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Characterization of High-Risk Human Papillomavirus Genotypes in Formalin-Fixed Paraffin-Embedded Cervical Tissues Using Genexpert®

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ABSTRACT

Backgrounds: There is a growing need for human papillomavirus (HPV) annotation of formalin-fixed paraffin-embedded (FFPE) tissues. This study was carried out to characterize high-risk human papillomavirus genotypes in formalin-fixed paraffin-embedded cervical tissues using GeneXpert®

Materials & Methods: In this study, 200 formalin-fixed paraffin-embedded (FFPE) cervical tissue samples were collected from women with pre-invasive and invasive cervical diseases and screened for high-risk human papillomavirus (hrHPV) using GeneXpert®

Findings: Analysis of the results showed that 47 (23.5%) samples were positive for hrHPV, with HPV 16 being the most common type (16.5%), followed by other high risk (OHR) types (31, 33, 35, 52, 58, 51, 59, 39, 56, 66, 68) (4.5%) and HPV 18/45 (2.5%). Ninety-six (48%) samples were negative for hrHPV, while 57 (28.5%) samples were invalid. GeneXpert® had low sensitivity for hrHPV in CIN 1 (cervical intraepithelial neoplasia) (9.1%), CIN 2 (33.3%), CIN 3 (22.2%), and cervical cancer (50.7%). However, the specificity of the test was much better, with values ranging from 64.9-83.8%. Apart from cervical cancer, which had a positive predictive value of 74.5%, the positive predictive value of all pre-invasive diseases was very poor. However, the negative predictive value of the test was very good, with CIN 2 having a high value of 97.9%.

Conclusion: This study findings show that FFPE cervical tissue is functional for HPV detection, although HPV detection and characterization by the GeneXpert® assay is still recommended only after full optimization..

Keywords: hrHPV, FFPE, GeneXpert®, XpertHPV®, Cervical tissues

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Introduction

Human papillomaviruses (HPVs) are a small group of non-enveloped viruses belonging to the *Papillomaviridae* family [1]. The viral particles consist of a genome in the form of a circular double-stranded DNA, encompassing eight open reading frames and a non-enveloped icosahedral capsid. Human papillomavirus (HPV) infection appears to be involved in the development of more than 90% of cervical cancer cases [2]. Over 200 HPV types have been identified, with approximately 40 types that preferentially infect genital mucosa [3, 4]. Oncogenic types of HPVs, especially HPV-16 and -18, are etiologic agents of cervical carcinoma (CC) and responsible for more than 70% of all cervical cancer cases [5, 6].

There is growing evidence showing that, apart from HPV types 16 and 18, a significant number of other high-risk human papillomavirus (hrHPV) types are continuously being uncovered to be implicated in cervical cancer [7-10]. This increases the need for more HPV testing so that these emerging types could be captured in vaccine development. Additionally, there is a need to adopt simpler and quicker HPV assay methods to ensure easy access to testing for diverse populations across various locations. While a variety of tests are available for the detection and genotyping of human papillomaviruses, varying in scope and automation, relatively few commercial assays have been validated for formalin-fixed paraffin-embedded (FFPE) materials [11, 12].

The Xpert®HPV assay (Cepheid®, Sunnyvale, USA) is a rapid, cartridge-based, real-time HPV PCR test validated for use on cervical cytology samples. The assay is targeted to amplify the viral E6/E7 oncogenes and automates the test process (including DNA extraction, amplification, and detection) into a fully integrated cartridge. The machine is optimized to detect 14 hrHPV types, including HPV16, HPV18/45, and other hrHPV types (31, 33, 35, 52, 58, 51, 59, 39, 56, 66, 68). Sample adequacy control (SAC) confirms that the patient sample contains human DNA. It has a sensitivity of 90.9% (83.9-95.6%) and a specificity of 43.5% (38.8-48.2%) for cervical intraepithelial neoplasia 2 (CIN 2) disease status. Xpert®HPV also has a sensitivity of 94.5% (86.6-98.5%) and a specificity of 41.3% (39.6-45.8%) for cervical intraepithelial neoplasia 3 (CIN 3) disease status.

