

RESEARCH COMMUNICATION

Comparison between Direct Sequencing and INNO-LiPA Methods for HPV Detection and Genotyping in Thai Women

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Abstract

Human papillomaviruses (HPVs) have been recognized as etiologic factors in cervical carcinoma and several other anogenital cancers in females and males. HPV are classified as low risk (LR), probable high risk and high risk (HR) on the basis of their oncogenic potential. HPV genotypes, which are crucial for diagnosis and relationship with carcinogenesis, have been determined by several genotyping methods. In this study, two genotyping methods were compared: direct sequencing and INNO-LiPA. In total, 2,494 cervical specimens were tested and 27.2 % of these were found to be HPV DNA positive with 24.5% showing normal cytology. Specimens were divided into four groups according to their pathological cytology as normal, LSIL, HSIL and cancer and 134 specimens were selected for HPV genotyping by both methods. HPV genotyping results showed 87.5% positive correlation. With 17 specimens, the results were discordant, 12 specimens showed different genotypes. Others had genotypes that could not be typed by the INNO-LiPA method. Neither did direct sequencing in 3 different regions yield unequivocal results. Both genotyping methods have advantages and disadvantages. Consequently, the method most suitable for the study objective, budget and predominance of HPV genotype in any given area should be selected.

Keywords: HPV - genotyping - direct sequencing - INNO-LiPA

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Introduction

Cervical cancer is the second most common cancer in women worldwide, with an estimated 493,000 new diagnoses and approximately 270,000 deaths annually (Parkin et al., 2005). It is now clear that human papillomavirus (HPV) is the etiological agent implicated in cervical cancer and its precursor lesions (cervical intraepithelial neoplasia (CIN)), with HPV DNA present in 99.7% of cervical cancers (Walboomers et al., 1999). More than 100 HPV genotypes have been identified, with 40 commonly infecting the anogenital epithelium, and 15 thought to be carcinogenic (Smith et al., 2007). Thus, these viruses have been classified as low- and high-risk types depending on their propensity to cause cancer (de Villiers et al., 2004; Lin et al., 2010).

Because of their biological properties, HPVs cannot easily be grown in tissue culture, which makes preparation of antigens for routine detection of HPV difficult (Doorbar, 2006). Furthermore, serological assays, which detect HPV-specific antibodies, cannot be used for diagnostic purposes, since these antibodies are markers of a lifetime's cumulative exposure to HPV types (Ho et al., 2004).

For diagnostic purposes, methods based on detection of HPV-specific nucleic acids are being used. In addition, the typing of HPV isolates is accomplished by means of molecular biological methods (Brink et al., 2007; Sabol et al., 2008).

HPV DNA detection and HPV type determination are important for studies of the natural history of HPV, vaccine development, and patient care (Bosch et al., 2008; Wheeler et al., 2009). Over the last few years, virus genotyping has become an important way to approach cervical cancer. Several groups have searched for an effective genotyping test for HPV, as this would vastly contribute to the diagnosis of infections and to a better understanding of the relationship between HPV and carcinogenesis, in addition to aiding in the development of type-specific vaccines (Ermela et al., 2010).

As no test has officially been approved for HPV genotyping (Meijer et al., 2003), several methods have been used to identify different virus types, including PCR with generic primers (Gravitti et al., 2000), RFLP (Astori et al., 1997), hybridization with specific probes (Mendez et al., 2005), reverse hybridization line probe assay - HPV-LiPA (Kleter et al., 1999), reverse line-blot

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hybridization (Mendez et al., 2005), nucleotide sequencing (Verteramo et al., 2006; Fontaine et al., 2007; Lee et al., 2007; Montaldo et al., 2007; Carvalho et al., 2010) and DNA Chip (Choi et al., 2005).

Although DNA sequencing is considered to be the “gold standard” for HPV genotyping, it is costly, time-consuming and difficult to apply in routine diagnostic settings [Feng et al., 2009]. However, nucleotide sequencing can not provide any information on mixed genotype infection. Currently, the most widely used HPV genotyping tests are based on reverse hybridization of amplified HPV products on a membrane strip containing multiple probes immobilized as parallel lines, such as INNO-LiPA HPV Assay (Innogenetics, Gent, Belgium) which can detect 28 different HPV genotypes (van Hamont et al., 2006; Coutlée et al., 2006). This study has been aimed at comparing two methods for HPV genotyping (direct sequencing and INNO-LiPA) to find an effective strategy for virus genotyping in clinical samples from the Thai population.

