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A new method of screening human papillomavirus genotypes and clinical validation

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1. ABSTRACT

Human papillomavirus (HPV) infection is a necessary factor in the development of cervical cancer. A new HPV screening method, "Human Papillomavirus Genotyping (HPG)", was developed to detect 29 HPV genotypes distribution in China. The utility of HPG was compared to Hybrid Capture 2 High-Risk HPV DNA test (HC2), and it was determined that the HPG test had been proven to be a more credible and sensitive screening HPV method than the HC2 test. HPV16, HPV 52, HPV 56, and HPV 58 were the four most common HPV genotypes in women who have suffered chronic cervicitis or abnormal vaginal bleeding in China. HPV 16 (28.57%) and 18 (17.86%) were more likely to infect multiple HPV genotypes than other HPV genotypes. Age group more than 50 years had a higher risk than other age groups.

2. INTRODUCTION

Cervical cancer is one of the most common cancer types in the world. According to a recent survey, an estimated 100,000 new cases of cervical cancer are diagnosed annually in China, representing about one-tenth of all cervical cancer cases worldwide, and resulted in 20000 deaths in 2001 (1). When cervical cancers are detected at an early stage, the five-year survival rate is approximately 92%. HPV infection is believed to be a necessary cause in the development of cervical cancer (2). More than 90% of cervical cancer is attributed to one or more high-risk HPV genotypes persistent infection, so the HPV DNA testing is listed as a powerful method of detecting the risk of cervical carcinoma and has become a part of the cervical cancer screening method for women (3).

Now there are more than 120 HPV genotypes. Furthermore, 15 different HPV genotypes are classified as high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82), which are demonstrated to be related to cervical cancer. Some research also showed that HPV26, 53 and 66 are probable high-risk HPV genotypes, that may be related to the risk of cervical cancer (4). In addition, multiple HPV infection of different genotypes might increase the risk of cervical cancer. Women with multiple genotypes of HPV infection usually have more chance to suffer cancer than those with single HPV infection (5-6). HPV 16 and 18 were recognized as the oncogenic HPV genotype by the World Health Organization (WHO), however, the rest of high-risk HPV genotypes prevalence results in cervical cancer varies greatly according to geographical areas. In China, HPV 16, 18, 58 and 52 are the four most common high-risk HPV genotypes in cervical cancer specimens, and the prevalence are 79.6%, 7.5%, 3.8% and 2.6% respectively (7), For Italy, the genotypes are HPV 16, 18, 31 and 45 (8). HPV 16 (64.9%),18 (12.2%),33 (4.7%) and 45 (4.1%) in Slovenia (9), and HPV 16 (73%) , 18 (19%) , 31 (7%), 33 (4.1%) in France (10). In addition, the HPV genotypes prevalence also varies in different continents. For example, HPV 35 and 31 are the second and the third most frequent genotypes in Sub-Saharan Africa, while the same position are HPV 33 and 56 in Asia, HPV58 and 31 in South America and HPV 31 and 18 in Europe, respectively (11).

HPV genotype detection systems mainly include a series of polymerase chain reaction (PCR) protocols employing type-specific or general primers and Southern hybridizations (12-13). The sensitivity and specificity of PCR protocols mainly depend on primer sets, the size of product, reaction conditions, the detection methods and so on. The primer sets used for HPV detection included GP5+/6+ (14), MY09/11 (15), PGMY09/11 (15-16) and SPF systems (17-18). Cross-contaminated and reaction of specimens, reagents or different polymerases can also affect the sensitivity and specificity (19), both of them greatly limit their applicability. Southern hybridizations is restricted by time-consuming and labor-intensive processes, so it is not suitable for high throughput screening (20). Gene chip assembly PCR, chip technology and reverse dot hybridization have the advantage of high throughput, accurate and rapid HPV genotypes detection of clinical specimens infected with HPV.

In our study, a highly sensitive method (HPG) is developed for detecting the distribution of 29 HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 6, 11, 26, 40, 42, 43, 44, 53, 54, 55, 57, 66, 67, 69, 73, 82) .in cervical swab samples. In brief, 29 HPV specific probes are attached to the chip's surface. After the DNA isolation, PCR amplification, and hybridization and image acquisition steps, the image acquisition system can automatically get the value of inspection (INS). The recommended INS cutoff value is 11, with dots on the gene chip with an INS value ≥11 considered positive. An INS value <11 showed either the absence of the specific HPV genotype DNA or HPV DNA levels below the threshold of detection. The utility of HPG for screening high-risk HPV genotypes is compared to HC2 (Digene Corporation, Gaithersburg, MD) approved by FDA in cervical swab samples and direct sequencing analyses.

The purpose of this study is to validate the accuracy of the established HPV genotypes method and to evaluate the distribution of HPV genotypes in China by the newly developed method, HPG. A total of 200 anonymous cervical swab samples who have suffered chronic cervicitis or abnormal vaginal bleeding are tested by both HPG and HC2 assays, with the results compared, followed by the results of both assays being compared with DNA sequence assay to assess the utility of the HPG method.

3. MATERIALS AND METHODS

3.1. Patients and specimen collection

Cervical swab specimens (n = 200) were collected from women who have suffered chronic cervicitis or abnormal vaginal bleeding aged 22 to 75 years (40.62±11.17) for routine cervical screening between April 2009 and August 2009 at our hospital. Specimens were collected by the Cervex brush (Rovers Medical Devices, Oss, The Netherlands) and then rinsed into ThinPrep vials containing PreservCyt fixative solution (Cytyc Corporation, Boxborough, MA). In the screening tests, specimens were aliquoted and stored at -20°C until analysis. HPG and HC2 tests were performed and compared using the same cervical swab specimens. Written informed consents were obtained from all the patients.

