

Genotyping test based on viral DNA, RNA or both as a management option for high-risk human papillomavirus positive women: a cross sectional study

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ABSTRACT

Aim This cross-sectional study of a group of women with abnormal cytology and high-risk human papillomavirus (hrHPV) infection compared genotyping HPV DNA and mRNA assays according to two age categories of women (S1: ≤ 30 and S2: > 30 years).

Methods The hrHPV DNA positive results of 105 cervical samples of women were pooled and those harbouring HPV-16, 18, 31, 33 and/or 45 DNA were tested for the type specific HPV oncogene E6/E7 overexpression (mRNA).

Results Although HPV DNA testing showed a higher proportion of women infected by any of five hrHPVs in S1 group, total agreement of hrHPV DNA and mRNA positive results was higher in S2 group of women (75.8% v. 83.9%). The most prevalent type in both age groups was HPV-16. A 100% agreement of positivity of both tests was noted for HPV-18 and 33 in S1 group, and for HPV-18 in S2 group. Increasing concordance of HPV-16 and 31 DNA and mRNA positive results with the severity of cervical cytology was observed in S1 group of women. Absolute matching (100.0%) of positivity of both diagnostic tests was recorded in S2_{ASCUS} group (for HPV-16, 18 and 33), in S1_{HSIL} (for HPV-16, 18, 31 and 33), in S1_{LSIL} category (for HPV-18 and 33) and in S2_{HSIL} group (for HPV-18).

Conclusion The results indicate the possibility of predicting the risk of persistent infection only by HPV DNA typing test, with no need for additional RNA testing in categories of infected women showing a high (absolute) agreement of positivity of both tests.

Key words: biomarkers, early detection of cancer, human papillomavirus DNA tests, human papillomavirus 16, human papillomavirus 18

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INTRODUCTION

For the detection of precancerous changes, human papillomavirus (HPV) DNA testing has a higher sensitivity which results in greater negative predictive value over a longer period of time (1,2). However, the low specificity of HPV DNA tests allows to identify transient infections that never lead to dysplasia or cancer, especially in younger women (3).

Various strategies are being considered that improve the specificity of the management decisions of the highly prevailing HPV DNA positive results, reducing unnecessary diagnostic procedures or treatments (4-5). Potential strategy improvement has been achieved by high risk (hr) HPV genotyping (HPV-16 and 18) (6). Furthermore, the test that utilizes HPV E6/E7 mRNA detection demonstrated significantly higher clinical specificities than the DNA-based hrHPV group tests (7-9). In fact, HPV mRNA testing is based on the detection of overexpression of viral oncogenes E6 and E7, which are direct biomarkers for the prediction of cervical cancer (10). The importance of mRNA testing suggests such an approach to be a primary cervical cancer screening option for women ≥ 30 years of age (11,12). In many cervical screening guidelines, hrHPV testing is recommended only for women over the age of 30, while younger women begin screening at age of 21 with cytology alone (13,14).

As a consequence, a certain percentage of younger women who develop high grade intraepithelial lesions (HSIL) remain unidentified (15). More than half of women from 25 to 29 years of age with CIN3+ were found to have normal cytology (2), which led some countries to include women below 30 years of age to start cervical screening based on HPV DNA testing (16).

However, spontaneous regression of CIN-2 is common among young women and adolescents. Factors associated with CIN-2 regression or progression to CIN-3, respectively, are correlated with HPV persistence, specifically HPV-16 and HPV-18 infections (17). As the incidence of cervical cancer is low in this age group of women, the progression of CIN-2 to cancer is also very rare.

Since an organized cervical cancer screening program has not been established in B&H yet, studies involving the population of infected women are important in order to determine the age category of interest and appropriate guidelines

for an adequate disease prevention.

The aim of this study was to compare two genotyping approaches based on the detection of five hrHPV DNA or RNA, respectively, in two age categories of women (≤ 30 and >30 years) with abnormal cervical cytology as a contribution to the management options for hrHPV DNA positive women in the cervical cancer screening program.

PATIENTS AND METHODS

Patients and study design

The women were recruited from outpatient clinic of the Institute for Health Protection of Women and Maternity of Sarajevo Canton, Bosnia and Herzegovina, during the period June 2010 to December 2012. Conventional Pap smears were taken by gynaecologists and examined by experienced cytologists independently of the HPV testing. Cervical specimens were referred for HPV testing either to the Clinical Centre University of Sarajevo (Bosnia and Herzegovina) or to the Institute for Biomedical Diagnostics and Research „Nalaz” in Sarajevo (Bosnia and Herzegovina).

