VALIDATION OF VAGINAL SELF-SAMPLING AS AN ALTERNATIVE OPTION IN PCR BASED DETECTION OF HPV IN CERVICAL CANCER SCREENING IN BOSNIA AND HERZEGOVINA

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Abstract

Cervical cancer represents a serious health problem affecting women worldwide especially in developing countries due to low socioeconomic status, inadequate health-care infrastructure, weaknesses in education on this particular issue and lack of effective screening programmes. The primary aim of this study was to assess alternative screening method for the improvement of cervical cancer prevention in conditions of Bosnia and Herzegovina (B&H), which could be applicable in other developing countries as well. The study was conducted on 101 subjects who provided their self-sampled vaginal swabs and/or cervical specimens collected by their gynecologists. Universal Human Papilloma Virus (HPV) primer set optimized to detect a wide range of HPV types was used for HPV genotyping from obtained swab samples in multiplex PCR. Amplicons were analyzed in agarose gel and *Agilent 2100* bioanalyzer – a platform based on microfluid technology. Inter-rater agreement *kappa* (MedCalc2) was used to assess concordance between results of cervical and vaginal sample analysis. Out of 39 subjects who provided their vaginal and cervical samples, results of HPV detection mismatched in 10% of the cases. *Inter-rater agreement* showed good strength of coincidence between the results of cervical and vaginal sample analysis (*kappa*=0,748, CI=95 %). We presented an alternative PCR method for the detection of HPV based on vaginal self sampling which is affordable, informative, simple and applicable with high coverage level of defined targeted population and potentially significant in the given cultural and socioeconomic context.

Key words: *HPV, cervical cancer, PCR, screening, self-sampling*

Introduction

In spite of the advancement and rapid improvement of technologies in the fields of medicine, molecular biology and genetics, cervical cancer still represents a serious health problem affecting women worldwide and causing death of approximately 250000 women every year (WHO, 2007). According to the data of World Health Organization from year 2002 (WHO, 2002), about half a million new cases

are diagnosed each year, 80 % of which are recorded in developing countries. Nobel laurate Harald zur Hausen was the first to discover that the cause of cervical cancer lies in persistent infection with oncogenic types of the human papillomavirus (HPV) (Boshart et al., 1984).

Human papilloma virus (HPV) is type of small DNA virus which infects epithelial tissue of higher vertebrates leading to cell proliferation, which often results in the formation of papillomas. Human papillomaviruses are species specific. So far, over a hundred types of HPV have been identified and most of them infect anogenital system; those were the focus of this research. Infection with these HPV types is often asymptomatic, which makes regular gynecologic examinations crucial in the detection of possible precancerous and cancerous lesions caused by this virus. Protein L2 is a structural molecule of HPV and has many important roles: it introduces HPV DNA in viral particles formed by self-assembling of L1 main structural protein (Munger et al., 2004; Zhou et al., 2004); it helps virus enter the epithelial cells; it incorporates viral components in nucleus and also represents a potential target for new generation of protective vaccines (Pereira et al., 2009; Peter et al., 2013).

HPV genome covalently binds to mitotic chromosomes (You, 2010) and thus ensures its transfer and distribution to daughter cells. Although HPV genome is usually found in episomal form in the infected cells, it can also be inserted into genome of the cell and viral integration seems to be a crucial step in progression of low-grade to high-grade cervical intraepithelial lesions and cervical carcinoma (Kalantari et al., 1998; Klaes et al., 1999). This can explain the strong association between some types of HPV and high-grade cervical lesions or cervical carcinoma. Transcripts of integrated genomes are more stable and, in case of HPV 16 it has been shown that infected cells with the integrated HPV genomes had selective advantage in their growth (Jeon et al., 1995). Epigenetic factors associated with the progression of HPV infection from subclinical stage to invasive carcinoma are being investigated as well. Demethylation of CpG region happens before or at the same time with neoplastic progression (Badal et al., 2003) and it is probable that methylation pattern changes during replication cycle of HPV.

