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# Comparison of the detection of HPV-16, 18, 31, 33, and 45 by type-specific DNA- and E6/E7 mRNA-based assays of HPV DNA positive women with abnormal Pap smears

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A B S T R A C T

This study compares the type-specific human papillomavirus (HPV) DNA test with E6/E7 mRNA detection assay because of their importance in cervical cancer screening programs. A total of 105 women with positive high-risk Hybrid Capture 2 or Abbott RealTime High Risk HPV screening test and an abnormal cervical Pap smear were enrolled in the study. HPV typing was performed by multiplex real-time PCR (HPV High Risk Typing Real-TM test). HPV-16, 18, 31, 33, and 45 E6/E7 mRNAs were determined by type-specific real-time NASBA assay (NucliSENS EasyQ® HPV v1.1). Infections caused by HPV-16, 18, 31, 33, and 45 types increased with severity of cervical cytology ($p = 0.008$). Global positivity of five HPV E6/E7 mRNAs was lower than DNA positivity within women with *atypical* squamous cells of undetermined significance ($p = 0.016$; $p = 0.008$). High agreement of the tests was found in the groups of women with low-grade ($p = 1.000$; $p = 0.063$) and high-grade squamous intraepithelial *lesion* ($p = 0.250$; $p = 0.125$). Type-specific agreement of both diagnostic approaches was high regardless of cytology. Based on the found differences between HPV-16, 18, 31, 33, and 45 E6/E7 mRNA and DNA positivity, further study is needed to test the role of mRNA testing in the triage of women with *atypical* squamous cells of undetermined significance in Pap smear.



## 1. Introduction

Screening programs are still the leading measure in prevention of cervical cancer development, for both non-vaccinated and vaccinated women (Arbyn et al., 2012a; Dillner et al., 2008; Moyer, 2012). The method of conducting the screening programs and management of women with abnormal cytological findings should adapt to the development of new technologies (Cuzick et al., 2012). This requires detailed clinical studies that would validate the new methods and their applicability, cost-efficiency and clinical feasibility of their implementation (Arbyn et al., 2012b; Poljak et al., 2012; Ronco et al., 2010; Szarewski et al., 2012). The introduction of human papillomavirus (HPV) DNA detection increased the sensitivity of cervical cancer screening program compared to cytology. On the contrary, the loss in specificity for the risk assessment of women, due to the large number of transient HPV infections especially among the younger population, has occurred (Boulet et al., 2008; Cuzick et al., 2006; Mayrand et al., 2007). Some of the promising methods could be: the determination of high-risk HPV types (HR-HPV) (Meijer et al., 2006), the detection of excessive synthesis of cellular proteins that are directly or indirectly activated by deregulated expression of viral oncogenes E6 and E7 in the basal and parabasal cells of the epithelium (Andersson et al., 2006; Wentzensen et al., 2006) or changes in the pattern of methylation in several cellular genes that could effectively predict the initiation of neoplastic transformation (Wentzensen et al., 2006). However, many of these approaches are still used for research purposes only. On the basis of the results of comparative studies and virus induced oncogenesis, the detection of type-specific HR-HPV E6/E7 mRNA, as a marker of productive and persistent infection, might serve as a better risk evaluation factor for monitoring HPV DNA positive women and predicting high-grade cervical intraepithelial neoplasia and invasive cervical cancer (Cuschieri et al., 2004; Molden et al., 2005). The aim of the present study was to compare the type-specific detection of HR-HPV DNA and mRNA of five types HPV-16, 18, 31, 33, and 45 in outpatient group of tested women with *atypical* squamous cells of undetermined significance, low-grade squamous intraepithelial *lesion* or high-grade squamous intraepithelial *lesion* in cytological Papanicolaou (Pap) smears.

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## 2. Materials and methods

### 2.1. Study subjects

In the study, 105 women with positive cervical cytology ranging from *atypical* squamous cells of undetermined significance (37/105), low-grade squamous intraepithelial *lesion* (33/105) to high-grade squamous intraepithelial *lesion* (35/105) and HR-HPV infection detected by HPV DNA screening test were included. The average age of women was 36.6 ± 9.5 years (range 19–62). Women were mainly recruited from outpatient clinics and some gynecologists in private practice in Sarajevo, Bosnia and Herzegovina, who were reffered to University Clinical Centre – Sarajevo (Bosnia and Herzegovina) or Institute for Biomedical Diagnostics and Research "Nalaz" in Sarajevo (Bosnia and Herzegovina) for HPV testing. Conventional Pap smears were taken by gynecologists and examined by experienced cytologists independently of the HPV testing.

