



Sero-Molecular Screening of Human Papillomavirus (HPV) Antibodies and Its Associated Risk Factors Among Female University Students in Port Harcourt



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Abstract

Human papillomavirus is a human-specific infection associated with malignancy in humans. The study was aimed to investigate HPV antibodies among young university female subjects in Port Harcourt. A total of 209 apparently healthy female subjects were recruited using a randomized cross-sectional research design. The study explored the potential of the double antibody sandwich ELISA kit by MyBiosource.com, United States and using the Staffax 2100 Microplate Reader by Awareness Technology, Japan to evaluate the low-risk and high-risk genome proteins in recruited subject's samples. The standards were analyzed in duplicate, and a concentration of ≥ 1.5 ng/ml was set as the cutoff value for positive cases. A well-structured questionnaire was used to generate their demographics and possible risk factors. The data generated was analyzed with SPSS version 25. The p-value of 0.05 was considered significant. Out of 209 female subjects assayed, 19 (9%) were positive, with a general prevalence rate of 0.091 (9.1%). The age range 20–24 (57.9%) recorded the highest positive frequency, followed by 15–19 (31.6%). With respect to marital status, single subjects recorded the highest frequency of 18 (94.7%), while married subjects recorded 1 (5.3%). Some demographics data showed that those with tertiary education had the highest frequency at 19 (100%), unemployed subjects at 18 (94.7%), and those who do not exercise stood at 13 (68.4%) ($P > 0.05$). The age range < 25 years were mostly at risk thus, there is need to build robust and sustainable intervention strategies, as lack of vaccine and awareness would potentially promote the spread of the infection in our communities

Keywords: Sero-Molecular Screening; HPV Antibodies; Risk Factors; Human papillomavirus; University Female Students; Port Harcourt

Introduction

Human papillomaviruses are non-enveloped icosahedral capsids and are small viruses (around 40 to 60nm). They belong to the Alpha-Papillomaviridae family. They possess circular double-stranded deoxyribonucleic acid (DNA) helices with a genome of about 8 kbp linked to histones [1]. However, concerning its pathogenesis to either cancerous or non-cancerous disease, there are two groups of HPV strains: low-risk HPV (LR-HPV) and high-risk HPV (HR-HPV) [1]. The high prevalence of HR-HPV disease and its malignant growth complication makes it critical to recognize significant analytic tests for prompt screening of the disease. Its identification depends on a conventional Pap smear or fluid-based cytology, electron microscopy, serological strategy, molecular procedures, and genotyping tests respectively [1]. Studies have

shown an association between the human papillomavirus (HPV) and cervical cancer. HPV genotypes 16 and 18 cause about 70% of precancerous lesions and cervical cancers worldwide [2]. In low-income countries such as those in Sub-Saharan Africa, the burden of HPV is high. Consequently, in these countries, 85% of deaths resulting from cervical cancer occur [3].

Nevertheless, amongst the sexually transmitted viruses, HPV transmission occurs the most (CDC, 2007). Most sexually active people come in contact with the virus at least once in their lifetime [4]. In most cases, the virus clears or remains latent in asymptomatic subjects for a long time. However, if the host's immune system cannot clear them, they cause cancers especially cervical cancer [5,6]. It is strongly believed that this virus infection

occurs in all age groups, but young women are more at risk, maybe due to their youthful exuberant traits that is highly linked to risky sexual behaviors [7,8]. According to previous studies, the occurrence of HPV increases with the onset of sexual activities [9,10]. In early adulthood and adolescence, the prevalence increases but decreases with an increase in age [11].

There is poor awareness of the relationship between cervical cancer and HPV and there is also visible evidence-based neglect in terms of vaccination, prevention, and screening, especially in developing communities of the world with most times with higher number of the vulnerable subjects [12]. Currently, there is a paucity of evidence-based robust research data on HPV among female students in Port Harcourt. The study would certainly add to the body of knowledge and existing literature, especially among the most vulnerable and neglected population groups from this part of the world. Furthermore, data generated from this study would also provoke much needed attention from relevant health care agencies and government to pay more critical attention towards providing sustainable prevention and treatment strategies, among the hard-to-reach population set group in our local settings. These would be achieved by exploring the capacity of the double sandwich ELISA technique to assay for HPV antigens among different age groups of female students in University Environment in Port Harcourt, Rivers State.

