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Impact of Six-Month Frozen Storage of Cervical Specimens in Alkaline Buffer Conditions on Human Papillomavirus Genotyping†

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Abstract

The impact of 6-month storage of cervical specimens under alkaline conditions that occurs as the result of Hybrid Capture 2 testing on human papillomavirus (HPV) genotyping is not well documented. To examine this issue, 143 frozen hc2-positive specimens in specimen transport medium were selected at random from each of the following groups: specimens stored for 6 months, 4 months, and 2.5 months under alkaline pH (pH 12–13) and specimens stored 1 month at neutral pH (pH 6–7) as controls (one control specimen was missing results). Specimens were tested in a masked fashion for 19 HPV genotypes (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73, and 82) using a prototype, research-use-only GP5+/6+ L1 consensus PCR method and multiplex hybridization using Luminex® xMAP® for detection of specific HPV genotypes. There were no statistical differences in the number of HPV genotypes detected, number of carcinogenic HPV genotypes detected, or in the signal strength among HPV-positive results across groups. Six-month frozen storage of cervical specimens at alkaline pH had little impact on testing for HPV genotypes among hc2-positive women using this HPV genotyping method.

Keywords

Human Papillomavirus (HPV); HPV genotypes; PCR

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The impact of short-term alkalinization of cervical specimens in STM (specimen transport medium; Qiagen, Gaithersburg, MD, USA) on the detection of human papillomavirus (HPV) genotypes by a commercial, research-use-only PCR assay was evaluated recently (LaMere et al. 2007). Specimens from women with low-grade squamous intraepithelial lesion (LSIL) cytology, highly likely to be HPV positive, were used in split specimen design, with one aliquoted treated by alkalinization and the other left untreated. There was no evidence that alkalinization of cervical specimens for up to 18 hours at 4°C negatively impacted PCR detection of HPV genotypes.

During the freezing and banking of these cervical specimens for a cohort study of HPV-positive women, failed neutralization of some cervical specimens led to frozen storage of specimens at an alkaline, denaturing pH of 12–13 rather than a neutral pH of 6–7. This provided an opportunity to examine the longer-term impact of alkaline conditions on stability of the HPV DNA and PCR-based HPV genotyping.

Hybrid Capture 2 (hc2; Qiagen)-positive cervical specimens in STM that had been stored frozen within 18 hours under different conditions and time (143 specimens per group[1]) were selected at random: (A) 6 months frozen storage at pH 12–13; (B) 4 months frozen storage at pH 12–13; (C) 2.5 months frozen storage at pH 12–13; and (D) 1 month frozen storage at neutral pH 6–7. The latter group served as control group. One control specimen was missing, resulting in only 142 control specimens. Specimens were de-linked from patient information and tested anonymously; 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 tested for 20 HPV genotypes (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) using a prototype, research-use-only GP5+/6+ L1 consensus PCR method, which amplifies a 150 base-pair target, and multiplex hybridization using Luminex® xMAP® for detection of specific HPV genotypes (Qiagen). DNA was purified from 100 μL of cervical sample collected in STM™ using QIAGEN DNeasy® Tissue Kit. PCR was performed using biotinylated GP5+/6+ primers (van den Brule et al. 2002) plus an additional 5′ primer specific for HPV genotype 68, Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), and 7.5×10^5 copies of an internal control. Purified DNA was amplified with 40 cycles of PCR cycling (denaturation, annealing, and elongation) as previously described (van den Brule et al. 2002). Denatured amplicons were neutralized and hybridized at 48°C for 30 minutes to HPV genotype-specific oligonucleotide probes that were coupled to Luminex®xMAP® microspheres. A Streptavidin-Phycoerythrin conjugate (Moss, Pasadena, MD, USA) was added to the hybridization mixture, and this mixture was incubated for 5 minutes at room temperature with shaking. Reactions were diluted by addition of 150 μL of PBS and read on the Luminex 200 IS instrument. Median Fluorescent Intensity (mfi) readings above 77 were considered positive. Specimens were tested masked to selected group of specimens.