3.2. HPG assay

3.2.1. DNA extraction and PCR

The HPG assay had been applied patent and approved by the State Intellectual Property Office of the People's Republic of China (patent number of 200820094742.5). The principle of HPG test was shown (Figure 1). HPV DNA was extracted according to the manufacturer's instructions for the isolation of DNA from cervical swab specimens. Firstly, the cell pellet was obtained by centrifugation for 5 min at 13000 rpm, followed by removal of the supernatant completely. At least 50μl DNA extracting solution for lysing the cells pellet was added into each tube. The solution was mixed by vortexing and centrifuged for 10 min at 13000 rpm, and then the supernatant was transferred into a new tube. Finally, DNA solution was eluted, and 2μl of the aliquot was used for PCR amplification. The concentration of primers, dNTP, Mg^{2+} and the additive BSA, annealing temperature and the number of PCR cycles had been optimized. The optimization reaction conditions were as follows: forward primers (0.4 μM), reverse primers (0.2 μM), dATP (200 μM), dCTP (200 μM), dGTP (200 μM), dUTP (400 μM), Mg^{2+} (3.5 mM), BSA (0.4 mg/ml), annealing temperature (52°C), the number of PCR cycles (40). The PCR was performed with a final reaction volume of 30μl, containing 2μl of the isolated DNA sample and 28μl of PCR master mix. The mixture was incubated for 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 30 s at 95°C ,45s at 52°C and 30 s at 65°C, with a final

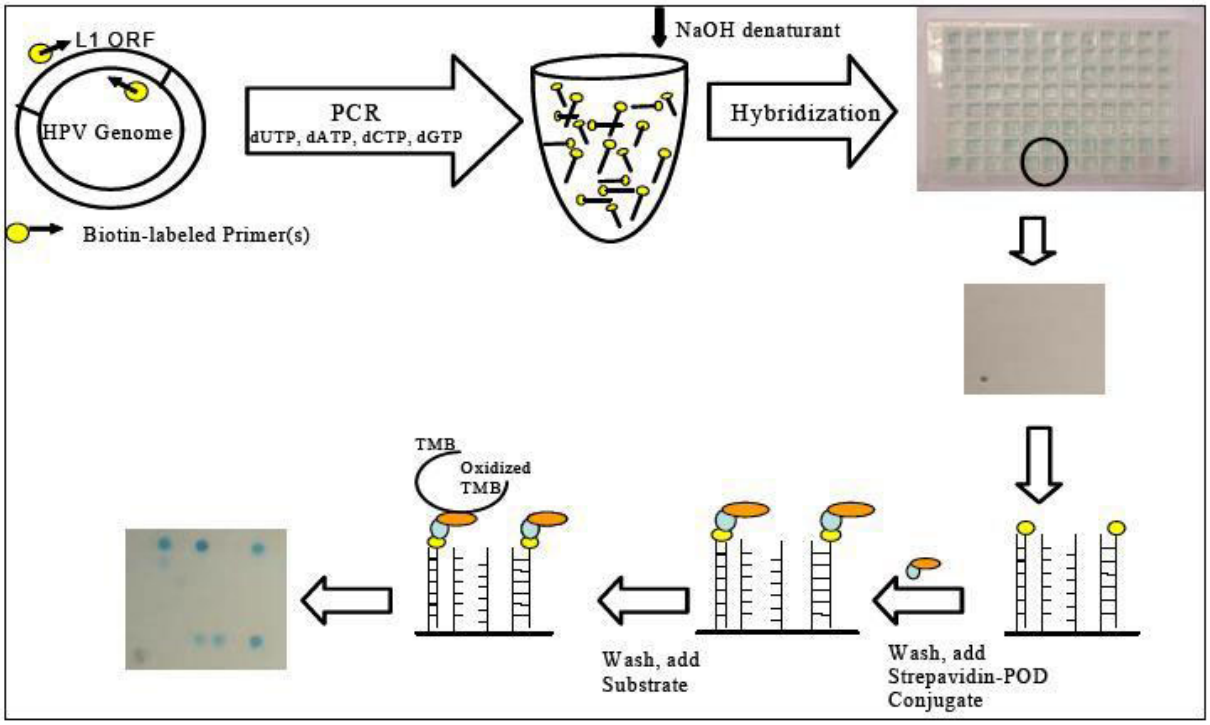

Figure 1. Principles of the Human Papillomavirus Genotyping (HPG) method

extension of 5 min at 65°C. Each experiment was performed with separate positive and negative PCR controls.

3.2.2. Hybridization and Color development

After PCR amplification, 30μl of HPG denaturation solution (0.1M NaOH) was added to each PCR tube, followed by incubation for 10 min at room temperature to allow complete denaturation. 100μl of hybridization buffer (5×SSC, 0.5 M NaH_2PO_4, 0.1% SDS, pH 6.0) was added to each well of 96-microwell plates and 60μl denatured amplified sample DNA was added to separated wells coated with high-risk HPV probes and an intern control probe. The 96-microwell plates were incubated for binding to the probes. Five different hybridization temperatures 52°C, 55°C, 58°C, 61°C and 65 °C were used for optimization at a fixed time 45 min, after the most suitable temperature of hybridization were chosen, four different times of hybridization 15min, 30min, 45min and 60 min at 55°C were compared in order to get the most suitable time of hybridization. The plates were then washed with 150μl of wash solution B (0.5×SSC, 0.1% SDS, pH 7.4) at room temperature for four different times at 5min×5, 5min×3, 3min×5 and 3min×3. Streptavidin-POD conjugate was diluted by three different dilution buffer wash solution A (2×SSC, 0.1% SDS, pH 7.4), PBST and TBST at a fixed dilution ration of 1:200. When the most suitable dilution buffer was conformed, four different dilution rations of 1:50, 1:100, 1:200 and1:300 were compared to get the most suitable dilution ratio, then 100μl Streptavidin-POD conjugate dilution was added into each well to link the biotin at room temperature for different reaction times for 15min, 30min, 45min and 60 min, after which plates were washed twice with 300μl of wash solution A and wash solution C (0.1 M citrate sodium, pH 5.0) to remove unbound conjugate. Color development was initiated by the addition of 100μl of four different compositions of color reagent. Reagent one consisted of 3% H_2O_2 and 1.8mg/ml tetramethyl benzidine (TMB), diluted by ethanol and wash solution C. Reagent two consisted by 0.12% H_2O_2 and 1.6mg/ml TMB, diluted by ethanol and wash solution C. Reagent three consisted of 3% H_2O_2 and 1.8mg/ml TMB, diluted by dimethylformamide (DMF) and wash solution C. Reagent four consisted of 0.012% H_2O_2 and 1.6mg/ml TMB, diluted by DMF and wash solution C. After incubation for 5 min at room temperature in the dark, the stop solution was added. After 45°C drying, the strips were scanned by the automatic scan system. Positive, negative and intern controls were performed in each run (The HPG test can detect 29 HPV genotypes, as mentioned previously).