Participating women ($n=105$) were grouped into two categories according to their age: women aged ≤ 30 years (group S1: 31/105, 29.50%) and >30 years (group S2: 74/105, 70.50%). The average age of women in the group S1 was 26.2 ± 3.2 years (range 19-30 years) and in the group S2 it was 40.9 ± 7.7 years (range 31-62 years). All women with positive cervical cytology starting from atypical squamous cells of undetermined significance (ASCUS) through low-grade squamous intraepithelial lesion (LSIL) to high-grade squamous intraepithelial lesion (HSIL) and hrHPV infection detected by HPV DNA screening test were included in the study. In S1 (younger) group ($n=31$) cytology results were distributed as follows: $n_{ASCUS}=13$ (41.94%), $n_{LSIL}=10$ (32.26%) and $n_{HSIL}=8$ (25.80%), while in S2 (older) group the distribution of cytological results was as follows: $n_{ASCUS}=24$ (32.43%), $n_{LSIL}=23$ (31.08%) and $n_{HSIL}=27$ (36.49%).

Methods

Cervical specimens were collected—using one of the following kits: digene Cervical Sampler- STM (Qiagen, Gaithersburg, MD, USA) (88/105), Abbott Cervi- Collect Specimen Collection Kit” (Abbott Molecular, Wiesbaden, Germany) (14/105) or ThinPrep Pap Test Preser-

vCyt Solution (Cytic Corporation, Boxborough, MA, USA) (3/105), respectively, as previously described (18,19).

DNA-based detection assays. Screening for hrHPV infection was performed by one of the two clinical assays: HC2 (Qiagen, Gaithersburg, MD, USA), which was done using the hrHPV probe cocktail and Abbott RealTime High Risk HPV test (Abbott Molecular, Wiesbaden, Germany), in accordance with the specific manufacturer's protocol.

DNA- based genotyping assays. Total nucleic acid extraction was performed by using NucliSENS® miniMAG™ Magnetic Extraction Kit (bioMérieux, Lyon, France) from the sample aliquots separated before HPV DNA screening test was done. The 400 µL aliquots of samples collected in digene Cervical Sampler- STM (Qiagen, Gaithersburg, MD, USA) and the 1 mL aliquots of samples collected in Abbott Cervi-Collect Specimen Collection Kit (Abbott Molecular, Wiesbaden, Germany) were transferred for extraction. Samples taken in ThinPrep Pap Test PreservCyt Solution (Cytic Corporation, Boxborough, MA, USA) were separated in 5 mL aliquots, then centrifuged 12 minutes on 1125 g, and finally 4 ml of supernatant were discarded while the pellet was resuspended in the rest of 1 mL of supernatant.

HPV genotyping was carried out by multiplex real-time PCR amplification (HPV High Risk Typing Real-TM test, Sacace Biotechnologies, Como, Italy) allowing identification of 12 hrHPVs: HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 according to manufacturer's instructions. Five samples with indeterminate results obtained by this method were identified using reverse line probe hybridization diagnostic kit INNO-LIPA HPV Genotyping Extra (Fujirebio Europe, Gent, Belgium) enabling the detection of 54 HPV types and identification of 28 specific HPV types among them.

Other HPVs belong to HPV-35, 39, 51, 52, 53, 56, 58, 59, 66, 70 and X determined by HPV High Risk Typing Real-TM (Sacace Biotechnologies, Como, Italy) or INNO-LIPA HPV Genotyping Extra (**Fujirebio Europe**, Gent, Belgium) assays. In the case of HPV-X, although the high-risk HC2 (Qiagen, Gaithersburg, MD, USA) screening test showed a positive result, genotyping assay HPV High Risk Typing Real-TM (Sacace Biotechnologies, Como, Italy) failed to identify this HPV type. Likewise, within INNO-

LIPA HPV Genotyping Extra (**Fujirebio Europe**, Gent, Belgium) assay, specific SPF10-PCR HPV product was obtained, but HPV type was not ultimately identified.