The data were analyzed in three ways. First, the distribution of the number of HPV genotypes and the prevalence of the individual HPV genotypes was compared for each group of selected specimens. Second, each infection was considered as an independent event; each woman could

[1]One-hundred forty-three hc2-positive specimens per time period were selected, estimating that 30% would contain multiple HPV types, predominately with two genotypes. Therefore, we *a priori* estimated 186 HPV genotypes out of a possible 2,860 possible genotypes (143 * 20 HPV genotypes = 2,860 possible infections) would be detected in the positive control group, equaling a mean prevalence of HPV genotypes of 6.50%. Sample sizes of 2,860 in group one and 2,860 in group two achieve 83% (α= 0.05) power to detect a non-inferiority margin difference between the group's mean prevalence of −1.9%. The treatment group proportion is assumed to be 4.6% (29% reduction in detection of HPV genotypes) under the null hypothesis of inferiority. The power was computed for the case when the actual treatment group's mean prevalence is 6.50%. The test statistic used is the one-sided Z test (unpooled).

potentially have 20 HPV genotype-specific infections and 14 carcinogenic HPV genotype-specific infections (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) (Castle et al. 2005). Treating each infection as an independent event, the mean prevalence of individual HPV genotypes was calculated for each group of specimens by summing all or all carcinogenic HPV genotypes detected and dividing by the number of possible HPV genotype-specific types (20) or possible carcinogenic HPV genotypes (14), respectively. For both analyses, contingency tables were used, with Pearson χ^2 and a test for trend (Cuzick 1985) to test for significant differences between groups of specimens. Finally, the signal strengths for the detected HPV genotypes for each group of specimens were examined as a qualitative marker of specimen viability, using non-parametric analysis of variance (Kruskal-Wallis) statistics to test for differences in the median between groups of specimens.

Table 1 shows distribution of HPV genotypes detected by group of specimens. There was no difference by group ($p = 0.3$, Pearson χ^2; $p_{trend} = 0.2$). Even the extreme comparison was not significant: for specimens stored for 6 months at pH 12–13, 6% had no HPV genotype detected, 62% had one HPV genotype detected, and 32% had two or more HPV genotypes detected. For the group of controls specimens stored for 1 month at pH 6–7, 8% had no HPV genotype detected, 63% had one HPV genotype detected, and 29% had two or more HPV genotypes detected.

For individual HPV genotypes, there was some minor variation in the prevalence of HPV genotypes among the hc2-positives between groups of specimens but it was generally not related to the duration of exposure to alkalinity (Table 2). HPV16 prevalence varied between groups of specimens ($p = 0.03$) but there was no significant trend by duration of exposure ($p_{trend} = 0.3$). HPV18 was more common in those specimens with greater duration of exposure ($p_{trend} = 0.02$), and HPV51 was marginally more common in those with less duration of exposure ($p_{trend} = 0.05$). There were no significant differences between exposure groups and prevalence of the other HPV genotypes.

There was also no difference in the mean prevalence for detection of any HPV genotype ($p = 0.5$, Pearson χ^2; $p_{trend} = 0.3$) or for detection of any carcinogenic HPV genotype ($p = 0.7$, Pearson χ^2; $p_{trend} = 0.6$) between groups of specimens (Table 3). There were no detectable differences between groups in the median signal strength (mfi) for all HPV genotypes detected for that group ($p = 0.1$, Kruskal-Wallis) (data not shown).

Thus, there was little effect of 6-month frozen storage of cervical specimens in STM under alkaline conditions on detection of HPV genotypes by PCR. These observations are consistent with a previous observation with a smaller sample size showing that long-term storage of denatured cervical specimens in STM at −20°C for 18 months did not reduce the viability of specimens for HPV genotyping (Rabelo-Santos et al. 2005).

One limitation of this study was that specimens were selected from different patients for the comparison of different treatment times, rather than applying the different treatment times to split aliquots from the same patients. A split aliquot design would have provided better control for nuisance variables such as different amounts of total DNA, viral DNA, and inhibitors of different nature, all of which influence PCR efficiency. In addition, it would have controlled for the distribution of HPV genotypes, which varied between groups of specimens by chance as is apparent in Table 2. The design used in this study undoubtedly added variability into this analysis, obscuring small differences between the different duration of exposures to alkalinity. It is also of note that the PCR method for HPV genotyping used in this study amplifies a relatively small 150 base pair target. PCR methods that amplify larger DNA regions might have been more sensitive to minor degradation of the target DNA.

In conclusion, HPV viral DNA is resilient for up to 6-months of frozen storage in the typical alkaline conditions employed for denaturing DNA for hc2 testing. Hybrid Capture 2-tested specimens stored under these conditions can be HPV genotyped using this PCR-based genotyping method.

