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Phylogenetic Analysis of Human Papilloma Virus Isolated from Women in Abuja, Nigeria

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ABSTRACT

Human Papilloma Virus (HPV) infection causes over 99% of cervical cancer and detection of HPV types might identify women at risk of development or progression of cervical cancer. This study was aimed at determining the phylogenetic relationship between the HPV strains isolated and identified. Cervical swabs were collected from the cervix and DNA was obtained by extraction and then used to identify HPV types by PCR method using consensus primer sets MY09/MY11 and GP5+/GP6+. Phylogenetic study was also carried out to determine the genetic association between strains discovered using CLUSTALW on Unipro Ugene software. All HPV isolated in this study showed 80-99% nucleotide identity with types related to the same species. The phylogenetic tree was constructed using 15 isolates from this study against 45 reference strains selected from NCBI data based on percentage similarity. Mutation was detected in HPV 6 and HPV 18 strain isolated in the study. Based on the results there is a need to increase the level of surveillance on females at risk of cervical cancer in this environment, since significant proportion of highly oncogenic strains with a high tendency to transform into malignancy were observed in this study. The results from this study also contributed to the epidemiological data on the distribution of HPV within this region.

Keywords: Papillomavirus infections, Cervical smear, Phylogenetic analysis, Nigeria.

INTRODUCTION

Since HPV persistence is an important risk factor of Cervical Intraepithelial Neoplasia (CIN) progression and cancer, identification of oncogenic HPV types is of significance. There is need in detection, typing and also discovering the evolutionary history of these viruses [1]. The most frequently used amplification systems for the detection of HPV DNA in clinical samples are based on MY09/MY11 and GP5+/GP6+ consensus primer sets mediated PCR, amplifying DNA fragments in the conserved L1 region of approximately 450 bp and 150 bp respectively [2]. Several studies indicate that any single method or technique for the detection of HPV may underestimate the true prevalence of HPV in cervical samples [3]. Since detection of HR HPV infections is crucial for identifying women who are at increased risk of development or progression of a cervical lesion, optimization of methods for HPV testing is an important task. In order to optimize the PCR-based assays, used for HPV identification, we compared the ability of different primer sets to detect and type HPV DNA in clinical materials [2]. About 500,000 new cases are diagnosed annually with approximately 85% of deaths occurring in developing countries of the world [4]. In Nigeria, the incidence of cervical cancer is 14,550 per 100,000 and the mortality rate is 9,659 per 100,000 [5]. Approximately more than 200 types of HPV have been identified. About 40 HPV types infect the genital mucosal and are categorized according to their carcinogenic potential [6]. Clinical manifestations of HPV infection include genital warts, recurrent respiratory papillomatosis, Cervical Intraepithelial Neoplasia (CIN), and cancers, including cervical, anal, vaginal, vulva, penile, head and neck cancer [7].

Vaccines for cervical cancer such as bivalent vaccine (Cervarix) that contains HPV 16 and 18 only, a quadrivalent vaccine (Gardasil) that has HPV 6, 11, 16, and 18, and a 9 valent vaccine with HPV 16, 18, 11, 6, 31, 33, 45, 52, and 58 have been discovered [8,9]. These vaccines are a breakthrough for cancer prevention since they have the HPV genotypes that are oncogenic [10,11].

Phylogenetic analysis is important for the analysis of viral genes that display high rates of mutation and for determining the patterns of viral evolution [12]. Therefore, HPV phylogenetic analysis is important for precise diagnosis and vaccine design. This study is aimed at constructing a phylogenetic tree to determine the evolutionary relatedness between the isolates and HPV sequence in the GenBank database.

MATERIAL AND METHODS

Study Population

The study involved 501 women within age range of 15-65 years attending the Obstetrics and Gynecology Clinic of some selected hospitals in Abuja, Nigeria. A structured questionnaire was administered to the women, after an informed consent had been obtained from the women. Ethical clearance was also obtained from the health management board of the Federal Capital Territory Administration.

