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Identification and Molecular Characterization of Alpha Papillomavirus from Male Olive Baboons (*Papio anubis*) Maintained in a Captive Colony

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Abstract Papillomaviruses (PVs) are ubiquitous highly diverse group of circular double stranded DNA viruses. Nearly all the human papillomavirus (HPVs) that cause cancer are clustered in *Alphapapillomavirus* (αPV*)* genera and have a common ancestor. The aim of the study was to isolate and perform molecular characterization of alphapapilloma virus from male olive baboons (Papio anubis) that are maintained in a captive colony at the Institute of Primate Research, Nairobi, Kenya and establish their evolutionary relationship with known strains responsible for various species causing cervical cancer in human. Twenty (n=20) different genital swabs from sexually active male olive baboons were collected. Positive samples for αPV by nested PCR were 9/20 (45%). The nested PCR primers targeted a conserved region of L1 major capsid gene and aided in generating amplicons of 134bp. Only three amplicons with good quality bands (1C, 2C, and 4C) were further sequenced and analysed using MEGA X, Clustal W algorithm and DnaSP 5.10.01 software. Phylogenetic analysis through Neighbour-joining method indicated a close evolutionary relationship between subtype 2C and Human papillomavirus (AB745694) which is associated with human cervical cancer. Subtype 2C was found to be more close to 1C than 4C and other sequences of JF304764, EU490515, EF558839, AB745694, FJ598133 as well as EF591300 blasted from NCBI and treated as outgroup. On analysis of genetic diversity using DnaSP software, sequences of subtype 2C and 4C were found to harbour synonymous SNPs at position four and eight respectively hence indicating that the region is more conserved. Male olive baboon harbor αPV and may be a good model for study of the pathogenesis of HPV and also for testing therapeutic agents that target αPVs in both humans and non-human primates.

Keywords: *alphapapillomavirus, male olive baboon, prevalence, non-human primates, SNPs*

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1. Introduction

Papillomaviruses (PVs) are a highly diverse group of circular double-stranded DNA viruses [1]. The viruses have the preferences of the epithelia and specific host characteristics [2]. Evolutionary changes have led to the adaptation of the virus to epithelia providing ecological niches for the infection in the vertebrates [3,4]. Furthermore, this evolutionary has contributed to the diversity of the virus creating an opportunity for a little cross transfer between species, and now found in birds, reptiles, marsupials, and mammals [2,5]. Alpha papillomavirus genus is the most commonly known for sexual transmission infection [6]. The viruses cause benign lesions such as warts as well as asymptomatic lesions that can progress to high-grade neoplasia and invasive malignant cancer [7]. Cancer infection is as a result of functional inactivation of the Protein, P53 and retinoblastoma (pRb) tumour suppressor protein and related "pocket proteins" p107 and p130, by the early genes E6 and E7 of the Papilloma virus [8,9,10,16] All cancer-causing papilloma virus are suggested to have a similar ancestors in existence [11]. The viruses have co-evolved gradually resulting in diversity causing chronic in apparent infections especially in Beta Human papilloma virus (β-HPVs) [12] and gamma human papilloma virus [13] (γ-HPV)and αPV that causes virtually all type of cervical cancer whose pathogenicity ranges from highly carcinogenic, moderately carcinogenic to not carcinogenic [14].

The virus is estimated to cause 5.2% of all human cancers [15]. Human papilloma virus contains three important regions: Upstream regulatory region (URR) which controls the transcription and replication of the viral, Early region Open Reading Frames (ORFs; e.g. E1, E2,

E4, E5, E6 and E7) the Open Reading Frame which is significantly involved in multiple functions such as *trans*-activation of transcription, transformation, replication and including viral adaptation to different cellular [11,16,17]. The late regions include L1 and L2 the capsid proteins which are necessary for the formation of virion structure and also facilitate viral DNA packaging and maturation [17,18]. The viruses have a genome of approximately 8000 nucleotide in size and typically contain eight genes of which L1 gene (Late region 1) is well conserved necessary for classification and construction of phylogenetic trees. Due to its conserved genes can be used in the alignment of the unknown papillomaviruses [19].

