PCR-BASED SCREENING OF HUMAN PAPILLOMA VIRUS (HPV) AND CERVICAL CARCINOMA IN DUTSE JIGAWA STATE.

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Abstract

Given the significance of cervical cancer (CC) as a neoplasia, it is a major health burden to the world and particularly in many developing countries like Nigeria. Formalin-Fixed Paraffin-implanted tissue samples were obtained at Federal University Dutse Teaching Hospital, Rasheed Shekoni Jigawa state, Nigeria. The samples were subjected to DNA extraction, quantification, polymerase chain reaction and DNA agarose gel electrophoresis. The system used GP5+/GP6+ and SPF 10 primers that amplify the viral genome's L1 region. The results showed equal number of positive samples using both primers separately. The viral DNA was found in 11% of our samples. This study recommends using multiple oligonucleotide primer types in clinical samples to boost the sensitivity of HPV detection and also HPV DNA testing should be incorporated into clinical practice with the intention of locating women who are at risk for cervical cancer.

Keywords: Cervical cancer, human papillomavirus, oligonucleotide primers and polymerase chain reaction.

Introduction

Human Papilloma Virus is a small, non-enveloped, epitheliotropic DNA virus which cause a variety of lesions of epithelial origin. Based on DNA sequencing, more than 200 different varieties of HPV have been identified (Bonnez and Reichmann 2000). Scientists have identified more than 40 types of HPVs that are sexually transmitted and primarily infect the cervix, vagina, vulva, penis, and anus. It is reported that, of these HPV types, four are most often found within the malignant cells of cervical cancers, this includes type 16, 18, 31, and 45 respectively (Harro 2001). According to their associations with genital tract cancer, these genital-type HPVs are further classified into high- and low-risk kinds. Types 6, 11, 42, 43, and 44 of low-risk HPV are typically responsible for benign anogenital warts. Anogenital cancer is caused by high-risk HPV strains, which include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 (Muoz et al., 2003). The main illnesses linked to HPV infections are anogenital malignancies.

In the early 1980s, German virologist Harold ZurHausen made the first direct association between genital HPV infections and cervical cancer (Zur hausen 1999). Since that time, many people are aware of the connection between high-risk HPV kinds and cervical squamous cell cancer. In the entire world, HPV is one of the most prevalent sexually transmitted disease (STD) causes in both men and women. (Cates 1999). The second most frequent cancer among women worldwide is cervical cancer, which is also the most common cancer in many developing nations. (Franco et al., 2001). In 99.7% of cervical squamous cell carcinoma cases worldwide, HPV has been found to be responsible. (Franco 1995). In Nigeria, cervical cancer accounts for a majority of female genital malignancies, 62.3–70.5% of gynecological cancers (Uzoigwe et al., 2004 and Oguntayo et al., 2011). Additionally, it is the most prevalent form of female cancer in Northern Nigeria and the commonest cause of death in the women due to cancer (Rafindadi et al., 1999). In some Nigerian studies, squamous cell carcinoma was also the most common with a prevalence of 95% in Zaria, (Mohammed et al., 2006) 93% in Ibadan, (Babarinsa et al., 1998) 92% in Maiduguri, (Kyari et al., 2004) and 85.7% in Ilorin. (Ijaiya et al., 2004).

Molecular diagnosis of HPV for viral infection screening is crucial, and the majority of the techniques used are hybrid capture (Söderlund-strand et al., 2005), in situ hybridization (Bagarelli et al., 2004), and PCR (Dinc et al., 2010; Seaman et al., 2010; Tsiodras et al., 2010). Due to its excellent ability to identify tiny DNA fragments, PCR is currently utilized the most in various areas of molecular diagnostics. The sensitivity and specificity of these procedures vary greatly (Chang et al., 1995 and Gravitt et al., 2000).

An essential molecular method that makes it possible to multiply a single DNA fragment into millions of copies is called PCR gene amplification. Various primer sets have been created, such as the GP5+/GP6+ bio primers and the SPF 10 primers which mostly target the virus's L1 region.
With a detection level as low as one viral copy, this method is extremely sensitive and precise. This technique is simple, safe, rapid and also accurate.
This study aimed at detecting HPV DNA in paraffin embedded cervical tissue using two different pairs of oligonucleotide primers respectively. considering this research we hope that concerned health organizations and agencies particularly in developing counties will take

quick actions to introduce HPV DNA testing into practical use to detect women at risk for cervical cancer, and this will help lower death rate due to cervical cancer and as well progress to total prevention of the disease.

MATERIALS AND METHODS

ETHICAL APPROVAL

Ethical approval was obtained from the Rasheed Yekoni Hospital Board Management, Ministry of Health, Dutse, Jigawa state, Nigeria for this study (RSH/GEN/226/V.11/1).