3.3. HC2 assay

High-risk HPV DNA testing was performed by HC2 assay according to the manufacturer's instructions (21). Briefly, specimens were denatured at 65°C for 45 min and then hybridized at 65°C for 1 hour with a mixture of high-risk HPV probes. The DNA-RNA hybrids were captured by anti-DNA-RNA hybrid antibody which was coated on the surface of the microliter plate. The hybrids then reacted with an alkaline phosphatase-conjugated anti-hybrid monoclonal antibody. Light intensity was measured with a luminometer. Results were reported with a value of relative light units (RLU). The recommended positive cutoff value was 1 pg/ml, and all samples with RLU of ≥1.0 were considered positive.

Table 1. Primers for DNA sequencing of HPV genotypes

HPV genotype	Forward Primers (5'-3')	Reverse Primers (5'-3')
16	GAGCACAGGGCCACAATAA	TCCTCCCCATGTCGTAGGTA
18	TGGTGTTTGCTGGCATAAT	TCATATTCCTCAACATGTCTGCT
31	CATGGTTACTTCAGATGCACA	GCAATTGCAGCACAAACAGA
33	TATTTGTTGGGGCAATCAG	CAAACTGTAGATCATATTCTTCAACAT
35	TGTCTGTGTGTTCTGCTGTGT	TCCTCTAAAATGGACGGGTTC
39	CTGTTGTGGACACTACCCG	ATCATACTCCTCCACGTGCC
45	TAATTTAACATTATGTGCCTCTACAC	AAATAAACTGTAAATCATATTCCTCCA
51	TAAGCCTTATTGGCTCCAC	TTCATACTCTTCCCCATGCC
52	AGGGCCACAATAATGGCAT	CTCGCCATGACGAAGGTATT
56	CTTATTGGTTGCAACGTGC	TCCTCCACATGTCTAAGGTACTGA
58	TAGGTAGGGGACAGCCATT	GCTCACCAGTGGGAGGTTTA
59	ATATTGGCTGCACAAGGCT	AATTCCTCCACATGTCTGGC
68	AGGCACAGGGACACAACAA	GCAAATCATATTCCTCAACATGC
6	CAAAAAGCCCAGGGACATA	TGGAAGATGTAGTTACGGATGC
11	CAGGGACATAACAATGGTATTTG	CAGATTTAGACACAGATGCACATAGTG
26	ATGGAGTGGATGCAGATGC	ACAACGTGCACAGGGTCATA
40	AGGGCCATAACAATGGCAT	CCCATGACGCAAATATTCCT
42	TGGTTACAACAAGCACAAGG	GTTGCAGTGGCACACAAAGT
43	AATGGCATTTGTTTTGGGA	CTGGGCACAGTAGGGTCAGT
44	GCAGGGCCACAATAATGGT	ACGGAGGGGACTGTGTAGTG
53	ACGTGCCCAGGGACATAAT	GTGGTTGCGGAAAGAGTCAT
54	GCCATACTGGTTACAACGG	TCCTCCACATGTCTAATATACTCCC
55	TGGAGACTGAGTTGTAGCAG	AAGCCTTTTTGGTTGCAAAG
57	TTAACAAGCCTTACTGGCTG	AAAGAGACATTTGTGCTGCG
66	AATGGCATATGCTGGGGTA	CTCCACATGGCGAAGGTATT
67	GTTACAACGCGCACAAGG	GGGATATTTTGCACAGCTGAA
69	CGTGCCCAGGGTCATAATA	AGTGGCAGATGCAGATTGTG
73	TATTGGTTGCAAAAGGCAC	TTGGCATACGTTGTAGTAGAGC
82	GAATCCATGGTGTGCAGGT	GCAGTACATTAGGCATGGGG

RLU<1.0 indicated either the absence of the 13 high-risk HPV genotypes DNA or DNA levels below the threshold of detection. Positive and negative controls (provided by the manufacturer) were performed in each run (22).

3.4. HPV genotyping by direct sequencing assay

HPV positive samples detected by HPG or HC2 tests were analyzed by direct DNA sequencing assay. Different HPV genotypes were classified according to L1 gene DNA sequence (23) and primers for the L1 gene of HPV were designed to determine the HPV genotypes (Table 1). The DNA sequences of L1 gene obtained from the samples were compared to the GenBank database by using the BLAST program at the National Center website (24).

3.5. Statistical analysis

The prevalence for each HPV genotype was expressed as the proportion of specimens harboring specific HPV genotype DNA among all specimens. Agreement between HPG and HC2 tests was measured by Cohen's kappa statistic, with values of 0.00 to 0.20 indicating poor agreement, 0.21 to 0.40 indicating fair agreement, 0.41 to 0.60 indicating moderate agreement, 0.61 to 0.80 indicating substantial agreement, and 0.81 to 1.00 indicating nearly perfect agreement. The McNemar test was used to compare paired HPG and HC2 positive rate. The calculations were performed with SPSS computer program software (version 11.5 for Windows).

4. RESULTS

4.1. Optimization of Hybridization and color development

Four different compositions of color reagent were compared according to the results of color development (Figure 2). There were three positive controls and one negative control shown on the gene chip. The positive control would have blue color after color development and the negative control had no color. The color development results of reagent one and three had deep background, and it would influence the scan system to judge the results. Reagent two and four had a suitable background; however, the composition of reagent two was unstable. Comparing the four different color reagents, reagent four was chosen as the most suitable composition of color reagent.

The genotypes and INS results of optimizing the composition of Streptavidin-POD conjugate dilution buffer at a fixed dilution ratio of 1:200 were shown (Table 2). Genotypes of 8 samples were known previously and shown (Table 2). Genotypes detected by wash solution A, PBST and TBST were conformed to the genotypes of 8 samples, except for N2 detected by TBST. The INS of N2 can not be detected by the scan system, so it was classified as false-negative. The INS of Solution A was higher than PBST and TBST, and the cost of Solution A was lower than PBST and TBST. Solution A was chosen as the dilution buffer of Streptavidin-POD conjugate.