Methodology

Study Population

The study population was focused on female university subjects of reproductive age (15-45 years old), being sexually active (penetrative or oral sex), those without the human immune deficiency virus and women who have given consent for their samples to be used for the study in the Rivers State University and the Ignatius Ajuru University of Education, all in Port Harcourt, Rivers State, Nigeria. A well-structured questionnaire was given to all subjects to determine their socio-demographics and their possible risk factor assessment. Port Harcourt is in the oil-rich Niger Delta region at 4.8472° N and 6.9746° E. The study was based on a randomized cross-sectional design conducted from December 2021 to February 2022.

Sampling Size Calculation

To determine the minimum sample size of the subjects recruited in the study, the prevalence of HPV infection as reported by Ashaka *et al.* was 87.59% in women with normal cytology, which was used as follows [13]:

$$\text{Using the formula: } N = \frac{z^2 pq}{d^2} \quad [14]$$

Where N = Minimum sample size

Z = Standard normal deviation corresponding to 95% confidence level set at 1.96

$$p = 87.59\% = 0.8759,$$

$$q = 1-p = 0.1241,$$

$$d = \text{desired precision, } 5\% (0.05)$$

$$\text{So, } N = 167$$

The sample size for this present study was increased to 209 to increase its statistical relevance and power.

Sample Preparation

Whole blood (2 ml) collected in plain bottles was spun in a centrifuge for 5 minutes at 5000 rpm. The serum supernatant was separated and transferred into similarly labelled Eppendorf bottles and stored in a refrigerator (4 °C) awaiting processing.

Human papillomavirus (HPV) ELISA KIT Principle

The Human papillomavirus ELISA kit (MyBiosource.com, United States) used is based on the principle of double antibody sandwich and relies on the characteristics of the target analyte with more than two possible epitopes that can be identified by both the pre-coated capture antibody and the detection antibody simultaneously. The pre-coated antibody is an anti-human HPV monoclonal antibody, while the detection antibody is a biotinylated polyclonal antibody. Samples and biotinylated antibodies are added into ELISA plate wells and washed out with PBS or TBS after their respective additions to the wells. Then avidin-peroxidase conjugates are added to the wells. TMB substrate is used for colouration after the enzyme conjugate has already been thoroughly washed out of the wells by PBS or TBS. TMB reacts to form a blue product from the peroxidase activity and finally turns yellow after the addition of a stop solution (Color Reagent C). The colour intensity and quantity of target analyte in the sample are positively correlated.

Test Preparation

A serial dilution of the stock standard solution was made to produce the following concentration values for the standard curve: 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 ng/ml. The serial dilution was made by first labelling 7 clean tubes with their expected concentrations (50, 25, 12.5, 6.25, 3.12, 1.56, 0 ng/mL). Using a pipette, 300 µL of the standard diluent was added to each tube. Furthermore, 300 µL diluent from the reconstituted standard was pipetted out into the tube labeled 50 ng/mL and mixed well. In the same manner, 300 µL diluent from the 50 ng/mL tube was pipetted out into the tube labeled 25 ng/mL and mixed well. The process was repeated from the subsequent tube up to the labelled 1.56 ng/mL tube. The standard diluent in the 0 ng/mL tube was used as the negative control. For the biotinylated antibody preparation, the antibody diluent was used to dilute it in a proportion of 1:100 30 minutes in advance before the start of the test. Similarly, the enzyme-conjugate was also diluted using the enzyme diluent in a proportion of 1:100 30 minutes in advance of the test. The color reagent was prepared by adding Color Reagent A and Color Reagent B in a proportion of 9:1.

Test Procedure for Human papillomavirus (HPV) ELISA KIT

The blank well on the ELISA plate was set aside with 100 µL of the 0 ng/mL labeled standard diluent added to it. 100 µL of the standards were added to their respective wells, as were the samples, which were added in duplicate. The ELISA plate was sealed with foil and incubated at 37°C for 90 minutes. The ELISA plate was washed twice by pipetting 350 l of the wash buffer into each well and allowing it to stand for 30 minutes. The plate was inverted, agitated to remove as much liquid as possible, and dabbed with absorbent paper. The prepared biotinylated antibody was pipetted into each well (100 µL per well) except the blank well. The plate was sealed with foil and incubated at 37°C for 30 minutes. The plate was then washed three times with a similar method as stated above. The prepared enzyme conjugate was pipetted into each well (100 µL per well) except the blank well. The plate was sealed with foil and incubated at 37°C for 30 minutes. The plate was then washed five times with a similar method as stated above. The prepared colour reagent was pipetted into each well (100 µL per well), including the blank well. Protected from light, the plate was sealed with foil and incubated at 37°C. The incubation was stopped when the highest standards became darker and the colour gradient appeared. 100 L of the colour reagent C was piped into each well, including the blank well. The plate was mixed well and read at an optical density (OD) of 450nm within 10 minutes. The OD of the blank well was subtracted from each sample and standard. A standard curve was produced automatically using a Statfax 2100 microplate reader (Awareness Technology, Japan).