### 2.2. Specimen collection for laboratory analysis

Cervical specimens were collected from June 2010 to December 2012 with digene Cervical Sampler-STM (Qiagen, Gaithersburg, MD, USA) (88/105) for Hybrid Capture 2 HPV DNA test (HC2, Qiagen, Gaithersburg, MD, USA), Abbott Cervi-Collect Specimen Collection Kit (Abbott Molecular, Wiesbaden, Germany) (14/105) for Abbott RealTime high risk HPV test (Abbott Molecular, Wiesbaden, Germany) or ThinPrep Pap Test PreservCyt Solution (Cytic Corporation, Boxborough, MA, USA) (3/105) for NucliSENS® EasyQ HPV v1.1 (bioMérieux, Lyon, France) and HPV DNA screening assays as well. All samples were stored up to 7 days at +4 °C or longer at −20 °C/−70 °C until testing. HPV testing was performed at: (a) University Clinical Centre – Sarajevo, Bosnia and Herzegovina, Department of Clinical Microbiology-Division of Virology and (b) Institute for Biomedical Diagnostics and Research "Nalaz" in Sarajevo, Bosnia and Herzegovina.

### 2.3. DNA-based detection assays

Beside cytology as one of the inclusive criteria of the study, screening for HR-HPV infection was performed by one of the two clinical assays: HC2 (Qiagen, Gaithersburg, MD, USA) done with HR-HPV probe cocktail and Abbott RealTime High Risk HPV test (Abbott Molecular, Wiesbaden, Germany), in accordance with manufacturer's protocol.

### 2.4. DNA-based genotyping assays

Clinical material that met the criteria for inclusion in the study was separated in appropriate aliquots required for extraction of total nucleic acids and stored at a temperature of +4 °C up to 7 days or longer at −20 °C/−70 °C until genotyping assay was performed.

Total nucleic acid extraction was performed by using NucliSENS® miniMAG™ Magnetic Extraction kit (bioMérieux, Lyon, France) from 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, centrifugated 12 min on 1125 × *g*, than 4 ml of supernatant were discarded and pellet was resuspended in the rest of 1 ml of supernatant. DNA/RNA was eluted in 55 μl elution buffer and stored for a week at +4 °C or a month at −20 °C prior to the testing.

HPV genotyping was carried out by following assays according manufacturer's instructions:

- HPV High Risk Typing Real-TM test (Sacace Biotechnologies, Como, Italy) was used for qualitative detection and genotyping of 12 HR-HPVs: HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 in the cervical swabs. Test is based on multiplex real-time PCR amplification running in four tubes for each sample. Each tube contained primers directed against regions of three HPV types and human b-globin gene used as internal control. The 20 μl of total nucleic acid extracts per sample were used in 4 PCR reactions (8 μl of master mix and 5 μl of eluate made 13 μl of each of 4 PCR mixes).
- Genotyping of five samples with indeterminate results obtained by High Risk Typing Real-TM (Sacace Biotechnologies, Como, Italy) was performed by reverse line probe hybridization diagnostic kit INNO-LIPA HPV Genotyping Extra (Innogenetics, Gent, Belgium). The 10 μl of the total nucleic acid extracts were used for the analysis. Assay works with short PCR fragment (SPF10-PCR) designed to discriminate a broad spectrum of HPV types by reverse line probe hybridization, thus allowing the detection of 54 HPV types and the identification 28 among them.

### 2.5. RNA-based assay

RNA extraction was performed simultaneously with the extraction of DNA, as described in Section 2.4, 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).

Those samples that contained some of HPV-16, 18, 31, 33, and 45 types were tested for the presence of viral oncogene transcripts (E6/E7 mRNAs) by type-specific real-time NASBA assay (NucliSENS EasyQ® HPV v1.1, bioMérieux, Lyon, France). This qualitative assay uses real-time multiplex NASBA which amplifies mRNA in a DNA background with real-time detection of the products by molecular beacon probes. The test provides monitoring of the effectiveness of all steps in the procedure by detection of an internal control (mRNA from U1A house-keeping gene present in human cells) during analysis. 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 index cytological Pap smear group (*atypical* squamous cells of undetermined significance, $n = 37$; low-grade squamous intraepithelial *lesion*, $n = 33$ and high-grade squamous intraepithelial *lesion*, $n = 35$) and the frequencies of HR-HPV infections caused by any of HPV types in each group of women (*atypical* squamous cells of undetermined significance, $n = 53$; low-grade squamous intraepithelial *lesion*, $n = 49$ and high-grade squamous intraepithelial *lesion*, $n = 45$).

### 2.6. Statistical analysis

Statistical analysis was done by utilizing the SPSS for Windows (version 15.0, SPSS, Chicago, IL, USA). Descriptive statistics was expressed by frequency, arithmetic mean, standard deviation (SD), minimum and maximum values and percentages.

The statistical method used for assessing agreement between HPV DNA and E6/E7 mRNA assays was McNemar's test. The relation of cytologic diagnosis and HPV type was evaluated by chi-square test of independence of variables. Significance was based on $p < 0.05$.