Identifying the type of HPV has a clinical significance with respect to different pathogenic potentials of various types. It has also been shown that the distribution of different HPV types varies in different geographic regions (Mammas et al., 2008), so knowledge of the frequencies of certain HPV types is important in creating appropriate screening strategies and policies of cervical cancer screening and prevention. Bosnia and Herzegovina, as many other developing countries, has no developed strategies of organized screening. Cervical carcinoma is the second most common carcinoma which affects women in Federation of Bosnia and Herzegovina with rate 6,9 per 100000 inhabitants for malignant neoplasms of cervix in 2011 (ZZJZ FBIH, 2011). Developing countries have a huge problem with cervical carcinoma because of factors such as: low socioeconomic status, limited health infrastructure, specific cultural context, inadequate health-care and lack of effective screening strategies. World Health Organization particularly points out the importance of well-organized screening in cervical carcinoma prevention. In the development of effective screening strategies special attention should be paid to their good integration into the existing health system, having in mind specific social, economic and cultural conditions in developing countries.

For all of the above-mentioned reasons, this research focused on the development of

alternative methods for HPV detection which could be potentially applicable for screening of women in developing countries. The primary aim of the study was optimization of the HPV detection based on the PCR methods with the special emphasis on the screening from self-sampled vaginal swabs. Multiplex PCR method was also developed to genotype some of the most common HPV genotypes in European population.

Materials and methods

In this study, the participants were volunteers who signed informed consent positively evaluated by Scientific Council of research institute where the study was conducted. The participants also provided relevant information for this study before biological sampling in the form of questionnaire, which contained no personal information.

The total of 101 female individuals accepted participation in study during the study period of two years (2009-2011). The sample was geographically limited to the Canton Sarajevo and divided into two groups:

1) general population (75 subjects who were randomly chosen; contacted in their firms/institutions or at home addresses),
2) special population (26 subjects addressed by gynecological consulting rooms because of the presence of certain cytological abnormalities or positive history of HPV infection).

Two methods of gynecological sampling were conducted in this study: self-sampling of vaginal swabs and sampling of cervical swabs provided by medical professional. Examinees were given detailed oral and written instructions on self-sampling. All sample swabs were taken using *DNAPapTM Cervical SamplerTM* (*QIAGEN GmbH*, Germany). DNA was isolated from all the collected cervical and vaginal samples using simple salting-out procedure (Miller et al., 1988). Agarose gel-electrophoresis was used for qualitative assessment of the obtained DNA. Concentration of the obtained DNA was determined by spectrophotometry (UV mini 1240, Shimadzu, Japan). Control PCR was performed with universal HPV primers as described before (Maki et al., 1991) which can detect a broad spectrum of HPV types yielding the product of around 270bp. The amplification reaction conditions were: 10% PCR buffer and 1 unit of Taq DNA Polymerase per reaction (TrueStart Taq Polymerase and Buffer, *ThermoScientific*, USA), 25mM MgCl2 (Fermentas, Latvia), 50 pM each of forward and reverse universal primer (Biotez GmbH, Germany). Thermal conditions of PCR were 30 cycles of chain reaction of denaturation step at 95°C for 60 s, annealing at 34°C for 45 s and elongation at 72°C for 45 s, after single initial denaturation step at 95°C for 5 min and followed by single extension step at 72°C for 5 min. PCR reactions were successfully optimized to the volume of 10 μl.

In addition to the optimization of universal marker PCR, multiplex PCR was optimized as well, in order to screen for the most common HPV types in European population further into the experiment. Positive controls for PCR reaction were plasmids with integrated HPV sequences and previously genotyped samples in standardized laboratory (*Genetic Lab*, Romania). Negative controls included validated HPV negative sample and blind control (water). Since we included two additional primer sets for detection of HPV types 31 and 35, amplification reaction was optimized with respect to the annealing temperature and concentrations of the chemicals and template.