Sample Collection

Cervical smear was collected from February 2018 to June 2018 by a gynecologist after visual inspection. Sample collection was performed as described by Mbamara et al. [13], in which each of the participants was placed in a dorsal position, with her legs flexed at the hip and knee abducted. The labia were parted with gloved thumb and index fingers. A Cusco's bivalve speculum which is not lubricated was passed and fixed to visualize the cervix, under a bright light source. The detachable end of the cervical brush was then inserted into the cervix and rotated through 360˚ movements, either in a clockwise or counterclockwise direction, to scrape the entire squamocolumnar junction of the transformation zone [14].

The smears collected was immediately placed in 20 mls of Liquid Prep collection vial, stored at freezing temperature before being transported to DNA laboratory in Kaduna for further analysis

DNA Extraction Procedures

Bioneer Accuprep Genomic DNA Extraction Kit (K-3032) was used for the extraction of the HPV DNA as describe by Aondona et al. [15], 2020. PCR technique and HPV sequencing procedure were achieved as described by Manga et al. [16]. 2015 and Aondona et al. [15], 2020. A total of 15 isolates were successfully sequenced.

Phylogenetic Analysis

After sequence analysis of DNA samples was carried out to confirm the genotype of HPV, the Finch TV (version 1.4.0) software was used to edit chromatographs. All nucleotide sequences obtained were screened using the online BLAST (Basic Local Alignment Search Tool) to search for similarity between sequences and previously reported sequence in the database that are closely related. Standardisation and classification of HPV types was according to the international HPV reference center [17,18]. The following are the accession numbers of different HPV reference sequences from data bank used in this study; JN617899.1, MH607475.1, LC155254.1, X94164.1, HQ834589.1, JQ902133.1, MK463926.1, JN617887.1, LC155235.1, DQ315392.1, MH028426.1, MK211166.1, KU707814.1, MF288657.1, KJ467243.1, KU550624.1, JN041072.1, JN617892.1, KU163584.1, KJ754580.1, KR674072.1, JN383600.1, AB601047.1, AB601048.1, JX912950.1, FJ797813.1, HQ834585.1, JN617895.1, MG195999.1, KU050127.1, EU911303.1, KR674075.1, KU961847.1, JN617890.1, EF140819.1, LC155223.1, GQ179959.1, DQ448184.1.

The evolutionary history was inferred using the Neighbor-Joining method [19]. The optimal tree with the sum of branch length=6.39480300 is shown and the percentage replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches [20]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances between the reference sequences and the isolated sequences were computed using the Maximum Composite Likelihood method [21] and are in the units of the number of base substitutions per site involving 60 nucleotide sequences. All ambiguous positions were removed for each sequence pair using pairwise deletion option. There were a total of 8112 positions in the final dataset with which evolutionary analyses were conducted in MEGA X [22].

Sequence alignments and Mutation analysis

The query sequence in FASTA format was analysed using UniproUgene v33.0 which allows quick identification and characterization of the viral strain. The tool identifies mutations for the gene of query genome of Sequence 3 and Sequence 4 with respect to the reference sequences. Multiple sequence alignment was performed using CLUSTALW on Unipro Ugene software.

RESULTS

Phylogenetic Analysis

A total of fifteen samples were successfully sequenced which includes HPV-6, 16, 18, 31, 58, 66, 70, 72 and 81; about 4 high risk HPV Type 16, 18, 31 and 58 were detected, HPV 66 which is categorized as probable carcinogenic was also identified and other HPV genotypes 6, 70, 72 and 81 were also identified under the low risk HPV Types. The phylogenetic tree was constructed using

