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Human Papillomavirus Genotyping After Denaturation of Specimens for Hybrid Capture 2 Testing: Feasibility Study for the HPV Persistence and Progression Cohort†

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Abstract

Human papillomavirus (HPV) genotyping could be clinically useful, depending on the results of large, prospective studies like the HPV Persistence and Progression cohort. The cohort is based on genotyping and follow-up of Hybrid Capture-positive women at Kaiser Permanente, Northern California. HPV DNA testing by Hybrid Capture 2 requires denaturation with alkali, possibly damaging the DNA for optimal PCR-based genotyping. A feasibility study was conducted on paired aliquots of anonymized specimens from 100 women with low-grade intraepithelial lesion cytology. Test aliquots were left in denaturant for 10 or 18 hours at 4°C and then neutralized; comparison aliquots were not denatured but diluted to match the timing, temperature, concentration and salt conditions of the treated specimens. The masked aliquots were tested using a commercialized PCR-based assay that detects of 37 HPV genotypes. There was no overall effect of treatment on test

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positivity or number of types. HPV16 was marginally more likely to be detected in untreated versus treated aliquots ($P = 0.09$) but HPV45 was marginally more likely to be detected in treated than untreated aliquots ($P = 0.07$), suggesting that these differences represented chance (intra-test variability). It can be concluded that residual Hybrid Capture-positive specimens can be accurately genotyped by PCR after Hybrid Capture 2 processing.

Keywords

Cervical Cancer; Human Papillomavirus (HPV); HPV genotypes; Cervical Precancer; Cervical Intraepithelial Neoplasia Grade 3 [CIN3]; screening; PCR

1. Introduction

It is now recognized that virtually all cervical cancer and its immediate precursors are the consequence of persistent infections by 15 or so cancer-associated (carcinogenic) human papillomavirus (HPV) genotypes. Carcinogenic HPV infections are extremely common and most are transient, lasting only 1–2 years at most. A number of large population cohorts of HPV and cervical neoplasia have been conducted (Bratti et al. 2004; Franco et al. 1999; Kjaer et al. 2002), providing much information on causality and HPV natural history. These studies have provided strong empirical support for 4-stage model of cervical carcinogenesis that replaces the older model of stepwise progression through increasing severe grades of cervical intraepithelial neoplasia: 1) HPV acquisition; 2) HPV persistence [vs. clearance]; 3) neoplastic progression to cervical precancer; and 4) invasion (Wright, Jr. and Schiffman 2003).

However, despite the size and breadth of these studies, there has been limited power to examine the later stages of cervical carcinogenesis because progression and invasion are relatively uncommon outcomes to a common exposure (HPV infection). For example, the cohort study in Guanacaste (Bratti et al. 2004), a true population sample of 10,000 women, yielded ~100 prevalently-detected cases of cervical precancer and cancer and another ~100 cases diagnosed in almost a decade of follow-up. Thus, new, more statistically-powerful studies are needed to focus on critical unanswered questions in cervical carcinogenesis: the viral, host, and exogenous determinants of carcinogenic HPV persistence and progression. In particular, carcinogenic HPV genotypes display wide variation in phenotypic behaviors, including substantial differences in their absolute and attributable risks for cervical precancer and cancer. The inter-typic differences in risk are possibly great enough to be clinically useful in patient management (Khan et al. 2005; Castle et al. 2005b).

To achieve powerful studies to address the aforementioned questions, it is rational to focus on populations of carcinogenic HPV-positive women rather than a sample of the entire population, in which most women will test carcinogenic HPV negative at any one time point. To accomplish this goal, screening programs in which women 30 and older are being co-screened by cytology and carcinogenic HPV testing, per U.S. screening guidelines, could be used to identify carcinogenic HPV-positive women. As of 2003, Kaiser Permanente of Northern California has instituted a co-screening program based on conventional Papanicolaou smears and using Hybrid Capture 2 (Digene Corporation, Gaithersburg, MD, USA) on a second cervical specimen. Hybrid Capture 2 is a DNA test that collectively targets 13 carcinogenic HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) (Schiffman et al. 2000). Hybrid Capture 2 also cross-reacts strongly with a 14th carcinogenic genotype (HPV66) (Castle et al. 2002; Schiffman et al. 2005b). While Hybrid Capture 2 does not distinguish the infecting genotype(s), it is possible to create efficiently a cohort study of carcinogenic HPV-positive women by banking their residual Hybrid Capture 2-positive specimens and follow their subsequent clinical course.

