

HPV Adjunct Testing to VIA as an Alternative to Cytology for Cervical Cancer Screening in Pakistan

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Abstract

Background: Developing countries often lack sufficient resources to use the Pap smear as a screening tool for cervical abnormalities. Visual inspection with acetic acid (VIA) is similar to Pap smears in terms of sensitivity for high-grade CIN. However, it is not very specific and adjunct testing with HPV can improve the test characteristics of VIA.

Aim: To evaluate VIA with adjunct Human Papilloma Virus (HPV) testing as an alternative to routine cervical cytology for cervical cancer screening in a low resource setting.

Methods: We conducted a cross-sectional study among the attendees of the gynecology clinics attached to our hospital. Each patient underwent three screening tests, viz., Pap smear, HPV DNA testing and VIA, sequentially. The patients who tested positive by any of these methods underwent colposcopy. Trained colposcopists, blinded to the results of initial screening tests collected biopsies from patients with colposcopic abnormalities. We tested the specimens for HPV by Polymerase Chain Reaction (PCR) using the general primers GP5+/GP6+, and checked for high risk HPV types in positive samples.

Results: The median age of participants was 38 years. Out of the 857 patients screened, 46 (5.36%), 4 (0.47%) and 13 (1.53%) tested abnormal/positive by VIA, Pap smear and HPV PCR, respectively. Sequential VIA and HPV PCR yielded a sensitivity and specificity of 80% and 93%, respectively.

Conclusions: We propose that sequential testing involving the use of VIA followed by HPV PCR could improve the test characteristics, in low-resource settings.

Keywords: Human papilloma virus; Visual inspection with acetic acid; Cervical cancer screening

Introduction

Cervical cancer is the third commonest cancer among women worldwide [1]. Statistics show that 528,000 new cases and over 266,000 deaths occurred in 2012, due to the disease. The low- and middle-income countries suffer almost 90% of cervical cancer burden [2]. Poor awareness, lack of effective screening programmes and late clinical presentation contribute to poor survival rates from the disease, in these countries.

The strategies for cervical cancer screening include cytological testing (the Papanicolaou or 'Pap' smear), HPV DNA testing, and Visual Inspection techniques using Acetic acid (VIA), or Lugol's Iodine (VILI). Pap smear represents the simplest of these, but needs a laboratory setup and trained manpower, and multiple clinic visits by the patient. Visual screening methods have high sensitivity but low specificity, and can facilitate diagnosis and treatment interventions in the same visit [3]. HPV DNA testing is cost-effective and sensitive for detection of pre-cancerous cervical lesions, but has a low specificity compared to cytology [4-6].

Gaffikin et al. reported earlier that single-visit visual inspection techniques are quite practical for cervical cancer screening in resource-poor countries [7,8]. Reports indicate that adjunct testing can increase the specificity and cost-effectiveness of individual screening methods, and can avoid false-positive results and unnecessary referrals for colposcopy.

A study by Nawaz et al. (2005) from our hospital revealed

discordant results in Pap smears and cervical biopsy in 10% of cases [9]. Another group reported an increase in screening sensitivity by 65.6% with the addition of care HPV testing in parallel to Pap smears and to 72% for the combination of HPV and VIA [10]. Blumenthal et al. (2001) reported increase in diagnostic sensitivity upon addition of HPV DNA testing to VIA, and Pap smears [11]. Studies from India, South Africa, Thailand [3,12,13] reveal that VIA and HPV DNA testing could be better alternatives to cervical cytology in poor countries. VIA followed by HPV testing yields fewer false positives, compared to VIA alone [12]. A comparative study of three screening techniques from Brazil recommended the testing for high risk HPV types as the most sensitive single test for identification of CIN 2 or worse lesions [14].

Despite these reports, no study so far has evaluated the utility of adjunct tests for cervical cancer screening in Pakistan, a country that shares the healthcare and logistic challenges common to the developing world. Herein we attempt to find out a suitable screening algorithm for cervical cancer screening in our geographical region by finding the test characteristics of different screening tests for identification of CIN.

