Chapter 2 - Human Papillomavirus Types

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The following chapter has most of the sections available as an e-learning course.

Please click here to see the course: Human papillomavirus taxonomy.

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2.1 TAXONOMIC CLASSIFICATION

Human papillomaviruses (HPVs) are a large group of non-enveloped double stranded deoxyribonucleic acid (DNA) viruses belonging to the Papillomaviridae family.

The classification and nomenclature of HPVs is based on DNA sequence analysis, more specifically on the nucleotide sequence similarity of the L1 open reading frame, which is the most conserved region of the viral genome.

When identifying an HPV isolate, the DNA sequence of the L1 open reading frame is compared with the sequences corresponding to the L1 open reading frame of other HPV types that are already established and classified. Depending on how similar or different the sequences are, the HPV isolate is classified in its corresponding taxon. The International Committee on Taxonomy of Viruses (ICTV) is responsible for the virus nomenclature, down to the level of Species (Order, Family, Genus and Species). Taxonomic classification of HPVs to the level of Family is shown in **Table 2-1**.

Table 0-1 Taxonomic classification of HPV

SUPERKINGDOM	CLADE	KINGDOM	PHYLUM	CLASS	ORDER	FAMILY
Viruses	Monodnaviria	Shotokuvirae	Cossaviricota	Papovaviricetes	Zurhausenvirales	Papillomaviridae

Within the Papillomaviridae family, there exist several genera. Each genus is designated with a greek letter. Papillomaviruses that have human as a host are human papillomaviruses and are classified into 5 different genera: *alpha, beta, gamma, mu and nu*. HPV types belonging to different genera within the family Papillomaviridae share less than 60% similarity: when comparing the L1 sequences from HPV types from different genera, the sequences are <60% similar to each other.[1-3]

Within each genus, HPVs are classified in different species. Different species share between 60% and 70% similarity[1-3] and are designated by a number attached to the genus, e.g. alphapapillomavirus-9 (alpha genus, species 9).

Below the species level, HPV types are named with a number based on the order of their identification. New type designation is the responsibility of the International HPV Reference Center (IHRC). A unique HPV type number, for example HPV16, (is assigned only after the whole genome has been sequenced and the full genomic sequence in a clone or extract has been confirmed by the IHRC. A novel HPV type shares less than 90% similarity to any other type.[1-3]

2.1.1 Simplified example to classify HPV type

To determine the genus/species/type of an HPV isolate, the isolate´s L1 sequence is compared with the L1 sequence from a known and classified HPV type.

For example, comparing the L1 sequence from the unknown isolate (isolate 2) with the L1 sequence of HPV16, which is a type belonging to the genus alpha species 9, identifies 6 differences in sequence (**Table 2-2**). This alignment shows that the sequences are 80% (24/30 nucleotides) identical. Using the rules shown in **Table 2-3**, it can be determined that isolate 2 is a different HPV type (it is not HPV 16 as it is <90% similar to this type) but it belongs to the same species (≥ 70% similar), thus it is an alpha-9 human papillomavirus.

Table 0-2 Nucleotide sequence alignment of unknown HPV type and HPV16

Isolate 1 (HPV16): ATGGCCCTGAATGGCTAGGGACTTGACTAA
Isolate 2 (unknown): ATGTCCCAGATTGGCTAGCGACATGGCTAA

Table 0-3 Rules for classifying HPV type

If nucleotide sequence similarity <60% → Different genera
If nucleotide sequence similarity <70% → Different species
If nucleotide sequence similarity <90% → Different type

Below the category of type, within the papillomavirus research community, isolates of the same HPV type are referred to as variants or subtypes when the nucleotide sequences of the L1 open reading frame (ORF) differ by less than 10%.[1,3] At the International Papillomavirus Workshop held in Quebec in 1995, scientists working on papillomavirus taxonomy and diagnosis agreed to the following definition: differences between 2% and 10% homology define a subtype and less than 2% a variant. Nevertheless, due to the large continuum of differences between existing and potential subtypes, as well as the huge advance in detection methods, including whole genome sequencing, some scientists use the term variants to include subtypes.