RNA- based assay. RNA extraction was performed simultaneously with the extraction of DNA, as described above (DNA- based genotyping assays), from the corresponding aliquots of samples. The 15 µL of total nucleic acid extracts per sample were used in three real-time NASBA reactions (10 µL of Primer-Probe Reagent solution and 5 µL of eluate made 15 µL of each of the three real-time NASBA mixes).

The samples containing some of HPV-16, 18, 31, 33 and 45 types were tested for the presence of viral oncogene transcripts (E6/E7 mRNAs) using the type-specific real-time NASBA assay (NucliSENS EasyQ® HPV v1.1, bioMérieux, Lyon, France). Assay was performed according to the manufacturer's instructions.

Data obtained by detecting HPV E6/E7 mRNA were calculated in relation to the frequencies of women in each age group: women 30 years and younger (S1; 31/105, 29.50%) and older than 30 years of age (S2; 74/105, 70.50%) and the frequencies of hrHPV infections caused by any of HPV types in each group of women (S1: ≤30 years, n=53 and S2: >30 years, n=94).

Statistical analysis

Descriptive statistics were expressed by frequency, arithmetic mean, standard deviation (SD), minimum and maximum values and percentages. Type-specific calculations were made without taking into account multiple HPV infections, but considering each HPV infection as a single one.

RESULTS

In S1 group (≤30 years) of women, HPV DNA testing showed a higher proportion of women infected with any of HPV-16, 18, 31, 33 and 45 types (83.9%, 26/31) in comparison with the S2 group of women (>30 years; 67.6%, 50/74).

As expected, the type specific E6/E7 mRNA testing followed similar pattern as HPV DNA testing results showing the larger percentage of positivity in younger women (71.0%, 22/31) compared to women in the S2 group (58.1%, 43/74).

Total agreement of the positive results of hrHPV DNA and mRNA tests was higher in the group of older women (83.9% v. 75.8%, Table 1). The most

Table 1. Descriptive comparison of type-specific HPV DNA and E6/E7 mRNA assays in two age groups of women (S1: ≤30 years and S2: >30 years).

	Age group															
	S1 (≤30 years)					S2 (>30 years)										
	HPV DNA		HPV E6/E7 mRNA			HPV E6/E7 mRNA v. HPV DNA (f2/f1)x100 (%)		HPV DNA		HPV E6/E7 mRNA v. HPV DNA (f2/f1)x100 (%)						
	f1	(%) [†]	f2	(%) [†]	(%) [†]	f1	(%) [†]	f2	(%) [†]	(%) [†]		f1	(%) [†]	f2	(%) [†]	(%) [†]
HPV-16	17	32.1	14	42.4	26.4	82.4	31	33.0	28	50.0	29.8	90.3				
HPV-18	3	5.7	3	9.1	5.7	100.0	8	8.5	8	14.3	8.5	100.0				
HPV-31	9	17.0	5	15.2	9.4	55.6	12	12.8	8	14.3	8.5	66.7				
HPV-33	2	3.8	2	6.1	3.8	100.0	5	5.3	3	5.4	3.2	60.0				
HPV-45	2	3.8	1	3.0	1.9	50.0	0	0.0	0	0.0	0.0	0.0				
Total of HPV-16, 18, 31, 33 and 45	33*	62.4	25	75.8	47.2	-	56*	59.6	47	83.9	50.0	-				
Other HPV [‡]	20	37.6	-	-	-	-	38	40.4	-	-	-	-				
Total HPV [‡] in the age group of women	53*	100.0	-	-	-	-	94*	100.0	-	-	-	-				