Six primer pairs (Karlsen et al., 1996) were included in simultaneous multilocus co-amplification reaction to detect for HPV types

11, 16, 16 a, 18, 31 and 35 complementary templates. Primer pairs were divided in two reactions: (I) types 11, 16, 16a and (II) types 18, 31, 35 in order to avoid possible mispriming and unspecific products.

PCR reaction was done in total volume of 10 µl using 2 µl of DNA template and 20pM oligonucleotide sequences from three primer pairs in each primer set –set A (sequence specific primers for HPV types 11, 16 and 16a) and set B (sequence specific primers for HPV types 18, 31 and 35). A touch-down PCR technique was used to obtain high specificity of annealing conditions for primer pairs in multiplex reaction as follows: initial denaturation at 94°C for 3 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and elongation at 72°C for 45 s, then 20 repetitions of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and elongation at 72°C for 45 s, completed with final extension at 72°C for 7 min.

A platform based on microfluid technology (*Agilent 2100*, *Agilent Technologies*, USA), was used for highly precise detection and evaluation of the size of obtained PCR products. Statistical analysis *Inter-rater agreement* (*kappa*) of collected data was done using MedCalc2 statistical software.

Results and discussion

Swab samples were collected from total of 101 subjects aged 20-60 from Canton Sarajevo. DNA was successfully isolated from 98 individuals which represent 98% of the samples. It was not possible to obtain DNA from three samples of vaginal swabs.

Of 103 women who were contacted, 101 expressed interest to participate in the study, which reflects high response rate (98%). There were 39 participants who provided both cervical and vaginal samples for the purpose of cross-validation of the results obtained from two different biological specimens (self-sampled and sample taken by medical professional) of the same individual.

The applicability of the plasmids with incorporated sequences of HPV 11, 16, 16a, 18, 31 and 35 used as positive controls has been confirmed with positive PCR signal with respective primers. The reaction for each plasmid was performed in duplicate with accompanying negative control. Figure 1 shows that, besides HPV detection in cervical swabs, it was also possible to detect HPV infection in self-sampled vaginal swabs using universal HPV primer pairs.

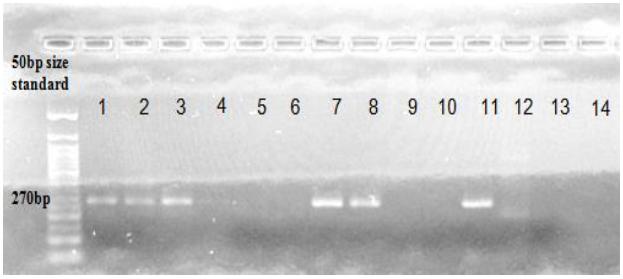

Figure 1. HPV detection in cervical and vaginal samples. DNA ladder (50 bp) in lane 1; lanes 2 to 7 – PCR products of self-sampled vaginal swabs (four HPV positive, two negative); lanes 8 and 9 – cervical and vaginal samples of the HPV positive subject; lanes 10 and 11 – cervical and vaginal samples of the HPV negative subject; lanes 12 and 13 – positive and negative controls previously genotyped by standardized laboratory; lane 14 – PCR negative control

Out of 101 collected vaginal samples we detected amplification of universal HPV sequence in 17 or 17,17% cases. Out of 17 HPV positive samples 11 (11% of overall study sample) had positive signal for high-risk HPV targeted DNA sequence: HPV type 16 in 6 cases, 31 in 2 cases and 35 was positive in one case.

Two specimens were positive for 2 high-risk genotypes- in one case 16 and 35 and in second

case 18 and 31. HPV type 11 was not detected in any of the analyzed samples. This finding also indicates that 65% of vaginal HPV infections are due to high-risk HPV genotypes.

