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Molecular Methods for Detection of Beta-and Gamma-Papillomaviruses: A Review

Alltalents T Murahwa[1,2*], **Monalisa T Manhanzva**[1,2] **and Babill Stray-Pedersen**[3]

[1]*Department of Medical laboratory Sciences, University of Zimbabwe College of Health Sciences, Avondale, Harare, Zimbabwe*

[2]*Institute for Infectious Diseases and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Division of Medical Virology, Cape Town, South Africa*

[3]*Institute of Clinical Medicine and Division of Obstetrics and Gynecology, Rikshospitalet, Oslo, University Hospital, Norway*

[*]**Corresponding author:** Alltalents T Murahwa, Department of Medical laboratory Sciences, University of Zimbabwe College of Health Sciences, Avondale, Harare, Zimbabwe,Tel: 00263-779-136342; E-mail: atmurahwa@gmail.com

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Abstract

Accumulating evidence on beta and gamma Human Papillomavirus (HPV) genera, show its association with non-melanoma skin cancers (NMSC). Current methods for detection of HPV in clinical specimens are molecular based. A variety of molecular techniques developed for diagnosis of HPV; focus more on detection of mucosal HPV types. A limited number of assays have been developed to detect and genotype cutaneous HPVs. These assays detect a proportion of the HPV types implicated in skin lesions with differing sensitivities and specificities. Conflicting literature on the prevalence of HPV in NMSC has resulted from the inconsistent diagnostic standards. This review discusses the methods available for detection and genotyping of HPVs with emphasis on cutaneous HPV genotypes. This information will provide researchers the relevant choices of molecular methods applicable to epidemiological surveys. The ultimate objective is to make automated, rapid and cheaper molecular approaches for resource poor settings.

Keywords: Human papillomavirus; Lesions; Skin cancers; Carcinoma

Introduction

HPV is by far the most commonly implicated virus in human malignancies with 5.2% of all cancers attributable to HPV infection [1]. The World Health Organization (WHO) showed that about 9-13% (~630 million) of the world population has an HPV infection [2]. The involvement of HPV in cervical, penile, oral, genital and laryngeal cancers and cutaneous lesions such as skin warts, squamous cell carcinomas (SCC) and basal cell carcinomas (BCC) has been documented extensively [3].

HPVs are mandatory intraepithelial viruses present in the skin, mucosa and genital areas [4]. They replicate at the superficial layers of the mucosa and epidermis where the cells are more differentiated [5]. Clinically HPVs are classified as mucosal types and cutaneous types. Mucosal types being those mostly implicated in cervical neoplasia and cutaneous types in skin warts and non-melanoma skin cancers.

HPVs are from the family Papillomaviridae, which to date contains 29 genera formed by 189 papillomavirus types. One hundred and twenty types are human papillomaviruses and the remaining 69 are animal and bird papillomaviruses [6,7]. HPVs are circular dsDNA viruses approximately 8 kbs in size, typically containing 8 genes [8]; namely, the E1 to E7 genes, and L1 and L2 genes.

The HPV genome consists of three main regions the early region (E) encoding: E1, E2, E4, E5, E6, E7 proteins; and the late region (L) encoding: L1 and L2 proteins; and the Long control region (LCR) also known as the upstream regulatory region [9,10]. The L1 region is the most conservative region of the HPV genome and maintains its integrity after integration of viral DNA into the host cell genome [11-13]. The classification, differentiation and molecular diagnosis of HPV is thus solely based on the nucleotide sequence variations within the L1 region of the genome.

Presented below is a selection of method evaluation and epidemiological studies, critically reviewed to provide information on the choices of methods available for use in different HPV applications.