15 isolates from this study and 45 reference strains selected from NCBI data based on percentage similarity. Clade 1 had most of the reference strains within except for reference strain MG195999.1 and GQ179959.1 that did not fall within this clad due to the fact that they clustered with isolates from this study. Clade 1 had a branch length of 100%. Most of the reference strains clustered together on the tree with each HPV strain been closely related to a similar HPV type from selected from the NCBI site (Figure 1). The two HPV 16 strains isolated in this study were in Clade 2 on the phylogenetic tree. Both strains had the same ancestral parents with a branch length of 92 indicating a high similarity between the two HPV 16 strains. Clade 3 had a branch length of 68 with the two HPV 58 isolated in this study found. These two strains also shared same ancestral parents. The fourth clade had most of the isolates from this study which included two HPV 18 strains, HPV 81, HPV 72, HPV 6, HPV 31, four HPV 70 isolates, reference strain MG195999.1 and GQ179959.1 from Nigeria and Iran respectively. From the phylogenetic tree as shown in figure 1, clade 4 had a branch length of 75 indicating the level of relatedness within the different HPV strains. HPV 70 strains found within this clade were closely related and clustered together. However, HPV 31 and HPV 6 shared the same ancestral parents but HPV 6 was more closely related to reference strain GQ179959. 1 isolated from Iranian women with a branch length of 67%. HPV 72, HPV 81 and HPV 18 also had the same ancestral parents but HPV 18 was more closely related to the reference strain MG195999.1 isolated in Bayelsa Nigeria with a branch length of 89 indicating a high percentage similarity between the two strains. HPV 66 also clustered with other strains in clade 4 as shown in (Figure 1).