The PV has been studied in cattle and rabbit [20] and other mammals such macaques, rhesus monkey [21] including avian(bird) reptile (python) and recently cervical cancer has been discovered in female olive baboons as described by Bergin [1]. This virus has not been studied in male olive baboons despite the fact that cervical cancer has been discovered in the female olive baboon. Due to cross-transmission of the virus, the male asymptomatic baboons could be carriers and can transmit it sexually to females. Evolution of the virus for many millions of years has resulted into divergence of the PV family and corresponding carcinomas [22]. The vaccine available only covers a few ranges of viruses, given to naïve females and still they have shown little efficacy. Efforts in developing new drugs and vaccines may require a PV animal model for evaluation. Efforts are ongoing to evaluate the female baboon as a model for study of cervical cancer [1] but so far no work has been done on PV in male baboons which could cause prostate cancer or be transmitted to female baboons and cause cervical cancer. Therefore, the current research focused on isolation and molecular characterization of alpha-papillomavirus from male olive baboon (Papio anubis) maintained in a captive colony.

2. Materials and Methods

2.1. Animal Background

The 20 sexually active male olive baboons maintained in captive at Institute of Primate Research (IPR) were sedated using the trapping net and given ketamine, anesthesia, to enable sample collection from the animals. This study was ethically approved by the Institutional Scientific and Ethics Review Committee (ISERC/09/16).

2.2. Sample Collection

In this study, 20 genital swabs from different sexually active male olive baboons maintained in the captive colony approximately weighing 8 kilograms were collected using cotton wool tipped swabs**.** Various genital sites, including the penile shaft, prepuce area, glans, and entire penile region were swabbed. The steady gentle pressure was applied repeatedly to abrade the entire surface of the genital site for collection of cells. Some of the swabs were put into viral transport media (Qiagen:CA,U.S.A.) to keep the cell viable while other swabs wetted with 0.9 % saline water and both the swabs were stored at −80℃ until further use.

2.3. DNA Extraction

DNA Extraction was carried out using QIAamp cador Pathogen Mini Kit (Qiagen; CA, USA). The genital swabs from male olive baboons were thawed and adjusted to 200µl with Phosphate buffered saline (PBS) before conducting DNA extraction using Qiagen QIAamp cador mini prep as per manufacturer's instructions. DNA extracts were then kept at negative -20℃ in the refrigerator awaiting the polymerase chain reaction (PCR) assay.

2.4. Nested PCR Amplification

Amplification was accomplished using the Primers based on 8-kilobase size genomic sequence of *Papio hamadryas papillomavirus* (PhPVs) that was previously sequenced and designed to target the conserved regions of the LI gene [23] as described by Bergin *et al.,* and Chai *et., al* respectively [1,24] and is publicly available as Gene bank accession number JF304764 type 1 isolate Mac 2085. These primer were synthesised by Enqaba Biotech company. The Oligonucleotide primers is indicated below in Table 1.

Table 1. The designed and synthesised oligonucleotide primers

Primers	Oligonucleotide Sequences 5'-3'end	Thermocycler Conditions	Fragment Size	References
1.Phpv1fr2.Fout	GGGTATGACGTGAGGCAGTT	40 Cycles:94℃120seconds Initial Amplification Denaturation at 94℃/60 Sec , Annealing at 50℃/60 Sec and	134base Pairs(Bp)	[1,24]
2.Phpv1fr1.Rout	TACGCAACTTTGGTGTTTCA	Extension at 72℃/60 Sec Plus Long Extension At 72℃/ 5 Minutes Accomplishing The Primary Amplification	134bp	[1,24]
3.Phpv1fr2.Fin	TGGCATAGGGTTTCATGAGC	The Primers 3 and 4 Were Used For Secondary Amplification Using 40 Cycles: 94℃/120 Sec,94℃/60sec,50℃/60 Sec,72℃/60 Sec And	134bp	[1,24]
4.Phpv1fr1.Rin	TGCAATGTGGCTCAATAAGG	Long Extension Of 72℃/5minutes For Nested Amplification.	134bp	[1,24]

