Predominant HPV Types From Cervical Swabs Determined by Molecular DNA Testing in a Period From 2018-2021 in Bosnia and Herzegovina

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ABSTRACT

Background: Human papillomavirus is a sexually transmitted infection and it is estimated that 75% of all women have been exposed to HPV infection in a certain period of life. High-risk types of HPV are considered to be one of the major causes of cervical cancer and its precursor intraepithelial neoplasia.

Objective: The aim of this study was to investigate the degree of HPV infections and to provide more data on HPV genotype distribution among women in Bosnia and Herzegovina (B&H).

Methods: Number of 375 samples were collected from different polyclinics in Sarajevo and were analyzed by Alea Genetic Center using Genomed f-HPV typing™ multiplex Fluorescent PCR kit for human papillomavirus genotyping. DNA required for this method is extracted from cervical swabs and amplified using a multiplex PCR reaction containing a set of 16 fluorescently labeled primers that recognize 16 HPV types. 14 HPV types are classified as high-risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and two are low-risk (6 and 11) HPV types.

Results: Results showed that in the years 2018, 2019, and 2021, HPV type 16 is predominant causing the high-risk factor for CIN1, CIN2, CIN3, and cervical cancer development. HPV 18 infection rates decreased during the last four years of study. HPV 6 infection rates increased during that period of time.

Conclusion: HPV 16 and HPV 18 are almost completely preventable by vaccination implying that the number of diagnosed cervical cancers in B&H could be much lower in the next decades if the HPV vaccination routine immunization program starts soon.

Keywords: HPV, fragmental analysis, cervical cancer, Real-time PCR.
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Molecular genotyping can determine multiple HPV infections as well as monitor individual HPV infections during a period of time. It is very important to determine if an infection is a low or high risk because of prognostic and patient follow-up in order to decrease the risk of the disease. High-risk HPV infection is a risk factor for precancer and cancer development. To predict high-risk HPV progression, a methylation test should be done. The methylation test determines whether an infection is actively transforming cervical cells into cancer detecting is the promoter of the tumor suppressor genes FAM19A4 and/or miR124-2 hypermethylated. Hypermethylation of these genes indicates a presence of advanced transforming CIN and a high-term risk of developing cervical cancer.

2. OBJECTIVE
The aim of the study were (1) to investigate the prevalence and incidence of HPV infections among B&H women during a period from 2018 to 2021; and (2) to provide more data on HPV genotype distribution

3. MATERIAL AND METHODS
The number of 375 clinical samples were collected from the year 2018-2021 and were retrospectively analyzed. DNA was extracted using QIAamp® DNA Mini Kit from cervical samples collected in dry swabs. Extracted DNA was amplified using a multiplex PCR reaction with a set of 16 fluorescently labeled primers that recognize 16 HPV types, from which 14 are high risk (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68) and two low risks (6 and 11) HPV types, using Genomed F-HPV typing™ multiplex Fluorescent PCR kit for human papillomavirus genotyping.

PCR program was set up according to the Genomed F-HPV typing™ multiplex Fluorescent PCR kit manual. PCR mix is prepared before PCR amplification by mixing 5µl F-HPV PCR Master Mix, 15µl Primers Mix, and 5µl of extracted DNA is added separately. Hot start polymerase is included in the kit for higher specificity which is inactive at room temperature. F-HPV typing™ is a rapid, specific, and sensitive method for HPV genotyping. The F-HPV typing™ kit generates amplicons between 156 and 489 bp which are efficiently separated by capillary electrophoresis (CE). For fragmental and software analysis, ABI PRISM 310 Genetic Analyzer and Applied Biosystems SeqStudio Genetic Analyzer were used. Each HPV type is identified by the size and color of corresponding amplicons.

Size and color of detected electropherogram peaks were as in the Genomed FHPV typing™ multiplex Fluorescent PCR kit manual.

4. RESULTS
In this study, 375 cervical swab samples from the female population of Bosnia and Herzegovina were analyzed in the period from 2018 to 2021. Human polymorphic sequence (STR) was used as an internal control to check the integrity of reaction and samples mishandling. Internal control (D18S386) can generate one peak (homozygous) or two peaks (heterozygous) of different sizes. In cases with high viral load, internal control can fail to amplify because of PCR reagents competition. In this case, we reduce template DNA input and repeat PCR reaction. For data analysis, GeneMapper software was used. In case of an invalid result caused by PCR inhibition, the whole procedure was repeated until an internal control peak is present.

