Original Article

Comparison of Human Papillomavirus Detection and Genotyping with Four Different Prime Sets by PCR-Sequencing*

CAI Yu Pin[1,3,†], YANG Yi[2,†], ZHU Bao Li[1], LI Yuan[2], XIA Xiao Yu[2], ZHANG Rui Fen[1,#], and XIANG Yang[2,#]

1. CAS Key Laboratory of Pathogenic Microbiology & Immunology, Institute of Microbiology, the Chinese Academy of Sciences, Beijing 100101, China; 2. Department of Obstetrics and Gynecology, Peking Union Medical College Hospital, Peking Union Medical College and the Chinese Academy of Medical Sciences, Beijing 100005, China; 3. Graduate University of the Chinese Academy of Sciences, Beijing 100101, China

Abstract

Objective To assess and compare the Human Papillomavirus (HPV) detection efficiency and the potential clinical utility of PCR sequencing-based technology.

Methods Four HPV consensus primer sets (GP5+/6+, MGP, MY09/11, and PGMY09/11) were used in order to amplify a broad spectrum of HPV types for HPV infection in 325 cervical samples and the PCR products were sequenced afterwards for the HPV genotyping.

Results The HPV-positive rate was 75.4%, of which 35.5% harbored more than one HPV genotype. A total of 36 different genotypes was found, with HPV 16 (24.1%) being the most prevalent, followed by HPV 58 (13.3%) and HPV 52 (9.6%). There were substantial to almost perfect agreements between different primer sets regarding HPV detection efficiency, with the kappa value varying from 0.751 to 0.925, MGP, and PGMY09/11 were the most effective in detecting multiple infections (*P*<0.001). With each of the primer sets, a board range of HPV types could be identified, though there were several differences for a few genotypes.

Conclusion The substantial agreement between PCR-sequencing and HC2 for the detection of high-risk HPV (kappa=0.761) indicated that PCR-sequencing is also suitable for routine HPV screening.

Key words: Human papillomavirus; Cervical cancer; Polymerase chain reaction; DNA sequencing

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INTRODUCTION

Cervical cancer is the third most commonly prevalent cancer among women, with an estimated 529 000 new cases and 274 000 deaths in 2008 worldwide, of which more than 85% occurred in developing countries[1]. In China, the age-standardized incidence and mortality rate are 9.6 and 4.3 per 100 000, respectively, while the mortality rate is even higher in some poor, and rural areas[1-2]. It is now well established that infection with the high-risk human papillomavirus

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#Correspondence should be addressed to: ZHANG Rui Fen, Tel:86-10-64807433, E-mail: Zhangrf@im.ac.cn; and XIANG Yang, Tel: 86-10-65296240, E-mail: xiangyang65@gmail.com

†These authors contributed equally.

Biographical note of the first authors: CAI Yu Pin, male, born in 1985, M.S, majoring in genetics; YANG Yi, male, born in 1970, MD, Ph.D, associate professor, majoring in obstetrics and gynecology.

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(HPV) is a necessary cause of cervical cancer and this virus has been found in 99.7% of cervical tumors[3-4]. More than 100 types of HPV have been identified and approximately 40 types from those 100 types can infect the genital tract[5]. According to their association with cervical cancer, HPV is divided into 3 categories: high-risk types, probable high-risk types and low-risk types[6]. The distribution of type-specific HPV in invasive cervical cancer (ICC) varies by geographic region. HPV 16 is slightly more prevalent in Europe and North America, HPV 31 is more prevalent in South/Central America, HPV 33 and 45 are more prevalent in Africa, and HPV 52 and 58 are more prevalent in Asia[7-8].