HPV Detection Methods

HPV has not been successfully grown in cell cultures and the application of serological assays has limited accuracy because of its inability to distinguish between present and past infections (HPV lab manual, 2009). The detection of HPV specific nucleic acids remains the best method for detecting HPV in clinical samples. Several molecular methods for HPV detection are available and can be categorized into amplification and non-amplification (or direct hybridization assays) techniques. Amplification techniques can be further categorized as: signal amplification and target amplification methods (HPV lab manual, 2009). However, most of these techniques have only been applied to mucosal HPVs. The laboratory detection of cutaneous HPV is still very underdeveloped with the few methods available not covering all the cutaneous HPV genotypes. The current review describes briefly the different molecular methods available for detection and genotyping of HPV with a particular emphasis on cutaneous (beta and gamma) HPV types.

Amplification methods

Target amplification assays make use of the polymerase chain reaction (PCR) to amplify nucleic acids and are the most widely used methods. PCRs can be done by type specific primers for amplification of individual HPV genotypes, or by consensus primers designed to

amplify a broad spectrum of HPV genotypes [14]. Consensus primers are usually designed to identify conserved regions of the HPV genome such as the L1 open reading frame or the E1 region [15,16]. The GP5+/6+ and MY09/11 primer pairs are some of the commonly used primers in HPV consensus PCRs. The amplicons generated from the PCR can be genotyped in various ways discussed below.

Restriction fragment length polymorphism (RFLP): The PCR product can be investigated by restriction fragment length polymorphism (RFLP) detection, using restriction endonuclease enzymes to generate a number of fragments that are then analyzed by gel electrophoresis [17-19]. The RFLP method is less cumbersome and less expensive than sequencing. It can also distinguish between high risk and low risk HPVs, as well as detect multiple infections.

Reverse line blotting (RLB): Alternatively, PCR products can be analyzed by hybridization with one or more type specific oligonucleotide probes layered on a filter paper pad or membrane strip (such as reverse line blot (RLB), linear array and INNO-Lipa) or bound on the walls of a micro titer well [20-22]. The advantage of hybridization techniques are their specificity to detect HPV types for which the probes are layered on the membrane strips and hence, it allows detection of multiple HPV types in a single run.

HPV genome sequencing: Another way of genotyping HPV is by direct sequencing of amplicons. The sequences will then be compared to reference sequences in the HPV database [23-24]. The advancement of sequencing techniques from Sanger sequencing, pyrosequencing through to the latest next generation sequencing (NGS) or high throughput sequencing has led to improved speed, quantitation, specificity and sensitivity. The cost of NGS technologies are however still high and not feasible for resource poor settings.

Real-time PCR: The application of real-time PCR, which simultaneously identifies and quantifies (via its ability to determine viral load) specific HPV types is extremely rapid and reproducible [25-27]. The other advantage of the real-time PCR is its ability to multiplex different nucleic acid targets.

Microarrays and DNA chips: Microarrays involve hybridization of a PCR product onto a chip and the hybridized signal is read on a DNA chip scanner. The main advantage is its ability to analyze numerous samples concurrently.

Signal amplification assays, the most widely used signal amplification method is, namely, the Digene hybrid capture assay 2 (HC2), and it is the only FDA approved HPV diagnostic test. This method is based on hybridization of target HPV-DNA to labeled RNA probes and subsequent attachment/capture of the hybrids on to micro titer wells, then, detection by an antibody-substrate system [28-31]. The test does not however genotype, but groups HPVs as either low risk or high risk types; and for this reason it has a wide application in epidemiological studies. The disadvantage is that HC2 does not genotype. HPV genotyping is essential in identifying single oncogenic types, if significant clinical interventions are to be made.

Non-Amplification Methods

The non-amplification methods were among the first to be used for HPV diagnosis before the advent of PCR. These include the southern blotting, in situ hybridization (ISH) and dot hybridization. These are all based on specific binding of probes to purified, but non-amplified DNA on a gel (southern blotting) or on the original sample, ISH [32-35]. Previously, the dot blot method was available as a commercial kit but it is no longer in use, namely, the Virapap and Viratype kits (Digene Corporation, Gaithersburg, USA). The ISH is still available in the form of the Kreatech commercial kit (Kreatech Biotechnology B.V, Amsterdam, Netherlands) and is in use for research purposes. Overall, the disadvantage of non-amplification techniques are their low sensitivity, which means large amounts of DNA is required for detection, and the DNA utilized has to be intact. These techniques are also cumbersome and time-consuming.

