

Genotyping of human papillomavirus high-risk types and correlation with potential risk factors

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Abstract

Human papillomavirus (HPV) is one of the most common sexually transmitted disease (STD) worldwide. HPV is a small, double-stranded DNA virus that infects mucosal and coetaneous epithelia through tiny cuts and abrasion, exposed by the cells of the basal layer. If diagnosed on time HPV can be successfully treated, however, in some cases it can lead to the development of tumor. Most of cervical tumors contain HPV DNA, and majority of them contain high-risk types HPV16 and HPV18. Different risk factors are associated with HPV infection, including behavioral and biological predispositions. Aim of this study is to genotype potentially infected patients on high-risk types HPV DNA and to correlate the results with patient's different biological and lifestyle factors. For this purpose 20 gynecological smear samples were collected from women, previously subjected to the survey. Methodology included DNA extraction and real-time polymerase chain reaction (RT-PCR). Results showed that out of 20 patients five were positive for high risk HPV. Four of five positive patients were positive on HPV16 type of which one had HPV16 together with others high risk types. One of five positive patients was positive on HPV18 type and other high risk types not identified. Final outcome indicates the correlation of potentially endangered patients with specific sexual behavior and lifestyles, and furthermore represent the general consensus and awareness level this disease has on the public.

Key Words: Human papillomavirus (HPV), HPV high risk types, RT-PCR

1. Introduction

Human papillomaviruses (HPVs) comprise a highly diverse group of small, non-enveloped double-stranded DNA viruses that belong to the Papillomaviridae family. They are agents of the most common sexually transmitted diseases that can infect both females and males. HPV infection is very common among men and women across all geographical, racial and socio-economic subgroup worldwide [1]. Probably the widespread of this infection, made HPV one of the most researched issue today. If diagnosed on the time this disease can be successfully treated, however in some cases it can progress to a cancer. Recent studies indicated that over 99% of cervical tumors contain HPV DNA, approximately 65% of them contain the most

common high-risk types HPV 16 and 18. Using DNA sequence determination and classification, genotypes such as 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are classified as high-risk types, meaning that they are prone to develop a cancer [2]. The International Committee on the Taxonomy of Viruses (ICTV) has classified the papillomaviruses as a distinct taxonomy family, the *Papillomaviridae*. Taxonomy status of papillomavirus types is based on the sequence of L1 gene, which are different from each other by at least 10%, 2-10%, and maximally 2% [3]. In benign cervical lesions, viral DNA is maintained in a free, extra chromosomal circular form termed episome, but, in many cervical cancers the DNA is integrated into the host genome. It is also demonstrated that the integration occurs more frequently in carcinomas associated with

HPV 18 than in those associated with HPV 16. Integrated HPV was detected in 72% of the HPV 16 associated carcinomas compared to 100% of the HPV 18 associated carcinomas. In some cases it is also possible that both the integrated HPV DNA and episomal HPV DNA coexist [4]. According to the information from Institute for Public Health FBH in 2009 year cervical cancer was third malignance of ten leading malignances among women suffering from cancer in Federation BiH. In the year 2009 out of 2715 women suffering from cancer, 215 is diagnosed with cervical cancer, in percentage 7,9% out of 2715 cases. In the year 2010, number of women suffering from cervical cancer decreased for 11.63% giving the nubmber of 190 cases. Risk factors for HPV infection can be of biological or behavioral nature. Biologically based risk factors primarily affect downstream transitions in the oncogenesis pathway rather than risk for acquiring HPV. They include HPV virus its self and intrinsic host factors affecting immune response. Known or proposed host factors include immunosupression and human immunodeficiency virus infection, cooccurrence of other sexually transmitted infections such as Chlamydia trachomatis, herpes simplex virus, micronutrient deficiency, genetic polymorphism in the human leukocyte antigen (HLA) system, and patient age. Different studies have demonstrated that younger individuals have increased risk for HPV infection. The highest prevalence of HPV occurs among adolescent and young adults between the age of 15 and 25, and it is estimated that more than 75% of new HPV infections occur in individuals of this age range. Behaviorally based risk factor primarily affects the acquisition of HPV infection. They include characteristics of women's sexual history (number of partners and contraceptive use) and substance use history (alcohol, cigarettes, illicit drugs). Taking in consideration sexual history characteristics, increased number of lifetime sexual partners and having had a recent new sexual partner are two factors that have been consistently shown to be associated with an increased risk of HPV infection. Impact of substances use on risk for HPV infection has been similarly difficult to assess. Some studies showed that current or past cigarette smoking can be associated with the acquisition of HPV infection, progression to precancerous lesions and cervical cancer, but most of other studies failed to support this connection. [5]

2. Objective

Aim of this study is to genotype potentially infected female patients on high-risk types HPV DNA and to correlate the results with patient's different biological and lifestyle factors. Final outcome should indicate the correlation of potentially endangered patients with specific sexual behavior and lifestyles, and furthermore

represent the general consensus and awareness level this disease has on the public.