After the Solution A was chosen as the dilution buffer of Streptavidin-POD conjugate, the dilution ratio was optimized. Genotypes of 8 samples were known previously and shown (Table 3). The genotypes and INS results of the dilution ratio of 1:50, 1:100, 1:200 and 1:300 were shown (Table 3). Compared to the genotypes of 8 samples, the genotypes detected at a dilution rate of 1:50 had false-positive, for example, HPV59 of N1, HPV40 of N3, HPV51 of N4 and HPV55 of N5. The results at a dilution rate of 1:100 were conformed to the genotypes of 8 samples. The results at a dilution rate of 1:200 had false-

Table 2. The genotypes and INS results of optimizing the composition of Streptavidin-POD conjugate dilution buffer

Sample No.	**Genotype**	**Solution A**	**PBST**	**TBST**
		Genotype (INS)	Genotype (INS)	Genotype (INS)
1	52	52 (23.30)	52 (15.87)	52 (8.46)
	56	56 (21.32)	56 (10.88)	56 (7.09)
	40	40 (38.78)	40 (46.21)	40 (48.25)
	44	44 (36.95)	44 (31.22)	44 (20.35)
	66	66 (23.87)	66 (15.69)	66 (14.82)
2	53	53 (34.12)	53 (21.92)	Negative
	66	66 (25.19)	66 (19.18)	Negative
3	45	45 (39.53)	45 (19.04)	45 (11.50)
4	35	35 (32.86)	35 (9.50)	35 (27.72)
	52	52 (29.88)	52 (15.69)	52 (26.21)
5	58	58 (32.91)	58 (27.31)	58 (23.96)
6	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative
8	Negative	Negative	Negative	Negative

Table 3. The genotypes and INS results of optimizing the dilution ratio of Streptavidin-POD conjugate dilution buffer

Sample No.	**Genotype**	**1:50**	**1:100**	**1:200**	**1:300**
		Genotype (INS)	Genotype (INS)	Genotype (INS)	Genotype (INS)
1	33	33 (43.57)	33 (33.84)	33 (31.42)	33 (34.98)
	52	52 (8.33)	52 (16.02)	52 (11.63)	Negative
	-	59 (23.66)	-	-	-
2	39	Negative	39 (15.90)	39 (29.57)	Negative
	51	51 (41.98)	51 (29.46)	51 (30.86)	51 (35.32)
	56	56 (17.38)	56 (13.75)	56 (8.95)	56 (14.06)
	68	68 (31.22)	68 (30.34)	68 (19.59)	68 (34.80)
	-	-	-	-	54 (9.21)
3	18	18 (39.86)	18 (33.26)	18 (25.36)	18 (28.51)
	45	45 (37.31)	45 (25.34)	45 (27.22)	45 (22.53)
	44	44 (23.03)	44 (19.31)	44 (22.00)	44 (14.69)
	54	54 (16.72)	54 (19.90)	54 (17.03)	54 (8.95)
	-	40 (12.68)	-	-	-
4	33	33 (43.65)	33 (33.49)	33 (25.05)	33 (24.14)
	52	52 (23.82)	52 (26.41)	52 (10.11)	52 (14.03)
	-	51 (9.91)	-	-	-
5	39	39 (16.28)	39 (20.57)	39 (35.79)	39 (18.20)
	51	51 (23.08)	51 (34.26)	51 (47.37)	51 (38.79)
	56	56 (13.00)	56 (18.09)	56 (32.46)	56 (14.91)
	68	68 (22.73)	68 (33.37)	68 (52.74)	68 (26.45)
	-	55 (8.57)	-	55 (21.96)	55 (16.55)
	-	-	-	54 (12.07)	54 (8.35)
6	Negative	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Negative
8	Negative	Negative	Negative	Negative	Negative

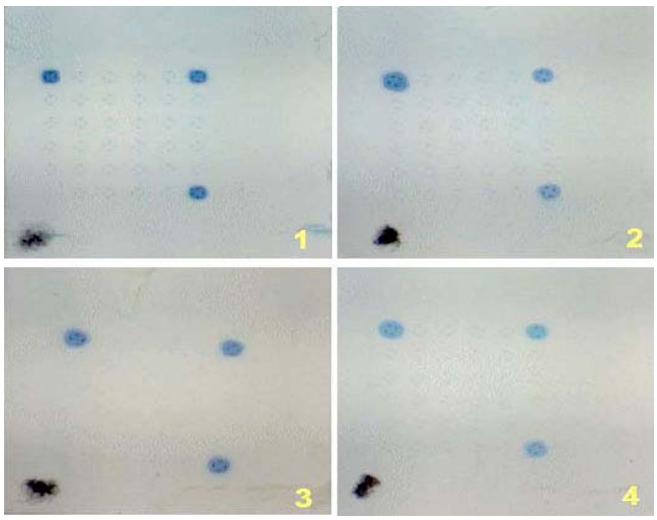

Figure 2. Color development results of optimizing the color reagents

Table 4. The genotypes and INS results of optimizing the temperature of hybridization

Sample No.	**Genotype**	**52**℃	**55**℃	**58**℃	**61**℃	**65**℃
		Genotype (INS)	Genotype (INS)	Genotype (INS)	Genotype (INS)	Genotype (INS)
1	56	56 (15.95)	56 (23.75)	56 (23.46)	56 (8.93)	56 (7.81)
	-	33 (18.25)	-	-	-	-
2	33	33 (17.18)	33 (33.20)	33 (19.56)	33 (18.19)	33 (19.36)
3	33	33 (32.22)	33 (43.48)	33 (35.35)	33 (28.42)	33 (25.44)
	52	52 (17.03)	52 (15.30)	52 (13.64)	52 (14.53)	52 (13.65)
	-	59 (8.50)	-	-	59 (7.55)	-
4	40	40 (10.94)	40 (17.71)	40 (10.95)	Negative	Negative
5	68	68 (44.20)	68 (54.25)	68 (37.51)	68 (22.24)	Negative
	-	67 (9.10)	-	-	-	-
6	Negative	Negative	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Negative	Negative
8	Negative	Negative	Negative	Negative	Negative	Negative