Isolates of the same HPV type are referred to as variant lineages and sublineages when the pairwise nucleotide sequences of their complete genomes differ by approximately 1.0%−10.0% and 0.5%−1.0%, respectively.[4-6] Please note that the whole genome is now taken into consideration and not only the L1 gene. The rationale for the use of complete genome sequences to define viral lineages instead of the L1 region is because the percentage of sequence homology may differ by DNA region and may make a difference when subtle differences are used for classification. As an example, HPV 44 and HPV 55 (which is now classified as a subtype of HPV 44 and not as a new type) share 6.7% difference when analyzing only L1, but 6.2% nucleotide difference when comparing the whole genome.

Lineages and sublineages are named with a capital letter and one number, respectively, e.g: isolate belonging to an HPV16 A2 sublineage. Reference genome sequences for each lineage/sublineage are available at the publication from Burk et al., 2013, Table 1.[4] An example of taxonomic classification below the level of Family is shown at **Table 2-4**.

Table 0-4 Taxonomic classification of HPV

GENUS	SPECIES	TYPE	LINEAGE	SUBLINEAGE	ISOLATE
Alpha	-9	16	A	A1	Unique ID given by the originating author

2.1.2 Procedure of taxonomic classification of HPV types

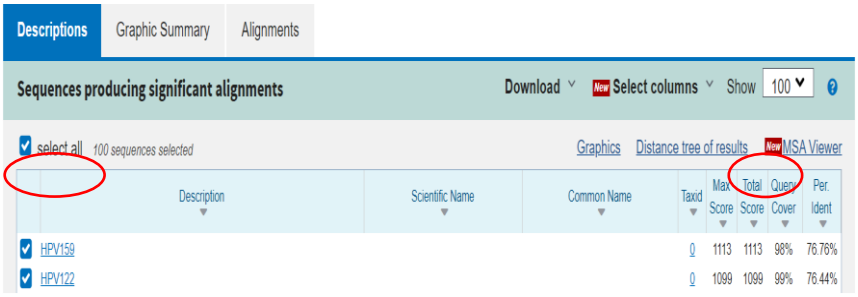
To classify the HPV isolate obtained in the laboratory, the whole L1 sequence must be compared with the L1 sequences from all the HPV types officially validated and confirmed by the IHRC. The L1 sequences comprise around 1550 nucleotides and therefore, manual comparison is impractical if not impossible.

Comparison can be easily performed using a Basic Local Alignment Search Tool (BLAST) by selecting the option of blastn (nucleotide blast) and aligning 2 or more sequences. The query sequence corresponds to the unknown sequence (the isolate identified in the laboratory), while the subject sequence should comprise all L1 sequences from all the officially established HPV types. An updated file with all L1 sequences from the officially established HPV types can be found at the bottom of Human Reference Clones of IHRC website. A full video explanation on use blastn to classify HPV sequences can also be found at IHRC website.

When performing blastn, the top hit (the HPV type that appears at the top position of the list of sequence alignments), will indicate the similarity to the closest official HPV type. The number obtained for percent identity, can be used to determine the isolate classification as done in the simplified example (Section 2-1-1). For example, nucleotide BLAST analysis of L1 sequence of an HPV isolate show HPV159 as the top hit with 76.76% percent identity (**Figure 2-1**). In this case, HPV 159 is the most closely related HPV. The last column shows the percent identity (Per. Ident. 76.76%), which is the similarity between the HPV isolate and HPV type 159. As the number is <90%, the isolate does not correspond to HPV159, but as the percent identity is above 60% and 70%, the HPV isolate does correspond to the same genus and species as HPV159, which is a beta-2 papillomavirus. The isolate is therefore a putative novel HPV type, belonging to species beta-2.

To know which genus/species each officially established HPV type belongs to, please visit the Human Reference Clones section of IHRC website.