Cutaneous HPV Specific Methods

The role of HPV in cervical carcinogenesis has been firmly established. Hence, diagnostic methods became urgently imperative [36]. Evidence to support the association of HPV and skin lesions were only ascertained relatively recently [37-39]. As a result, there has been a lag in diagnostic techniques for cutaneous HPVs.

According to recent classification, cutaneous HPVs belong to the beta-and gamma- papillomavirus genera [7], while the mucosal are from the alpha genus. Moreover, it has been observed that cutaneous HPVs are phylogenetically distinct from the mucosal types [40], on the other hand, cutaneous HPVs infect the external skin. Beta- and gamma- papillomaviruses consist of epidermodysplasia verruciformis (EV) related HPVs, and phylogenetically related cutaneous HPVs. Their genotypes consist of HPV types 4, 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 2, 23, 24, 25, 36, 37, 38, 47, 48, 49, 50, 60 and 65.

Previously, the role of beta-and gamma- HPVs in NMSCs was established, but diagnosis has been limited by the methods of detection and typing. Most available methods rely on the amplification of DNA by consensus PCR, followed by sequencing [41-43]. The specimen of choice in detection of cutaneous HPVs is a skin biopsy taken from patients suspected of having NMSCs. After collection, they are either snap frozen or formalin fixed. HPV DNA can be extracted using several commercial kit methods, following the relevant manufacturer's instructions.

Consensus primer PCR and hybridization methods

Beta- and gamma- cutaneous PCR (BGC-PCR) [40] is based on HPV amplification by consensus primers using a mixture of six overlapping forward and eight overlapping reverse primers. Both are targeting the L1 open reading frame, generating a 72 bp amplimer and the reverse primers are all biotinylated. In this case, amplification is followed by RLB probes for multiple beta- and gamma- HPV types, that have been fixed on a carboxyl coated nylon membrane strip. Their PCR products are applied perpendicular to the oligonucleotide probes layered in the membrane. PCR products are then hybridized to the strip, and finally, undergo visual detection. Consequently, visual detection is achieved by incubation of the membrane in anti-biotin conjugate and chemiluminescence detection. This assay detects 25 different HPV types and the improved version adds to the conserved region genotypes 75, 76, 80, 92, 93, 96 [44]. This method has got the advantage of genotyping 25 beta- and gamma- HPV types.

Another variation of the BGC PCR was also developed known as the PM-PCR reverse hybridization assay (RHA) [Diassay, Netherlands] [45]. This method uses the same principle but uses a reduced amount of broad spectrum primers, i.e. two forward and seven reverse, targeting the E1 region. As a result, this variation generates an 117 bp amplimer as compared to the 72 bp version by the BGC-PCR; altering specificity. The hybridization method is the same, however, the PM PCR RHA method has oligoprobes for HPV types 75, 76, 80, 92, 93, 96

(layered on strips), unlike the BCG PCR, which identifies 4, 48, 50, 60, 65 HPV types. PM-PCR is thus designed for beta-papillomaviruses, whilst the BGC-PCR covers both genera.

Consensus primer PCR and sequencing methods

Several PCR methods for cutaneous HPVs have been developed that use sequencing as the genotyping method. The 'hanging droplet' PCR technique is based on a single tube nested PCR procedure [46]. Its first PCR reaction mixture with degenerate FAP59/64 primers targets the L1 region of the HPV genome, and is placed in a tube, where, the reaction goes on while the second round PCR mixture is a hanging 25 ul droplet on the lid of the first round tube. After the first round, the same tube is centrifuged, and the hanging droplet falls back in the tube, which initiates the second round PCR. Amplicons generated are separated by electrophoresis and then, cloned before sequencing can be done. Finally, generated sequences are then compared to existing sequences in a relevant database.