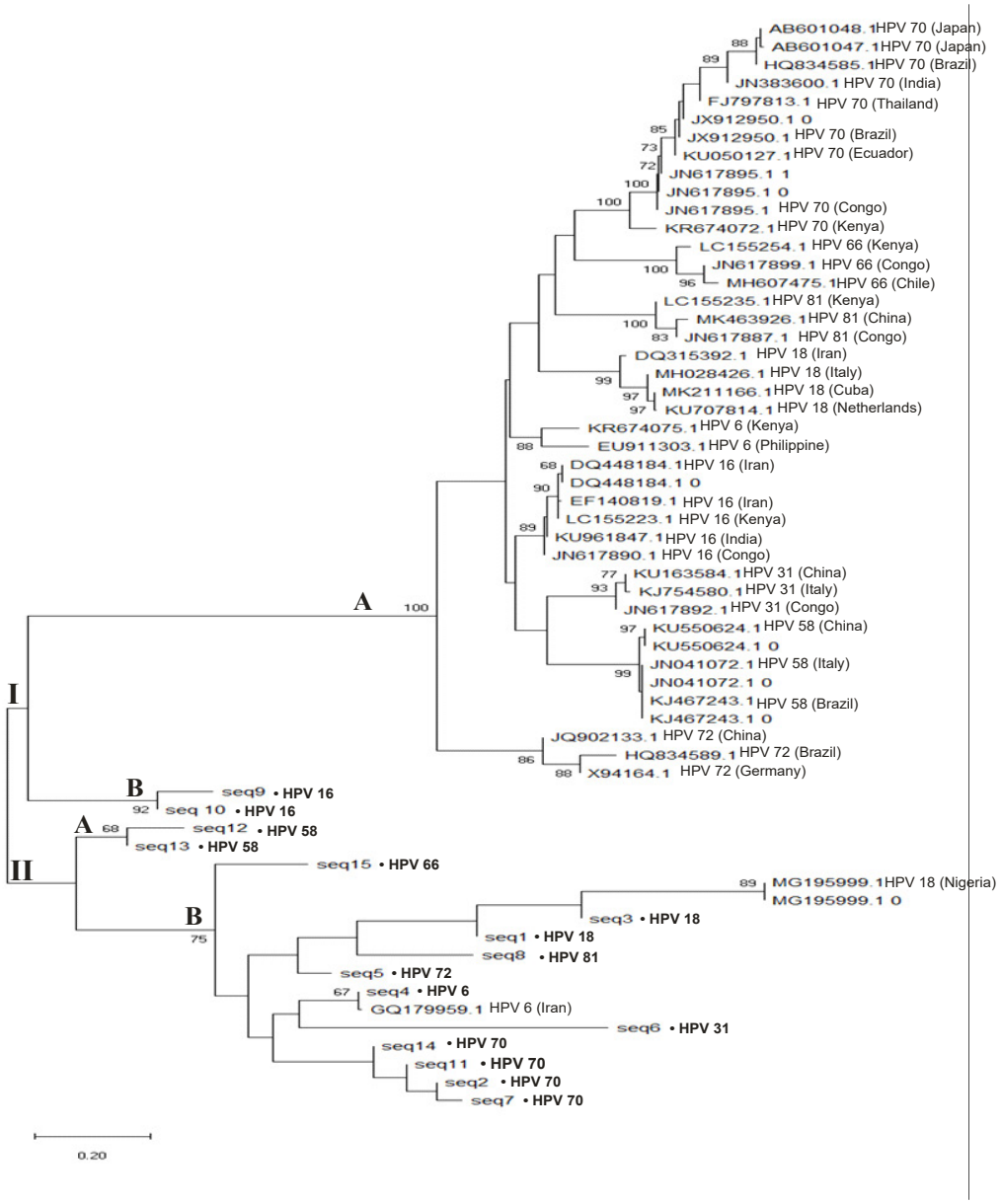

Figure 1. Bootstrapped Neighbour-Joining Phylogenetic Tree.

From the variation analysis between HPV 6 isolated in this study and the reference strain GQ179959.1 it was found that both strains differed by Intercalary deletion mutation at position 101, 102, 120 and 133 of the sequence (Figure 2). Intercalary deletion mutation is a deletion of nucleotide sequences that occurs from the interior of a gene (Lewis, 2004) as a result from unequal crossing over or breaking without re-joining during DNA replication. Therefore, there was no sequence dissimilarity between HPV 6 and GQ179959.1 (Table 1).

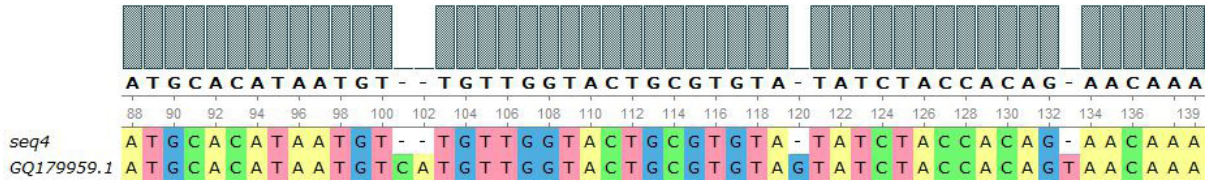

Figure 2. MSA displaying point mutations between sequence 4 and reference sequence

Table 1. Hamming dissimilarity between sequence

	seq4	GQ179959.1
seq4	0	0
GQ179959.1	0	0

HPV 18 in addition to deletion mutation at position 263 and 304 experienced a base substitution mutation or point mutation with respect to the reference sequence. The point mutation occurred at position 336, where Guanine is replaced with Adenine, and position 357, where Thymine is replaced with Adenine (Figure 3). Therefore, there were two sequence dissimilarities between HPV 18 and MG195999.1 (Table 2).