Beyond its easy-to-use interface, it also has a high level of accuracy and could deliver results in 58 min. GeneXpert® technology could be very instrumental in the prompt delivery of results, which in turn enables quick commencement of treatment for patients. Its easy-to-use interface enables its utilization across a wide range of populations and geographical locations, which is very fundamental in the fight to eradicate cervical cancer.

Cepheid GeneXpert® has been optimized to detect high-risk human papillomavirus in only cervical cytology samples. To enhance the effectiveness and usability of the assay, there is a need to optimize it for a wide range of sample types. There is also an increasing demand for HPV annotation of formalin-fixed paraffin-embedded tissues. Formalin-fixed paraffin-embedded tissue samples have been proven to be very steady at room temperature over decades, allowing for repeated sampling. Cervical cytology samples may not always be available and may require certain conditions to remain viable. Hence, there is a need to optimize paraffin-embedded samples for GeneXpert® assay. This study aimed to detect high-risk human papillomaviruses in formalin-fixed paraffin-embedded cervical tissues using Xpert®HPV.

To the best of our knowledge, this study might be the first (1st) of its kind in Nigeria and the third (3rd) worldwide.

Objectives: The findings of this study enhance the potential for eradicating cervical cancer by enabling rapid HPV testing and diagnosis, leading to prompt treatment initiation.

Materials and Methods

This hospital-based cross-sectional study was done on women attending three different hospitals in northern Nigeria. A total of 200 cervical tissues were collected from patients who were histologically diagnosed with pre-invasive and invasive cervical diseases and gave their consent to participate in the study. The number of participants in this study depended on the prevalence of human papillomaviruses in the study population. Ethical approval was obtained from the ethics committee of the selected hospitals before the commencement of the study. Cervical tissues were collected from these patients and processed into a formalin-fixed paraffin-embedded state for subsequent detection of high-risk human papillomavirus (hrHPV) genotypes.

About 10 µm of formalin-fixed paraffin-embedded samples were sectioned, deparaffinized by two changes of Xylene (1 mL) each for 5 min, and then hydrated serially by two changes of decreasing concentration of alcohol (1 mL of each absolute ethanol, 80% ethanol, and 70% ethanol). The tissue was transferred to distilled water for 10 min, rinsed in phosphate buffered saline (PBS) for 2 min, and finally suspended in 2 mL of PBS [13].

The suspended tissue was centrifuged at 10,000 rpm for 2 min, and the supernatant was discarded. Tissue digestion was carried out by adding 180 µL of DNAse/RNAse-free water, 15 µL of Tween 20, and 25 µL of proteinase K. The sample was spun at 10,000 rpm for 1 min and then incubated in a water bath at 56 °C overnight. Enzyme inactivation was done by raising the water bath temperature to 90 °C for 30 min.

Detection of high-risk human papillomavirus genotypes in tissue samples: All cervical tissue samples were assayed using GeneXpert® (Cepheid®, Sunnyvale, CA, USA) to detect the presence of some high-risk human papillomavirus types. About 1.2 mL of PBS was added to the digested tissue, then 1 mL of the dilution was aspirated and transferred to a pre-labelled test cartridge (Xpert®HPV). The cartridge was inserted into the GeneXpert system, the reaction was started and run for 58 min, and the results were read. The hrHPV test results were displayed on an Excel sheet in real-time.

Statistical analysis: The obtained data were analyzed using Epi-Info statistical package Version 7.2.5 at a 0.05 significance level and a 95% confidence interval. The performance of the GeneXpert® assay was evaluated by calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The association between demographic/clinicopathologic factors was calculated using Pearson Chi-square test. Categorical variables were summarized by frequencies and percentages. The results were recorded and presented in tables and charts where appropriate.

Findings

Out of the 200 cervical tissue samples analyzed, 47 (23.5%) samples were positive for high-risk human papillomavirus (Table 1), 96 (48%) samples were negative, and the remaining samples (n=57, 28.5%) had invalid results. Repeated tests were done for these invalid samples, but the same error message was observed. Therefore, these invalid samples were excluded from subsequent analyses. Of the 200 samples analyzed, 143 (71.5%) samples had valid XpertHPV® results and were included in the statistical analysis (47 positive and 96 negative results), indicating a prevalence of 32.9% (47 of 143) for high-risk human

Table 1) Prevalence of high-risk human papillomavirus types in formalin-fixed paraffin-embedded cervical tissues (N=200)