A previous variation of the hanging droplet method was a single round FAP PCR utilizing a pair of degenerate PCR primers (FAP59/64) to amplify a broad spectrum of cutaneous HPV types. This is followed by cloning and sequencing for genotyping [41]. The disadvantages are that sequencing fails to detect cases of multiple infections and it is also very laborious. It has the additional requirement that PCR products be cloned before direct sequencing is performed, to avoid it giving inconsistent results in multiple infections [41,43].

Multiplex PCR and new genotyping methods

A relatively new group of molecular techniques allow for detection of almost all the 50 HPV types in the beta- and gamma- genera. One such is the Bead based Multiplex genotyping of 58 HPVs from the gamma, beta, mu and nu genera implicated in cutaneous lesions [47]. This method is based on a mixture of FAP59/64 primers (i.e. the hanging droplet method) coupled by an additional 18 primers, also targeting the L1 region, via a nested PCR method [41] that also amplifies a broad spectrum of HPVs implicated in cutaneous lesions. Biotin labeled PCR products, hybridize to type specific oligonucleotide probes conjugated to fluorescence–labeled Luminex beads and the results are read in a Luminex analyzer.

Another type of multiplex PCR method uses HPV type specific primers for amplification of the E7 region with array primer extension (APEX) method for genotyping [48]. APEX assays are based on carbon-6 oligonucleotides, labeled with a fluorescing dye, immobilized on a slide [49]. PCR amplicons are then placed on the chip and incubated to allow for hybridization to take place, and detection is achieved by measuring fluorescence intensity. The application of novel DNA sequencing techniques such as NGS is being attempted for use in HPV detection [50]. These new technologies allow for fast, accurate diagnosis, detection of new HPV types and variants, and use in large epidemiological studies [51]. Among these novel methods on the market is the 454 pryosequencing method (that has been tested for use in HPV genotyping) [52], the ion proton sequencing technologies [53], and a whole range of other NGS technologies; that have a high throughput.

Discussion

Choice of methods

Choice of an amplification and genotyping method is dependent on the objective of the study and intended outcomes. In a wide epidemiological survey to determine the presence of HPV DNA on the skin of healthy individuals; it is usually conducted by the use of a consensus primer PCR system. This is then, followed by gel electrophoresis to check for the right band size of amplicons, which would suffice to distinguish between HPV DNA positive or negative. On the other hand, to check for HPV DNA in a group of immunocompromised patients (either HIV positive or organ transplant recipients), it would be prudent to employ a system that uses consensus primer PCR. Subsequently, a genotyping method employing hybridization of amplicons onto labeled oligonucleotide probes layered on a chip, nitrocellulose membrane, gel or one of the many available platforms can be used. This protocol would identify known HPV genotypes that are previously known to be associated with such conditions. To confirm EV HPV types in a child with EV, use of a method that has got EV associated HPV oligoprobes only, would be cost effective. In a study to determine the skin microbiome were novel HPV types maybe expected, use of NGS methods would be helpful to identify the new HPV types. HPV DNA detection and genotyping for a private/commercial laboratory which performs routine HPV testing, will either select a commercial kit with primers (that cover the range of the common HPV types for efficient amplification) or a cost-effective genotyping method (that gives clinically relevant results). Whereas, a survey intended to identify the most common HPV genotypes, with the view of identifying epitopes for vaccine development, would employ a deep sequencing method amplifying L1 and E1, or other regions of the genome; from which the epitopes of choice would be designed. Studies to identify HPV variants would also use deep sequencing methods, as they would inform decisions on appropriate vaccine development. Surveys to assess vaccine efficacy, after a vaccination roll out program, would make use of the HC2 assay to determine presence or absence of high and low risk types, without necessarily genotyping. Studies to determine the burden of HPV multiple infections in individuals would not use direct sequencing methods, but rather cloning into TOPOTA or other vectors; followed by sequencing to facilitate identification of more than one HPV genotype in one specimen. If multiple infections are expected, then reverse hybridization or line blot platforms layered with oligoprobes of known HPV types can be used.