PCR protocol with universal HPV primer modified according to (Maki et al., 1991) could be a valid template to detect targeted HPV genotypes relevant for European population in both cervical and vaginal – self-collected samples (Figure 2). PCR reactions were successfully optimized to the volume of 10 µl using TrueStart Hot Start Taq DNA Polymerase (ThermoScientific, USA) as minimum volume reliable for succesful HPV detection. As the reduction of volume reduces cost by 3-5 x per reaction this is also qute cost-effective approach.

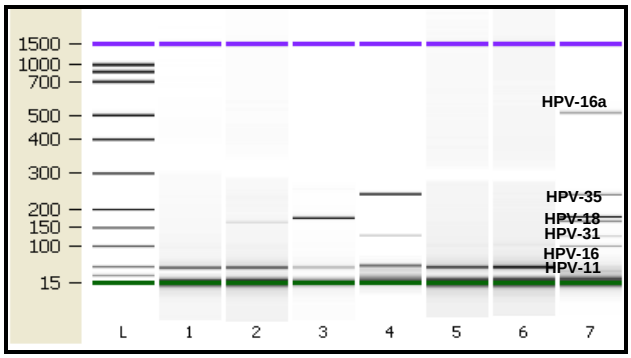

Figure 2. HPV genotyping; L – DNA ladder; lane 1 – HPV negative sample; lane 2 – HPV positive sample, type 31 (153 bp); lane 3 – HPV positive sample, type 18 (172 bp); lane 4 – HPV positive sample, type 35 (230 bp) and 16 (119 bp); lanes 5 and 6 – HPV negative samples; lane 7 – post-PCR mix of plasmid controls (HPV 11 – 80 bp, HPV 16 – 119 bp, HPV 31 – 153, HPV 18 – 172 bp, HPV 35 – 230 bp; HPV 16a – 499 bp)

Out of 39 vaginal samples that had a cervical sample counterpart, 35 (around 90%) showed concordance in the results of cervical and vaginal samples analysis. In four samples, HPV was detected in cervical smears, while it was not possible to detect it in vaginal swabs probably due to low quantities of viral DNA template retrieved during sampling. The quality of the band signals from vaginal swabs varied among the samples. Analysis of the disputed four samples using Real-Time PCR based method showed that the viral infection was present in the vaginal swabs as well, but in the lower quantity, which can be seen from the amplification diagram (Figure 3) where signal of the vaginal swab crosses threshold in the later stages compared to the cervical smear of the same patient.

Inter-rater agreement-kappa (Altman, 1991) of 0,74 (CI=95 %) showed good agreement between the results of cervical and vaginal sample analysis.

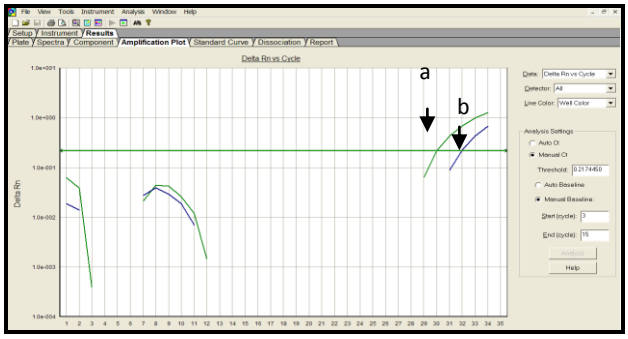

Figure 3. Real Time PCR Amplification Plot – cervical (a) and vaginal (b) sample of the same examinee showing successful PCR amplification in both.