The strong causal relationship between HPV and cervical cancer revealed to us that regular screening for high-risk HPV types is necessary for the prevention and control and management of this cancer. Furthermore, HPV genotyping plays a critical role in determining the prevalence and relative risk of each type of the virus, monitoring the recurrence after the cancer treatment and evaluating the efficacy of prophylactic vaccines. At the present, the most widely used methods for the detection of genital HPV types are the FDA approved Hybrid Capture 2 (HC2, Digene)[9] and PCR-based assays. HC2 has been shown to be a robust and reproducible test for the detection of relevant high-risk HPV DNA. However, the disadvantage of this assay is that it cannot differentiate the 13 different specific high-risk HPV types. Further, the detection limit is 1.0 pg/mL or approximately 5000 genome equivalents, which is less sensitive than PCR- based methods. For PCR-based methods, the most frequently used primers for HPV detection are GP5+/6+[10], MY09/11[11] and its modified version PGMY09/11 which target the highly conserved region of the HPV L1 gene[12-13]. Genotyping for HPV followed by general primer-mediated PCR can be accomplished by restriction-fragment length polymorphism[14], type-specific oligonucleotide hybridization[15-17], mass spectrometry[18-19] and nucleotide sequencing[20-21]. Probe-based genotyping assays have been shown to be more efficient in detection of multiple infections, while their detection spectrum is restricted to a given range[22-23]. To a certain extent, the sequencing of PCR products is considered as the “gold standard” for HPV genotyping[22]. Sequencing-based assays have advantage of identifying a broader range of HPV types and eliminating the risk of false detection due to the cross-hybridization[20,24]. In recent years, the cost of sequencing has decreased dramatically, enabling a widespread use of it for HPV detection in cervical cancer screening. However, there are few studies analyzing the HPV detection efficiency and the potential clinical utility of PCR sequencing-based technology. And it is therefore in the present study, we compared the HPV detection performances of PCR-sequencing assays based on four general primer systems [GP5+/6+, MY09/11, PGMY09/11 and the newly modified GP5+/6+ (MGP)[18]] in order to evaluate their possible use for large-scale HPV screening.

SUBJECTS AND METHODS

Study Material

The collection of specimen was approved by the Ethics Committee of Peking Union Medical College Hospital. A total of 338 specimens was collected from the outpatients aged 21-66 years (mean age: 40.4±9.2; median age: 41.0) attending the Department of Obstetrics and Gynecology of Peking Union Medical College Hospital between June 2009 and March 2010. The endocervical Cytobrush were used for the collection of the samples, which were placed then into 20 mL of PreservCyt Solution (Cytyc Corp., Boxborough, Massachusetts, USA) and stored at -4 °C.

Cytological Examination

Cytological examination was performed for all the samples by senior cytopathologists according to the Bethesda System 2001[25]. All squamous cell carcinoma (SCC) samples were determined and confirmed by histopathological analysis.

DNA Extraction

The residual ThinPrep liquid-based cytology samples were used for total genomic DNA extraction with a QIAamp DNA Mini kit (Qiagen, Hilden, Germany). DNA was eluted from the columns in 100 μL of AE buffer afterwards and then stored at -20 °C. The concentration of the DNA samples was measured by a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Montchanin, DE, USA) and the DNA quality was assessed by the amplification of the β-globin gene using the GH20 and PC04 primers.

Consensus PCR and Direct Sequencing

The consensus primers GP5+/6+[10], MGP[18], MY09/11[11], and PGMY09/11[13] were used in the present study to amplify HPV DNA. Each PCR was

performed on a Labcycler thermal cycler (Sensoquest, GmbH, Gottingen, Germany) in a final volume of 50 μL containing 2 μL of DNA, 1×PCR Buffer (Mg^{2+} plus), 200 μmol/L of each dNTP, and 1.25 U of Ex Taq HS polymerase (TaKaRa, Dalian, China). The final concentrations of primers for GP5+/6+, MGP, MY09/11, and PGMY09/11 were 1.0 μmol/L, 0.3 μmol/L, 0.3 μmol/L, and 0.2 μmol/L, respectively. A plasmid containing full length of the HPV 16 genome (ATCC 45113) was used as the positive control. Negative and positive controls were then added in each amplification procedure. A 5-μL aliquot of the PCR products was analyzed by ethidium bromide staining after gel electrophoresis. The PCR products with a visible band were then purified with the AxyPrep™ DNA Gel Extraction Kit (Axygen, California, USA) according to the instructions of the manufacturer. Sequencing was performed on the ABI 3730xl DNA Analyzer using a BigDye Terminator kit v3.1 (Applied Biosystems) with the corresponding forward primer as the sequencing primer. Infection with a single HPV type produces readable chromatograms with sharp peaks and sufficient signal height (quality values≥20) and, little or no background noise was observed. When the samples contain more than one HPV type, direct sequencing results in mixed chromatograms, with overlapping peaks or two or more fluorescent signals in positions where the L1 gene differs. Internal regions of 360 bp for MY09/11 and PGMY09/11 or 110 bp for GP5+/6+ and MGP were aligned to the GenBank database using the BLAST server (http://www.ncbi.nlm.nih.gov/blast/) and the HPV type was confirmed when the identity was at least 95%. The PCR products without visible bands or without confirmed sequence homology to HPV types were considered as negative.