Figure 2-1 Nucleotide BLAST analysis of L1 sequence of HPV isolate



2.2 ESTABLISHED HPV TYPES AND OTHER HPV SEQUENCES

Currently, 225 HPV different types have been completely cloned, sequenced and given an official number by the IHRC (last accessed October 1, 2024). However, the current highest type number is 231 because 6 previously assigned HPV type numbers (HPV 46, 55, 64, 79, 217 and HPV 218) were withdrawn due to re-classification as subtypes of other HPV types. Phylogenetic tree of officially established is shown in **Figure 2-2** and more information can be found at Human Reference Clones section of IHRC webpage.

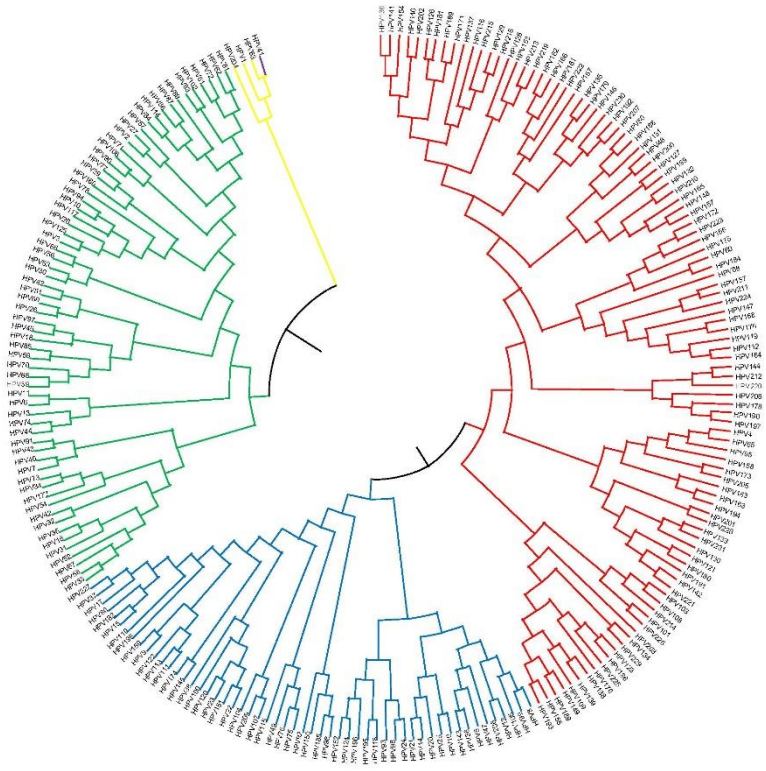
Furthermore, complete sequences of another 222 putative novel HPV types which have not been cloned nor investigated by the IHRC and therefore do not have an HPV type number, can be found in a public database named Papillomavirus Episteme. In addition, many partial genomic sequences of HPV isolates with no reference to HPV types have been deposited in public databases, with GenBank alone showing >50 000 hits when searched using “human papillomavirus” as query (data accessed on April 4th, 2021). It is estimated that the total number of different HPV types with public sequence information is around 800 and novel types are being continuously identified.[7-9]

2.3 RISK ASSOCIATION

The World Health Organization (WHO) and the International Agency for Research on Cancer (IARC) systematically review epidemiological and mechanistic evidence on possible oncogenicity of environmental exposures and classifies them as either oncogenic (Group 1), probably oncogenic (Group 2A), possibly oncogenic (group 2B) or not oncogenic (Group 3). IARC has classified 12 HPV types as carcinogenic (Group 1).[12] All of them belong to the alpha genus and comprise HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. One HPV type (HPV68) is classified as probably oncogenic (Group 2A) and HPV types 26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85, 97 are classified as possibly oncogenic (Group 2B) together with the non-genital HPV 5 and 8 found in patients with epidermodysplasia verruciformis.[12]

To classify HPV types as oncogenic, probably/possibly oncogenic or non-oncogenic, the IARC Working Group chose to use the association of HPV6 with cancer as the threshold for oncogenic

Figure 2-2 Phylogenetic tree comprising all official HPV types established until 2024-10-02 (available at https://www.hpvcenter.se/human_reference_clones/).