However, to test ~500,000 specimens per year efficiently, the Kaiser lab processes the entire specimen with alkali to denature DNA, leaving no untreated aliquot. Exposure to alkaline conditions could slowly cause DNA damage by backbone cleavage and thereby reducing the viability of the specimens for PCR-based HPV genotyping assays. To consider banking these specimens to examine viral factors such as HPV genotype-specific risk for cervical precancer and cancer, it was important to test whether HPV DNA in residual Hybrid Capture 2-tested specimen can be sufficiently preserved if the tested specimen is neutralized prior to storage. Hence, a feasibility study was conducted to examine the detection of HPV genotypes on treated aliquots of specimens, using a second, untreated aliquot as the control, as the basis for starting the HPV Persistence and Progression Cohort.

2. Materials and Methods

2.1. Specimens

One-hundred cervical specimens collected into specimen transport medium (STM; Digene) per routine clinical practice at KPNC from women under age 30 were selected. Specimens were selected from women who were under the age of 30 years and whose cytology was called low-grade squamous intraepithelial lesion (low-grade squamous intraepithelial lesion). Thus, these specimens were not tested by Hybrid Capture 2 and therefore not already processed, but were highly likely to be HPV positive. Specimens were de-linked from patient information and therefore anonymous, and their use was approved by the Kaiser Permanente Northern California institution review board and deemed exempt from review by the National Cancer Institute institution review board. Specimens were stored at -20°C until used.

Two equal aliquots of approximately 400 μL were produced from each specimen. One aliquot was processed by denaturation using an alkali solution (200 μL of 8% sodium hydroxide) using the same ratio of specimen volume and denaturant as the Hybrid Capture 2 testing protocol. Per the Hybrid Capture 2 protocol, the aliquot and denaturant were mixed and then incubated for 45 minute at 65°C . Half the treated aliquots ($n = 50$) were allowed to incubate for 10 hours at 4°C and the other half ($n = 50$) were allowed to incubate for 18 hours (overnight) at 4°C in the denaturing conditions to simulate the times specimens remain alkalized before being neutralized (200 μL of 0.2M 2-Morpholinoethanesulfonic acid and 1M Acetic Acid in a 9:1 ratio) before being banked. The other aliquot of the pair ("untreated aliquot") was unprocessed, left for same length of time at 4°C , and then diluted by the same volume as the treated aliquots using a premix of the alkali solution and the neutralizing buffer in the same proportion that was used to treat specimens. Thus, the untreated aliquots were similarly diluted and had the same salt and buffer composition as the treated aliquots.

2.2. HPV testing

Banked aliquots were tested, masked which of the aliquots were paired and which of the pair was denatured, using the commercially-available Linear Array HPV Genotyping Test (LA), according to the manufacturer's instructions in the product insert. Briefly, DNA was extracted from clinical specimen aliquots using the QIAamp MinElute Media Kit (QIAGEN, Inc., Valencia, CA), and target DNA amplified by PCR. LA utilizes the PGMY09/11 L1 consensus primer system and includes co-amplification of a human cellular target, β -globin (Gravitt et al. 2000), as an internal control. Detection and HPV genotyping are achieved using a reverse line-blot method (Gravitt et al. 1998; Peyton et al. 2001), which includes probes to identify 37 anogenital carcinogenic and non-carcinogenic HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51–56, 58, 59, 61, 62, 64, 66–73, 81, 82 subtype [IS39], 82 subtype [W13b], 83, 84, and 89). The only deviation from the LA product insert protocol was to implement an automated sample preparation for extraction of up to 96 specimens at a time on the QIAGEN

MDx platform (using the MinElute Media MDx Kit and manufacturer's instructions) rather than processing 24 specimens per batch with the manual vacuum method (Castle et al. 2006).