3. Materials and Methods

3.1 Patients. Twenty women being at risk for HPV infection were tested at Institute for Biomedical Diagnostics and Research "NALAZ". Only after their consent and patients agreement, tested samples were implemented in this study. Samples were collected from *cervic uteri* (cervix). For the collecion and transport of cervical specimens and detection of HPV, *Abbot Cevi-Collect Specimen Collection Kit (USA)* was used. Collection tubes are stored at 2°- 8°C before the DNA extraction, and at -20°C after the DNA extraction.

3.2 DNA Extraction. To extract HPV DNA ABBOTT *mSample Preparation System_{DNA} for RealTime High Risk HPV extraction kit (USA)* was used. The purpose of sample preparation is to extract, concentrate and purify the target DNA molecules for amplification. The process is based on magnetic particle use, that capture nucleic acids and washes the particles to remove unbound sample components. After the nucleic acids are eluted PCR amplification was preformed.

Real-Time PCR (*RealTime High Risk HPV, Abbott, USA*) amplifies and detects High Risk (HR) HPV DNA in cervical cells collected in liquid media. The detection of HR HPV genotypes is achieved through a primer mix targeting a conserved L1 region of HPV genome and single stranded DNA probes. The assay can differentiate between HPV 16, HPV 18 and non-HPV 16/18 genotypes. A primer mix consists of three forward and two reverse primers targeting a conserved L1 region of HPV genome (approx. 150bp). Signal for HR HPV genotypes is generated with the use of fluorescent labeled probe (*TaqMan probe*). Internal Control (IC) amplicons are generated with a primer set targeting an endogenous human β -globin sequence and are detected with the IC specific probe. Probes for HPV 16, HPV 18, non-HPV 16/18 genotypes and IC are labeled with different fluorophores allowing their signals to be distinguishable in a single reaction. In each of 96-wells, 25 μ l of the amplification master mix is added, together with 25 μ l of sample elute giving the total volume of 50 μ l. 25 μ l of the amplification master mix contains: 11,6 μ l Activation Reagent, 16,8 μ l HPV oligonucleotide reagent (primers) and 2,9 μ l AmliTaq Gold Enzyme.

3.3 Results

In the period of three months (March-May, 2013), out of twenty tested women five were positive for HPV infection. Three patients (p1261, p1105, p1062) had HPV16 type. One patient (p1427) had HPV16 types and was co-infected with others HPVs that were not identified. One patient (p1066) had HPV18 type and others HPVs detected as well (Fig. 1). Important factors

such as sexual behavior and lifestyle were analyzed for HPV positive patients. Patients were questioned about other sexual transmitted diseases (STDs) and four of infected patients had *Candidiasis*, commonly known as fungal infection. One patient (p1105) was negative for other STDs. Age of first sexual intercourse for four positive patients was between fifteen and twenty (15-20) years old. One patient (the same that was negative for other STDs) had first sexual intercourse with older than twenty (>20) years old. Question about contraceptive use, was positive for all infected patients. Four patients had more than one sexual partner during their lifetime; one patient (p1261) had one sexual partner (Table 2). Alcohol and cigarette consumption can increase the risk for HPV infection. Out of five positive patients four of them were positive for cigarette consumption, one patient was negative (p1105) (Fig. 2). Answers for alcohol consumption were classified as Never, Rare (1-2 glass per month), Often (1-2 Glass per week) and Very often (1-2 glass per day). All of the HPV positive patients were positive for alcohol consumption of which four were Rare, and one (p1261) was Often (Fig. 3 and table 3).

Table 1: HPV genotyping results. Results of HPV testing was classified as HPV negative, HPV16 positive, HPV16 and other type positive, and HPV18 and other type positive.