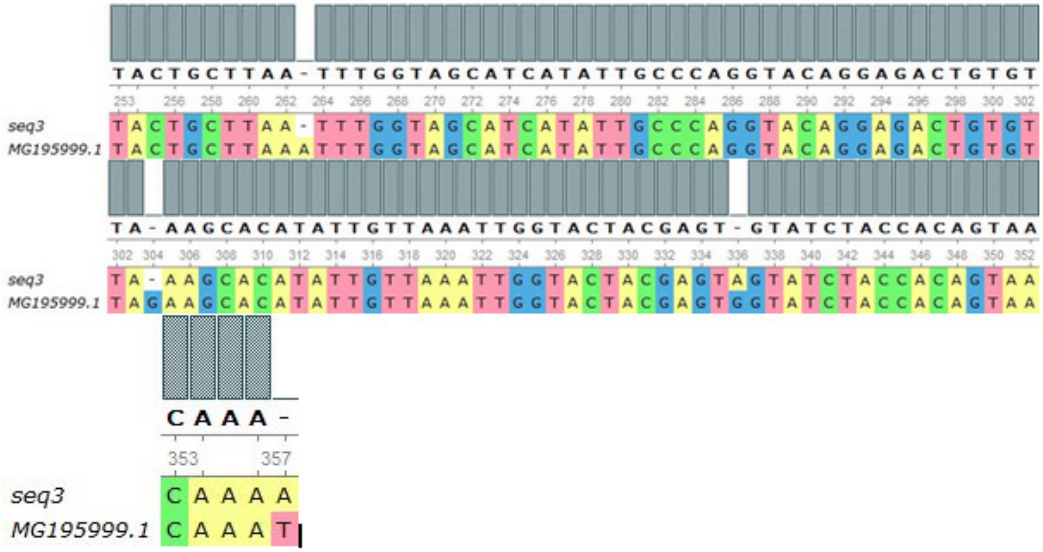

Figure 3. MSA displaying substitution and deletion mutations between sequence 3 and reference sequence

Table 2: Hamming dissimilarity between sequences

	seq3	MG195999.1
seq 3	0	2
MG1959991	2	0

DISCUSSION

The phylogenetic analysis was able to cluster some HPV types in this study based on the genotypes in addition to their oncogenic potential and pathogenicity. Genotypes that group together share the same ancestry and have common carcinogenic properties and the kind of host tissues they infect [23]. The HPV 16 phylogenetic tree depicts the origin of the isolates and groups them by their geography as European, African 1, African 2, and Asian American [24]. In this study, HPV 16, 18, and 58 had two variants each while HPV 70 had four variants. There was a strong relationship between other HPV 16 types and other reference sequence on the phylogenetic tree. This suggests that the majority of African HPV 16 circulating in might be very virulent. HPV variants are thought

to have diverged through genetic drift which is evidenced by the grouping of HPV 16 based on geography or human ethnic group [23]. HPV 6,18,31,70,72 and 81 which formed a distinct cluster in this study.

The phylogenetic tree had shown that HPV 6 isolated in this study was closely related to reference strain GQ179959.1 (HPV 6) isolated from tissue sample of cervicitis cases in Iranian women. In Africa, HPV 6 has an estimated prevalence of 0.8% [25]. HPV 6 isolated from this study was from a HIV positive woman within the age range of 25 to 35 years who also practiced monogamous marriage. The suppressed immune system as a result of HIV infection could be the reason for the HPV positivity and likely also lead to future persistence of the virus by reactivating latent infection as Denny et al. [26] also reported that HPV infection were more common among women who were HIV positive. Another study by Isaakidis et al. [27] also stated that due to immune-suppression HIV positive women tend to have persistent HPV infection and increased sexually transmitted infections. Also, she was within the sexually active age group 25-45 years which explains the positivity for HPV as studies have shown that within this age range, most women test positive for HPV even if doesn't persist to cancer as reported also by Ebrahim et al. [28]. Denny et al. [26] also reported that women are exposed to HPV infection when they become sexually active and remain at risk throughout their lives. Another factor been considered was the use of oral contraceptive by most of the women who tested positive in this study. This explains the percentage of positivity among women as the use of oral contraceptive has been established to be a risk factor in previous studies on HPV. Manga et al., (2015) also reported in his study that persistent use of oral contraceptive has shown to be a risk factor in HPV infection. One similarity between the HPV 6 isolated from this study and the closely related reference HPV 6 strain isolated in Iran by Mirzaie-Kashani et al. [29] is the fact that both strains were isolated from women with same social behavior of one partner throughout their marriage. This agrees with a study by Vaccarella et al. [30] who reported that marital status played a role in an increased risk of HPV positivity. A study by Aziz et al. [31] also recorded a low prevalence among women due to the customary monogamous behavior in a Pakistan society.

HPV 6 have been classified as low risk HPV and cause genital warts but an infection could also be asymptomatic and persist for a while [29]. This justifies why the patient isolated in this study was a healthy individual since infection can be asymptomatic and persistent. Majority of genital warts are associated with HPV 6, this affects the ectocervix causing inflammation of the cervix know as cervicitis [32], this could be the reason why reference strain HPV 6 was isolated from samples of cervicitis tissues among the Iranian women.