Result Category	Number of Samples	Percentage (%)
Positive for hrHPV	47	23.5
Negative for hrHPV	96	48.0
Invalid results	57	28.5

hrHPV: High-risk human papillomavirus

Table 2) Distribution of high-risk human papillomaviruses based on age of the studied individuals (N = 143)

Age Group (years)	Screened N	Positive N (%)	*P*-Value
20-29	9	1 (11.1)	.3937
30-39	23	6 (26.1)	
40-49	45	16 (35.6)	
50-59	25	5 (20.0)	
60-69	32	17 (53.1)	
70-79	7	1(14.2)	
≥80	2	1(50.0)	
Total	143	47 (32.9)	

Table 3) Distribution of histological diagnoses and percentages of positive results for high-risk human papillomavirus detected by Xpert®HPV (N = 143)

Grade of Neoplasia	Screened N	Positive N (%)	*P*-Value
CIN 1	12	1 (8.3)	.0334
CIN 2	3	1(33.3)	
CIN 3	9	2(22.2)	
Cervical cancer	69	35 (50.7)	
Others	50	8 (16.0)	
Total	143	47 (32.9)	

Others: Cervicitis, polyps, and nabothian cysts

papillomavirus in this study.

Table 2 shows the age distribution of women with high-risk human papillomavirus infection. The highest prevalence of high-risk human papillomavirus infection was among women in the age group of 60-69 years (53.1%: 17 of 32), while the lowest prevalence was observed among those in

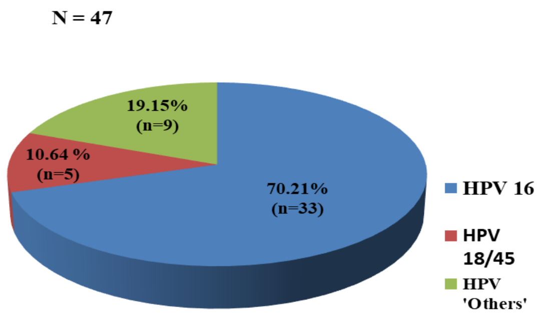

Figure 1) Characterization of high-risk human papillomavirus genotypes in formalin-fixed paraffin-embedded cervical tissues
HPV 'Others': HPV other types (,51 ,58 ,52 ,35 ,33 ,31 68 ,66 ,56 ,39 ,59)

the age group of 20-29 years (11.1%: 1 of 9). There was no statistically significant association between age and high-risk human papillomavirus infection (χ^2 = 4.0917, *df* = 6, *p*= .3937).

The Xpert®HPV assay detected 14 high-risk human papillomavirus types, which were categorized as HPV16, HPV18/45, and "others" (31, 33, 35, 52, 58, 51, 59, 39, 56, 66, and 68). Figure 1 shows the distribution pattern of these human papillomavirus types. Human papillomavirus type 16 was detected in 33 samples, showing the highest prevalence (70.21%), HPV18/45 was detected in five samples, representing the lowest prevalence (10.64%), and other high-risk human papillomavirus types were collectively detected in the remaining samples (n=9, 19.14%).

Out of the 143 samples that had valid results, 74 had various benign cervical conditions, while 69 had cervical cancer. Considering the distribution of high-risk human papillomaviruses based on histological findings (Table 3), it could be observed that the highest prevalence of high-risk human papillomavirus was among women with cervical cancer (41.6%: 37 of 89), and this

Table 4) Clinical performance of GeneXpert for the detection of high-risk human papillomaviruses based on neoplasia grades

	Sensitivity (%) (95 % CI)	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
CIN 1	9.1 (0.4-40.2%)	64.9 (56.0-72.9%)	2.1 (0.1-12.7%)	88.5 (80.0-93.9)
CIN 2	33.3 (1.7-87.4%)	67.1 (58.6-74.7%)	2.1 (0.1-12.7%)	97.9 (92.0-99.6%)
CIN 3	22.2 (3.9-59.8%)	66.4 (57.7-74.2%)	4.3 (0.7-15.7)	92.7 (85.0-96.8%)
Cervical cancer	50.7 (38.5-62.6%)	83.8 (73.0-91.0%)	74.5 (59.4-89.6%)	64.6 (54.1-73.9%)

association was significant.