Patient's Code	Year	Place	HPV
p1608	20-25	KS	Neg
p1487	25-30	KS	Neg
p1427	20-25	KS	HPV16/others
p1372	25-30	KS	Neg
p1356	20-25	KS	Neg
p1340	25-30	KS	Neg
p1294	25-30	KS	Neg
p1289	30-40	KS	Neg
p1263	30-40	KS	0neg
p1261	30-40	KS	HPV16
p1235	30-40	KS	Neg
p1163	30-40	KS	Neg
p1133	25-30	KS	Neg
p1105	25-30	KS	HPV16
p1104	30-40	KS	Neg
p1086	25-30	KS	Neg
p1080	>40	TK	Neg
p1066	20-25	KS	HPV18/others
p1064	25-30	KS	Neg
p1062	30-40	KS	HPV16

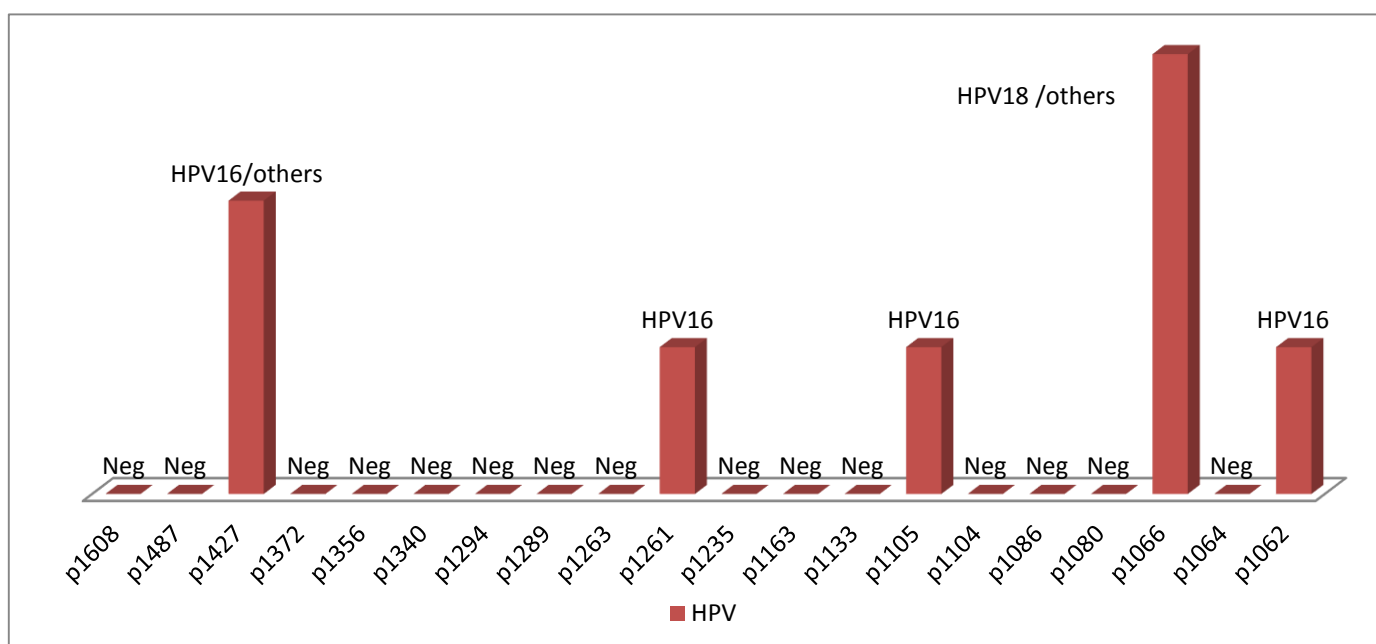


Figure 1: Schematic representation of HPV positive patients

Table 2: Correlation of HPV positive patients with the type of STDs and sexual behavior

Patient's Code	HPV type	Other STDs	1. sexual intercourse	Contraception	> 1 sexual partners
1427	HPV16/others	Candida	15-20	Condom and Pills	Yes
1261	HPV 16	Candida	15-20	Contraceptive Pills	No
1105	HPV16	Neg.	>20	Condom	Yes
1066	HPV18/others	Candida	15-20	Condom	Yes
1062	HPV16	Candida	15-20	Condom and Pills	Yes