Table 5. The genotype and INS results of optimizing the time of hybridization

Sample No.	**Genotype**	**15min**	**30min**	**45min**	**60min**
		Genotype (INS)	Genotype (INS)	Genotype (INS)	Genotype (INS)
1	16	16 (15.06)	16 (19.20)	16 (30.77)	16 (11.68)
	52	52 (11.87)	52 (13.57)	52 (20.95)	52 (10.35)
2	18	18 (32.03)	18 (24.68)	18 (31.52)	18 (39.25)
	45	45 (24.58)	45 (25.73)	45 (24.62)	45 (35.56)
	44	44 (16.05)	44 (22.37)	44 (13.20)	44 (20.85)
	54	54 (17.96)	54 (17.14)	54 (8.14)	54 (14.63)
	-	-	-	-	40 (12.05)
3	33	33 (20.30)	33 (37.14)	33 (33.78)	33 (10.69)
	-	-	-	-	52 (7.51)
4	39	Negative	39 (24.25)	39 (18.70)	Negative
	51	51 (36.88)	51 (26.64)	51 (34.92)	51 (34.08)
	56	56 (16.00)	56 (9.39)	56 (14.12)	56 (14.10)
	68	68 (25.25)	68 (16.12)	68 (34.42)	68 (32.80)
	-	-	-	-	54 (8.94)
5	52	52 (14.76)	52 (16.14)	52 (12.48)	52 (22.13)
	-	-	-	58 (23.42)	35 (25.27)
6	Negative	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Negative
8	Negative	Negative	Negative	Negative	Negative

positive HPV55 and 54 of N5, and the results at a dilution rate of 1:300 had false-negative HPV 52 of N1, false-positive HPV 54 of N2 and false positive HPV55 and 54 of N5. After comparing the genotypes detected at different dilution ratios, the dilution ratio of 1:100 was chosen as the most suitable dilution ratio.

Five different temperatures were compared to optimize the temperature of hybridization at a fixed time 45 min. The genotypes and INS of the five temperatures 52°C, 55°C, 58°C, 61°C and 65°C were shown (Table 4). Genotypes of 8 samples were known previously and shown (Table 4). Genotypes detected at 52°C had some false-positive, for example, HPV33 of N1, HPV59 of N3 and HPV67 of N5. Genotypes detected at 55°C and 58°C were all conformed to the 8 samples, but the INS of 55°C was higher than at 58°C. Genotypes detected at 61°C had false-positive HPV59 of N3 and false-negative HPV40 of N4. Genotypes detected at 65°C had false-negative HPV40 of N4 and HPV68 of N5. Comparing the genotypes and INS detected of five different temperatures, 55°C was chosen as the most suitable temperature of hybridization.

When the optimized temperature was fixed, the different time of hybridization was compared and shown (Table 5). Genotypes of 8 samples were known previously and shown (Table 5). Genotypes detected after 15 min hybridization had false-negative HPV39 of N4. Genotypes detected after 30 min hybridization were conformed to the 8 samples, and the genotypes were also conformed to the 8 samples except HPV58 of N5 after 45 min hybridization. Genotypes detected after 60 min hybridization had false-negative HPV39 of N4 and false-positive HPV40 of N2, HPV52 of N3, HPV54 of N4 and HPV35 of N5, so the 30 min time was chosen as the most suitable time of hybridization.

After the hybridization procedure, the hybridization product reacted with the Streptavidin-POD conjugate dilution. The genotypes and INS of four different reaction times; 15min, 30min, 45min and 60 min were compared and shown (Table 6). After 15 min reaction, there were some false-negative genotypes, for example, HPV56 of N2, HPV56 of N3 and HPV 55 of N4. The genotypes detected after 30 min and 45 min reaction were all conformed to the 8 samples. In addition, after 60 min there were false-positive genotypes, HPV40 and 59 of N1, HPV54 of N2 and HPV31, 40, 51 and 66 of N4. The 30 min and 45 min were the two most suitable reaction times between hybridization product and Streptavidin-POD conjugate dilution. In order to reduce the whole time of HPG tests, 30 min time was chosen as the most suitable reaction time.

After the hybridization product reacted with the Streptavidin-POD conjugate dilution at 55°C for 30 min, the wash time and the number of times were optimized.

Table 6. The genotype and INS results of optimizing the reaction time between hybridization product and Streptavidin-POD conjugate dilution

Sample No.	Genotype	15min	30min	45min	60min
		Genotype (INS)	Genotype (INS)	Genotype (INS)	Genotype (INS)
1	33	33 (33.27)	33 (30.01)	33 (29.73)	33 (36.00)
	52	52 (15.51)	52 (13.11)	52 (16.32)	52 (19.94)
	-	-	-	-	40 (7.23)
	-	-	-	-	59 (11.06)
2	39	39 (23.40)	39 (23.05)	39 (19.86)	39 (23.77)
	51	51 (25.05)	51 (30.75)	51 (37.59)	51 (40.54)
	55	55 (10.50)	55 (20.02)	55 (14.59)	55 (11.17)
	56	Negative	56 (16.77)	56 (16.15)	56 (23.27)
	68	68 (26.74)	68 (25.75)	68 (27.65)	68 (34.96)
	-	-	-	-	54 (9.19)
3	56	Negative	56 (17.14)	56 (19.78)	56 (19.65)
4	52	52 (15.12)	52 (18.16)	52 (16.89)	52 (31.31)
	55	Negative	55 (10.65)	55 (7.00)	55 (16.70)
	-	-	-	-	31 (7.05)
	-	-	-	-	40 (12.05)
	-	-	-	-	51 (8.17)
	-	-	-	-	66 (9.07)
5	16	16 (37.76)	16 (28.49)	16 (40.77)	16 (32.87)
6	Negative	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Negative
8	Negative	Negative	Negative	Negative	Negative

Four different wash times at 5min×5, 5min×3, 3min×5 and 3min×3 were compared. The genotypes and INS were shown (Table 7). When the wash time was 5min×5, there was a false-negative HPV43 of N3. The INS of 5min×3 was lower than 3min×5 and 3min×3, and the INS of 3min×5 was similar to the INS of 3min×3. In order to reduce the whole time of HPG tests, 3min×3 was chosen as the most suitable wash time and the number of times.

4.2. The distribution of HPV genotypes

The distribution of HPV genotypes measured by HPG method was shown (Figure 3). Among the 29 HPV genotypes, 18 different single HPV genotype infections were detected. HPV 16 was the most common genotype (13.04%), followed by HPV52 (10.87%), HPV56 (6.52%) and HPV58 (5.43%). The other HPV genotypes (HPV 66, 51, 45, 39, 35, 31, 18, 11, 43, 44, 53, 54, 55, 6) were lower than 5%. In addition, the multiple infections were higher than any single HPV genotype and account for 30.43% in the HPV infection in our observed cases. The distribution of HPV genotypes in the positive sample for multiple HPV genotypes was shown (Figure 4). HPV 16 (28.57%) and 18 (17.86%) were more likely to infect multiple HPV genotypes than other HPV genotypes, followed by HPV56, HPV 52, HPV33, HPV11, HPV51, HPV43, HPV39 and HPV31, respectively.