2.3. Statistical Analysis

An exact McNemar's χ^2 was used to test for statistical differences ($P < 0.05$) between treated and untreated aliquots in the prevalence of each individual HPV genotype, all HPV genotypes combined, and all carcinogenic HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). Comparisons were made considering all specimens and also stratified on the duration of alkalization (10 hours vs. 18 hours).

A different kind of analysis at the infection level was conducted by combining the results for all women as follows. Each infection was considered as an independent event, and each woman could "contribute" up to 37 HPV type-specific infections and 14 carcinogenic HPV type-specific infections. Therefore, the total numbers of possible HPV infections and carcinogenic HPV infections for 100 women were 3,700 and 1,400, respectively (Castle et al. 2005a). Detection of all HPV genotypes combined and all carcinogenic HPV genotypes combined were compared for treated and untreated aliquots. Kappa values, percent total agreement, and percent positive agreement with 95% confidence intervals (95%CI) were calculated.

3. Results

The individual test results for the treated and untreated aliquots for 100 women with low-grade squamous intraepithelial lesion cytology are shown in Table 1. Half the specimens were incubated for 10 hours and the other half for 18 hours.

Table 2 shows the prevalence for any HPV genotypes, any carcinogenic HPV genotypes, and the 37 individual HPV genotypes detected by Linear Array. All or nearly all of the aliquots, whether treated or untreated, 10 hours or 18 hours of incubation, were positive for at least one HPV genotype, demonstrating as previously reported that low-grade squamous intraepithelial lesion cytology is a specific cytopathic marker of HPV infection (Zuna et al. 2006). Approximately 90% were positive for at least one carcinogenic HPV type, which is higher than previously reported using a less sensitive PCR assay (Schiffman et al. 2005b). There was virtually no difference between treated and untreated aliquots, with regard to testing positive for any HPV genotype type or any carcinogenic HPV genotype

For individual types, there were no statistical differences between any types, although HPV16 was marginally more likely to be detected in untreated than treated aliquots ($P = 0.09$) and conversely HPV45 was marginally more likely to be detected in treated than untreated aliquots ($P = 0.07$). The marginal difference in detection of HPV16 was primarily due to the marginal difference in detection of HPV16 in the untreated aliquot compared to the treated aliquot with 10 hours incubation ($P = 0.07$). The marginal difference in detection of HPV45 was due to the significant difference in detection in the treated aliquot compared to the untreated aliquot with 18 hours incubation ($P = 0.03$).

Finally, in Table 3, testing results at the level of infection for all HPV genotypes (Table 3, A) and all carcinogenic HPV types (Table 3, B) were combined. There was a good agreement for both. For detection of all HPV genotypes, the Kappa was 0.67 (95%CI = 0.62–0.72), the percent total agreement was 95.5% (95%CI = 94.8%–96.1%), and percent positive agreement was 53.3% (95%CI = 48.0%–58.6%). There was no difference in detection of all HPV genotypes between treated and untreated aliquots ($P = 1.0$). For detection of all carcinogenic HPV genotypes, the Kappa was 0.63 (95%CI = 0.56–0.69), the percent total agreement was 91.6% (95%CI = 90.0%–93.0%), and percent positive agreement was 51.0% (95%CI = 44.5%–

57.5%). There was no difference in detection of all carcinogenic HPV genotypes between treated and untreated aliquots ($P = 1.0$).