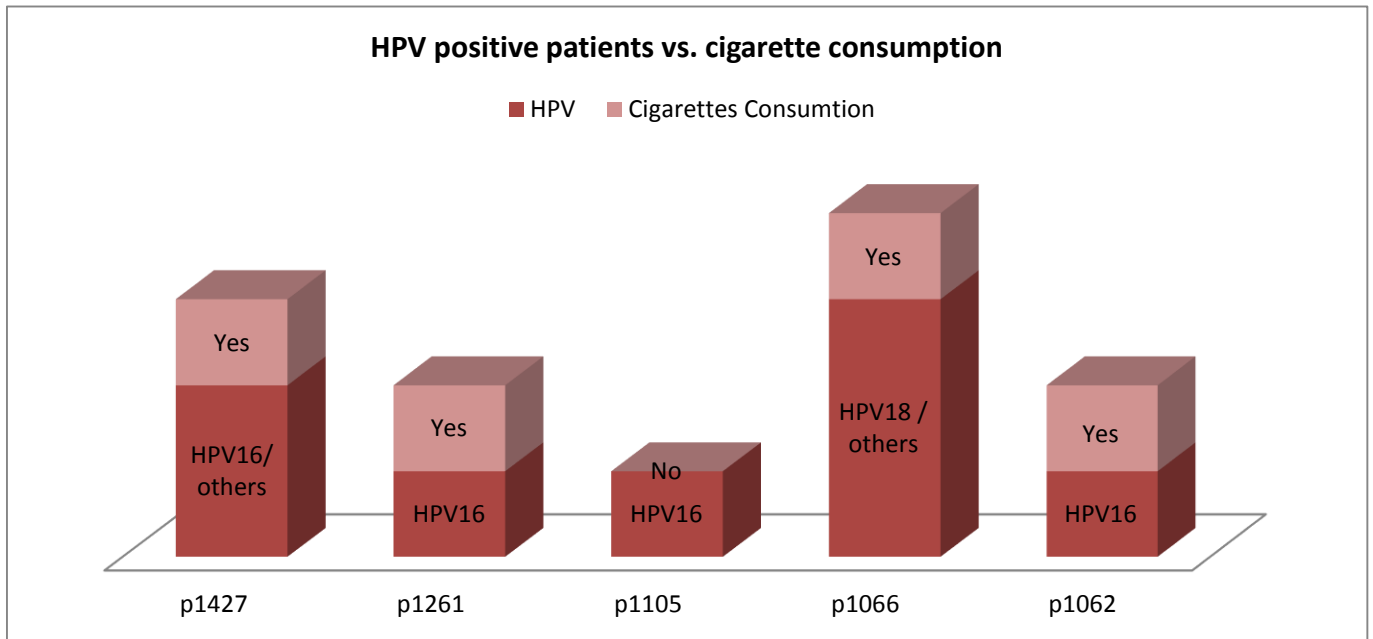


Figure 2: Schematic representation of HPV positive patients vs. cigarette consumption