Materials and Methods

Study population and clinical specimens

The Ethics Committee of the hospital and Faculty of Medicine, Chulalongkorn University, approved all study protocols. The HPV positive samples were chosen from among the specimens obtained during the patients' routine check up, investigation or treatment. All the studied specimens were anonymous with a coding number for analysis and permission was granted by the director of the hospital. The samples included in this study comprised 2,494 cervical specimens obtained from Thai women routinely checked for cervical cancer and newly diagnosed cervical cancer from April 2008 to October 2010. All of these specimens were collected as anonymous at Chulalongkorn hospital, National Cancer Institute Thailand, Bangpakok 9 International hospital and Samitivej Srinakharin hospital. All specimens were collected as anonymous in LBC buffer (ThinPrep®, Hologic, West Sussex, UK) or in phosphate buffered saline (PBS) and stored at 70°C until used.

DNA extraction

Cell suspensions were centrifuged (4,000xg, 10 min), resuspended in 6% Triton X-100 and Proteinase K (400 µg/ml) buffer and incubated at 56°C for 2 hours. After incubation, the suspension was heated to 90°C for 10 min for inactivation of Proteinase K and then stored at -20°C until tested.

HPV DNA detection

The HPV DNA was detected by PCR amplification with consensus primers in the E1 region based on a previous study [Lurchachaiwong et al., 2009]. The PCR amplification program comprised an initial denaturation step at 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were examined by 2% agarose gel electrophoresis and stained with ethidium bromide. The amplified products were

visualized by UV transillumination (Gel Doc 1000, BIO-RAD, CA). Human DNA in all specimens were also identified by using β-globin PCR analysis.

HPV genotyping

Direct sequencing. Typing of the E1, E6 and L1 amplicons (amplified by in-house primers as indicated in a previous study [Lurchachaiwong et al., 2009]) was performed with direct sequencing by FirstBASE Laboratories SDNBHD (Selangor Darul Ehsan, Malaysia). The sequences were analyzed by BLAST from the NCBI website and compared with HPV sequences in the database.

INNO-LiPA method. One-hundred thirty-four randomly selected HPV positive DNA samples from four groups were genotyped by INNO-LiPA HPV genotyping Extra (Innogenetics N.V., Ghent, Belgium), following the manufacturer's instructions. This kit has been designed for the identification of 28 different genotypes of the human papillomavirus (HPV) by detection of specific sequences in the L1 region of the HPV genome. The assay covers all currently known high-risk HPV genotypes and probable high-risk HPV genotypes (16,18,26,31,33,35,39,45,51,52,53,56,58,59,66,68,73,82) as well as a number of low-risk HPV genotypes (6,11,40,43,44,54,70) and some additional types (69,71,74). Amplified products were denatured under alkaline conditions and immediately incubated with the test strip in hybridization buffer. The results were interpreted by comparison with the provided template. The hybridization patterns were interpreted by two independent readers.

Data Analysis

Genotyping results by 2 methods were compared and defined as concordance, partial concordance and discordance when HPV genotypes from both methods were totally identical, partially identical and non-identical, respectively.

Results

Characterization of the study groups and HPV DNA detection

The 2,494 cervical specimens were divided into four groups according to their pathological cytology as normal (n = 1,756), low grade squamous intraepithelial lesions: LSIL (n = 486), high grade squamous intraepithelial lesions: HSIL (n = 111) and cervical carcinoma (n = 141).

Upon amplification of the HPV E1 region, HPV DNA was detected in 678 of 2,494 specimens (27.2%). Of these specimens, 24.5% (166 of 678) showed normal cytology. In total, 134 specimens were selected from all groups in order to compare between genotyping methods.

HPV genotyping

All 134 specimens were genotyped by using both methods direct sequencing and INNO-LiPA for comparison. The comparative results are shown in Table 1. The genotype comparison was divided into 3 categories: concordance (both methods indicated identical

Table 1. Comparison of HPV Genotyping by Direct Sequencing and INNO-LiPA Methods

Groups	Genotyping concordance		HPV-ve*		Total
	concordance	partial discordance	DS	IN	
Normal	4	6	5	-	8
LSIL	6	26	6	-	38
HSIL	14	21	1	-	37
Cancer	16	12	3	1	4
Total	40	65	15	1	13

*DNA negative/untypeable; DS, direct sequencing; IN, INNO-LiPA

Table 2. Clinical Characteristics of Cervical Specimens and HPV Genotyping by INNO-LiPA

Parameter	Normal	LSIL	HSIL	Cancer	Total
No.	23 (17.1)	38 (28.4)	37 (27.6)	36 (26.9)	134 (100)
HPV-ve	7 (70.0)	0	1 (10.0)	2 (20.0)	10 (7.5)
HPV genotype (124)					
typeable	15	38	36	32	121 (97.6)
untypeable	1	0	0	2	3 (2.4)
HPV infection (120)					
single	6	13	16	18	53 (44.2)
multiple	9	24	20	14	67 (55.8)

genotypes), partial concordance (both methods indicated at least 1 overlapping genotype) and discordance (both methods indicated different genotypes).