HPV prevalence was estimated in four broad age groups (20-30 years, 31-40 years, 41-50 years and more than 50 years) (Figure 5). HPV prevalence ranged from 20-30 years (25%) to peak prevalence of more than 50 years (53.57%). Among the age-specific HPV prevalence in China, there was the lowest HPV prevalence in the 20-30 years group, with an upward trend as age group increased. The HPV prevalence of 31-40 years (36.67%) was a little higher than the 41-50 years group (34.62), showing age-specific HPV prevalence was essentially constant between the 30 to 50 years group. The age group of more than 50 years showed the highest HPV prevalence (53.57%) in our studied population.

4.3. Comparison between HC2 test and HPG test

The results of HPG were compared to HC2 tests available for 200 patients (Table 8). The high-risk HPV positive rates by HC2 and HPG tests were 72/200 (36%) and 70/200 (35%). Positive agreement rate, negative agreement rate and overall agreement rate between HPG and HC2 tests were 91.67%, 96.88% and 95.00% respectively. The concordant results between HPG and HC2 tests were obtained for 190 (95%) of the 200 samples (kappa statistic, 0.89±0.03), showing nearly perfect agreement. However, 10 (5%) of the 200 samples had discordant results between HPG and HC2 tests (Table 9). The 10 discordant samples were analyzed by the DNA sequencing assay, with the results of DNA sequencing assay recorded (Figure 6). The HC2 test detected positive, but negative by HPG in 6 samples and HPG detected positive, but negative by HC2 in 4 samples. Of the 6 samples (HC2+, HPG-), 3 samples (N181, N195, N197) were low-risk HPV genotypes (HPV6, 53, 66) demonstrated by DNA sequence assay. The remaining 3 samples (N149, N168, N187) were high-risk HPV genotypes (HPV18/66, 16/67, 52/82). Of the 4 samples (HC2-, HPG+), 3 samples (N84, N89, N100) were high-risk genotypes (HPV58, 33/39, 56/67), the HPV genotype of the remaining sample (N70) detected by HPG was HPV 56, but can not be detected by DNA sequence assay, so we justified it as a false-positive high-risk HPV genotype.

The results of HPG and HC2 tests for the detection of high-risk HPV were compared to the DNA sequencing assay test (Table 10). DNA sequencing assay

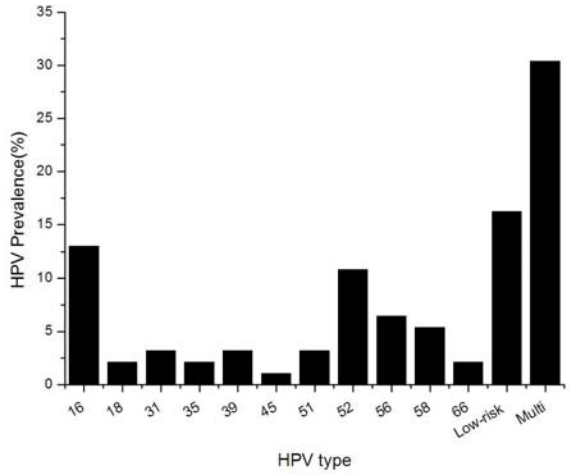

Figure 3. Distribution of HPV genotypes in the population of China.

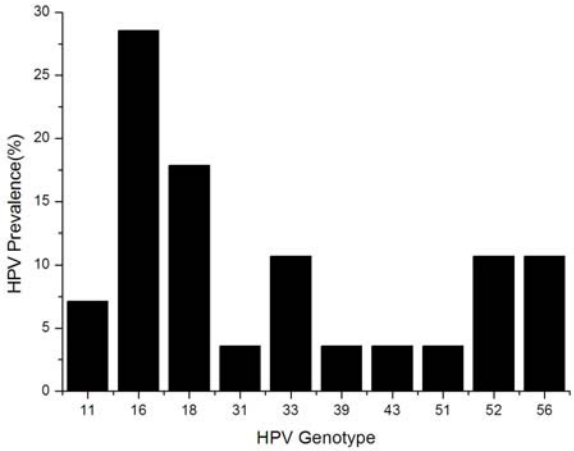

Figure 4. Distribution of HPV genotypes in the population positive for multiple HPV infection in China.

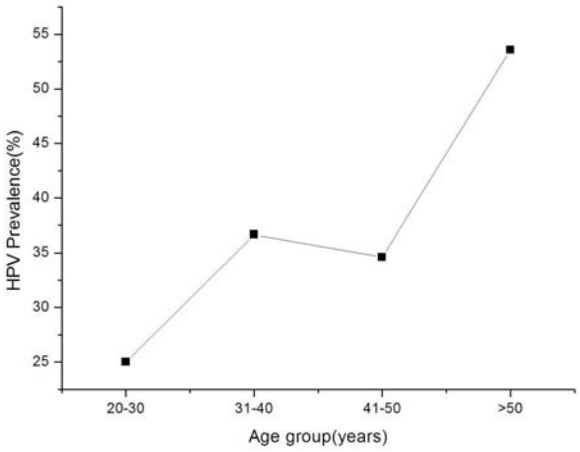

Figure 5. Distribution of age specific HPV genotypes in China.

was justified as the final test result. HPG test had 3 missing detection samples and 1 false-positive sample. The sensitivity and specificity of the HPG were 95.83% and 99.22%, respectively. The concordant results between HPG and DNA sequencing assay were obtained for 196 (98%) of the 200 samples (kappa statistic, 0.96±0.02), showing nearly perfect agreement; HC2 test had 3 missing detection samples and 3 false-positive samples. The sensitivity and specificity of the HC2 were 95.83% and 97.66%, respectively. The concordant results between HC2 and DNA sequence assay were obtained for 194 (97%) of the 200 samples (kappa statistic, 0.94±0.03), showing nearly perfect agreement. The sensitivity of HPG test was higher than HC2 tests, and the specificity of HPG equaled to the HC2 tests.