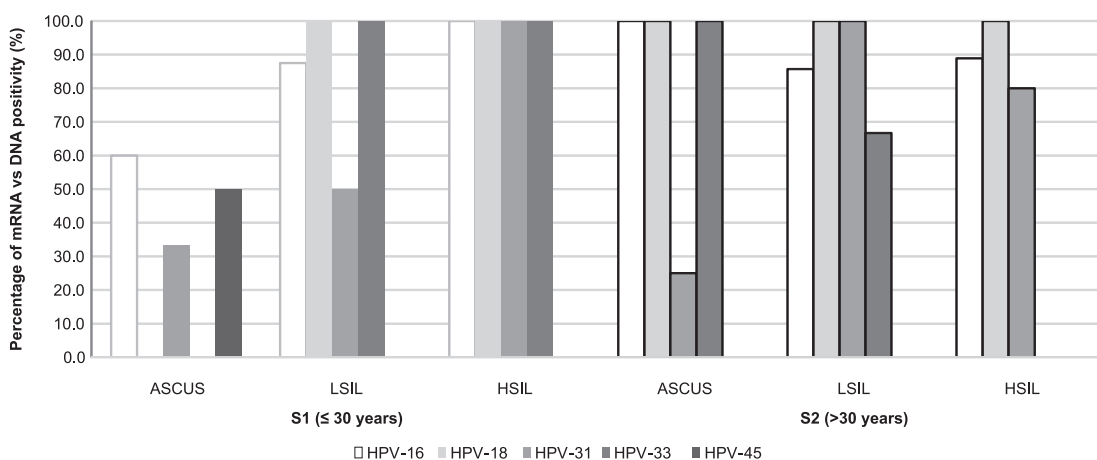
f1, frequency of HPV type detected by HPV DNA testing, f2, frequency of HPV type detected by HPV mRNA testing.

*Calculations were made according to the global frequencies of HPV-16, 18, 31, 33 and 45 types in each age group of women; †Calculations were made according to the total frequencies of all HPV types in each age group of women; ‡Other HPV[‡] belong among HPV-35, 39, 51, 52, 53, 56, 58, 59, 66, 7 and X;

prevalent type in both age groups was HPV-16 detected either by DNA or mRNA test (S1: ≤30 years, 32.1% and 26.4%; S2: >30 years, 33.0% vs 29.8%). The type-specific concordance of hrHPV DNA and mRNA tests revealed a 100.0% matching for detection of HPV-18 regardless of age and HPV-33 and -45 in the group of younger women (Table 1). These results were followed by HPV-16 in both age groups of women (S1: ≤30 years, 82.4% and S2: >30 years, 90.3%) (Table 1).

Increasing concordance of DNA and mRNA positive results, with the severity of cervical cytology, was observed in women ≤30 years of age harbouring HPV-16 or HPV-31 infections. In older women with ASCUS cytology, higher rates of matched positivity reaching 100% were detected in comparison with the same category of younger

women, specifically for types HPV-16, 18 and 33. In the group of younger women exhibiting HSIL cytology, absolute matching (100.0%) of both diagnostic tests was recorded for all four of five hrHPV types detected (except HPV-45) as well as for HPV-18 and HPV-33 in women with LSIL cytology. The same matching was revealed for HPV-18 DNA and mRNA positive results in women over 30 years of age. The E6/E7 mRNA of HPV-18 was detected in each infection it caused, starting from younger women with LSIL to older women exhibiting HSIL cytology. HPV-45 was detected only in younger women with ASCUS cytology (50.0%, 1/2 were mRNA positive). Furthermore, in older women with HSIL cytology, mRNA test showed positivity for HPV-16, 18 and 31 types (Figure 1).

**Figure 1. Five type-specific HPV E6/E7 mRNA v. DNA positivity according to age groups of women (S1: ≤30 years and S2: >30 years) and cervical cytology;**

ASCUS, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion

DISCUSSION

This cross-sectional study demonstrates the comparison of agreement rates of five hrHPV type positivity in two age groups of women with abnormal cervical cytology as a contribution to the management options for hrHPV DNA positive women in the cervical cancer screening program. Since the current cervical screening guidelines differ mainly in the management of women below 30 years of age (employing HPV testing or not), due to the nature of HPV infection and the rate of clearance/progression of cervical abnormalities to cancer (14,16,20), two groups of women (≤ 30 years and >30 years) were of interest in our study.

Five hrHPV types targeted by this study have been found in 75.1% of women with CIN2/3 and 88.5% of squamous cell carcinomas (SCC) (21,22). Among all five types detected, HPV-16 was the most prevalent type in both investigated age groups of women. Although type-specific HPV DNA testing showed a higher proportion of women infected with any of five hrHPV types in younger group, total positivity agreement of hrHPV DNA and mRNA tests was higher in older group of women (83.9%). The difference in the prevalence of five hrHPV types between the two age groups of women can be explained by the clearance rate of type-specific hrHPV infection. In fact, a study that evolved from POBASCAM (Population-Based Screening Amsterdam) (23), involving women aged 30 to 60 years to assess the type-specific clearance of hrHPV infection (24), showed the lowest clearance rates for HPV-16, 18, 31 and 33 and normal cytology. Significantly reduced 18-month clearance rates were observed for HPV-16 and 31 in women with normal cytology, and for HPV-16 in women with borderline/mild dyskaryosis. Women with HPV-16 persistence displayed an increased detection rate of \geq CIN3.