Detection of HPV genotype by direct sequencing

In this study, 3 regions of HPV DNA (E1, E6 and L1) were selected for amplification and sequencing by in-house primers. The genotype results were quite different for each region and thus, it was quite difficult to conclude whether this was due to multiple infection, intratypic recombination or error in the sequencing method. However, this problem was solved upon comparing these results with the results from INNO-LiPA. With four specimens direct sequencing of 3 regions could not unequivocally determine the HPV genotype. Yet, applying INNO-LiPA, this was accomplished.

Detection of HPV genotype by INNO-LiPA

Of 134 specimens, 10 were negative for HPV DNA by using INNO-LiPA HPV genotyping Extra Amp from Innogenetics. With three, the results were untypeable. Of all specimens, 98.33 % (118/120) showed at least one HR-HPV genotype infection. Single and multiple HPV genotype infections were similarly frequent (44.2 and 55.8%, respectively). Clinical characterization and genotype results are summarized in Table 2.

Discussion

Detection of HPV sequences and determination of HPV genotypes in clinical specimens is important for epidemiological studies, development of HPV vaccines, and has a potential role in screening for cervical cancer and for management of women with cervical dysplasia. At present there are several different HPV DNA detection methods available for clinical and research use, but no "gold standard" does exist due to the strengths and

limitations of the individual assays. HPV detection methods differ in many aspects including sample preparation, primers used in the assay (for PCR-based assays), and interpretation methods. In addition, there are variations in both sensitivity and specificity of the assays (Ermela et al., 2010). In the current study, the results of two HPV genotype determination methods (direct sequencing and INNO-LiPA) were compared to decide on a method suitable for HPV genotyping and distribution study among the Thai population.

In our study, we found 27.2% of cervical specimens HPV DNA positive. Interestingly, specimens with more severe pathological cytology also displayed HPV DNA more frequently. Thus, HPV DNA could be detected in 9.5, 64.2, 69.4 and 88% of the normal, LSIL, HSIL and cervical cancer cytology specimens, respectively. Our finding may confirm that HPV is the major causative agent of cervical cancer (Grm et al., 2009; Bharadwaj et al., 2009). To effectively prevent cervical cancer, yearly Pap smear test is as crucial as screening for HPV DNA, as HPV DNA could be detected in 24.5% of normal cytology specimens.

Eleven specimens proved HPV DNA negative upon amplification applying INNO-LiPA and one specimen proved negative by direct sequencing. HPV DNA amplification using in-house primers proved more sensitive than with the INNO-LiPA Extra Amp kit. HPV genotyping by direct sequencing which was used as the gold standard also yielded more typeable results than by INNO-LiPA. This may be due to the larger size of the amplified product as opposed to the 65-bp product obtained with the INNO-LiPA method.

Upon comparing both methods, 87.5% (105/120) of specimens showed a positive correlation in genotype results (concordance & partial concordance). As for the specimens with discordant results, 12 specimens showed different genotypes. Another five specimens had genotypes that could not be typed by the INNO-LiPA method. These genotypes were typeable as 30, 32, 34, 67 and 91 by direct sequencing. Unfortunately, these genotypes are commonly found and could be detected in approximately 31.7% of the Thai population (Chansaenroj et al., 2010). Hence, by exclusively relying on INNO-LiPA for HPV genotyping in the Thai population, some genotypes may go unnoticed.

Direct sequencing of various regions can also cause a problem in HPV genotyping. As only the predominant genotype is shown, this method may indicate different genotypes. Typing by INNO-LiPA can solve this problem because it is suitable for detection of multiple HPV infection. INNO-LiPA uses the technique of reverse hybridization with multiple probes for different genotypes. Yet, interpretation of the results can be quite difficult, depending on the number and density of the bands shown on the strip. Thus, some genotypes which are not commonly distributed worldwide may prove untypeable as found in our previous study (genotype 30, 32, 34, 42, 55, 81, 85, 90, 91) (Chansaenroj et al., 2010). However, this point should not be ignored because the distribution of HPV genotypes in different areas has proven different which is important for the clinical outcome in each area. The next generation of INNO-LiPA test should include

additional probes for detecting more genotypes per area.

Infections with multiple HPV genotypes can be found in 10-40 percent of HPV positive cases (Nielsen et al., 2008; Hlaing et al., 2010; Tsao et al., 2010; Sukasem et al., 2011). Interestingly, in our study approximately 50% of HPV infected cases displayed multiple genotype infection based on INNO-LiPA, yet not correlated with cytological findings. In conclusion, the HPV genotyping method selected should be suitable for clinical specimens and HPV genotype predominance in each area. INNO-LiPA is good for detecting multiple HPV infection but not all HPV genotypes were typeable. Direct sequencing can determine more genotypes but cannot show multiple HPV infection and is also time-consuming.

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