The clinical performance of the GeneXpert® assay was evaluated (Table 4). The sensitivity and specificity of the assay were observed to increase with increasing the disease severity. The test had the highest positive predictive value in cervical cancer cases (74.5%), while pre-invasive diseases had a very low positive predictive value. The negative predictive value was the highest in CIN 2 (97.9%) and the lowest in cervical cancer (64.6%).

Discussion

To assay high-risk human papillomavirus genotypes in formalin-fixed paraffin-embedded cervical tissues, clinical samples of 200 women were analyzed using GeneXpert®. A total of 143 samples had valid results, of which 47 (32.9%) samples were positive for high-risk human papillomavirus. This prevalence is slightly lower than the prevalence of 48.7% reported by Virtanen et al. (2017) [14]. The obtained prevalence indicates that high-risk human papillomavirus is actively circulating in the study area. This result also represents a mirror image of the cervical cancer burden in the area, given the fact that high-risk human papillomavirus is known to cause 90% of cervical cancer cases [2].

In this study, the rate of invalid test results (28.5%) was higher than those reported by Guerendiain et al. (2016) (9.1%) [15] and Virtanen *et al.* [14] (2017) (16.7%). The high number of invalid results is attributable to sample inadequacy, given that only 10 µm of formalin-fixed paraffin-embedded cervical tissue had to be sectioned out and used in the assay. It was observed that for most of the samples that were found to be invalid, the sample adequacy control (SAC) sent an error message. An invalid result is obtained when the internal SAC could not amplify any DNA at all. This is mainly assumed to be due to insufficient DNA for amplification. Therefore, there is a need to optimize the Xpert®HPV assay for use as an independent testing method for formalin-fixed paraffin-embedded tissues.

Considering the Xpert®HPV type detection spectrum, this study demonstrated that the assay was capable of detecting all genotypes pre-optimized in the assay; HPV 16 and HPV 18/45 were found in 70.2 and 10.6% of the positive samples, respectively, which are much higher than the results reported by Virtanen et al. (2017) (20.5 and 2.6%, respectively) [14]. Human papillomavirus types 16 and 18 account for about 70% of cervical cancer cases, with HPV type 16 implicated in about 50% of cervical cancer cases [5, 16]. Human papillomavirus type 16 accounting for 50% of cervical cancer burden explains the result of this study.

Contrary to previous studies, women were more likely to be infected with HPV 'Others' or other high-risk (OHR) types than with type 18/45. This represents a new trend in hrHPV distribution and has implications for vaccine development.

The sensitivity of a test refers to the ability of a test to correctly identify individuals who truly have the disease [17]. In other words, sensitivity looks at the proportion of individuals with the disease, who also test positive, no matter how many people test positive. For a test to be considered good in terms of sensitivity, it must have at least 80% sensitivity [18]. This study recorded low sensitivity for Xpert®HPV in examining formalin-fixed paraffin-embedded tissues, although the test had better specificity for all grades of neoplasia. The low sensitivity of the assay could probably be due to the poor quality of DNA, which could result from tissue fixation.

Fixation of pathology specimens and their embedding in paraffin wax are essential steps for processing tissues for microscopic evaluation and long-term preservation. During the tissue fixation process, protein cross-linkages are formed, stabilizing the cells and preventing the tissue as a whole from putrefaction. Human papillomavirus could be detected in formalin-fixed paraffin-embedded samples up to 70 years post-fixation, provided that the target molecules are preserved [19].

Unfortunately, over-fixation could cause excessive formation of protein crosslinks, thereby reducing the efficiency of DNA detection by polymerase chain reaction (PCR) [19]. In this study, fixation of the tissues might have affected the quality of DNA, thereby reducing the sensitivity of the assay. Kocjan and colleagues (2016) [19] reported that the amount of protein cross-linking was proportional to the fixation time. This finding could also be considered to generally improve the quality of nucleic acids in fixed tissues. For this reason, it is recommended that in future studies, tissue samples be increased threefold or fourfold and cut into 30-40 μm sections and used, resulting in an increase in the quantity of proteinase K and Tween 20 to 75-100 and 45-60 μL, respectively. The incubation period could also be extended to further remove crosslinks and improve amplification.