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Materials and Methods

After the approval of our institutional review board we did a cross-sectional study among the attendees of the gynaecology clinics of the Aga Khan University Hospital, Karachi, Pakistan, and its three satellite family medicine clinics at Kharadar, Garden and Karimabad. All sexually active women presenting at these clinics between 2008 and 2010 were included in the study. The exclusion criteria consisted of age less than 20 years, history of previous genital tract malignancy and irradiation, pregnancy and refusal to be included in the study. We obtained informed consent from the patients after explaining the study and its purposes, and collected their socio-economic, demographic and reproductive data using a questionnaire.

Pap smear, sampling for HPV DNA and VIA

Following history taking and clinical examination, the gynecologists performed three screening tests in succession. Per speculum examination was performed and Pap smear was taken from cervix with Ayer's spatula. The slide was fixed with alcohol spray and it was dispensed for cytological examination. The specimen for HPV was collected by Ayer's spatula and was dispensed in 15 ml biophosphate saline solution and was stored at 4°C. Finally the patients underwent VIA, consisting of cervical examination after 1 minute of application of 5% acetic acid. VIA results were classified as positive, negative or suspicious, as per the guidelines of the Alliance for Cervical Cancer Prevention (AACP) [15].

Women positive on any screening test were referred for colposcopy. During colposcopy, the gynecologists collected punch biopsies from abnormal cervical areas and submitted the material (fixed in 10% formalin) for histopathological evaluation.

DNA extraction

The receiving laboratory extracted total DNA from 200 µL of the cell suspension (within 3 days of sample receipt), using QIamp DNA mini kit (QIAGEN, Hiden, Germany) as per manufacturer instructions.

HPV DNA polymerase Chain Reaction (PCR)

We performed HPV DNA PCR on the extracted DNA, using GP5+/GP6+ primers (general primers for HPV). [16].

The PCR reaction mixture for HPV detection (25 µL) included 5 µL sample DNA, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM MgCl₂, 200 µM deoxynucleotide triphosphates (dNTPs), 0.4 pmol of each primer, and 0.2U of Taq Polymerase. The cycling proceeded through the steps of 95°C for 5 min.; followed by 40 cycles of 94°C for 30 sec., 45°C for 30 sec., and 72 °C for 30 sec.; and a final extension step at 72°C for 5 min.

The master mix for amplification of the β-globin gene included 0.2 pmol of PCO3 and PCO4 primers [17] and the rest of the reaction components as above. The cycling conditions were: 94°C for 5 min., followed by 40 cycles of 94°C for 30 sec., 51°C for 30 sec., and 72°C for 30 sec., and a final extension step at 72°C for 5 min.

The housekeeping gene β-globin served to ensure successful DNA extraction. DNA extracted from cervical carcinoma samples were included as positive control for the PCR, while reaction mixes with elution buffer instead of DNA served as negative control. The primer sequences are listed in Table 1.

HPV genotyping

We evaluated the HPV-positive specimens for presence of high risk

HPV types (including type 16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, and 66), using a commercial kit (Sacace Biotechnologies, Italy), following manufacturer guidelines.

Data analysis

We analyzed the data using SPSS (Version 16).

Results

Table 2 summarizes the socio-demographic data of our study subjects. The median age of the patients was 38 years, and 735 (85.8%) of them were housewives. Among the patients, 188 (21.9%) had completed at least primary education. Fourteen (1.6%) patients had married twice, while 842 (98.2%) reported only one marriage. A total of 436 (50.9%) patients expressed awareness of cervical cancer screening. While 285 (33.3%) of the subjects visited for a routine gynecological evaluation, 182 (21.2%) had complaints of menstrual disturbances. Seventy-seven (9%) patients had vaginal discharge, and 81 (9.5%) and 41 (4.8%), suffered from primary and secondary infertility, respectively.

