HPV testing in first-void urine provides sensitivity for CIN2+ detection comparable with a smear taken by a clinician or a brush-based self-sample: cross-sectional data from a triage population

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ABSTRACT

Objectives: To compare sensitivity of high-risk HPV (hrHPV) and genotype detection in self-collected morning (U1) and later (U2) urine samples, brush-based self-samples (SS) and physician-taken smears (PTS) for detecting CIN2+ in a colposcopic referral population.

Design: Cross-sectional single centre study.

Setting: A colposcopy clinic in Spain.

Population: 113 women referred for colposcopy after an abnormal Pap-smear.

Methods: Women undergoing colposcopy with biopsy for abnormal Pap-smears were sent a device (Colli-Pee™, Novosanis, Wijnegem, Belgium) to collect U1 on the morning of colposcopy. U2, PTS and SS (Evalyn brush™, Rovers Medical Devices B.V., Oss, The Netherlands). All samples were tested for HPV DNA using the analytically sensitive SPF10-DEIA-LiPA25 version1 assay and the clinically validated GP5+/6+-EIA-LMNX.

Main outcome measures: Histologically confirmed CIN2+ and hrHPV positivity for 14 high-risk HPV types.

Results: Samples from 91 patients were analysed. All CIN3 (N=6) were hrHPV positive in PTS, SS, U1 and U2 with both HPV assays. Sensitivity for CIN2+ with the SPF10 system was 100%, 100%, 95% and 100% respectively. With the GP5+/6+ assay, sensitivity was 95% in all sample types. The sensitivities and specificities for both tests on each of the sample types were not significantly different. There was 10-14% discordance on hrHPV genotype.

Conclusions: CIN2+ detection using HPV testing in first-void urine shows sensitivity similar to that of physician-taken smears or brush-based self-samples and is convenient. There was substantial to almost excellent agreement between all samples on genotype with both hrHPV assays. There was no advantage in testing morning first-void urine over later samples.

INTRODUCTION

Cervical cancer is the fourth leading cause of cancer death in women worldwide. Its incidence and mortality has decreased in countries with organized cytology screening. [1,2] However, the sensitivity of a single smear for high-grade cervical intraepithelial neoplasia (CIN 2+) varies and requires a visit to a physician, leading to frequent repeats of Pap-smears over a life-time. [3] Testing for high-risk human papillomavirus (hrHPV) in physician taken smears (PTS) is more sensitive than cytology for CIN2+ and CIN3+ and results from randomized controlled trials have led to foreseeable implementation of the hrHPV test in several screening programmes. [4-7] Yet, its requirement for visits to a primary care centre may still be a barrier for participation.

Participation is the main impediment of a programme's effectiveness, since even in countries with well-organized screening programmes, half of all potentially detectable carcinomas are found in women who have not attended screening programmes. [2] Self-sampling has been proposed to increase participation as non-responders are more likely to hand in a self-collected sample of cervico-vaginal cells (SS) than to respond to recall for a Pap smear. [8] Testing SS for hrHPV was found to be well accepted among these women and comparable in performance to a PTS [9, 10, 11].

A novel, alternative method of self-sampling is collecting urine. The main advantage of this method over SS is that the procedure is non-invasive. A recent study showed that first-void urine contained higher concentrations of human and HPV DNA than midstream urine. This fraction contains most cervico-vaginal cells, because it is the first to pass the external genitalia, taking mucus adherent to the surface. [12] Whether the first fraction of urine of the first void of the day, after the mucus has remained unmoved during the night (U1), contains a higher concentration of DNA than that of later during the day (U2) remains to be determined.

The objective of this study was to determine the sensitivity and specificity of hrHPV-testing in first-void urine for the detection of CIN2+ in women with an abnormal Pap-smear. In addition, we compared HPV detection results of morning first-void urine (U1) with either urine voided later that day (U2), physician taken sample (PTS) or cervico-vaginal self-sampled specimen (SS). Finally, two different HPV assays were used and compared.