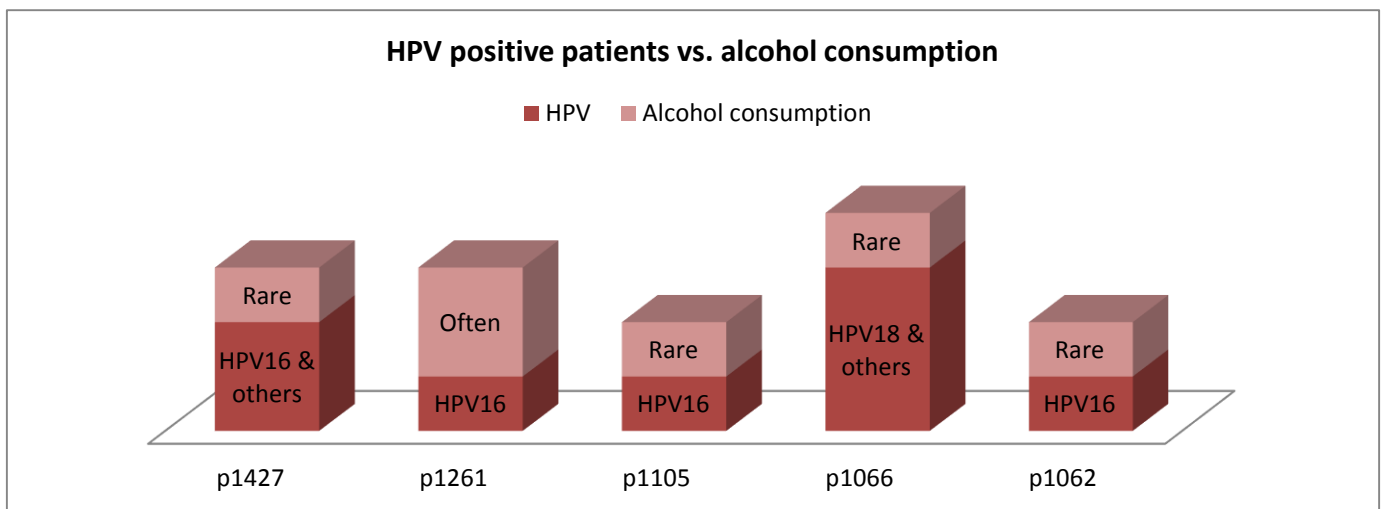


Figure 3: Schematic representation of HPV positive patients vs. alcohol consumption

To estimate the general consensus and awareness level that HPV infection has on the public, tested patients were subjected to the survey. Results are showing that the level of general information about HPV infection is relatively low (Fig. 4). Furthermore, only three of twenty patients knew about HPV HR types 16 and 18 (Fig. 5) and twelve patients never have heard about HPV vaccines existence, and three patients didn't know that HPV infection can lead to cervical cancer development (Fig. 6 and 7). Only two patients get the idea to do HPV testing by themselves, while other eighteen patients were sent by their gynecologists (Fig. 9).

Table 3: Correlation of HPV positive patients with cigarettes and alcohol consumption

Patient's Code	HPV	Smoker	Alcohol use
1427	HPV16/others	Yes	Rare
1261	HPV16	Yes	Often
1105	HPV16	No	Rare
1066	HPV18/others	Yes	Rare
1062	HPV16	Yes	Rare

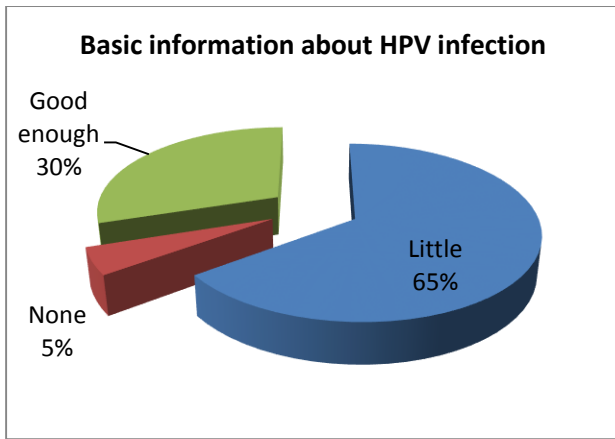


Figure 4: Basic information about HPV infection among twenty tested patients: good enough 30% (6patients), little 65% (13 patients) and none 5% (1patient).

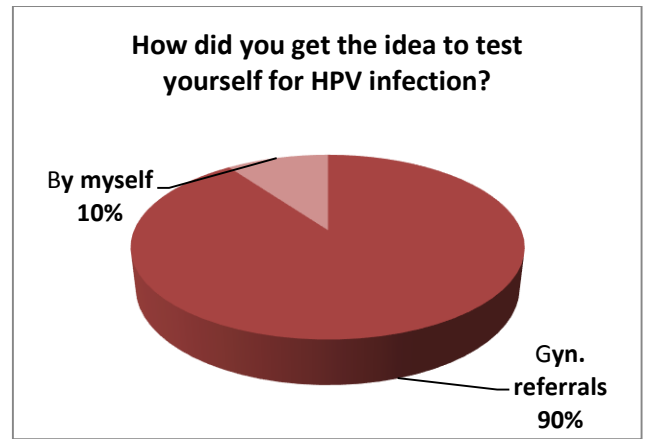


Figure 7: Patients referrals: Only 10% (2 patients) of patients get the idea to do HPV testing by themselves, while 90% (18 patients) of them get gynecological referrals.