| Type | 2018. | 2019. | 2020. | 2021. |
|------|-------|-------|-------|-------|
| 33   | 3     | 6     | 2     | 0     |
| 59   | 7     | 2     | 2     | 2     |
| 18   | 8     | 5     | 2     | 1     |
| 56   | 3     | 6     | 2     | 3     |
| 31   | 4     | 8     | 5     | 1     |
| 39   | 5     | 4     | 3     | 0     |
| 35   | 2     | 2     | 1     | 3     |
| 51   | 9     | 6     | 1     | 2     |
| 68   | 0     | 3     | 2     | 0     |
| 16   | 11    | 18    | 8     | 6     |
| 45   | 2     | 0     | 0     | 0     |
| 58   | 1     | 6     | 2     | 2     |
| 6    | 6     | 8     | 9     | 5     |
| 52   | 6     | 9     | 3     | 2     |
| 11   | 1     | 3     | 0     | 2     |
| 66   | 6     | 8     | 3     | 2     |
| HPV negative samples | 36    | 94    | 53    | 24    |
| HPV positive samples | 43    | 69    | 34    | 22    |
| Multiple HPV infection | 20    | 20    | 9     | 7     |
| Total number of samples | 79    | 163   | 87    | 46    |
| Table 1. Number of each HPV types per year |
| HPV type | 2018. | 2019. | 2020. | 2021. |
|---------|-------|-------|-------|-------|
| 33      | 3,79% | 3,68% | 2,29% | 0%    |
| 59      | 8,86% | 1,22% | 2,29% | 4,34% |
| 18      | 10,12%| 3,06% | 2,29% | 2,12% |
| 56      | 3,79% | 3,68% | 2,29% | 6,52% |
| 31      | 5,06% | 4,90% | 5,74% | 2,12% |
| 39      | 6,52% | 2,45% | 3,44% | 0%    |
| 35      | 2,53% | 1,22% | 1%    | 6,52% |
| 51      | 11,39%| 3,68% | 1%    | 4,34% |
| 68      | 0%    | 1,84% | 2,29% | 0%    |
| 16      | 13,92%| 11,04%| 9,19% | 13,04%|
| 45      | 2,53% | 0%    | 0%    | 0%    |
| 58      | 1,26% | 3,68% | 2,29% | 4,34% |
| 6      | 7,59% | 4,90% | 10,34%| 10,86%|
| 52      | 7,59% | 5,52% | 3,44% | 4,34% |
| 11      | 1,26% | 1,84% | 0%    | 4,34% |
| 66      | 7,59% | 4,9%  | 3,44% | 4,34% |
| HPV negative samples | 45,56%| 57,66%| 60,91%| 52,17%|
| HPV positive samples | 54,43%| 42,33%| 39,08%| 47,82%|
| Multiple HPV infection | 25,31%| 12,26%| 26,47%| 31,81%|
| Total number of detected HPV types | 20    | 20    | 34    | 22    |
| Total number of samples | 79    | 163   | 87    | 46    |
| Table 2. Percentage of each HPV type per year |
Data used for this study is obtained from the data of Alea Genetic Center in four years and was retrospectively analyzed. Based on that information, clinical findings of HPV progression to CIN or cervical neoplasia are unfortunately unavailable.

5. DISCUSSION

In the years 2018, 2019, and 2021, HPV type 16 is a predominant type which is a high-risk factor for CIN1, CIN2, CIN3, and cervical cancer. 50% of cervical cancers and CIN1, CIN2, CIN3 are caused by HPV type 16 (8). Different epidemiologic studies have demonstrated that HPV 16 and 18 are considered strong carcinogens and that they progress to cervical cancer faster compared to other HPV types (9, 10).

The main advantages of capillary electrophoresis over an RT-PCR technique are multiplexing, internal control per sample, and detection of multiple HPV infections. Capillary electrophoresis combined with PCR containing type-specific primers provides accurate genotyping of different HPV loci seen in HPV cancer cases. Once (multiple) HPV infection is diagnosed it helps to redesign screening programs and improve their quality, which is necessary for eliminating cancer as a life-threatening disease. Internal control that was used for CEHPV genotyping consisted of human STR primer set of target cDNA template for each primer set and it provided the assurance that clinical specimens are successfully amplified, detected, and were not cross-contaminated. This type of control is far superior over a single control sample per batch (Figure 1).

At Figure 1 internal control is present on the electropherogram, which confirms the accuracy of the procedure. An analysis of the results showed the presence of multiple HPV infections. Detected HPV types 16, 31, 39, and 56 belong to high-risk virus types.

During the last decade, many studies implied a strong correlation between HPV infection and the spectrum of diseases and cancers including head and neck cancers, cervical cancers, and several skin and oral diseases. The two most common HPV infections are 16 and 18, which are the main types directly linked to carcinogenesis, promoting chromosomal anomalies and cellular immortalization (11). In this study, we saw the growing incidence of HPV 16 infection during 2018-2021 among tested patients from Bosnia and Herzegovina, while HPV 18 infection rates decreased during the last 4 years of study. It is estimated that in B&H yearly, 556 women are diagnosed with cervical cancer out of which 141 patients die. In this case, it is important to note that HPV 16 and HPV 18 are almost completely preventable by vaccination (12) implying that the number of diagnosed cervical cancers in Bosnia and Herzegovina could be much lower in the next decades if the HPV vaccination routine immunization program starts soon (13).

Besides factors -16 and -18 other HPV types including -45, -31, -33, -52, -58, and -35, are shown to be responsible for approximately 90% of all cervical cancers worldwide. While other HPV genotypes did not show that strong correlation with cervical cancer. Types -6 and -11 are low-risk factors causing malignant lesions (anogenital warts and recurrent respiratory papillomatosis) that are most commonly sexually transmitted (14). Among 6 tested patients from Bosnia and Herzegovina, these two factors showed a slightly growing incidence during the last two years. Also, multiple HPV infections were higher in 2020-2021 than in 2018-2019.

| Predominant HPV types | 2018. | 2019. | 2020. | 2021. |
|-----------------------|-------|-------|-------|-------|
| 1. 16 (13.92%)        | 16 (11.04%) | 6 (10.34%) | 16 (13.04%) |
| 2. 51 (11.39%)        | 52 (5.52%) | 16 (9.19%) | 6 (10.86%) |
| 3. 18 (10.12%)        | 31, 6, 39 (4.90%) | 31 (5.74%) | 35, 56 (6.52%) |

Table 3. Predominant HPV types with percentage per each year
6. CONCLUSION

Future studies of HPV infections among the Bosnia and Herzegovina population should include histopathological examination of HPV infection that progressed to CIN or cervical neoplasia to establish a correlation between HPV genotypes and different types of tumor. Also, once high-risk HPV infection is detected, furthermore methylation test should be considered since it helps to reduce unnecessary medical treatments and save time and money for diagnostics. Age, high parity, use of hormonal contraceptives, tobacco smoking, and eating habits should be considered as well since they are susceptible cofactors of HPV progression.

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