METHODS

EVAH study

Women included in the study were part of a prospective cohort of women included in a multicenter study of triage and colposcopic management of women with abnormal smears conducted between August 2010 and September 2015 (EVAH study [13]). The EVAH study aims to evaluate colposcopic visual appearance of cervical lesions in relation to underlying histology, HPV genotype(s) and molecular parameters, and to study cervical HPV at the tissue level. Women included were aged 18 years and older and had been referred for colposcopy to the Hospital Clínic, Barcelona, Spain because of abnormal cervical cytology.

For the present study, a total of 113 women between 18-60 years of age were recruited from the EVAH study. Specimens were collected between September 2014 and March 2015 in Hospital Clínic, Barcelona, Spain. The medical ethical board of the Hospital Clínic approved this study. All women gave signed informed consent.

Sample collection

For urine sample collection a device that allows the collection of first-void urine (Colli-Pee™, Novosanis, Wijnegem, Belgium) was sent to participating women. This device was delivered to home, accompanied by an information letter and instructions on use. Collection tubes contained 4mL of a buffered lithium dodecyl sulfate solution containing RNA preservative. Women were asked to collect a urine sample of the very first void on the morning of their visit (U1), and to take the sample to the outpatient clinic. The day before their visit women received a telephone call to clarify ambiguities and answer questions.

At the outpatient clinic, women were asked to hand in another first-void urine sample (U2) using the same device and a brush-based self-sample of cervico-vaginal cells with the Evalyn brush™ (Rovers Medical Devices B.V., Oss, The Netherlands).

Women then underwent a pelvic examination with a PTS using a Cervex-Brush (Rovers Medical Devices B.V., Oss, The Netherlands). Women also underwent colposcopy-directed biopsy after application of acetic acid 5% to elicit the acetowhite epithelial response. Colposcopy findings were described following the criteria of the IFCPC. [14] Up to four colposcopy directed biopsies (CDB) were collected from different lesions or different regions presenting different colposcopy patterns within one lesion. Distinct areas within a large complex lesion were biopsied separately. When the transformation zone (TZ) was not visible, an endocervical curettage (ECC) was collected. If fewer than four directed

biopsies were taken, a biopsy was taken from normal appearing epithelium of the SCJ (non-directed biopsy).

Questionnaires

To investigate the acceptability of the Colli-Pee and the Evalyn Brush, all women were asked to fill out a short questionnaire (Appendix S1) using a 5-point ordinal scale on their general experience, the instructions, and the convenience of both. Participants were also asked for their preferred sampling method.

Urine processing

Samples of 16 mL urine were stored in 4 mL of an in-house RNA preserving medium containing a buffered lithium dodecyl sulfate solution at -80 degrees Celsius within 48 hours of collection for up to three months before shipping to the Netherlands on dry ice. Molecular testing was performed at DDL Diagnostic Laboratory, Rijswijk, The Netherlands. For DNA isolation from the urine samples, 1000 µL was used to obtain 50 µL of eluate with the MagNa Pure 96 instrument.

SS processing

Brush-based self-samples were stored dry at room temperature for up to 3 months. For shipment to DDL Diagnostic Laboratory, brushes were placed in a vial containing 1 ml of Thinprep. Vials were vortexed twice for 15 seconds, stored at room temperature for 30 minutes and then vortexed again, twice for 15 seconds. Vials were shipped to DDL Diagnostic Laboratory, where 250 µl was used for DNA isolation using NucliSENS easyMAG to obtain 100 µl DNA.

PTS processing

Cervical samples were transferred to PreservCyt solution (Hologic Corp, Marlborough, MA, USA) for ThinPrep liquid-based cytology and hrHPV testing.

Liquid-based cytology's were aliquoted (2mL) and stored at room temperature until shipment to DDL Diagnostic Laboratory for molecular testing. Thin-layer cytology slides were prepared using the Thinprep T2000 slide processor (Hologic), stained using the Papanicolaou method, evaluated by a cytotechnologist and confirmed by a pathologist using the revised Bethesda nomenclature. [15] At DDL Diagnostic Laboratory, 250 µl of the aliquot was used for DNA isolation using the NucliSENS easyMAG obtaining 100 µl of DNA.