Nested PCR was conducted using the primers listed in Table 1. PCR reaction mix consisted of Nuclease free water from Qiagen; CA, U.S.A. company,10X PCR Buffer, 50M Mgcl2, 10MdNTPs, a Platinum Taq enzyme and the DNA template were added to a final volume of 25µl in the PCR tubes. The primer sets target the conserved regions within the L1 gene [25] and screen for a wider range of alpha papillomaviruses as described by Bergin [1]. PCR mix were subjected to the reaction conditions indicated in Table 1 using a thermocycler Q-tower machine (by Analytik Jena) followed by examination of the products by electrophoresis using 1.5% agarose gel in the trans-illuminator machine, ultra violet product, UVP upland CA USA. PCR amplicons from male olive baboons were sequenced by Macrogen Company Biotech, South Korea where sequencing reaction were performed in a MJ research PTC-225 Peltier thermocycler using ABIPRISM® Big Dye™ terminator cycle sequencing kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems) following the protocols supplied by the manufacture. Single-pass sequencing was performed on each template using the secondary primers above (Table 1 number 3&4 primers). The fluorescent-labelled fragments were purified from the unincorporated terminators with BigDye® XTerminator™ purification protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730sequencer (Applied Biosystems).

2.5. Sequence Alignments and Phylogenetic Analysis

The amplified conserved regions of the major capsid, L1, gene (134bp) sequences were obtained through the sequence matching of Fr2Fin and Fr1Rin with flanking sequences removed. Sequences were aligned using ClustalW, a platform offered by the MEGA X software (version10.0.4) permitting the construction of phylogenetic tree through Neighbour-joining method using the same software. Bootstrap values were indicated for each node from 999 replicates. The amplified conserved regions of L1 capsid gene sequences were compared with the known PVs strains from the NCBI Genbank database using Basic Local Alignment Search Tool (BLAST) searching algorithm for inclusion in the phylogenetic analysis.

3. Results

3.1. Alphapapillomavirus Detection and DNA Extraction

Alpha papilloma virus (αPvs) was detected from 9 out of 20 (45%) olive baboons in the captive colony. Using QIAamps cador pathogen mini kit Deoxyribonucleic acid (DNA) was extracted and were detected using gel electrophoresis. The detected viral DNA in the swabs obtained from the hosts samples were visualized for confirmation.

3.2. Amplification of Conserved Regions of the L1 Capsid Gene

Out of the extracted viral DNA samples, a nested PCR was carried out using Q tower thermocycler machine with the designed primers in Table 1 targeted 134bp of conserved regions of the major capsid, L1, gene. Amplified targeted a conserved region of the gene each isolate were detected as illustrated in Figure 1.

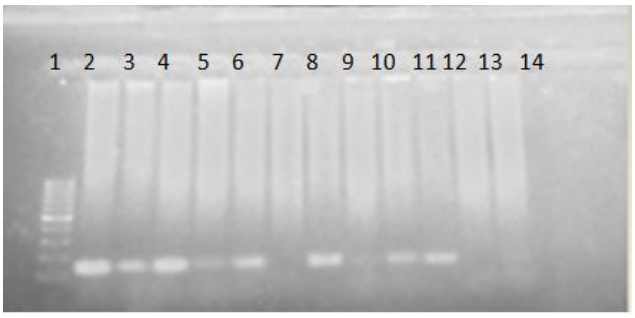

Figure 1. Amplified conserved region of the Major capsid, L1, gene. The first well (1) represents a 1kb ladder whereas the band in well labelled 2 and 3 (Sample 1C) acted as a positive control indicating the 134 bp which is the conserved region of L1 capsid gene. Well labelled 4 and 5 represents the conserved region amplified from DNA of Sample 2C while well labelled 6, 7 and 10 were from Sample 3C. Well labelled 8, 9 and 11 were amplified from DNA of Sample 4C. Well, 12 and 13 were obtained amplified from DNA of sample 2 and 3 respectively whereas well labelled 14 had been loaded with a negative control. Well, 7 had no band whereas well 9 had a faded one