4. Discussion

The goal of this study was to examine whether HPV genotyping was feasible on a cervical specimen stored in STM after it had been processed for Hybrid Capture 2 testing (denatured by alkalization). Using conditions that accurately mimic standard processing, neutralization of the processed STM specimen within 18 hours preserved the HPV DNA sufficiently well to permit HPV genotyping, with no apparent ill effects within the ability of this analysis to detect it in this analysis*. These observations are consistent with a previous observation showing that long-term storage of STM specimens with denaturing reagent at -20°C for 18 months did not reduce the viability of specimens for HPV genotyping (Rabelo-Santos et al. 2005). Repeat testing of the specimens a second time confirmed these observations (data not shown).

There were very few differences in the detection of individual HPV genotypes. HPV16 was marginally more likely detected in untreated versus treated aliquots but the converse was true for HPV45. Given the number of HPV genotypes, and the threshold for significance ($P < 0.05$ or $<1/20$), it is not unexpected that a couple of genotypes might vary by treatment status due to random chance (intra-test variability) alone. Nor was there any evidence that the incubation time of 18 hours had a significantly greater impact than 10 hours.

It can be concluded that these processed specimens can be used to create a carcinogenic HPV-positive cohort to examine with greater power viral, host, and exogenous factors associated with HPV persistence and progression. It can also be suggested that clinical HPV genotyping using PCR-based methods could be conducted on these residual specimens to identify the carcinogenic HPV-positive at greatest risk of cervical precancer and cancer, such as those women who HPV16-positive (Khan et al. 2005) or those with persistent carcinogenic HPV infections (Schiffman et al. 2005a; Kulasingam et al. 2002), when a validated HPV genotyping test is available (Stoler 2006; Stoler et al. 2007). Although Hybrid Capture 2-positive specimens could easily be neutralized and stored until used for genotyping relatively soon after Hybrid Capture 2 was complete, future studies should examine whether specimens could be stored longer periods of time and under what conditions (4°C or frozen) to make the process more practical. Finally, the variability in HPV genotype detection in these split low-grade squamous intraepithelial lesion specimens is notable, raising concerns regarding the clinical reliability of current HPV genotyping assays to detect persistent carcinogenic HPV predicting cervical precancer development. Future studies will need to address the ability of HPV genotyping assays to identify women with persistent carcinogenic HPV infection accurately and reliably.

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* A *post-hoc* power analysis at the level of infection for all possible HPV genotypes found that a sample sizes of 3,700 in the treatment group and 3,700 in the reference (untreated) group achieve 90% power ($\alpha = 0.05$) to detect equivalence. The margin of equivalence, given in terms of the difference in prevalence for all types (i.e., mean prevalence), extends from $\pm 2\%$ when there is no actual difference

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Table 1

Individual results of Linear Array testing for 37 HPV genotypes on paired aliquots, one treated by alkalization/neutralization and the other untreated but diluted equivalently to the treated aliquot. Bold italicized type indicates discordant types detected.