Histological processing

All biopsy specimens were collected in separate vials and fixed in 10% formalin before processing and embedding in paraffin wax. Hematoxylin-eosin (H&E) sections were examined by a local pathologist and classified as normal, CIN1, CIN2, CIN3, or invasive carcinoma. In this study, the overall histological diagnosis per case was based on the worst diagnosis found in any specimen from each woman. All biopsies were independently reviewed by a second central gynaecological pathologist. In case of disagreement between the original and review diagnosis, a third central pathologist reviewed the discordant cases independently. Diagnosis was determined by the agreement of two of three interpretations. In the case of three different diagnoses, the two central pathologists came to a consensus after joint review of the discordant case.

HPV DNA testing

SPF10 PCR-DEIA-LiPA25 version 1

HPV SPF10-LiPA25 version 1 (Labo Bio-medical Products, Rijswijk, the Netherlands, based on licensed Innogenetics technology) uses a short-PCR-fragment assay to perform broad-spectrum HPV DNA amplification. With this assay a 65-bp fragment of the L1 open reading frame of HPV genotypes is amplified, allowing us to detect at least 69 anogenital HPV genotypes using 9 conservative probes in a microtiter hybridization assay (DNAenzyme immunoassay: DEIA). [16] Line probe assay (LiPA25) was then used to analyse the samples found positive for HPV by DEIA by reverse hybridization with type-specific probes for 25 hrHPV and low-risk types: HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. [17]

GP5+/6+-LMNX

The GP5+/6+ primer-mediated PCR assay (Labo Bio-medical Products, Rijswijk, Netherlands) detects DNA from 14 hrHPV genotypes: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. From each DNA isolate, 10 µL was used for DNA amplification with the biotin-labelled GP5+/6+ primer set. [18] Genotyping of the amplimers was done using the LMNX kit HPV GP HR test using xMAP technology for high-throughput screening (Labo Bio-medical Products, Rijswijk, Netherlands). [19, 20]

RNase P

The adequacy of amplifiable human DNA in the urine samples was assessed using a qPCR of the reference human gene RNase P. [21] The PCR mix contained a plasmid spiked at a fixed concentration that functioned as an internal control to detect PCR-inhibition. All samples were positive for RNase P and no PCR inhibition was observed.

Comparison of sampling methods and HPV tests

All samples were tested with the same algorithms. In order to evaluate the concordance of the HPV testing results of all four (PTS, SS, U1, U2) samples, positivity for 14 hrHPV genotypes detected with the SPF10 algorithm were compared with the 14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) detected with the GP5+/6+ algorithm and considered important for the detection of CIN2+ lesions. For comparison of hrHPV positivity, results were concordant, when both samples were either hrHPV positive, or hrHPV negative, or discordant, when one of the sample types was hrHPV positive and the other was not. For comparison of detected genotypes, results were then classified as identical, compatible (at least one type found in both samples) or discordant.

Statistical analysis

Results were analysed using IBM SPSS version 22.0 for Windows (Chicago, IL). The level of statistical significance was set at $p \leq 0.05$ for all tests. The level of agreement was determined using Cohen's kappa statistics. For comparison of positivity rates the two-tailed McNemar's test was used.

The κ value with a 95% confidence interval (CI) was calculated as a measure of agreement between the HPV genotypes observed in the different sampling methods. HPV agreement was defined as slight (κ ≤0.20), weak (κ = 0.21- 0.40), moderate (κ = 0.41 -0.60), strong (κ = 0.61-0.80), near perfect (κ 0.81-0.99), and perfect (κ = 1.000) [22]. The efficacy of hrHPV testing for CIN2+ detection of PTS, SS, U1 and U2 was evaluated as sensitivity, specificity and positive (PPV) and negative predictive value (NPV).