Type-Specific PCR

Samples with multiple infections were subjected to type-specific PCR (TS-PCR). The TS-PCRs were performed as described previously with several modifications[26]. In brief, all of the TS-PCRs were performed using the 12 high-risk HPV primers (HPV 16, 18, 31, 33, 35, 39, 51, 52, 56, 58, 66, and 68) in separate reactions. Each reaction was performed in a final volume of 25 μL containing 1 μL of DNA, 1×PCR Buffer (Mg^{2+} plus), 200 μmol/L of each dNTP, 0.5 μmol/L of each primer, and 1 U of Ex Taq HS polymerase (TaKaRa, Dalian, China). The amplification conditions were 94 °C for 10 min in order to activate the HotStart Taq polymerase, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s, with a final extension being at 72 °C for 4 min. The TS-PCR genotypes were further confirmed by nucleotide sequencing.

Hybrid Capture 2 (HC2) Assay

A subset of 111 cervical samples was tested by HC2 using the high-risk cocktail according to the instructions of the manufacturer (Digene, Gaithersburg, USA), in which 13 high-risk HPV types were detected,namely: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. A positive result refers to a relative light units/cut-off (RLU/CO) ≥1.0 for the sample and all the tests were performed blindly with regards to the PCR results.

Data Analysis

Statistical analysis was performed using SPSS version 13.0 for Windows. The kappa statistics were calculated in order to evaluate the agreement among different primers for HPV detection, and the agreement between the PCR and HC2 assays. The chi-squared test was performed in order to assess the discordant results caused by different primer sets.

RESULTS

General Overview

338 specimens were collected from Peking Union Medical College Hospital. Thirteen specimens were excluded because of insufficient quantity and therefore, leaving 325 specimens valid to the cytological or histological diagnosis for further analysis. Among the patients, 97 (29.85%) cases showed normal cytology, 50 (15.38%) cases were atypical squamous cells of undetermined significance (ASCUS), 97 (29.85%) cases were low-grade squamous intraepithelial lesions (LSIL), 48 (14.77%) cases were high-grade squamous intraepithelial lesions (HSIL), 26 (8.0%) cases were SCC, and 7 (2.15%) cases were classified as others, including inflammation.

Among 325 samples tested by four primer sets, 245 (75.4%) were HPV positive, of which 87 (35.5%) showed multiple infections. The prevalence of high-risk HPV types increased with the severity of cervical lesions, from 19.6% in normal cytology to 77.3% in LSIL, 87.5% in HSIL, and 88.5% in cervical cancer.

All the HPV-positive samples were typed by

direct-sequencing, and multiple infections were further typed using TS-PCR-based sequencing. A total of 36 different HPV genotypes and 353 HPV sequences were identified, HPV 16 (24.1%, 85/353) was the most prevalent type, followed by HPV 58 (13.3%, 47/353) and HPV 52 (9.6%, 34/353). The remaining seven common types were HPV 68, 56, 33, 66, 53, 90, and 51 respectively. These ten types of HPV accounted for 75.1% of all sequence types (Figure 1).

Comparative Analysis of HPV Detection and Genotyping

All the samples were tested with four consensus primer sets, and the number of HPV positive samples detected by GP5+/6+, MGP, MY09/11, and PGMY09/11 was 217, 227, 220, and 228, respectively (Table 1). The kappa value of HPV detection varied from 0.751 (GP5+/6+ *vs*. PGMY09/11) to 0.929 (GP5+/6+ *vs*. MGP). PGMY-PCR and MGP-PCR were more efficient in HPV detection but slightly and the difference was not statistically significant (*P*=0.619). The proportions of high-risk types ranged from 56.4% for MGP to 64.5% for MY09/11 (Table 1).