The HPG genotypes were compared to DNA sequencing assay results. To multiple HPV infection genotypes, at least 2 HPV genotypes which were consistent with the result of DNA sequence assay were thought to be accurate. We had detected 90 specimens for DNA sequence assay, 85 (94.44%) of the 90 samples were accurate genotype, including 62 simple infection specimens and 23 multiple infection specimens. The remaining 5 specimens were inaccurate genotypes, including 2 simple infection samples and 3 mixed infection samples. There were results of 5 discordant samples between HPG genotypes and DNA sequencing assay (Table 11).

5. DISCUSSION

In our study, a newly developed HPV screening method, HPG, was used to detect the distribution of HPV genotypes in 200 Chinese females, whom suffer chronic cervicitis or abnormal vaginal bleeding. HPG method was based on using sets of probes followed by revert blotting in a gene chip It was a highly sensitive method that discriminated 29 HPV genotypes in a single chip, not 29 separate reactions. HPG was used to detect the distribution of HPV genotypes in women in China. The utility of HPG was compared to HC2 tests and DNA sequencing assay. DNA sequence assay had been used as the final detection of HPV not only because of an accurately determined HPV genotype, but also to unveil a novel HPV genotypes (24).

Our studies showed that the four most common HPV genotypes in China were HPV 16, HPV 52, HPV 56 and HPV 58, representing 35% of all HPV infections. The most common HPV genotype was HPV 16. However, the following HPV genotypes varied greatly according to different regions. HPV 58 and 52 were the priority HPV genotypes in Chinese women (1, 25), and they were relatively uncommon in Africa, America and Europe (26). HPV 52, HPV56 and HPV 58 were demonstrated as high-risk HPV genotypes, our study indicated that HPV 52, HPV56 and HPV 58 had priority HPV prevalence in China. It is valuable to pay more attention to the research on its relationship with the development of cervical cancer. HPV 16 and HPV 18 were more likely to infect multiple HPV genotypes than other HPV genotypes, indicating that HPV genotype screening should be done if the patients were infected by HPV 16 or HPV 18. Studies had showed that women infected with multiple HPV genotypes had a higher risk than single HPV genotype and multiple HPV types seemed to act synergistically in cervical cancer (5).

Table 7. The genotype and INS results of optimizing the wash time and the number of times

Sample No.	Genotype	5min×5	5min×3	3min×5	3min×3
		Genotype (INS)	Genotype (INS)	Genotype (INS)	Genotype (INS)
1	6	6 (15.64)	6 (20.44)	6 (21.91)	6 (22.71)
2	53	53 (14.13)	53 (27.72)	53 (36.18)	53 (38.22)
	54	54 (19.51)	54 (23.34)	54 (24.54)	54 (30.13)
	55	55 (24.04)	55 (25.01)	55 (27.90)	55 (29.72)
	57	57 (28.79)	57 (21.32)	57 (30.47)	57 (29.22)
3	59	59 (36.32)	59 (52.15)	59 (34.86)	59 (42.66)
	43	Negative	43 (10.24)	43 (24.35)	43 (31.79)
	44	44 (12.30)	44 (17.93)	44 (40.40)	44 (44.21)
	67	67 (12.81)	67 (15.53)	67 (39.96)	67 (45.33)
4	33	33 (18.07)	33 (19.77)	33 (22.56)	33 (26.19)
5	16	16 (17.28)	16 (20.06)	16 (19.08)	16 (37.32)
6	Negative	Negative	Negative	Negative	Negative
7	Negative	Negative	Negative	Negative	Negative
8	Negative	Negative	Negative	Negative	Negative

Table 8. Results of HPG tests and HC2 tests for the detection of 13 high-risk HPV genotypes in 200 samples

	HC2 tests results		
HPG tests results	Positive	Negative	Total #.samples
Positive	66	4	70
Negative	6	124	130
Total	72	128	200

Table 9. Final high-risk HPV test (combined HC2 tests and DNA sequencing assay) in 10 discordant samples by comparing HC2 tests and HPG tests

Sample No.	HC2 tests results	HPG tests results	HPV genotype	DNA sequencing genotype	DNA sequencing results
N149	Positive	Negative	HPV66	HPV18, HPV66	Positive
N168	Positive	Negative	HPV67	HPV16, HPV67	Positive
N181	Positive	Negative	HPV6	HPV6	Negative
N187	Positive	Negative	HPV82	HPV52, HPV82	Positive
N195	Positive	Negative	HPV53	HPV53	Negative
N197	Positive	Negative	HPV66	HPV66	Negative
N070	Negative	Positive	HPV56	HPV56 (Negative)	Negative
N084	Negative	Positive	HPV58	HPV58	Positive
N089	Negative	Positive	HPV33, HPV39	HPV33, HPV39	Positive
N100	Negative	Positive	HPV56, HPV67	HPV56, HPV67	Positive

Table 10. Results of HPG tests and HC2 tests comparing to DNA sequencing assay for the detection of high-risk HPV

HPG tests results	DNA sequencing assay Positive	DNA sequencing assay Positive	Total #. of samples
HPG Positive	69	1	70
HPG Negative	3	127	130
HC2 Positive	69	3	72
HC2 Negative	3	125	128
Total	144	226	400

Table 11. Inaccurate genotype specimens between HPG tests and DNA sequencing assay

Sample No.	HPG test	DNA sequence assay
N100	HPV56, HPV67	HPV56
N149	HPV66	HPV18, HPV66
N168	HPV67	HPV16, HPV67
N179	HPV16,HPV58	HPV16
N187	HPV82	HPV52, HPV82

However, little research had been done on the relationship between different HPV genotypes. Age specific HPV genotypes prevalence showed that HPV genotype infection was the most common in women more than 50 years which might be one of the important reasons for increased cancer risk in aged females, and a second peak of HPV prevalence was seen in women aged 31 to 40 years, followed by age groups 41 to 50 years and 21 to 30 years. The highest prevalence of HPV was in the age group of 25 to 29 years in Kenya (27), but the same condition has not been found in China. There might be several reasons for the difference between Kenya and China. First, sexual behaviour might be the principle reason. Second, The wide variation has been found across geographical regions in the 35 to 50 years group (28).