RESULTS

Study Cohort

From 91 out of 113 women, a complete set of PTS, SS, U1 and U2 samples were available for analysis. Twelve women were excluded because at least one of the samples had not been collected. All samples were positive for RNase P and suitable for analysis. The human DNA concentrations of the morning first-void urine sample and the first-void urine sample from later during the day were compared. The average human DNA concentration in U1 was 15 ng/µl and 21 ng/µl in U2 (p=0.225, 95% CI (-16.61;3.93)).

All patients completed the questionnaire and had a cytological diagnosis: 28 (30.8%) were classified as negative, 11 (12.1%) as ASC-US, 9 (9.9%) as ASC-H, 28 (30.8%) as LSIL and 15 (16.5%) as HSIL. The worst histological diagnosis was negative in 50 women (54.9%), CIN 1 in 22 (24.2%), CIN 2 in 13 (14.3%) and CIN 3 in 6 (6.6%).

Detection of hrHPV

Comparison of hrHPV tests

Without consideration of genotype, 68 women tested hrHPV positive on the PTS, 66 women tested positive on the SS, 67 on U1 and 70 on U2 with the SPF10-assay. With the GP5+/6+-assay, 62 women tested positive on the PTS, 59 on the SS, 60 on U1 and 60 on U2. The comparison of hrHPV detection between SPF10 and GP5+/6+ for PTS, SS, U1 and U2 is shown in Table 1. Good agreement for all sample types was found, with kappa values between 0.74-0.82. The SPF10 test showed more HPV positivity than the GP5+/6+, which was expected given the difference sensitivity. Nonetheless, the agreement between both hrHPV assays was strong to near perfect for all sample types.

Furthermore, all samples were compared for concordance of positivity for the 14 hrHPV genotypes detected by the SPF10 and GP5+/6+ systems (Table 2) between sample types (PTS vs U1, U2 or SS, SS ys U1 or U2, U2 vs U1) . With the SPF10 system the agreement between all sample types was near perfect with kappa values between 0.81-0.92. With the GP5+/6+ system, strong to near perfect agreement was found, with kappa values 0.73-0.85.

Table 1. Agreement between the analytically sensitive SPF10 test and the clinically validated GP5+/6+ test on the detection of 14 hrHPV genotypes, for clinician-taken smear (CTS), self-sample (SS), morning first-void urine (U1), and first void-urine from later during the day (U2)

Sample type	Both tests positive	SPF10 only positive	GP5+/6+ only positive	Both tests negative	j (95% CI)	P
CTS	61	6	1	23	0.81 (0.76–0.88)	0.125
SS	59	7	0	25	0.82 (0.76–0.89)	0.016
U1	59	8	1	23	0.77 (0.70–0.84)	0.039
U2	60	10	0	21	0.74 (0.66–0.81)	0.002

n = 91.

Table 2. Agreement on hrHPV positivity, not considering genotyping, in clinician-taken smear (CTS), self-sample (SS), morning first-void urine (U1), and first-void urine from later during the day (U2), tested by SPF10 and GP5+/6+

Comparison of samples (n = 91)	SPF10				GP5+/6+			
	Concordant	Discordant	j (95% CI)	P	Concordant	Discordant	j (95% CI)	P
CTS versus U1	85	6	0.83 (0.70–0.96)	1	81	10	0.75 (0.61–0.90)	0.754
CTS versus U2	86	5	0.85 (0.73–0.98)	0.375	82	9	0.78 (0.64–0.92)	1
CTS versus SS	88	3	0.92 (0.822–1.01)	1	84	7	0.80 (0.68–0.93)	0.727
SS versus U1	84	7	0.81 (0.67–0.94)	1	78	13	0.68 (0.53–0.84)	1
SS versus U2	85	6	0.83 (0.69–0.96)	0.219	80	11	0.73 (0.59–0.88)	1
U1 versus U2	88	3	0.91 (0.81–1.01)	0.25	85	6	0.85 (0.740–0.97)	1

hrHPV Genotype Concordance between specimen types

Comparisons between different sample types for detection of 14 individual hrHPV genotypes by SPF10 and GP5+/6+ were made. There was near perfect agreement on genotype between U1 and U2 with both the SPF10 system (kappa value=0.90) and the GP5+/6+ system (kappa-value=0.85). The comparison of the GP5+/6+ results of SS and PTS for any genotype shows near perfect agreement (kappa-value=0.81). All other comparisons for genotype between sample types result in strong agreements (kappa-values 0.74-0.77).