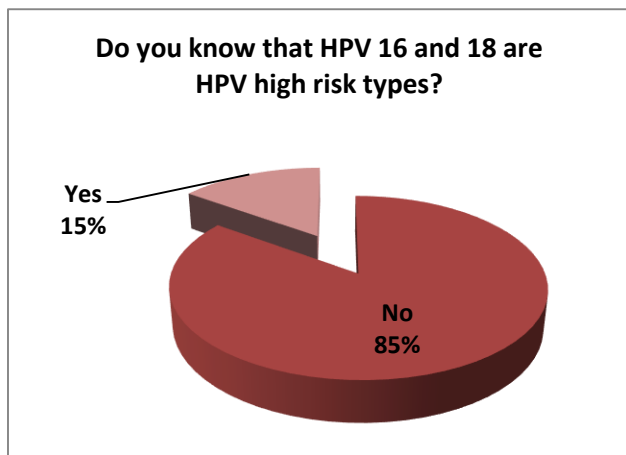


Figure 5: Knowledge about HPV high risk types: 15% (3 patients) knew about HPV HR 16 and 18 types, and 85% (17 patients) didn't know about HPV HR 16 and 18 types.

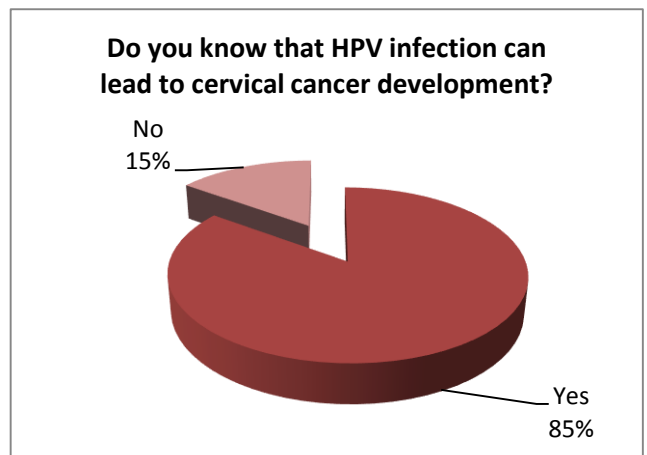


Figure 8: Knowledge about how the HPV infection is correlated with cancer: 15% (3 patients) didn't know that HPV infection can progress to cervical cancer, and 85% of them (17 patients) knew that fact.

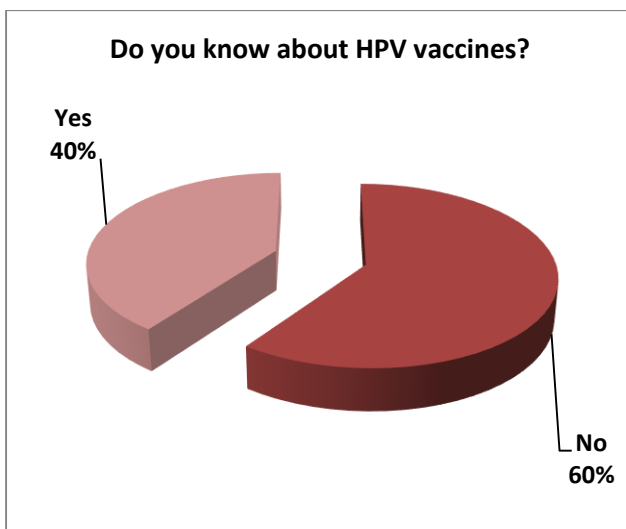


Figure 6: Awareness about HPV vaccines: 40% (8 patients) knew about HPV vaccines, 60% (12 patients) didn't know.

4. Discussion

In the period of three months during which time this study was conducted, out of twenty patients, five of them were positive for HPV infection. The majority of positive results were identified as HPV HR 16 type. This proves the frequency of this particular HPV type among HPV infected patient. One patient was identified as HPV HR 18 type positive. Two of five infected patients were also co infected with other HR HPV types that were not identified. They had a combination with HR HPV other types and HPV HR 16 or HPV HR 18 type. All of the results suggest that HPV HR types, and especially HR HPV 16 and 18 are commonly found among HPV infected patients, leading to an increased risk for cervical cancer. As previously stated, over 99% of cervical tumors contain HR HPV DNA, and 65% of them contain HR types HPV 16 and 18. All of the infected patients were from Canton Sarajevo. The reason for this could be that people from rural areas are less informed about this type of infection. Age difference among the infected patients is pretty high (from 20 to 40 years old). Two of the infected patients were in the age