Specimen #	Treated	Untreated	Specimen #	Treated	Untreated
#1	51	6, 16, 18, 40, 51, 52, 66, 68	#51	33, 40, 51, 52, 56, 62	40, 51, 56, 62
#2	6, 51, 66, 84	6, 51, 66, 84	#52	51, 53, 82v	51, 53, 82v
#3	16, 51, 82v	16, 51, 67, 82v	#53	39, 51, 52, 62, 66	6, 39, 66
#4	18	18, 68	#54	6, 68, 84	68, 84
#5	35, 52*, 53	16, 35, 39, 45, 51, 52*, 53	#55	31	31, 39
#6	51, 82	6, 18, 51, 82	#56	16	16
#7	39, 68	16, 18, 39, 42, 51, 52, 66, 68, 82v	#57	55, 84	55
#8	16, 51, 66	16, 51, 66	#58	16, 39, 53	16, 39, 53
#9	6	6	#59	39	16, 59
#10	-	16, 35, 39, 52*	#60	6, 16, 18, 39, 45, 51, 52, 59, 61, 66, 68, 70, 82	-
#11	51, 53, 84	42, 51, 53, 84	#61	39, 55, 61, 66	39, 52, 55, 61, 66
#12	66	66	#62	39	6, 39
#13	16	16	#63	66	66
#14	39	39, 42, 52*, 58, 82v	#64	6, 53	6, 53
#15	18, 51, 82v	16, 18, 39, 51, 52, 55, 66, 68, 82v, 84	#65	31, 53	31
#16	39	39	#66	18, 39	-
#17	-	52, 56, 82v	#67	35, 52*, 56, 59, 67	35, 52*, 56, 59, 67
#18	53	53	#68	-	-
#19	16	16	#69	33, 39, 51, 67, 82v, 84	33, 39, 51, 67, 82v, 84
#20	16	16, 40	#70	35, 52*, 62	35, 52*
#21	45, 53, 62	45, 53, 62	#71	39, 82v	39, 82v
#22	31	31, 62	#72	31	31, 39, 51, 52, 59, 66
#23	53, 66	16, 18, 53, 55, 66	#73	39	39
#24	35, 52*	35, 52*, 73	#74	52, 70	70
#25	6, 51, 84	6, 51, 84	#75	59, 66, 84	59, 66, 81, 84
#26	45	45	#76	16, 39, 52, 59, 61, 62, 66	16, 62
#27	66	66	#77	35, 45, 51, 52*, 66, 82	35, 52*

Specimen #	Treated	Untreated	Specimen #	Treated	Untreated
#28	33, 52 *, 53, 82v	33, 53, 82v	#78	39, 52 *, 58, 59 , 61	52*, 58
#29	66	66	#79	6	6
#30	16, 45	16	#80	6, 16, 45 , 52 *, 58, 59, 62, 81, 82, 82v , 84	6, 16, 52 *, 58, 59, 62, 81, 82, 84
#31	6, 16, 82v	6, 16, 66 , 68	#81	40	39, 40, 66 , 82
#32	16 , 18, 51 , 52 , 66 , 68 , 84	18, 84	#82	59, 66	51 , 59, 62 , 66
#33	39, 56, 67	39, 56, 67	#83	31, 53, 70	31, 53, 70
#34	11 , 16, 52 , 56	6 , 16, 18	#84	-	66
#35	53	53	#85	52 *, 58, 73	52*, 58, 73
#36	56	56	#86	51, 53, 59 , 62, 66, 83	6 , 16 , 18 , 35 , 39 , 51 , 52 *, 53, 62, 66, 83
#37	56	56	#87	56	56
#38	6 , 18, 31 , 53, 55 , 68, 81	16 , 18, 53, 68	#88	39	59
#39	16, 59	16	#89	82	66 , 82
#40	16	16	#90	42, 45, 51, 66, 82	42, 45, 51, 66, 82
#41	52	52	#91	16, 18, 51 , 53, 66 , 82v	16, 18, 39 , 53
#42	51, 66	51, 66	#92	39 , 53	53
#43	31, 35 , 52 *, 66, 83	31, 66	#93	16 , 39 , 45 , 56, 59 , 81, 82 , 84	56, 81, 84
#44	16, 18 , 40, 42, 51, 52, 82v , 84	16, 40, 42, 51, 52, 82v, 84	#94	52	52
#45	35, 52 *	35, 52 *	#95	31, 39 , 59 , 66	31
#46	51	51	#96	6, 16, 51	6, 16, 51
#47	16, 68	16, 68	#97	11, 45 , 61	11, 31 , 61
#48	51, 53	51	#98	51 , 59 , 61	59
#49	82	82	#99	6 , 39 , 42 , 45 , 51, 59	51
#50	52	52, 70	#100	51 , 52 *, 56, 58, 66	16 , 52, 53 , 56, 58, 66

* indicates that HPV52 could be not be confirmed or rule out. Treated aliquots were alkalized for 10 hours (Specimens #1-#50, black type/white background) or 18 hours (Specimens #51-#100, white type/grey background).