Alpha, beta, gamma, mu and nu papillomaviruses are presented in green, blue, red, yellow and purple colors, respectively. The phylogenetic tree is based on the L1 part of the genome. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model[10] and analyses were conducted in MEGA7.[11]

classification. It is widely known that HPV6 causes benign condyloma acuminata (external genital warts) and is detected only rarely in cervical cancers. HPV types 39, 51, 56 and 59 showed a stronger association with cervical cancer compared to HPV6, and thus were classified as oncogenic. However, several types such as HPV66, HPV68 and HPV73 showed association with cancer similar to HPV6 making their oncogenic classification uncertain.

All HPV types, and especially HPV types with uncertain oncogenicity, was further evaluated as new data and evidence became available. For example, IARC Working Group in 2009 classified HPV66 as carcinogenic[12] largely based on its detection in cervical intraepithelial neoplasia grade 3 (CIN 3), the immediate precursor of invasive cervical cancer. However, subsequent data from IARC and the Catalan Institute of Oncology first on nearly 30,000 typed cervical cancers[12, 13] and then on 40,000 cancers,[14, 15] indicates HPV66 is rarely found in invasive cancer and its association with cancer is greater than HPV 6. As a result, HPV 66 was classified in 2012 as only possibly carcinogenic (Group 2B). Furthermore, the recent report by IARC classifies HPV66 non-oncogenic as it does not comprise any measurable attributable fraction in cervical cancer (**Table 2-5**).

For HPV68 and HPV73, HPV types with uncertain oncogenicity, it was noted that detection was affected by assay selection. One of the commonly used polymerase chain reaction (PCR)-based method using short PCR fragment (SPF10) primers cannot distinguish between these two types due to same size of PCR amplicon.[16] Furthermore, neither of these two types is optimally detected by MY09-MY11 dot blot.[16] As shown in **Table 2-6**, the IARC Working Group now classifies HPV 68 as a probable carcinogen (Group 2A) as subtype of HPV68 (ME180) conferred immortalization to a cell line and HPV 73 as possible carcinogenic (Group 2B).

There are profound differences in carcinogenicity also among the oncogenic HPV type from Group 1 (**Table 2-5, Table 2-6**). HPV16 has by far the highest oncogenic potential, causing more than half of cervical cancers. HPV16 together with HPV 18 are responsible for about 70% of cervical cancer cases worldwide. Besides HPV 16 and HPV18, there are an additional 5 types (HPV31, 33, 35, 45, 52 and 58) that are found in >2% of cervical cancers and jointly account for an additional 20% of cervical cancers.[12, 16- 18] These 9 HPV types are included in the second generation nonavalent vaccine. HPV35 is responsible for about 1.4% of cancers and is common in Sub-Saharan Africa. Most of the cases of HPV58-associated cancers are from China. The least carcinogenic types (HPV 39, 51, 56 and 59) each contribute less than 1% of cervical cancer cases (**Table 2-5)**.

The strong differences in carcinogenicity among HPV types are the reason why some of the HPV tests detect HPV16 and HPV18 separately and report an aggregated result for other oncogenic, probably oncogenic and possibly oncogenic HPV types. It has been suggested that screening only for HPV16/18/31/33/45/52 would detect about as many cancers as when screening for more HPV types, but with only about half as many women testing positive in the screening.[19]

The HPV-type distribution is rather similar across the world, with a few exceptions. In China HPV52 and HPV58 have, in contrast to European distribution, a high prevalence in low- and high-grade cervical lesions.[20] In Europe, HPV 58 is rarely found in cancer.[19,21] In Sub-Saharan Africa, HPV35 is the most common HPV type in cervical neoplasia.[22] In Europe, it has been reported that HPVs 35/39/51/56/59/66/68 are found in around 3% of all women, but only 1.5% of the cancer cases detected in screening contain anyone of these HPV types. Some HPV types (in particular HPV51 and 66) are rather common in the general population and contribute greatly to lowering specificity, without noticeable gain in sensitivity.[18,19]

2.4 REFERENCE CLONES AND CDC REPOSITORY

The IHRC maintains all original reference clones of officially established HPV types (n=225). The Center distributes samples of these original reference clones for academic research use, free of charge, under Material Transfer Agreements agreed upon with the originator, supporting comparability and reproducibility of global HPV research. More information can be found at IHRC webpage.