Data Analysis

The collected data was organized via Microsoft Excel. They were subsequently exported into Statistical Product and Service Solutions (SPSS) version 25 to properly analyze the data obtained through the questionnaires at a 0.05 level of significance. The frequencies, percentages, prevalence, and chi-squares of the results analyzed were presented using tables.

Result

Prevalence of HPV Antigen among the Study Population Based on Socio-demographics and Risk Factors

The study was performed on 209 female university subjects in Port Harcourt. The E6 gene is the primary target of the double-sandwich ELISA technique used in this study. It was revealed that the prevalence of HPV antigen concentration was 9.1%, with 90.9% negative subjects.

Frequency of HPV Antigen among the Study Population Based on Risk Factors

Table 4.2 shows the risk factors associated with HPV and how they relate to the presence of HPV antigen concentration among the study population based on the pre-defined cut-off value. Subjects who were smokers had a significantly increased positive frequency of 16 (84.2%) than those who were not

smokers, and none for those who had smoked previously. Based on alcohol intake, subjects who did not respond to the item on the questionnaire had a significantly higher positive frequency of 17 (89.5%) compared to those who drank alcohol based on the level 1 and 2 categories. Based on secondary diseases that might be associated with HPV, subjects devoid of any secondary disease had a significantly higher positive case with 94.7% compared to other risk factors. Based on the inflammatory disease risk factor, subjects who reported not applicable (N/A) responses to the question had a significantly higher positive case 89.5% than the variables in the question. No positive case was recorded for a subject who had a heart condition. With regards to the case of subjects with sexually transmitted diseases (STD), subjects with no present or previously treated STD had a significantly decreased positive frequency of 17 (89.5%) compared to subjects with a present or previously treated STD of (10.5%).Based on subjects' awareness of HPV and its mode of transmission, subjects who had not heard of HPV and did not know how it is transmitted had a marginally increased positive frequency of 11 (57.9%) compared to subjects who knew about HPV and its mode of transmission 4 (21.1%) and subjects who know about HPV but do not know how it is transmitted (Yes/No) 4 (21.1%)(Table 1).

Table 1: Frequency distribution of risk factors and HPV antigen among the study population.

Risk Factors	Category	Frequency (%)	
		Negative	Positive
Smoking	Never	7 (3.7)	3 (15.8)
	Current	174 (91.6)	16 (84.2)
	Previous	9 (4.7)	0 (0)
Drinking	1	41 (21.6)	1 (5.3)
	2	5 (2.6)	1 (5.3)
	3	3 (2.6)	0 (0)
	4	1 (0.5)	0 (0)
	5	1 (0.5)	0 (0)
	N/A	139 (73.2)	17 (89.5)
Awareness	No	118 (62.1)	11 (57.9)
	Yes	50 (26.3)	4 (21.1)
	Yes/No	22 (11.6)	4 (21.1)

Descriptive statistics of the relationship between HPV Antigen among the Study Population and its Associated Risk Factors

A chi-square test of independence was performed to examine the relationship between the various associated risk factors as presented in (Tables 2-4). Apart from smoking (χ^2 (2, N = 209) = 0.043, $P= 6.293$), which had a significant association, drinking (χ^2 (5, N = 209) = 3.821, $P= 0.575$, LR = 5.038), and awareness (χ^2 (2, N = 209) = 1.481, $P= 0.477$, LR = 1.299), were not significant. Therefore, there is an independent association between smoking and HPV antigen concentration in the study population.

Table 2: Chi-square and Likelihood Ratio of Subjects Based on Smoking.

Test	Value	Degree of freedom	Asymptotic Significance (2-sided)
Pearson Chi-Square	6.293a	2	0.043
Likelihood Ratio	5.326	2	0.07
N of Valid Cases	209	-	-

Table 3: Chi-square and Likelihood Ratio of Subjects Based on Drinking.