VIA, Pap smear, HPV, colposcopy, and biopsy results

Sixty three (7.35%) of the 857 subjects tested positive in VIA, Pap smear or HPV PCR. Of these, 9 patients were lost to follow-up, 5 underwent hysterectomy and 1 was pregnant, and hence were all excluded from the study. The remaining 48 (5.6%) patients underwent colposcopy, and 18 (37.5%) of them had abnormal results. Biopsies from 5 (27.7%) of these patients showed CIN 2 disease.

Among the 63 patients with abnormal findings in the screening tests, 46 (5.37%) were positive by VIA, 4 (0.46%) had abnormal Pap smears, and 13 (1.54%) tested positive for HPV DNA. (Due to failure in β-globin gene amplification, we excluded 11 (1.28%) samples from analysis of PCR results, leaving only 846 samples in this category). Out of 46 VIA positive women 4 were positive by HPV testing.

The results for individual and sequential testing parameters are provided in Table 3. It is obvious that test parameters showed significant improvement in sequential setting when compared with stand alone testing.

Prevalence of oncogenic strains of HPV

We detected HPV type 16 in 8 (61.5%) and type 18 in 3 (23.1%) samples (Table 4). One (7.6%) sample had HPV type 45 and another (7.6%) was positive for both HPV type 18 and 45.

Discussion

Cytological screening for cervical cancer shows variable sensitivity, prompting frequent screening, which can be a difficult proposition in low-resource settings. A report by Sankaranarayanan et al. (2007) suggested no benefit of cytological screening per se in reducing mortality from cervical cancer [18]. VIA does not require laboratory facilities, yields immediate results, has better patient compliance and cost savings but has a low specificity [3,19]. Studies have revealed its

| Primer name | Sequence (5'→3') | Target gene | Amplimer length | Reference |
|-------------|-----------------------|-------------|-----------------|---------------------------------|
| GP5+ | TTTGTTACTGTGGTAGATAC | L1 | 155 bp | Baay MF et al., 1996 [16] |
| GP6+ | GAAAAATAAACTGTAATCA | | | |
| PCO3 | ACACAACCTGTGTTCACTAGC | β-globin | 110 bp | |
| PCO4 | CAACTTCATCCACGTTCCACC | | | Mikaelsdottir et al., 2003 [17] |

Table 1: Primers used for PCR amplification.

utility for cervical cancer screening in resource-poor settings [18,20]. HPV DNA testing, in comparison, is an objective method and a reasonable proxy for clinical specificity [21].

Herein we present results from a study on 857 gynecological patients that evaluated the utility of addition of HPV DNA testing to VIA in cervical cancer screening in Pakistan.

| Characteristics | Category | Number (n) | Percentage (%) |
|--|-------------------|------------|----------------|
| Age in years | <25 | 52 | 6.06 |
| | 25 - 34 | 274 | 31.9 |
| | 35 – 44 | 288 | 33.6 |
| | 45 – 54 | 175 | 20.42 |
| | 55 – 64 | 58 | 6.76 |
| | >64 | 10 | 1.16 |
| Religion | Muslim | 834 | 97.3 |
| | Christian | 11 | 1.3 |
| | Hindu | 12 | 1.4 |
| | | | |
| Socio-economic status | Low | 192 | 22.4 |
| | Middle | 534 | 62.3 |
| | High | 131 | 15.3 |
| Duration of Marriage | > 1 year | 19 | 2.21 |
| | 1 – 5 years | 147 | 17.1 |
| | 5-10 years | 121 | 14.1 |
| | 10 – 20 years | 265 | 30.9 |
| | >20 years | 305 | 35.58 |
| Number of marriages | 1 | 842 | 98.2 |
| | 2 | 14 | 1.6 |
| | 3 | 1 | 0.1 |
| Parity | Nulliparous | 116 | 13.5 |
| | Multiparous | 741 | 86.5 |
| Contraception | Nil | 560 | 65.3 |
| | Coitus interrupts | 4 | 0.5 |
| | Condom | 171 | 20.0 |
| | Pills/injection | 38 | 4.4 |
| | IUCD | 33 | 3.9 |
| | Tubal ligation | 49 | 5.7 |
| | Others | 2 | 0.2 |
| Awareness about cervical cancer screening | Yes | 421 | 49.1 |
| | No | 436 | 50.9 |
| Source of information related to cervical cancer | TV | 1 | 0.1 |
| | News | 1 | 0.1 |
| | | | |
| | Doctor | 840 | 98.0 |
| | Internet | 11 | 1.3 |
| | Friend | 4 | 0.5 |
| Partner's number of marriages | 0 | 1 | 0.1 |