Some of the original HPV plasmids were cloned by linearizing coding regions of the HPV genome resulting in plasmid clones with interrupted gene. The Center has re-cloned some HPV types to ensure *L1* gene is intact, for use in the International Proficiency Panels that are regularly issued. The Center for Disease Control and Prevention (CDC) is currently developing a plasmid repository for all known HPV types, with each clone containing the whole genome without interruptions in any coding region and inserted in a standard vector.

Table 2-5 Recent data as of 2022 on the importance of different HPV types in cervical cancer

HPV Type	Species	IARC Group	% in Cancer tissue	% in Normal tissue	Odds Ratio	% Attributable Fraction
HPV16	a9	Group 1	55.8	2.6	47.6	62.4
HPV18	a7	Group 1	14.3	1	15.7	15.3
HPV45	a7	Group 1	4.8	0.6	8.3	4.8
HPV33	a9	Group 1	4	0.6	7.1	3.9
HPV58	a9	Group 1	4	0.8	5.1	3.7
HPV31	a9	Group 1	3.5	1	3.7	2.9
HPV52	a9	Group 1	3.2	1	3.3	2.6
HPV35	a9	Group 1	1.6	0.4	3.9	1.4
HPV59	a7	Group 1	1.2	0.4	2.9	0.9
HPV39	a7	Group 1	1.3	0.6	2	0.8
HPV68	a7	Group 2A	0.6	0.4	1.5	0.2
HPV51	a5	Group 1	1	0.9	1.2	0.2
HPV56	a6	Group 1	0.8	0.6	1.3	0.2
HPV73	a11	Group 2B	0.5	0.3	1.8	0.2
HPV26	a5	Group 2B	0.2	0.1	4.1	0.2
HPV30	a6	Group 2B	0.2	0.1	2.6	0.1
HPV69	a5	Group 2B	0.2	0.1	1.4	0.1
HPV67	a9	Group 2B	0.3	0.2	1.2	<0.1
HPV82	a5	Group 2B	0.2	0.1	1.2	<0.1
HPV34	a11	Group 2B	0.1	0.1	1	Not attributable
HPV66	a6	Group 2B	0.3	0.6	0.4	Not attributable
HPV70	a7	Group 2B	0.2	0.8	0.3	Not attributable
HPV53	a6	Group 2B	0.5	1.1	0.4	Not attributable

*Table adapted and used with permission from Clifford G, et al. “IARC Handbooks Volume 18: Cervical Cancer Screening” (2022).[23]

Table 2-6 Classification of HPV types based on oncogenic risk

Group	HPV types
Group 1 (carcinogenic)	More than 60% of cancers:16 More than 5% of cancers: 18, 45 More than 2% of cancers: 31, 33, 52, 58 Less than 2% of cancers: 35, 39, 51, 56, 59
Group 2A (Probable carcinogenic)	68
Group 2B* (Possibly carcinogenic)	26, 30, 67, 69, 73, 82

*HPV 34, 53, 66, 70, 85, 97 do not cause any measurable fraction of cervical cancers and therefore are not listed in Group 2B.

2.5 PUTATIVE NOVEL HPV TYPES

A putative novel HPV type is identified when blastn (Nucleotide BLAST) analysis of L1 sequence of unknown HPV isolate show less than 90% identity with any of the official HPV types. When the novel HPV type is identified, the laboratory is advised to contact the IHRC to confirm its novelty as genomes of putative novel HPV types may be in the IHRC waiting for confirmation. A set of frequently asked questions can be found at the IHRC webpage.