Detection of mRNAs of HPV oncogenes E6/E7 in harvested epithelial cells during sample collection may be indicative of a deregulated expression (25). Evidence that expression levels of E6/E7 mRNA are linked with the persistence of HPV infections and the severity of cervical lesions have already been reported (26,27). Moreover, it was shown that the investigated five-type specific mRNA test has lower sensitivity for detecting cervical disease but the higher specificity in comparison with other HPV tests (28,29).

Following the data from our study, a 100.0% agreement of type-specific hrHPV DNA and mRNA tests was recorded for HPV-18 (both groups) and HPV-33 (younger group) identifying the women with the deregulated expression of transforming viral genes without taking into the consideration the cytology diagnosis at this point. It was shown previously that women who were DNA positive and also positive for mRNA transcripts at baseline were significantly more likely to harbour persistent infection compared to those in whom DNA only was detected at baseline (27).

Comparing an age and cervical cytology with type-specific HPV DNA and mRNA assays we observed an increasing concordance of HPV-16 and HPV-31 DNA and mRNA positive results with the severity of cervical cytology (ASCUS-LSIL-HSIL) in younger group of women. Absolute agreement of both diagnostic tests was recorded for HPV-16, 18, 31 and 33 in younger HSIL and for HPV-18 and 33 in LSIL group. In older ASCUS group, higher agreement reaching 100% (HPV-16, 18 and 33) was recorded in the comparison with younger ASCUS group reaching at best 60% for HPV-16 infections. In older HSIL group, mRNA test showed positivity for HPV-16, 18 and 31 types.

Supporting our results, previously described data showed that HPV-16, but also HPV-18, 31 and 33, conferred an increased risk of \geq CIN3 (30,31), which reflects the combined effects of differences in persistence and oncogenic potential of these types in regards to other types.

The majority of young and immunocompetent women clear HPV infection spontaneously with the regression of cervical precancerous lesions. On the other hand, persistent infection can be detected by repeating the genotyping HPV DNA test or by using the variant of HPV mRNA assay (for a better prediction of persistent infection, if no repeat testing is applied) (19). However, the type-specific differences in clearance rates indicate the value of hrHPV genotyping in screening programs (24).

Exclusion of women under the age of 30 from HPV testing resulted in 45% lower detection rate of HSIL (15). These results highlight the advantage of the typing capability of the mRNA test used in the study, especially for HPV-16 and/or 18 genotypes, as the estimated relative risk for the

high-grade cervical disease is significantly higher for women who test HPV-16 and/or -18 positive, compared to general hrHPV positivity (31).

Interestingly, HPV-18 mRNA was detected in each infection it caused (starting with younger LSIL to older HSIL). Iftner et al., using data from the German Aptima- and HC2-based screening study (12), and Wright et al., using data from the U.S.-based ATHENA study evaluating the cobas assay (2), found that optimal triage strategies seemed to involve HPV-16/18 genotyping at baseline. In fact, if one positive finding of the type-specific E6/E7 mRNA test indicates the persistence of infection, then the typing of the virus by a DNA test (which is already included in some IVD screening platforms, such as m2000 System (Abbott Molecular) or Cobas 4800 (Roche)), in the categories of women with the highest agreement of both assays could also be considered a hint of the persistence of infection. Further studies should investigate these indications.

There are several potential limitations of the study: positive results obtained from different HPV DNA detection and genotyping assays were pooled for comparison with mRNA assay, relatively

limited sample size; and participation of women younger than 21 years of age, even though according to the current guidelines, this age group should not have been screened regardless of the age of sexual initiation or other risk factors.

In conclusion, absolute agreement of genotyping hrHPV DNA and mRNA test results was observed in investigated age groups of women and their cervical cytology. Accordingly, the results indicate the possibility of predicting the risk of persistent infection only by HPV DNA genotyping test, which is already integrated in many screening tests (at least for HPV-16 and HPV-18), with no need for additional RNA testing, in categories of infected women showing a high (absolute) agreement of both tests.

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TRANSPARENCY DECLARATION

Competing interests: None to declare.

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