Salting out procedure is a simple, effective and economical method for the extraction of DNA from cervical and vaginal samples. The three samples from which DNA extraction proved impossible serve as a reminder that the subjects should be thoroughly informed on the importance of strict adherence to the instructions on self-sampling. Compared to the standard PCR, Papanicoulaou test (Papa-test), the golden standard in detection of precancerous changes, has lower sensitivity (61,3%) with wider dispersion interval (18,6-94%); sensitivity of PCR is far greater (90%), with more compact interval (84,9-100%) (De Guglielmo et al., 2010) which does not change with the age of patients. However, the clinical importance of such high PCR sensitivity in identification of women with the increased risk for developing

cervical cancer (women who already have CIN2) is questionable and should be discussed. PCR specificity is lower, because it detects also transient HPV infections which are so common among the younger population.

Since previous studies have already confirmed high concordance rate between PCR and other sequence based detection - Hybrid Capture (HC) test (Soderlund-Strand et al., 2005), the multiplex PCR method described here could be validated in small-scale setting for the purpose of evaluation of reliability of multiloci detection sensitivity and specificity prior to further application.

Nevertheless, it should be emphasized that: 1) the link between viral load and progression toward cervical carcinoma is still examined; 2) in socioeconomically weak areas, especially rural, some women rarely have a chance for screening (Peter et al., 2013; Chou et al., 2016). Having this in mind, HPV detection using less costly methods can serve as an early indication and warning and also motivate women to inform themselves about this topic, pay attention to their life-style and improve their overall health (Arbyn et al., 2015). Combination of cytology and molecular-genetic methods can have very significant, high predictive value for cervical cancer (98%) which can decrease screening intervals for HPV negative patients and thus contribute to cost reduction for health-care programmes (De Guglielmo et al., 2010; Zehbe et al., 2011; Vassilakos, 2016).

It was shown here that the universal HPV primer pair can detect genotypes 31 and 35 as well. Some of the HPV positive samples could not be genotyped with the HPV specific primers used in this study which corresponds with the assumption that universal HPV primer pair can detect wide range of HPV genotypes.

The fact that it was not possible to genotype HPV from 35% of HPV positive examinees could indicate the presence of more relatively common HPV types in population of Bosnia and Herzegovina. The most common type detected in this study was HPV 16, but having in mind that the sample was geographically limited it cannot be concluded that this is the most common HPV type in Bosnia and Herzegovina. Since there is no official data for Bosnia and Herzegovina, there is a need to conduct a serious, large population study which would provide a basis for taking the appropriate preventive measures.

The discordance in results of four cases of examined cervical and vaginal samples can be explained by the lower amount of viral template in vaginal swab since the highest concentration of viral particles is in transformation zone of the cervix.

The potential use of self-sampled vaginal samples analysis for screening is concordant with the earlier findings (Karwalajtys et al., 2006; Petignat et al., 2007). In every study which examined the acceptance of self-sampling, women prefered self-sampling (Bidus et al., 2005) – it is far less uncomfortable and less embarrassing (Petignat et al., 2007). Analysis of self-sampled vaginal swabs for HPV detection could encourage women to participate in screening programmes. This could be particularly significant for women who do not regularly attend gynecological examinations for different reasons. The test could play role in early detection of pathological changes of cervix. Lower specificity of PCR test could be overcome by increasing combination of direct HPV detection and standard cytological procedures or by applying this test to women older than 35 (when prevalence of HPV positive infections decreases and prevalence of cervical intraepithelial neoplasias increases). While standard diagnostic kits mostly require high technical, financial and logistic support, this test is affordable and could be significant in

screening strategies of developing countries, but should be validated against the golden standard and validated diagnostic kits. In that sense, tests based on PCR technology could represent more simple and economic alternative in screening for cervical cancer prevention. It could be particularly important in regions which lack adequate infrastructure and cytologic analysis of high quality and critical areas where women do not have approach to health-care system or, for some cultural or traditional reasons, do not go to gynecological examinations.

For efficient screening programme it is necessary to: define targeted population and insure high rate of its coverage, offer health-care services of high quality which will enable identification, treatment and monitoring of the patients, provide trainings for health-care workers, inform and educate women and develop strategies which can be incorporated into national programme for tumor prevention.