SAMPLES COLLECTION

Cervical cancer tissue blocks (FFPE) were obtained from the histology laboratory, pathology department, Federal University Dutse Teaching Hospital, Rasheed Shekoni Jigawa state, Nigeria. The study included 20 samples. Only samples that were shown to be positive to cervical cancer based on histology studies collected. The tissue block sectioned into about 10 microtome thick, using a microtome and a forceps, 8-10 section of the block transferred into a spin tube and stored until DNA extraction. The age of the patients and the lab number for the samples collected presented in Table 1.

Table 1: Lab number and patient's age for the samples obtained for the study

S/N	Lab No	Age
1	002	28
2	022	80
3	031	60
4	045	70
5	047	40
6	054	70
7	055	50
8	060	17
9	095	70
10	100	65
11	103	50
12	109	30
13	122	70
14	160	52
15	198	50
16	258	35
17	311	70
18	338	50
19	461	50
20	470	50

DNA EXTRACTION AND QUANTIFICATION

Materials

Centrifuge, Laminar Air Flow, pipette and pipette tips, water bath, incubator, vortexer, QIAamp MinElute® Column and Collection Tubes.

Reagents

Xylene, 96% ethanol, Buffer ATE, Proteinase K, Buffer ATL, Buffer AL, Buffer AW1 (concentrate), and Buffer AW2 (concentrate) TE buffer. Using the Qiagen QIAamp® DNA FFPE Tissue kit, DNA was extracted (Hamburg, Germany). With the exception of Proteinase K digestion, which was left overnight, extraction was carried out in accordance with the manufacturer's instructions. Using a NanoDrop 2000C spectrophotometer, DNA was measured (Thermos Scientific, USA)

DNA QUANTITATION OUTCOME

S/N	Sample ID	Nucleic Acid Conc.	A260	A280 ng/µl	260/280	260/230
1	2	99.9	1.999	1.074	1.86	0.5
2	22	780.1	15.602	8.001	1.95	1.9
3	31	77.3	1.547	0.82	1.89	0.61
4	45	426.4	8.529	4.494	1.9	1.31
5	47	34.2	0.683	0.419	1.63	0.37
6	54	21.4	0.428	0.257	1.67	0.42
7	55	56.9	1.138	0.618	1.84	0.37
8	60	674.8	13.495	6.965	1.94	0.41
9	95	1299.4	25.988	12.897	2.02	1.19
10	100	157.8	3.155	1.755	1.8	0.84
11	103	2458.7	49.174	29.462	1.67	1.43
12	109	368.4	7.369	3.801	1.94	0.86
13	122	1058.4	21.167	10.642	1.99	1.97
14	160	1816.6	36.331	18.314	1.98	1.79
15	198	943.4	18.868	10.25	1.84	1.74
16	258	24.3	0.486	0.272	1.79	0.86
17	311	603.2	12.063	6.23	1.94	0.89
18	338	382.4	7.648	4.003	1.91	0.99
19	461	456.3	9.125	4.69	1.95	1.4
20	470	9.2	0.184	0.113	1.62	0.16

Keys: Sample type: DNA; A260= Absorbance at DNA; A280= Absorbance at Protein; 260/280= Absorbance at Purity Determination

POLYMERASE CHAIN REACTION (PCR) AND GEL ELECTROPHORESIS.

BETA HAEMOGLOBIN DETECTION

Human Hb-beta subunit PCR was performed to check the purity of extracted DNA, the tissue's viability for PCR-based HPV detection and as an essential control gene for human tissues. A primer, which targets a 122 bp sequence of the Hb beta sub-unit, was used;

Forward: 5'CTTCTGACACAACTGTGTTCACTAGC 3'
Reverse: 5'TCACCACAACTTCATCCACGTTCACC 3'

Primers were acquired from Inqaba Biotech West Africa.
PCR was performed using One Taq Quick-load Master Mix in a 25 µL reaction (New England Biolab). It was added to the PCR reaction mixture, 1.25 U Taq polymerase in standard buffer containing 1.8 mM MgCl2, 22 mM NH4Cl, 22 mM KCl, 0.2 µM each of forward and reverse primers and 200 µM dNTPs. 2µL of extracted template DNA were added to each reaction. The thermocycler (Eppendorf, Germany) settings used for the amplification were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30s, annealing at 56 °C, and extension at 68 °C for 45s.

AGAROSE GEL ELECTROPHORESIS

According to the procedure recommended by (Green and Sambrook, 2012) 1.2% agarose gel electrophoresis was performed in TAE (Tris-acetate-EDTA) buffer to validate amplification of the 120 bp Hb beta sub unit.