The positive predictive value of a test indicates how many people who test positive actually have the disease [20]. The low number of human papillomavirus-positive cases could explain the very low positive predictive value.

The positive predictive value was shown to increase with increasing sensitivity. In this study, Xpert®HPV had a very good negative predictive value, meaning that samples that tested negative for high-risk human papillomavirus were very unlikely to have the virus.

Many studies have reported good performance of different assays for detecting human papillomavirus in formalin-fixed paraffin-embedded tissues [21-24], although they may not be without challenges. Castro et al. (2015) [21] expressed concern that the reliability of tissue-based genotyping may be worse in less experienced and less rigorous laboratories, as indicated by a WHO HPV laboratory comparison study involving 29 centers worldwide, which reported lower proficiency measures for HPV detection despite using standardized DNA samples [25]. Most HPV nucleic acid amplification tests (NAAT) are complicated to use, and some require batch testing, which could delay results and commencement of treatment. Indeed, Xpert® HPV set-up in service laboratories will be most beneficial when fully optimized for all sample types due to its flexibility in testing samples received ad hoc. The assay also provides a convenient platform for high-risk HPV detection and partial genotyping in individual samples. This is especially beneficial in places where the volume of test requests is unpredictable, and rapid turn-around times are essential. However, the Xpert®HPV assay has some disadvantages. It does not offer a very broad type detection spectrum and detects only 14 high-risk human papillomavirus types, whereas there are more than 40 human papillomavirus types that preferentially infect the genital mucosa [3, 4].

Xpert®HPV is also unable to specify which of the other 11 high-risk types are present in a particular sample. Nevertheless, the fact that it detects HPV types 16, 18, and 45, which account for more than 76% of cervical cancer cases [16], is a major milestone.

A limitation of our study was that the human papillomavirus infection status of formalin-fixed paraffin-embedded samples was not assessed and compared with other validated methods.

Xpert®HPV is an option for high-risk human papillomavirus detection and partial genotyping in formalin-fixed paraffin-embedded samples and may be particularly helpful in differential diagnosis and risk assessment of cervical cancer. Therefore, further studies are needed to establish adequate sensitivity and much better specificity, which will help reliably differentiate between hrHPV-positive and hrHPV-negative cervical neoplasias and hopefully help validate Xpert®HPV for formalin-fixed paraffin-embedded samples. There is also a need for scientific researchers to work on the GeneXpert® type detection spectrum to identify more high-risk human papillomavirus types and specifically identify each high-risk type.

Conclusion

The findings of this study showed that FFPE cervical tissue is functional for HPV detection by the GeneXpert® assay, though as discussed earlier, a larger amount of tissue (30-50 µm) should be sectioned out and used as a sample, and the enzyme incubation time should also be extended to mitigate the effect of cross-linkages. The results obtained could be compared with the gold standard method or other approved assays and properly evaluated. The GeneXpert® assay is relatively easy and fast, which is instrumental in the prompt implementation of treatment. Also, its easy-to-use interface contributes to its widespread use in diverse conditions and geographical locations. However, its use is recommended only after full optimization

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Ethical Considerations: Before the commencement of the study, ethical approval was obtained from the research ethics committee of the selected hospitals: Ahmadu Bello University Teaching Hospital (ABUTH), Zaria, Kaduna State, Abubakar Tafawa Balewa Teaching Hospital, Bauchi, Bauchi State, and University of Abuja Teaching Hospital (UATH), Abuja, FCT.

Conflicts of interests: None.

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Authors' contributions: Sheba Joseph MAGAJI conducted this work as part of her doctorate thesis. She was involved in the conceptualization, definition of intellectual content, design, literature search, experimental studies, data acquisition and analyses, and manuscript preparation. Maryam AMINU supervised the work and was involved in the conceptualization, design, definition of intellectual content, and the manuscript editing and reviewing. Muhammad H.I. Doko supervised the work and was involved in the design, data analysis, and the manuscript editing and reviewing. Adekunle O. OGUNTAYO supervised the work and was involved in the design, definition of intellectual content, and the manuscript editing and reviewing. Jigo D. YARO assisted in sample processing (including tissue processing, tissue sectioning, deparaffinization of tissues and staining of slides at all stages) as well as editing and reviewing the manuscript.

Consent to participate: Participants were satisfied to participate in the study.

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