Traditionally, the IHRC requested all originating authors to clone the putative novel HPV type and send the clones to IHRC for validation and confirmation. At IHRC, the complete L1 gene and some other random HPV DNA region is re-sequenced for sequence confirmation and clone viability is verified. Due to the advances in next generation sequencing, the IHRC now offers the alternative method allowing laboratories to send an aliquot of the specimen from which the novel HPV type was detected. The IHRC will then perform the cloning, sequence analysis and confirmation of the novel HPV putative type.

The submitting author will receive an email as soon as the IHRC has finished confirmation of the sequence. At the same time, the novel type is posted as an officially established type in the IHRC webpage. Although IHRC recommends publication of new HPV sequence in GenBank, it is not a requirement for obtaining an official HPV type designation from IHRC. The IHRC encourages all authors to promote open access.

2.6 HPV CLONING

Cloning of an HPV isolate can be done following standard cloning procedure but with modifications specific for HPV sequence. Several steps are involved in cloning starting with primer design, followed by PCR amplification, vector ligation, transformation, and finally performing sequence confirmation. Each step has its peculiarities.

2.6.1 Primer Design

Primer design may seem easy when there is only one HPV detected within a sample, but operators must be aware that multiple infection with other HPVs as well as other genomes may exist within the sample. Primers should only bind to the HPV type to be cloned to avoid possible chimeras. Most programs for designing primers include the possibility of adding "undesirable sequences, non-targets or mispriming libraries" where operators can include the coexisting microorganisms such as other HPV types and/or host genomes that are not to be amplified with the designed primers. If an operator has HPV16 and HPV18 in a human sample, and wants to clone only the HPV16, he/she should add HPV18 and the human genome as a mispriming library, to make sure that the primers will not amplify HPV18 nor human regions and will only amplify HPV16.

While the operator may choose to amplify whole HPV genome as a single amplicon (around 8000 bp), amplification of such large PCR amplicon can be challenging. HPVs genome can be amplified by generating several fragments of the HPV genome. However, when fragmenting the genome, it is important to generate overlapping fragments, in order not to miss any region afterwards, and to keep the L1 gene in only one of the fragments. L1 should not be fragmented as it is the region most used for detection of HPV. If the L1 is fragmented, the detection methods may not identify the HPV type and false negative result may be obtained. There are many programs for primer designing as well as web-based applications. Frequently used applications such as Primer3web and Primer-BLAST are freely available.

2.6.2 PCR Amplification

The operator must choose a long PCR amplification kit which is designed to amplify long amplicons using a high-fidelity polymerase and always use negative controls to detect background amplification. If possible, negative controls should include same components as the index sample excluding the target to be cloned. For example, if an operator wants to clone HPV16 from a human sample, the negative control should be a human sample free of HPV16. A standard operating procedure for PCR reactions to amplify large amplicon can be done using Long-range PCR with Takara LA Taq DNA polymerase. Amplicons should be subjected to a quality analysis with a bioanalyzer or agarose gel to confirm amplification and size. Special attention should be given to the negative controls, which may have the same background but should not have peak or DNA band at the desired size.

2.6.3 Amplicon Isolation and Purification

The IHRC uses the TOPO XL PCR cloning kit to perform amplicon isolation and purification following the manufacturer's recommendation.

2.6.4 Vector Ligation, Transformation, and Isolation of Clones

The IHRC uses the ZERO-blunt TOPO PCR cloning kit to perform vector ligation and uses one shot chemical transformation with *E.coli* competent cells and selection is done using Luria Broth agar plates containing kanamycin antibiotic.

2.6.5 Sequence Confirmation

Traditionally plasmids were re-sequenced to confirm putative sequences using Sanger sequencing. If high throughput sequencing is readily available, operator may choose this method but be careful and be strict with all parameters when performing the bioinformatic analysis with special attention given to quality trimming and variant calling steps, to avoid false calling of HPV types.[24]

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