The reference strain HPV 6 had 139 nucleotide base pair, while the study strain had 91 base pair. Both strains were from the same ancestral origin with a branch distance of 67% indicating percentage similarity and had no hamming dissimilarity among the sequences (Table 1) after subjecting to variation analysis. But it was found that HPV 6 in this study differed from reference strain HPV 6 by Intercalary deletion mutation at position 101, 102, 120 and 133 of the sequence (Figure 2) which could be the possible cause of variation on number of nucleotide base pairs.

There was a close relationship on the phylogenetic tree between HPV 18 isolated in this study to reference strain HPV 18 isolated in Bayelsa Nigeria. Both strains were isolated from the cervix of asymptomatic women. This supports a study that indicated that HPV 18 African (Af) variants showed more similarity to one another than non-African variant. Also, a study by Arias-Pulido stated that a high prevalence of African HPV 18 variants was noted in asymptomatic population [33] which indicates additional risk associated with HPV 18 strain. Bruni et al. [25] also reported a high prevalence of HPV 18 among women with normal cytology across the world and were consistently among the most common HPV type. HPV 18 has been classified as high risk, therefore Nejo et al. [34] reported in study in Ibadan Nigeria among women, that high risk HPV 18 is found in healthy individuals this buttress the fact that most HPV infections don't manifest clinically.

HPV 18 in this study was isolated from a married woman within the age range of 35-44 years, which also falls within the sexually active age (25-45 years) as reported by Ebrahim et al. [28]. Studies by Erdem-Yayla et al. [35], Kolawole et al. [14] and Castro et al. [36] also indicated a high prevalence of HPV 18 among women within the age 35-44 years which falls within the sexually active age and so the reason for the prevalence. A study by (2016) and Avci et al. [7] also reported that HPV infection increase and once it reaches peak, it decreases with increasing age between 41-50 years. This is similar to this study by Aondona et al. [15] that describes a decrease in infection from age 45 years and above.

The variation analysis indicated there were two sequences dissimilarity between HPV 18 in this study and reference strain HPV 18 (Table 2). However, HPV 18 in addition to deletion mutation at position 263 and 304 experienced a base substitution mutation or point mutation with respect to the reference sequence HPV 18. The point mutation occurred at position 336, where Guanine is replaced with Adenine, and position 357, where Thymine is replaced with Adenine (Figure 3).

This however agrees with study by Ramas et al., Zhang et al., Cui et al., and Condo-Ferraez et al, [37-39] that distribution of HPV types to an extent is not affected by geographical distance but emphasizes on the importance of nucleotide polymorphism and mutational changes in viral genes. This could be the reason for differences between the base pairs of HPV18 in this study (103) and the reference strain HPV 18 with 400 base pairs. Also, the variation analysis supports a study by Bernard et al. [18] who indicated that disparity in HPV strains is as result of mutation either by point mutation, deletion and insertion. Another factor causing variation of both HPV 18 in this study and reference strain (HPV 18) could be as result of difference in the genetic makeup of the women from whom HPV 18 was isolated from as Xi et al. [40] indicated in a study that genetic makeup of host causes disparity in HPV strains isolated.

CONCLUSION

About fifteen different HPV types were detected with the most predominant type being HPV 70. Other types identified in the study included HPV 6, 16, 18, 58, 72 and 81 which play an important role in cancer etiology but are not included in the HPV vaccine. The prevalence of the HPV genotypes as reported in this study exposes the level of the burden of HPV infection in the study area. There is therefore a need to increase the level of surveillance on females at risk of cervical cancer in this environment, since significant proportion of highly oncogenic strains with a high tendency to transform into malignancy were observed in this study. There is need to improve the current vaccine by including the other high-risk types prevalent in this region.

DATA AVAILABLITY

The HPV sequence data in this study have been deposited at the GenBank with accession numbers MT793787-MT793796. Personal data of the patients including filled questionnaires was restricted by the Ethical committee (federal capital territory administration and the hospital Ethics Review Committee) in order to protect patient privacy.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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