Table 2

A comparison of individual HPV genotypes detected in paired aliquots, treated (T) and untreated (U), of specimens from 100 women with LSIL cytology. The results were then stratified on the time of alkalization (treatment), 10 hours (n = 50) or 18 hours (n = 50). Bold type indicates statistical significant greater ($P < 0.05$, McNemar's χ^2 test) detection in one aliquot versus the other.

	All N = 100	10h Alkalization		18h Alkalization	
		N = 50		N = 50	
		T	U	T	U
Any HPV	96%	97%	100%	96%	94%
Any Carcinogenic HPV	86%	87%	92%	84%	82%
HPV6	12%	14%	14%	14%	14%
HPV11	2%	1%	0%	2%	2%
HPV16	21%	28%	38%	16%	18%
HPV18	8%	11%	18%	6%	4%
HPV26	0%	0%	0%	0%	0%
HPV31	8%	8%	4%	10%	12%
HPV33	3%	2%	2%	4%	2%
HPV35	7%	8%	8%	6%	8%
HPV39	21%	19%	14%	34%	24%
HPV40	3%	5%	6%	4%	4%
HPV42	3%	5%	8%	4%	2%
HPV45	10%	4%	6%	14%	2%
HPV51	26%	23%	28%	26%	18%
HPV52 [†]	23%	23%	24%	26%	22%
HPV53	17%	16%	16%	16%	16%
HPV54	0%	0%	0%	0%	0%
HPV55	3%	4%	4%	4%	4%
HPV56	9%	9%	8%	10%	10%
HPV58	4%	5%	2%	8%	8%
HPV59	13%	8%	0%	24%	16%
HPV61	6%	2%	0%	12%	4%
HPV62	7%	7%	4%	12%	10%
HPV64	0%	0%	0%	0%	0%

	All N = 100		10h Alkalinization N = 50		18h Alkalinization N = 50	
	T	U	T	U	T	U
HPV66	22%	24%	18%	24%	26%	24%
HPV67	3%	4%	2%	4%	4%	4%
HPV68	6%	8%	8%	14%	4%	2%
HPV69	0%	0%	0%	0%	0%	0%
HPV70	3%	3%	0%	2%	6%	4%
HPV71	0%	0%	0%	0%	0%	0%
HPV72	0%	0%	0%	0%	0%	0%
HPV73	1%	2%	0%	2%	2%	2%
HPV81	3%	3%	2%	0%	4%	6%
HPV82	8%	6%	4%	4%	12%	8%
HPV82v	10%	10%	10%	14%	10%	6%
HPV83	2%	1%	2%	0%	2%	2%
HPV84	11%	11%	10%	12%	12%	10%
HPV89	0%	0%	0%	0%	0%	0%

[†] Considered HPV52 positive for those samples in which it could not be confirmed or ruled out

Table 3

Tabulation of all individual HPV types (A) and all carcinogenic HPV types (B), considering each type as an independent infection and that any woman could potentially carry up to 37 HPV types including 14 carcinogenic HPV types. Thus, in 100 women, there were potentially 3,700 type-specific HPV infections and 1,400 type-specific carcinogenic HPV infections.

A. All HPV Types				
		Untreated		
		Neg	Pos	Total
Treated	Neg	3,342	83	3,425
	Pos	84	191	275
	Total	3,426	274	3,700

B. All Carcinogenic Types

Untreated				
		Neg	Pos	Total
Treated	Neg	1,161	58	1,219
	Pos	59	122	181
	Total	1,220	180	1,400

P = 1.0, exact McNemar's χ^2 ; Kappa = 0.67 (95% CI = 0.62–0.72); % Agreement = 95.5% (95% CI = 94.8%–96.1%); % Positive Agreement = 53.3% (95% CI = 48.0%–58.6%)

P = 1.0, exact McNemar's χ^2 ; Kappa = 0.63 (95% CI = 0.56–0.69); % Agreement = 91.6% (95% CI = 90.0%–93.0%); % Positive Agreement = 51.0% (95% CI = 44.5%–57.5%)