Comparison of cutaneous HPV methods

Table 1 shows the choice of molecular methods available for cutaneous HPV types from the beta- and gamma- genera that are essential in the detection of HPV types from all types of skin lesions.

Essential to the detection of HPV in skin lesions, is the nature of the specimen from which the DNA is extracted. Specimens are usually in the form of skin biopsies and skin swabs. Biopsies are both formalin fixed and paraffin embedded (FFPE) or snap frozen, on collection and stored at -80 C. It has been observed; FFPE tissues usually give a low DNA yield as compared to frozen tissue. Formalin has also been shown to have genotoxic effects.

Molecular Method	Reference	Primer(s)	Region Amplified	Aplimer Length	Sensitivity Copies number	Cloning	Genotyping Method	HPV Spectrum Detected
PM-PCR (Diassay)	de Koning et al. [45]	PM Consensus primer set	E1 Region	117 bp	10-100copies	-	RHA	25 beta types only
Multiplex PCR-APEX	Gheit et al. [48]	Type specific primers	E7 Region	186-286 bp	10-100copies	-	APEX	25 beta types only
FAP PCR	Forslund et al. [41]	Degenerate FAP 59/64 set	L1 Region	480 bp	1-10 copies	TOPOTA vector	DNA sequencing	>73 beta and gamma types
FAP Hanging Droplet Nested PCR	Forslund et al. [46]	Degenerate FAP 59/64 set	L1 Region	235 bp	1 copy	TOPOTA vector	DNA sequencing	37 beta 1 and beta 2 types
Multiplex Cutaneous PCR Genotyping (McPG)	Michael et al. [47]	FAP59/64 set + 18 more primers	L1 Region	235 bp	<100 copies	-	Bead Base Luminex Assay	58 beta and gamma, mu and nu cutaneous types
Beta and Gamma PCR (BGC-PCR)	Brink et al. [40]	Consensus primer set	L1 Region	72 bp	10-1000copies	-	RLB	25 beta and gamma types
BGC-PCR Extension	Nindl et al. [44]	BGC consensus primer set	L1 Region	72 bp	10-100copies	-	RLB	25 beta types only

Table 1: Molecular methods for cutaneous HPV types

On the other hand, the DNA yield in frozen tissues is reduced by intermittent freezing and thawing. It is thus imperative to choose a method that has got a high sensitivity, in order to detect the lowest DNA amounts. Skin swabs are usually stored and transported in commercial media to preserve the DNA. However, an assay to increase the DNA yield pre-PCR has been developed .i.e. the rolling circle amplification (RCA) method. RCA is an isothermal non-specific amplification of circular dsDNA using phi-DNA polymerase. Binding of non-specific primers initiate elongation reactions that produce similar DNA strands called concatemers. After 18 hours of isothermal amplification the DNA yield is markedly increased, then type specific PCR can follow. Choice of method is thus dependent on the nature of the specimen and the expected DNA yield. Therefore, the lower the expected DNA yield, the greater the need for a highly sensitive method that detects the lowest DNA copy numbers (see Table 1).

Method of genotyping cutaneous HPV post-PCR is another important choice that has to be made. While HPV genotypes to be detected are a result of the type of primers used in the PCR, the detection method is about the specificity of a technique to distinguish one HPV type from the other using the different detection signals. Careful considerations regarding the different methods have to undergo cost-effective and cost-benefit analyses. Recently, the prices of DNA sequencing are markedly reducing, and considering its utility in typing microbes by comparison to reference sequences; it's slowly becoming the ideal genotyping method. DNA sequencing is less burdensome and less time consuming, in comparison to the use of Luminex and fluorescence detection methods, which require extra complicated equipment. Moreover, DNA sequencing also allows for discovery of novel HPV types; by comparison to known sequences using bioinformatics.

The methods discussed here are not the best because several in-house PCR and genotyping systems can be developed to suit the needs of certain laboratories and studies. However, this review provides a range of currently available methods for HPV detection and when they can be best utilized.

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