Table 1. A comparison of Four Consensus Primer Sets for HPV Detection

HPV Category	Consensus Primer Sets [n (%)]			
	GP5+/6+	MGP	MY09/11	PGMY09/11
High-risk HPV[a]	136(62.7)	128(56.4)	142(64.5)	129 56.6)
Low-risk HPV[b]	21 (9.7)	15 (6.6)	21 (9.5)	13 (5.7)
Undetermined-risk HPV[c]	23(10.6)	25 (11.0)	18 (8.2)	20 (8.8)
Multiple infections	37 (17.1)	59 (26.0)	39 (17.7)	66 (28.9)
Total	217	227	220	228

Note. [a]High-risk (HR) HPVs include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. [b]Low-risk (LR) HPVs include 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108. [c]Undetermined-risk HPVs include 26, 30, 32, 53, 55, 62, 67, 69, 71, 74, 84, 87, 90, and 91.

The frequencies of HPV genotypes in single infection samples are summarized in Table 2. HPV 67, 90, and 91 were identified only by GP-PCR and MGP-PCR. However, MY-PCR and PGMY-PCR detected eight and seven samples infected by HPV 53, respectively, and only one sample that was detected by GP-PCR and MGP-PCR. Therefore, compared to GP-PCR and MGP-PCR, the MY-PCR and PGMY-PCR were more likely to detect HPV type 53 while GP-PCR and MGP-PCR were more effective for detection of HPV type 90, and possibly HPV types 67, 74, and 91.

Table 2. The Frequency of HPV Genotypes in 158 Single Infection Samples by the Chi-squared Test Assessing Difference in Positive Rates Obtained by the Four Consensus Primer Sets

HPV Genotype	Consensus Primer Sets (*n*)				*P*-value
	GP5+/6+	MGP	MY09/11	PGMY09/11	
6	1	1	1	1	1.000
11	3	3	3	3	1.000
16	46	48	55	49	0.723
18	6	6	4	6	0.904
26	1	1	1	1	1.000
31	3	3	2	3	0.964
33	9	8	9	6	0.852
35	3	3	2	2	0.939
39	4	4	1	4	0.548
42	1	1	1	1	1.000
51	1	1	–	2	0.570
52	7	10	11	11	0.759
53	1	1	8	7	0.016
54	–	–	–	1	0.391
55	–	1	–	3	0.110
56	5	5	5	5	1.000
58	13	13	16	14	0.925
61	–	1	1	1	0.800
62	1	1	1	3	0.568
66	2	2	2	2	1.000
67	1	1	–	–	0.571
68	5	7	6	7	0.928
69	1	1	1	1	1.000
74	2	2	–	1	0.529
81	6	6	5	5	0.979
84	1	1	2	3	0.662
90	10	10	–	–	<0.001
91	1	1	–	–	0.571
Total	133	140	135	139	0.619

Regarding the performance of each individual primer system, MY-PCR detected HPV types 16 and 58 easier than the other three primer sets while the primer less easier detected types 18, 39, and 51. PGMY-PCR detected more infections by HPV types 55, 62 and possibly 54, but fewer by type 33. GP-PCR detected fewer infections by HPV type 52 and possibly types 16, 61, and 68.

Of the 158 single infection samples, 109 showed complete agreement between HPV detection and genotyping by four primer sets, whereas 37 were missed by at least one primer but showing no discrepancy in HPV genotypes. Inconsistent genotype patterns existed in 12 samples (Table 3).

Table 3. Inconsistent Genotyping Results by Different Primer Sets

Number of Cases	Consensus Primers Sets				Cytology
	GP5+/6+	MGP	MY09/11	PGMY09/11	
2	90	90	16	16	SCC, Normal
1	18	18	52	18	SCC
1	39	39	58	39	ASCUS
1	74	74	52	52	ASCUS
1	33	33	33	62	ASCUS
1	90	90	53	53	LSIL
1	31	31	16	31	HSIL
1	—	—	58	55	HSIL
1	90	90	—	62	Normal
1	33	52	33	52	ASCUS
1	18	18	16	18	LSIL

HPV Genotyping in Multiple Infections

A total of 87 samples were identified having multiple infections with at least one primer test. GP5+/6+, MGP, MY09/11, and PGMY09/11 identified 37, 59, 39, and 66 multiple infections, respectively (Table 1). MGP and PGMY09/11 showed greater ability in detecting multiple infection samples ($P<0.001$).