There are several criteria which need to be fulfilled in order to have a successful screening based on HPV testing (WHO, 2012). The applicability of the method used in this study will be examined with regards to the given criteria:

1) minimally invasive sampling – since it has been shown that the virus can be detected in self-samples vaginal swabs, self-sampling could be applied as the minimally invasive procedure which is the least uncomfortable and economical as well.
2) high sensitivity and specificity of method to detect wide range of genital HPV types – although sensitivity and specificity of this particular test should be validated against standard diagnostic procedures, it has already been shown that PCR itself is highly sensitive method. Sensitivity can be increased by amplification of internal control (such as beta-globin gene) which could enable detection of potential PCR inhibition. Lower specificity, which is the main disadvantage of HPV PCR test could be overcome by using this test for screening women older than 30-35. The main advantage of the test with universal HPV primer pair is possible detection of wide spectrum of genital HPV types.
3) high level of intra- and interlaboratory reproducibility – reproducibility of the test can be checked by repeating the experiment and comparing results with other laboratories. Interlaboratory reproducibility in this study was checked by comparison of several samples with partner laboratory Genetic Lab (Romania). Additional three samples were analyzed by HC II test at the Clinical Centre University of Sarajevo. The complete concordance of the results was obtained, although the number of compared samples was limited. Functionality of the method was examined by repeating experiments in the laboratory – series of samples were analyzed under the same, controlled experimental conditions, with controls.
4) suitability of the method for automatic procedures and reading – PCR is automated and widely used method. Agilent Bioanalyzer also provides quick and precise analysis of given PCR products.
5) possibility to analyze large number of samples – this considers the possibility of collecting large number of samples and the existence of capacities for their analysis. This study indicates that there is a possibility that women actively take part in the screening programmes through self-sampling. It is necessary to standardize the optimized PCR method for HPV detection on the state level, to provide training of the staff and to improve effectiveness of existing laboratories and available human and material capacities.

There are potential capacities to apply this method but screening strategies need to incorporate timely and planning measures for effective usage of the available capacities on the state level.

6) short testing time – in this research, it is empirically estimated that the analysis of 20-25 samples, on average, takes 2 days per one analyst. The testing time could be improved through the distribution of work within the team.

7) economy – molecular-genetic HPV testing within the screening programme could quickly address HPV positive women to colposcopy in order to detect precancerous and cancerous lesions in time. HPV negative women would have longer screening intervals which would reduce the costs to health-care system. Hybrid Capture tests are validated but technically and financially demanding for developing countries. According to the study conducted in Brasil, HPV testing costs with Hybrid Capture technology are 3,84 times higher compared to PCR method (Nomelini et al., 2007). Detection of HPV based on PCR is more accessible, affordable, sensitive and simple method with high negative predictive value and, according to some studies (Morris, 2005; Nomelini et al., 2007; Romero-Pastrana, 2015) it will be the best option for primary screening.

Conclusions

Based on our research results, it is possible to successfully isolate DNA from cervical and vaginal samples using Miller's salting-out procedure. The modified PCR method with universal HPV primer presented here is sensitive to the level required to detect HPV infection presence in both cervical and vaginal samples. Having in mind that most of the developed molecular-genetic tests require high technical, financial and logistic support, detection of HPV based on PCR technology is simple, highly sensitive method with high negative predictive value which is also more affordable so it could represent a good alternative for screening in developing countries. Validated and clinically most confident method, previously approved by the relevant institutions, should be chosen for this purpose. *Kappa* value recorded in this study (0,784; CI=95%) indicates good agreement between the results of cervical and vaginal samples analysis. The optimized PCR method with universal HPV primer enables HPV detection in self-sampled vaginal swabs. This is particularly important for screening in developing countries. Analysis of self-sampled vaginal swabs for HPV detection could motivate women to participate in the screening programs which would be extremely important in the prevention of cervical cancer.

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