, on the other hand clinical performance of HR-HPV E6/E7 mRNA testing is more specific and has batter PPV in diagnosing HPV infection. Therefore, it may be suggested from this study that these two tests can supplement each other in clinical diagnosis, prognosis and treatment of HPV infection and diagnosis of CC in suspected patients.

Conclusion

Though test for detection of HPV DNA and HPV E6/E7 RNA both are highly sensitive and specific for diagnosis of HPV infection in VIA positive women, for specific diagnosis of HPV oncogenesis process, test for HPV E6/E7 mRNA is better than HPV DNA test. Though both these tests have some limitations, they can supplement each other and can be used in diagnosis, prognosis and treatment of HPV infection.

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