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Table 1

A comparison of the distribution of number of HPV genotypes detected in hybrid capture 2 (hc2)-positive specimens stored frozen at pH 12–13 for 6 months, 4 months, and 2.5 months (n = 143 for each time point). The results were compared to hc2-positives specimens stored at pH 6–7 for one month (n = 142) (“Control”).

		Storage Time at pH 12–13							
		6 Months		4 Months		2.5 Months		Control	
		n	%	n	%	n	%	n	%
Number of HPV Genotypes Detected	**0**	8	6%	3	2%	12	8%	11	8%
	1	88	62%	103	72%	94	66%	90	63%
	2	27	19%	25	17%	26	18%	27	19%
	3	15	10%	9	6%	6	4%	11	8%
	4	3	2%	2	1%	4	3%	2	1%
	5	0	0%	1	1%	0	0%	0	0%
	6	0	0%	0	0%	1	1%	0	0%
	7	2	1%	0	0%	0	0%	1	1%

$p = 0.3$, Pearson χ^2; $p_{trend} = 0.2$

Table 2

A comparison of the HPV genotypes detected in hybrid capture 2 (hc2)-positive specimens stored frozen at pH 12–13 for 6 months, 4 months, and 2.5 months (n = 143 for each time point). The results were compared to hc2-positives specimens stored at pH 6–7 for one month (n = 142) ("Control").

	Storage Time at pH 12–13					
	6 Months	**4 Months**	**2.5 Months**	**Control**	**p**	**p_{trend}**
HPV6	1.4%	4.9%	1.4%	0.7%	0.1	0.3
HPV11	0.7%	0.0%	0.0%	1.4%	0.2	0.4
HPV16	22.4%	23.1%	11.2%	21.1%	*0.03*	0.3
HPV18	14.0%	8.4%	8.4%	5.6%	0.1	*0.02*
HPV26	0.0%	0.0%	0.0%	0.0%	n/a	n/a
HPV31	7.7%	9.8%	10.5%	12.0%	0.7	0.2
HPV33	2.8%	1.4%	4.9%	3.5%	0.4	0.4
HPV35	4.2%	5.6%	7.0%	2.8%	0.4	0.7
HPV39	11.2%	7.7%	6.3%	9.2%	0.5	0.5
HPV45	6.3%	7.0%	6.3%	8.5%	0.9	0.5
HPV51	4.9%	5.6%	9.8%	9.9%	0.2	0.05
HPV52	12.6%	11.2%	16.8%	11.3%	0.5	0.9
HPV53	7.0%	2.1%	7.0%	4.9%	0.2	0.9
HPV56	14.0%	11.2%	9.1%	11.3%	0.8	0.5
HPV58	7.0%	6.3%	7.0%	9.2%	0.8	0.5
HPV59	5.6%	5.6%	4.9%	3.5%	0.8	0.4
HPV66	7.7%	9.8%	6.3%	4.9%	0.5	0.2
HPV68	13.3%	11.9%	10.5%	12.7%	0.9	0.8
HPV73	3.5%	2.8%	2.1%	0.7%	0.5	0.1
HPV82	2.8%	0.7%	0.7%	2.1%	0.4	0.7

Table 3

A comparison of the mean prevalence of all HPV genotypes detected and all carcinogenic HPV genotypes detected from all time periods versus the control. Mean prevalence is calculated by summing all infections detected within group and dividing by the total number of possible infections detected, assuming independence of infections. For example, 213 HPV genotype-specific infections were detected in the specimens stored for 6 months. The HPV genotyping assay detects 19 HPV genotypes for a total of 2,717 possible HPV genotype-specific infections, yielding a mean prevalence of 213/2,717 or 7.84%.

Storage Time	N (specimens)	Total Number of Infections	Mean Prevalence	Total Number of Carcinogenic Infections	Mean Prevalence
6 Months (pH = 12–13)	**143**	213	7.84%	180	8.99%
4 Months (pH = 12–13)	**143**	193	7.10%	164	8.19%
2.5 Months (pH = 12–13)	**143**	186	6.85%	161	8.04%
Control (1 Month) (pH = 6–7)	**142**	192	7.07%	171	8.60%

$p = 0.5$, Pearson χ^2; $p_{trend} = 0.3$ (all HPV genotypes)

$p = 0.7$, Pearson χ^2; $p_{trend} = 0.6$ (carcinogenic HPV genotypes)