3.3. Prevalence of the Virus

Prevalence of Alphapapilloma virus in sampled baboons based on Nested PCR detection was as follows. 45.00 % (9/20) of male olive baboons from captive colony harboured the virus. The conserved region of L1 capsid gene was only amplified in 3 out of 9 and accounted for 33.33%. The locations of the animals (baboons) harbouring the virus were detailed in Table 2.

Table 2. Displays of the animal model number, location in which it was obtained and gender

Animal	Location	Sex
1 Positive control	Laikipia	Female
2	Muringa abadare	Male
3	Laikipia	Male
4	Colony born	Male

3.4. Sequencing Results

Only three amplicons labelled 1C, 2C and 4C were sequenced. 1C was a positive control and represented amplicon from DNA present in isolate obtained from female Baboon (Table 2). 2C and 4C amplicons were both from animal labeled 2 and 4 as described in Table 2. Sequence 1C had a read length of 106bp after assembly whereas 2C and 4C had read length of 105bp and 107bp respectively.

3.5. Alignment and Phylogenetic Analysis

On blasting of the 2C and 4C sequences, a total of six related sequences were retrieved from the NCBI database (Table 3). These sequences were equally present on BLAST results obtained using each of the two sequences.

Table 3. Tabular display of related sequences from the NCBI database

Sequences producing significant alignments from NCBI	Accession Number
Papio hamadryas papillomavirus type 1 isolate Mac2085	JF304764.1
Macaca fascicularis papillomavirus type 10 isolate Mac616	EU490515
Macaca fascicularis papillomavirus type 3 Mac26	EF558839
AB745694.1 Human papillomavirus	AB745694
Rhesus papillomavirus type 1b isolate Mac686	FJ598133
Rhesus papillomavirus type 1b isolate Mac170	EF591300