Test	Value	Degree of Freedom	Asymptotic Significance (2-sided)
Pearson Chi-Square	3.821a	5	0.575
Likelihood Ratio	5.038	5	0.411
N of Valid Cases	209	-	-

Table 4: Chi-square and Likelihood Ratio of Subjects Based on Awareness.

Test	Value	Degree of Freedom	Asymptotic Significance (2-sided)
Pearson Chi-Square	3.821a	5	0.575
Likelihood Ratio	5.038	5	0.411
N of Valid Cases	209	-	-

Discussion

The objective of this study was to determine the seroprevalence of HPV antigen concentration (based on a cut-off of 1.5 ng/mL for positive cases) and the associated risk factors amongst the sample population. These results showed an HPV antigen prevalence of 9.1% among the 209 female subjects tested. A study carried out in Port Harcourt by Okonko & Ofoedu with a similar methodology as this study recorded a prevalence of 4.9% for IgG antibodies against HPV type 6, 11, 16, and 18 virus-like particles [15]. Another study in Port Harcourt by Nyengidiki et al. also showed a prevalence of 4.88% [16]. However, a study in Kenya by Sweet et al. reported an increase in the prevalence of 20.4%, as it was conducted among high-risk subjects (female sex workers) [17]. Unlike the present study, which was based on a randomized, cross-sectional design. However, the variations in different prevalence rate results as shown above from different studies conducted at different locations, may be due to different techniques in sample collection and research design, methods of assay, sample size and influence of potential risk locations and subjects sampled. In this study, subjects presently involved in smoking had a higher test positivity frequency distribution compared to subjects who previously smoked or never did. This agreed with a retrospective study performed among 611 oropharyngeal squamous cell carcinoma patients in the United States by Aguayo et al., which reported a reduction of positive progression associated with HPV-positive patients because of tobacco smoking [18]. In agreement, an epidemiological study by Auguste et al. concluded that tobacco smoke has a reduced impact as a co-factor for progression from pre-cancerous to full-blown cervical carcinogenesis when compared with non-smokers [19]. More studies with HPV

genotyping need to be performed to assess the contribution of smoking as a risk factor to the progression of HPV carcinogenesis. In this study, there was no statistical relationship ($P > 0.05$, 95% confidence interval) between drinking and HPV infection which contrasts with a cross-sectional study where alcohol consumption was shown to contribute as an associated risk factor for HPV infection [20]. Alcohol dependence can contribute to a general systemic decrease in immunity, which is a major contributor to HPV persistence and progression to malignancy.

This study showed that the awareness level of subjects who lacked knowledge about HPV prior to the test was higher compared to those who had. However, there was no statistical significance demonstrated between awareness and test positivity for HPV antigen concentration ($P > 0.05$, 95% confidence interval). This contrasts with a descriptive cross-sectional study by Ojimah and Maduka performed in Rivers State, Nigeria to assess the awareness level of HPV among 780 women in 3 universities where awareness of HPV infection and HPV vaccine among female undergraduates in Rivers State was poor (33.6%) [21]. The reduced level of awareness about the virus in Rivers State may probably be due to the poor interest given to HPV as an STI, which should be considered a public health menace as people are more concerned about HIV, syphilis, etc., and other STIs, which have gotten more public health recognition.

Conclusion

This study investigated using double antibody sandwich ELISA techniques, HPV, and its associated risk factors among female subjects in Port Harcourt. The samples were run in duplicates, and the prevalence of HPV antigen concentration among the

209 female subjects recruited for the study was 19.1%. Although drinking and awareness among study participants showed no statistical significance using the Chi-square analysis, there was a statistical relationship between viral infection and smoking.

Recommendations

In light of the growing health burden of HPV and its relatedness to cervical cancer, there is a need for a broader study, focusing on young unvaccinated female students in Nigeria, especially in the South-South region, which has a paucity of data in this direction. This would help to drive better public health policymaking. Also, a more robust molecular diagnostic approach with higher sensitivity and specificity that targets the HPV DNA directly is required for future studies to help reduce this menace to global health in general. A nationwide awareness program needs to be brought to our local schools and colleges about the dangers of HPV infection and safer sexual practices, as information about the virus and route of infection remains very sketchy among the public, especially among young girls in our universities, who are among the high-risk groups, given their lifestyles and drive for risk-taking.

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