Electrophoresis was performed at 90 V for 30 minutes and viewed through a UV trans-illuminator.
The standard size DNA marker was a 50 kb size ladder (Promega) stained with Ethidium Bromide.

PCR AND GEL ELECTROPHORESIS FOR HPV DNA DETECTION

SPF 10 and GP5+/GP6+, two distinct pairs of oligonucleotide primers, were employed in this study to identify HPV.
PCR was performed using the protocol recommended by (Evans et al., 2005), the primer GP5+/GP6+ (Snijder et al., 1990 and de Roda et al., 1995) with the following sequence; GP5+ [5'-TTTGTTACTGTGGTAGATACTAC-3'] and GP6+ [5'-GAAAAATAAACTGTAAATCATATTC-3'] and SFP 10 [5'-GCiCAGGGiCACAATAATGG-3'] oligonucleotide primer was used. The primer set amplifies all forms of HPV and specifically targets the L1 region of the HPV genome. Using One Taq Quick-load Master Mix (New England Biolab), PCR was performed in a 25 µL reaction. 1.25 U of Taq polymerase is used in a standard buffer that also contains 200 M dNTPs, 0.2 M forward and reverse primers, 1.8 M MgCl2, 22 M NH4Cl, and 22 M KCl. 2µL of the extracted DNA from the template DNA were added to each reaction.

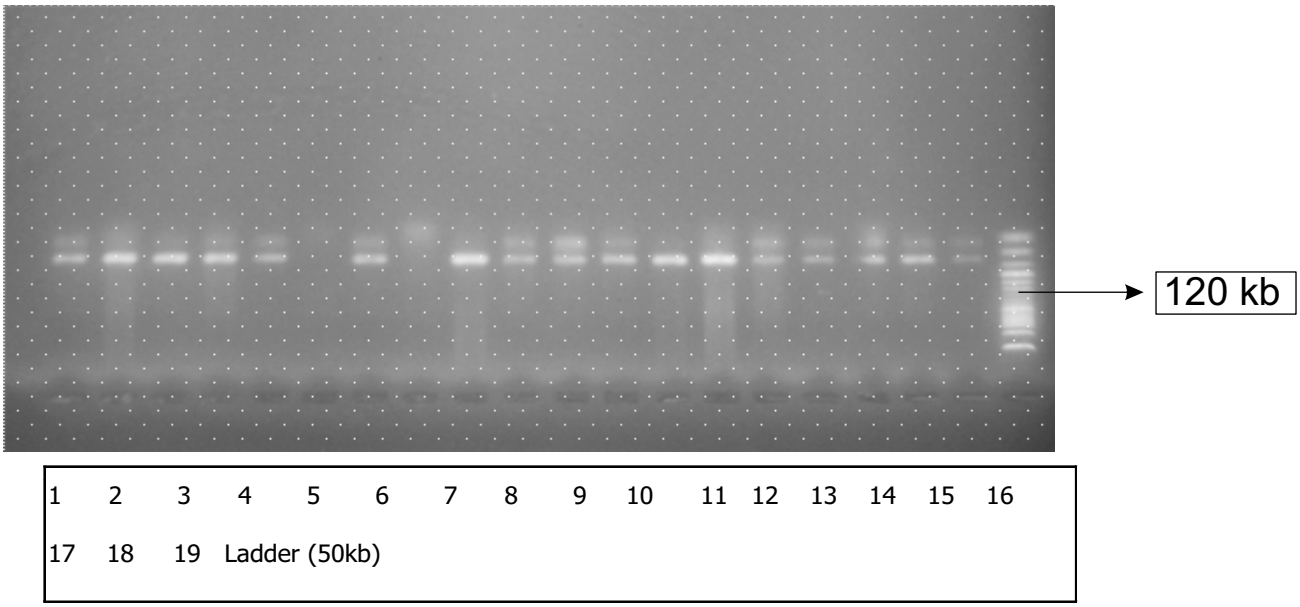


Figure 1: Gel image of Agarose gel (1.2%) of a 120 bp section of Haemoglobin beta sub-unit gene. Lanes 6 and Lane 8 did not show any amplification. Samples here were arranged serially for ease of identification, the actual sample number is the equivalence of a serial arrangement of our samples. The twentieth sample was not included in this gel because the 20 wells comb can only take 19 samples and 1 ladder.

AGAROSE GEL ELECTROPHORESIS

With the aid of agarose gel electrophoresis, amplification was observed, after 45 minutes of electrophoresis at 90 volts on 1.8% agarose in TAE buffer, a 150 bp band (GP5+/GP6+) was observed as positive. As previously mentioned, ethidium bromide was used for staining, and the results were seen under a UV trans-illuminator.