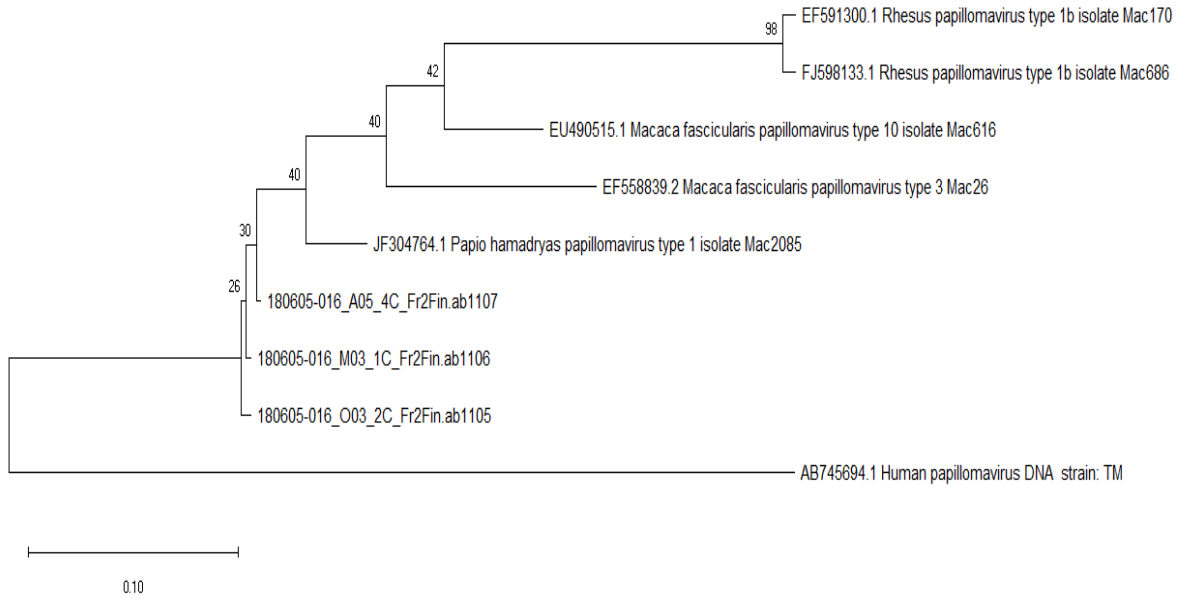

Figure 2. Phylogenetic tree of αpapilloma virus variants. Rooted neigbour joining phylogenetic tree of αpapilloma virus isolates (1C, 2C, and 4C) compared to six sequences retrieved from NCBI database after blasting. A total of 108 positions were noted in the final dataset prior to evolutionary analysis after removal of all ambiguous position. The sum branch length was 0.94512001 and the percentage of the replicated trees where the each of the associated taxa clusters within a bootstrap test of 1000 replicates is indicated on each branch. Each of the evolutionary distance was computed through Kimura 2-parameter method in units of the number of base substitutions per site [26]. The variation rate among sites was modeled through gamma distribution

The three sequences 1C, 2C, 4C and the retrieved 6 sequences were aligned through Clustal-w algorithms present in MEGA X (version 10.0.4) [27]. The phylogenetic analysis included all the 3 sequences obtained in this study together with the six sequences retrieved from the NCBI database.

The phylogenetic tree segregated into two major branches (Figure 2). Human papillomavirus (AB745694.1) sequences retrieved from NCBI constituted a monophyletic branch whereas the remaining eight sequences formed the second branch which was subdivided into two sub-clusters. One of the sub-clusters was monophyletic and comprised of Alphapapilloma virus isolate 2C sequences obtained from a male baboon in Muringa Abadare (Figure 2). The second sub-cluster comprised of isolates 1C, 4C and the five sequences retrieved from the NCBI database through blasting.

3.6. Genetic Diversity of α Papilloma Isolates

DnaSP software (Version 5.10.01) was used in identifying segregating sites within the three isolate sequences. The sites considered to be segregating are those that differed from other aligned sequences and included sites with 2 noted mutations. The two synonymous changes were detected at position 4 and 8 in sequences of sample 2C (pan 4061) and 4C (pan 4058) respectively. The singleton variable site or SNP at position 4 in sample 2C was as a result of the substitution of Guanine (G) by Adenine (A) whereas, in sample 4C, a thymine (T) was substituted by an Adenine (A) (Table 4).

Table 4. Nucleotide substitution at position 4 and 8 of the isolates sequences

Sample Id	Nucleotide at Position 4	Nucleotide at Position 8
1C	G	T
2C	A	T
4C	G	A

4. Discussion

Genital αPV is associated with types of cervical cancer among women, female baboons, cynomolgus and rhesus macaques [1]. Despite findings on the relationship between the occurrence of cervical cancer and αPV, there is limited research that has provided linkage between the αPV present in the olive male baboon to αPV isolated in the humans, female baboons, cynomolgus and rhesus macaques. Out of the extracted 20 αPV DNA where one isolate from female baboon and acted as a control, 9 αPV DNA were from isolates in male olive baboons. Based on this research, the prevalence of αPV in male olive baboons

was 45% (9/20) an indication that there is a high number of male infected by the virus. The noted prevalence may be attributed to infection through sexual contact [28,29]. The results reaffirm existence αPV in male olive baboons in Kenya and provide the basis for analysing isolates diversity and establishing how close they are to αPV in both humans and other primates.