Table 2: Demographic, socio-economic, gynecological and other relevant data of subjects in the study.

| Measure (%) | VIA | PAP SMEAR | HPV | VIA HPV Sequential testing |
|-------------|-----|-----------|-----|----------------------------|
| Sensitivity | 40 | 20 | 40 | 80 |
| Specificity | 94 | 99 | 98 | 93 |
| PPV | 4 | 25 | 15 | 30 |
| NPV | 99 | 99 | 99 | 96 |

Table 3: Test Mesasures with individual and sequential testing.

| HPV Type | Number of positive specimens | Prevalence of the viral subtype |
|----------------------|------------------------------|---------------------------------|
| 16 | 8 | 61.5% |
| 18 | 3 | 23.07% |
| 45 | 1 | 7.6% |
| Multiple (18 and 45) | 1 | 7.6% |

Table 4: Types of Human Papillomavirus detected in the study.

The majority of our patients were in the age group of 35-44 years, housewives, married once, and multipara. Among the subjects, 50.9% conveyed limited awareness about cervical cancer.

We identified a total of 5 cases of CIN 2 among the study subjects. These patients belonged to the 25-34 and 35-44 age group(s) (Table 4).

Shastri et al. (2005) in a large study reported sensitivities of 57.4%, 62% and 64.9% for Pap smear, high-risk HPV testing, and VIA respectively, in identifying CIN 2 disease [22]. In our series, Pap smear, HPV testing and VIA had sensitivities of 20%, 40% and 40%, respectively.

Studies have indicated that upto 70% of young women may show transient positivity for HPV DNA, which declines significantly after 30 years of age [23,24]. Sherman et al. (2003) have reported an average sensitivity and specificity of 89% and 90% with NPV more than 97%, for HPV testing in women older than 30 years [25]. Kuhn et al. and Bhatla et al. had earlier reported an HPV positivity of 6-18% among women older than 30 years [26,27]. In our study we used the general primers GP5+ and GP6+ which can detect a wide range of genital HPV types [28,29]. We observed only a low rate of (1.54%) HPV DNA positivity in our subjects, and only two patients among them had histopathology-confirmed CIN 2 disease.

HPV type 16 was the predominant viral type in our series, with 8 (61.5%) positive cases, followed by HPV 18 and 45, contributing 3(23.07%) and 1(7.6%), respectively. One sample tested positive for HPV types 18 and 45. The predominance of HPV 16 and 18 in cervical cancer specimens is in agreement with several earlier reports [30-32].

Our results indicate that when VIA was used in series with HPV the sensitivity was significantly improved with minimal compromise on specificity. Sequential testing with VIA and HPV testing achieved a sensitivity of 80% and specificity of 93% for CIN 2, in our study.

We conclude that adjunct testing for HPV DNA testing along with VIA can enhance the detection of CIN 2 disease, and would be a suitable screening approach for cervical cancer in low-resource settings. This strategy could be evaluated further in settings with different prevalence rates of the disease, and using larger sample sizes. The limitation of our study is that only screen positive patients by any of the test underwent the reference standard (Colposcopy and biopsy) and hence there is an indirect estimation of sensitivity and specificity.

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