In our study, HC2 test and HPG test showed nearly perfect agreement, with a kappa of 0.89±0.03, however, there were 10 discordant samples. In the 10 discordant samples, 6 samples were HC2 test positive, but negative by HPG; and 4 samples were HC2 test negative, but positive by HPG. HC2 was missing 3 samples, and HPG was also missing in 3 samples. In addition, HC2 had 1 false-positive sample, and HPG had 3 false-positive samples. Although these HPV types were identified by

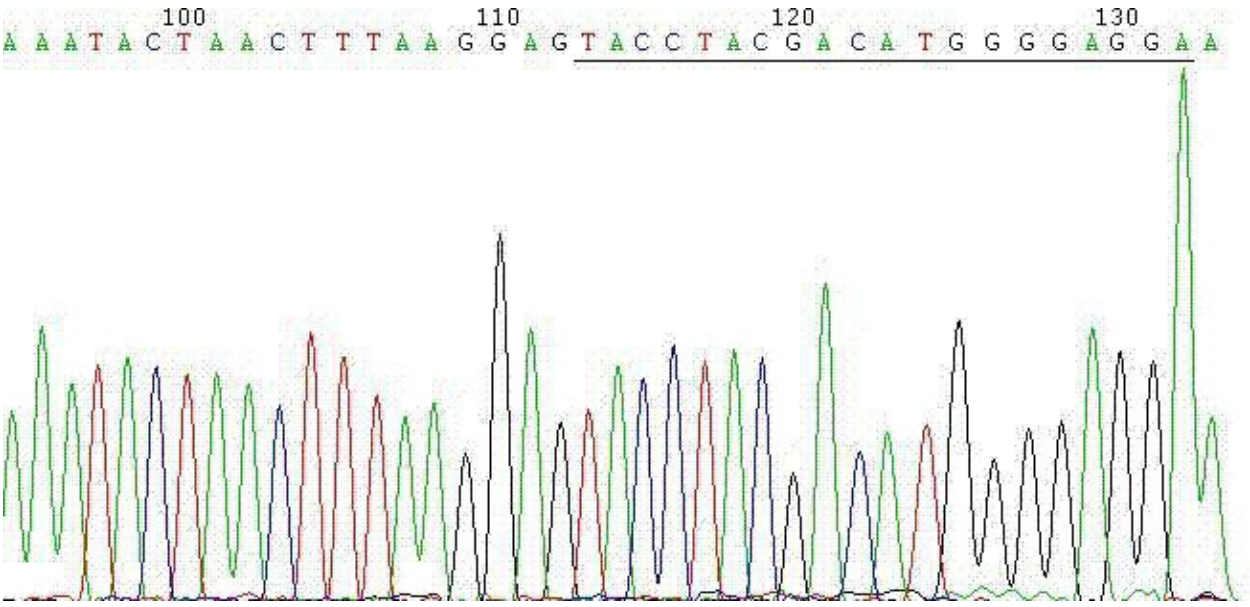

Figure 6. A typical signature sequence of HPV 16 by direct DNA sequencing, including 20 bases of the HPV 16 reverse priming site (underlined).

DNA sequence assay and can be explained by the cross-hybridization, these HPV genotypes had to be studied carefully. Different HPV genotypes with greater molar amounts were preferentially amplified in a multiple infections sample by comparing PGMY and SPF10 assay in 400 cervical swab specimens (29). Systematic studies demonstrated that HC2 test can occasionally detect untargeted HPV types 53, 66, 67, and 71 (7).After the DNA sequence assay, the sensitivity and specificity of the HPG were 95.83% and 99.22%, with a kappa of 0.96±0.02, indicating nearly perfect agreement. The sensitivity of HPV detection methods such as HC2 was based on the threshold value of the viral load/viral concentration, but the criteria to define the reference threshold value for the presence of HPV infection may be a problem in lots of studies (30-31). The 3 samples (N84, N89, N100) which HC2 missed may be because the HPV genotype 58, 33, 39 concentration was below the threshold value. After lots of studies, the mechanism of HPV infections was not understood fully; the concentration of HPV was probably low during the first phase of infection but increased with the development. Therefore, lots of studies should be done in the threshold value of HPV (31).

Most current HPV genotype screening protocols were developed in recent years. Developed fluorescence in situ hybridization (FISH) high-risk HPV assay to Hybrid Capture 2 (HC2) showed that FISH was concordant with HC2 and PCR in 120 (85%) (32). Real-time polymerase chain reaction for quantitative analysis of 14 types of HPV was used as a useful tool to screen high-risk HPV (33). Micro-array assay for detection of HPV had also been proved to be a sensitive, reproducible, robust molecular assay for HPV genotyping (34). Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS)-based assay can also be used for HPV genotyping, and can accurately detect and identify at least 74 different HPV genotypes (35). MALDI-TOF MS platform had a high-throughput method for detecting 14 HPV genotypes in 532 cervical cell samples. Compared to other detection methods, MALDI-TOF MS platform had a capacity of 10×384 samples within 2 working days and a lower cost (36). Different magnetic beads can also be used to detect HPV genotype (37).

HPG test not only had basically the same test result to HC2 assay in detecting 13 high-risk HPV genotypes, but also provided the information of 29 HPV genotypes, which account for about 90% of cervical cancer. It was important to know the genotype for clinical therapy, such as, the presence of HPV 16 and 18 would be helpful to triage the patients who are needed to get frequent follow-up. In addition, persistent infection with high-risk HPV genotypes may be a critical condition for cervical cancer. Large epidemiological studies had found that HPV26, 53, 66, 73, and 82 types to be present in cervical swab specimens, and they had been classified as probably HR (26, 53, 66) or HR (73, 82) types of the potential of HPV genotypes. In addition, HPV genotype is a necessary piece of information for patients deciding whether to vaccination against HPV (38-40). HPV vaccines also could generate antibodies against HPV 52 and HPV 58 (41).

In conclusion, HPG test had been proved to be a more credible and sensitive screening HPV method compared to HC2 test. After HPG tests, we found that HPV16, HPV 52, HPV 56 and HPV 58 were the four most common HPV genotypes in China. HPV 16 (28.57%) and 18 (17.86%) were more likely to infect multiple HPV genotypes than other HPV genotypes. Age groups more than 50 years had a higher risk than other age groups. Our study provided crucial information of HPV genotypes distribution in women in China to the HPV vaccination program and policy decisions.

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Abbreviations: HPV: Human papillomavirus; HPG: Human Papillomavirus Genotyping; HC2: Hybrid Capture 2 High-Risk HPV DNA test; INS: inspection; RLU: relative light units

Key Words: Human papillomavirus, Human Papillomavirus Genotyping, Hybrid Capture 2 High-Risk HPV DNA test, genotype

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