Eighty-seven multiple infection samples were further genotyped by TS-PCR, of which 40 (46.0%) were infected by two different HPV types, 18 (20.7%) were triple infections. Quadruple or more infections were identified in six samples. However, in 20 samples only one type of HPV was detected and three samples failed to be genotyped. Thus, a total of 183 sequences were obtained. The two most commonly HPV types identified were 58 and 16, which were found in 17.0% and 16.5% of the samples, respectively.

Interestingly, the prevalence of multiple infections appears to correlate negatively with the grade of cervical lesions, decreasing significantly from 48.4% in LSIL to 13.0% in SCC ($P<0.001$).

Comparison Between HC2 and PCR-Sequencing Analysis

The HC2 test was performed in 111 specimens, of which 42 (37.8%) were HC2-negative and 69 (62.2%) were HC2-positive. Overall, 92 of the 111 samples showed concordant results when compared with at least one PCR result (percent agreement, 82.9; kappa value, 0.613), including 66 samples that were positive for both tests and 26 samples that were negative for both tests. Discrepant results were predominantly PCR-positive but HC2-negative ($n=16$; Table 4).

Table 4. A comparison of the HC2 Assay with HPV Detection and Genotyping Results

HC2 Assay	PCR Results		Sequencing Results	
	Positive[a]	Negative	Positive[b,c]	Negative
Positive	66	3	58	11
Negative	16	26	2	40

Note. [a]A positive result refers to the detection of HPV using PCR assay regardless of the genotype. [b]A positive result refers to the detection of high-risk HPV genotype by direct PCR sequencing. [c]Multiple infections were categorized as positive if at least one high-risk HPV type was identified by TS-PCR.

While comparing the results of HC2 and high-risk HPV genotyping by PCR sequencing (HR-PCR) and as shown in Table 4, the concordant samples were slightly more numerous ($n=98$; percent agreement, 88.3; kappa value, 0.761). In contrast, the majority of differences resulted from HC2-positive but HR-PCR negative samples ($n=11$).

Of the 11 samples that were HC2 positive but HR-PCR-negative, eight samples were PCR positive including six samples of single infection by non-high-risk HPV types (HPV 11, 67, 69, 81, and 90) and two samples of multiple infections failed to be genotyped by TS-PCR. The RLU/CO values for the three other HC2-positive and PCR-negative samples were 1.09, 1.14, and 2.23, respectively, and the cytological diagnosis was normal or LSIL for these

three samples.

Most of the HC2 positive samples (92.8%) demonstrated less severe cytology, including 14 normal, 24 ASCUS and 26 LSIL samples.

DISCUSSION

In this study, the performance of four consensus primer sets were compared for HPV detection and genotyping by a PCR-sequencing strategy, and the primer sets were assessed for their potential clinical utility in 325 cervical samples. In general, a large spectrum of HPV types was found, HPV 16 was the predominant type in both normal and abnormal cervical lesions, which is in consistent with reports from previous studies[27-28]. HPV 58 was the second most prevalent type, which is in accordance with a meta-analysis in China and a recent population-based study in Beijing[27-29]. The high prevalence of HPV suggests that specific polyvalent vaccines, including HPV 58, may be more suitable for the Chinese population.

When analysis was restricted to samples that had single infections, as reported in other studies, a discrepancy in HPV genotyping was found among consensus primer sets[30-33]. These differences are caused mainly by the mismatches between the primer sets and the spectrum of HPV types detected. Similar results were observed in this study, as shown by HPV 53, which was detected particularly with primers MY09/11 and PGMY09/11, and HPV 90, which was detected with GP5+/6+ and MGP. HPV 53 was found to be fairly common in China and classified as a probable high-risk type in a large meta-analysis, because only one case was found in 1739 cancers and no cases were found in 259 controls[6,34]. However, HPV 53 was defined as non-oncogenic based on its absence in cervical intraepithelial neoplasia 3 (CIN 3) and cancer cases[35]. In the present study, HPV 53 was the eighth most prevalent genotype and was detected mainly in the normal and LSIL groups and that is why it was defined as an underdetermined-risk type. The prevalence of HPV 90 has been seldom reported since it was firstly identified as a low-risk type in 2002[36], mainly because it was not included in the spectrum of type-specific probes. HPV 90 infection was not uncommon and was detected exclusively by GP5+/6+ and its modified version MGP in the present study, which was in consistent with other reports using GP5+/6+ and nucleotide sequencing–based genotyping[21,37].