RESULT AND DISCUSSION

FFPE tissue can be utilized to get DNA for the identification and genotyping of papillomaviruses, although fixation damages nucleic acids, induces protein-protein and protein-nucleic acid cross-linking, and can interfere with PCR. (Cannova et al., 2012).
Both of the Primers used in this research, amplify the L1 region of the viral genome and are known as general primer

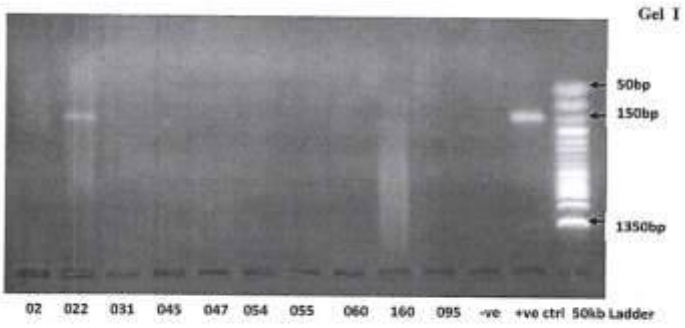


Figure 2: PCR electrophoresis profile utilizing GP5+/GP6+ oligonucleotide primer corresponds to approximately 150 bp (HPV-L1 gene) on agarose gel (1.8%) stained with ethidium bromide.
-ve: negative control; +ve: positive control; well 022: positive sample; wells 02, 031, 045, 054, 055, 160 & 095: negative samples.

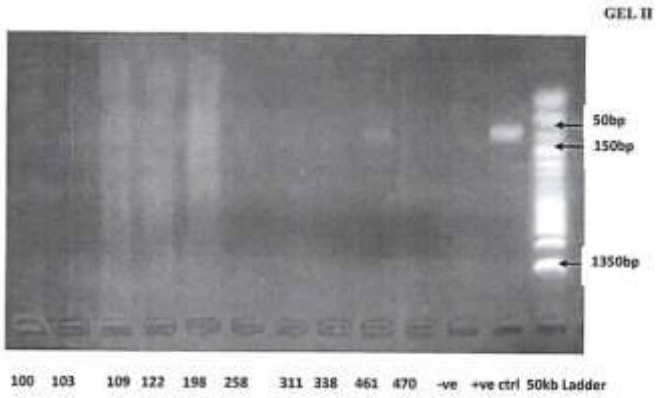


Figure 3: Agarose gel (1.8%) Ethidium Bromide showing PCR electrophoretic profile using SPF10 oligonucleotide primers for HPV-L1 gene detection corresponding ~65bp
HPV: human papillomavirus; PCR: polymerase chain reaction; -ve: negative control; +ve: positive control; well 461; positive sample. Wells 100, 103, 109, 122, 198, 258, 311, 338, 470: negative sample.

It was used as a positive control (DNA extracted from a condyloma sample) and a negative control (H20) water. It was observed that, overnight incubation of sample in protenaise K yields a better result. The samples were divided into two equal sets and each sets subjected to one of the two primers respectively. Equal number of positive samples were obtained using both primers accordingly.

The first step in the Management of any type of cancer is early detection. One major concern about cervical cancer is that symptoms like (vaginal discharge characterized by an offensive smell and bleeding either after normal menstrual cycle or after menopause) manifest only when the disease or the viral infection has progress to cancer, thereby making treatment difficult and expensive and complete cure sometimes impossible resulting in slow painful death. For this reason, more developed countries are adopting HPV DNA testing ahead of Pap cytology as part of their national cervical cancer screening programs and it has proven to be an effective method of screening. This method is important not only in identifying women with the disease but also identifies those at risk of developing the disease. Moreover, Molecular methods are accurate, rapid, safe, simple and reliable than the conventional method.

With this disease in focus there is need to enlighten women particularly those uneducated women living in poorer, unhygienic environment having little or no access to clinical services about their body in general, changes that might occur, symptoms of this disease and how deadly it could be when not properly managed.

CONCLUSION AND RECOMMENDATION

PCR as a molecular method was used in this study for HPV detection from cervical cancer tissue blocks using two different pairs of oligonucleotide primers (GP5+/GP6+ and SPF 10). Introduction of such methods into clinical settings can help overcome the limitations associated with the conventional method of cervix screening. The successful development of such tests especially in developing countries would ensure that very sensitive alternatives to Pap smear testing would be available to women, and they would have quicker access to test results. A woman will be able to undergo medical therapy the same day if rapid-results testing proved she has a high-risk kind of HPV infection, dramatically lowering her risk of developing cervical cancer, and this can be an important strategy for preventing the disease.

RECOMMENDATION

The materials and reagents used in this research are costly, therefore it will be of great importance if government and concerned bodies can put efforts to achieve its production locally with required standards.

DECLARATION OF INTERESTS

All authors declare that they have no conflict of interest.

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