From the extracted αPV DNA, Amplified conserved region of L1 capsid gene of only samples labelled 1C (female baboon also acting as control and obtained from Laikipia), 2C, 4C (each representing male olive baboon obtained from Muringa abadare and colony born) were further subjected for sequencing and analysis (Table 1; Table 2). The three samples gave a presentation of 33.33% (prevalence is similar to description of Chai [24]) of the 9 samples with positive αPV DNA and their amplicons were within the required range of 134bp (Figure 1). The capsid L1 gene sequences have widely been used in classifying PVs into genera and demonstrate their diversity [19]. Therefore, the gene remains to be the traditional criteria that can easily be used to show the linkage between various subtypes and variants of αPV [13]. By targeting the conserved region of L1 capsid gene, this research ensures that there is a high probability of linking the isolated strains to each other and other subtypes or variants that have closely been linked to cervical cancer in both primates and humans. The study targeted the 115bp that lies between 486bp to 598bp of the full-length HPV-L1 major capsid coding region that is made of 1706bp (Figure 1) [30].

Despite sample 1C, 2C and 4C having short read length of 106bp, 105bp, and 107bp respectively, two segregating sites were identified in sample 2C and 4C which represents the isolated αPV DNA from male baboons. The SNPs were however identified to be synonymous changes thus implying that they do not alter the express protein in full length. The findings correspond to King [31] results on Dutch HPV-16 and 18 where the highest number of identified SNPs as compared to the reference strain used was synonymous. Based on their findings, HPV-16 had 72% synonymous SNPs while HPV-18 had 59%. These results show that L1 major capsid gene is more conserved with the αPV variants or strains across all host species. One of the identified SNPs (Guanine substituted by Adenine) was in position four of the targeted conserved region of L1 major capsid gene from isolate 2C and corresponded to position 489 on the full-length sequence of the gene. The second SNP was at position eight on the target sequence of 4C isolate and is positioned at 493bp on the L1 major capsid gene. The absence of non-synonymous SNPs confirms that the targeted region is somehow conserved and stands to provide insight on evolutionary relationship of isolated αPV to already identified strains in NCBI database.

For the past years, the conserved ORF of L1 capsid gene have aided in the establishment of evolutionary relationships and identification of new strains or variants [32]. The difference of 2% to 10% homology has been considered to define a subtype whereas less than 2% has been used to identify new variants [33]. In terms of genetic variation and phylogeny, isolate 2C is closely related to AB745694.1 Human papillomavirus commonly found in flat wart on immunocompromised patients and is also linked to cervical cancer [34]. Isolate 1C which is from female olive baboon is more closely related to 2Cas compared to 4C (Figure 2). 4C however, is more related to clades of other primates such as Papio hamadryas papillomavirus type 1 isolate (Mac2085), *Macaca fascicularis* papillomavirus type 3 (Mac26), type 10 (Mac616), Rhesus papillomavirus type 1b (isolate Mac170) and isolate Mac170. Based on the homological differences, the isolates can be referred to as subtypes which evolved from a common ancestry. The closeness of subtype 2C to Human papillomavirus (AB745694) demonstrates that male olive baboon from Muringa abadare may be a good model for testing therapeutic agents that target αPV in both humans and primates.

5. Conclusion

The finding of this research shows that there is a relatively high prevalence of αPV among the olive male baboon in Kenya. Further, the use of L1 capsid sequences in the identification of new strains or variants among αPV is reaffirmed. By targeting the conserved sequences of the gene, at least two SNPs have been identified to exist in isolates 2C and 4C and they have a role in determining how close the isolates are to other strains or variants in databases. Isolate 2C was therefore identified to be close to Human papillomavirus (AB745694.1) as compared to isolate 1C and 4C. The results presents male olive baboon as a possible animal model for studying the pathogenesis of αPV. Nevertheless, we recommend more studies to isolate and evaluate more PV isolates from baboons from various regions. This may lead to identification of more novel PV isolates which will aid efforts in development of baboon as a model for cancer studies.

Disclosure

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Conflict of Interest

The authors declare no conflict of interest. The findings and conclusions in this paper are those of the authors and do not necessarily represent the official position of the participating institutions.

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