Notably, MY09/11 detected more HPV 16 and 58 infections than the other three primer sets in this study, demonstrating its advantage not only by detecting positive DNA samples with lower viral loads but also by preferentially detecting these two types in discordant genotype samples (Table 3). A serial dilution of the HPV 16 plasmid test confirmed that MY09/11 could detect a lower number of plasmid copies (data not shown). These findings are in accordance with a previous study in which MY-Gold and PGMY-Gold were compared[38]. In contrast, other studies have revealed that the PGMY09/11 appeared more sensitive than the MY09/11 in type-specific amplification[13,39] and more researches must be therefore performed to reach a meaningful conclusion. As indicated by other investigators, GP5+/6+ was less likely to detect HPV 52 compared with MY09/11 or PGMY09/11[30,33]. This situation may be due to mismatches of the five base pair of GP5+ and two mismatches of GP6+. Furthermore, three out of four samples that were missed by GP5+/6+ had a nucleotide substitution at position 6764 of T→C compared to the prototype. This substitution caused an additional mismatch of GP6+[33]. Fortunately, the modified version MGP overcame this shortcoming by expanding the single pair of primers to four forward and reverse primers so as to minimize the mismatches. This modification is very useful because HPV 52 is more prevalent among women with all types of cervical lesions in China[29,40-41].

The most remarkable difference among the four primers is their abilities to detect multiple infections. PGMY09/11 and MGP detected multiple infections on almost twice as many occasions as GP5+/6+ and MY09/11. Chan also found that PGMY09/11 detected more multiple infections than MY09/11 and GP5+/6+[33]. The discrepancy in detecting multiple HPV types may result from the primer design. Both MGP and PGMY09/11 has a set of forward and reverse primers, which may minimize the mismatch and improve the type-specific sensitivity compared to the prototypes of GP5+/6+ and MY09/11. The redesigned primer sets also allow a wider range of HPV types to be amplified. Additionally, the increased detection of multiple infections could possibly be due to the use of a mixture of sequencing primers, because the strategy for genotyping was direct sequencing. However, when suspected multiple infections were genotyped with type-specific primers, the majority were detected as multiple infections, leaving only 20

(23.0%) samples as one HPV type and three samples failed to be typed. This result was not paradoxical because there may be co-infections with other HPV types that were not included in the 12 type-specific types.

In the present study, 35.5% of mixed infections were detected among HPV positive samples. This incidence is similar to that of a previous study in which 136 out of 446 HPV-positive samples had multiple infections[24]. This proportion of ico-infection is much higher than those of a comparative study in Belgium and a population based study in the West German area, where the co-infection rates were found to be only 16.3% and 14.1%, respectively. This incongruity may be related to the combined consensus primer and the direct sequencing strategy which could identify some uncommon HPV types. In addition, most of the cervical samples in the present study were diagnosed as ASCUS and LSIL, which contain more multiple infections compared with severe cervical lesions[42].

The inconsistency in the 12 samples, where the PCR typing with four different sets of primers showed different results, is due to that these 12 samples have multiple infections; each pair of the primer set would amplify preferentially the target sequence that best matched to its primer sequence.

Comparing the results of the PCR and genotyping with the HC2 assay, the overall concordances were 82.9% and 88.3%, respectively, though 8 of 11 HC2-positive samples were found to be non-high risk HPV types, demonstrating cross-hybridization of HC2 with low-risk HPV that has long been discussed[43-45]. In the present study, two HC2-positive samples cross-reacted with HPV 69 and 90, which has never been described previously. Recent studies also found that a number of HC2-positive samples with a lower RLU/CO value failed to yield any HR HPV, indicating the introduction of a grey zone for the HC2 test, where false-negatives and false-positives are common[45-47]. Nested PCR combining MY09/11 and GP5+/6+ showed a higher sensitivity compared with HC2, while it was impractical in the clinic because of its higher risk of contamination[22,48].

In conclusion, although the four primer sets could amplify a wide spectrum of HPV types, a detection preference for each individual primer system existed. MGP and PGMY09/11 seemed to be more efficient in detecting multiple infections and combining consensus primer PCR and TS-PCR allowed the genotyping of nearly all of the samples. Compared with HC2, sequencing is much more accurate and cost-effective and, based on our results, it can be used as a standard method for HPV testing in large-scale cervical cancer screening.

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