group from 20 to 25 years, one from 25 to 30, and the other two patients from 30 to 40 years old. HPV infection is present among the young as much as in middle aged individuals. Two younger patients in the range from 20 to 25 were co-infected with other HR HPV together with HPV16 or HPV18 HR type. A possible reason for this is that young individuals tend to change their sexual partners more frequently, or it can be caused by lack of adaptive immune response. Sexual behavior and other risk factors can increase predisposition for HPV infection. All of the HPV positive patients used contraception. Four positive patients used condom as contraception, while two of them use condom in combination with contraceptive pills. Only one patient used just contraceptive pills. Using condoms as contraception in most cases can protect us from getting this type of infection, however some level of risk exist. Only one positive patient used contraceptive pills that could increase the risk for infection. Pills can protect individuals from unwanted pregnancy, but they can't prevent the infection with sexually transmitted disease (STD).

Becoming sexually active at early age and multiple numbers of lifetime sexual partners are important risk factors. Age range of first sexual intercourse for four of infected patients was between 15 to 20 years old. Only one patient was more than twenty years old. Year range between 15 to 20 years can't be estimated as to early period for becoming sexually active, taking in consideration that most of individuals become sexually active with eighteen or nineteen years old. What is important here to mention is that individuals that became sexually active earlier have higher predisposition for HPV infection. This was indicated also with our data, since four out of five infected patients became sexually active in the period from 15 to 20 years old, while only one patient was over twenty years old. Four of five positive patients had more than one sexual partner during their lifetimes, and only one positive patient had one sexual partner. Again our data is supporting the fact that increased number of sexual partners, increase predisposition for HPV infection. The risk factors also increases with recent change of sexual partner, but this information was not included in our survey. Being co-infected with other STDs, primarily affects downstream transition in the oncogenesis pathway, rather than risk for acquiring HPV. Four HPV positive patients had Candidiasis or fungal infection in their lifetime. *C. albicans* is the most prevalent species in the majority of cases of asymptomatic colonization and vulvovaginal candidiasis. Certain species of *Candida* are more pathogenic than others, and they can induce hyphal and pseudohyphal formation, and enhance proteolytic activity and antigen modulation. These properties enable *Candida* to penetrate the mucosal surface and induce mucosal swelling, erythema, and exfoliation of cells. Individuals with abnormal vaginal microbiota, can have

higher risk for formation of cervical cytologic abnormalities than woman without these conditions [6]. Behavioral risk factors such as alcohol consumption and cigarette smoking can be also associated with higher risk for HPV infection. Our results are showing that all five infected patients were positive for alcohol use, and four of them were positive for cigarette smoking. General information about HPV infection was obtained by the patient's survey answers. Most of the patients (65%) were poorly informed about this infection. Only one patient wasn't informed at all. We can estimate that overall knowledge about HPV is relatively poor. Positive fact is that most of the patients knew that HPV infection can lead to cervical cancer development. Keeping this in mind one should reconsider the level of seriousness that this infection can bring. Only three patients knew about HR HPV types. Considering our environment and educational system this can be caused by lack of readily found information's about HPV infection. Situation about HPV vaccines information wasn't as bad as expected, since HPV vaccines are relatively new approach in medicine.

Eight tested patients knew about HPV vaccines existence. Low awareness level about this infection is also estimated, by the information that of all twenty patients only two of them got the idea to do HPV test by themselves, while other eighteen were sent by their gynecologists. This proves us that people from this area are not concerned about their sexual health. Since we don't have any sexual education in our elementary or high schools, many people are not concerned because they don't know that this disease even exists.

5. Conclusion

HPV is one of the most common sexually transmitted disease in the world. Variations that exist among HPV viruses, make this disease harder to treat. Its silent infection and evasion can cause this disease to proliferate without any signs or symptoms, eventually leading to cervical cancer development. Because of this it is important to know the seriosity that this infection can bring. If it is estimated that 90% of cervical tumors contain HR HPV DNA, than public awareness and regular testing on HPV infection could drastically decrease the risk for cervical cancer. Solution for HPV infection in the future can be in HPV vaccines. Since people from our country still don't have the accessibility to this type of vaccination; increasing the awareness level through our education system about STD can be the alternative solution. If people are not informed about STD, and if they don't know what are the risk factors, they can't protect themselves from this type of disease. Awareness about HPV infection will not only decrease the level of infected patient, but will also decrease the number of patients suffering from cervical cancer.

6. References

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