We compared sample types at genotype level with the SPF10 assay and with the GP5+/6+ assay. With both tests, most samples show identical genotyping results. Most discordant results rely on one of the two samples being hrHPV negative, with the other sample being hrHPV positive rather than both samples showing different hrHPV genotypes.

Detection of CIN lesions

All women have been biopsied. In this group of 91 women, CIN3 was detected in 6 (6.6%) women and CIN2 was detected in 13 (14.2%) women. All CIN 3 lesions were found hrHPV positive by SPF10 and GP5+/6+ in PTS, SS, U1 and U2 (Table 3). All four types of sample show a high sensitivity for the detection of CIN2+ lesions with both SPF10 and GP5+/6+ (Table 4), without any significant differences. The specificities for CIN2+ detection in PTS, SS, U1 and U2 when using SPF10 or GP5+/6+ were not significantly different.

Table 3. Overall positivity for 14 hrHPV genotypes by SPF10 and GP5+/6+ assay in clinician-taken smear (CTS), self-sample (SS), morning first-void urine (U1), and first-void urine from later during the day (U2)

Histological diagnosis	CTS		SS		U1		U2	
	SPF10	GP5+/6+	SPF10	GP5+/6+	SPF10	GP5+/6+	SPF10	GP5+/6+
Negative (n = 50)	58.0%	52.0%	56.0%	50.0%	60.0%	46.0%	64.0%	50.0%
CIN1 (n = 22)	86.4%	81.8%	86.4%	72.7%	86.4%	86.4%	86.4%	77.3%
CIN2 (n = 13)	100.0%	92.3%	100.0%	92.3%	92.3%	92.3%	100.0%	92.3%
CIN3 (n = 6)	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%	100.0%
Total (n = 91)	n = 67	n = 62	n = 66	n = 59	n = 67	n = 60	n = 70	n = 60

Table 4. Sensitivity and specificity of hrHPV testing for CIN2+ detection in clinician-taken smear (CTS), self-sample (SS), morning first-void urine (U1), and first-void urine from later during the day (U2), tested with SPF10 and GP5+/6+

Sample	SPF10				GP5+/6+			
	Sensitivity		Specificity		Sensitivity		Specificity	
	%	95% CI	%	95% CI	%	95% CI	%	95% CI
CTS	100	83–100	33	24–45	95	75–99	39	28–50
SS	100	83–100	35	25–46	95	75–99	43	32–55
U1	95	75–99	32	22–43	95	75–99	42	31–53
U2	100	83–100	29	20–41	95	75–99	42	31–53

Questionnaire

Overall rating of the PTS, SS and urine sampling by all 91 women resulted in an average score of 7.6 out of 10 for the PTS, 8.1 for the SS and 8.6 for the urine sampling ($p<0.005$). A total of 82 (90.1%) women rated the convenience of the SS compared to a PTS as good to excellent and 81 (89.0%) women rated the urine sampling good to excellent when compared to a PTS. Two women rated the convenience of the SS compared to the PTS as poor. No other sampling issues were scored poor.

DISCUSSION

Main findings

Physician-taken smears, brush-based self-sampling, morning first-void urine and first-void urine from later during the day all showed similar high sensitivity for the detection of CIN2+ in this referral population, measured with two different hrHPV tests (the highly sensitive SPF10 LiPA 25 version 1 assay and the clinically validated GP5+/6+ assay). None of the samples or assays missed CIN3.

For SPF10 the sensitivity for CIN2+ detection in PTS, SS and U2 was all 100%, with a near perfect agreement between the four different types of samples (Kappa=NC-1.00). With the GP5+/6+ assay, the sensitivity was 95% for all sample types, with a strong agreement between all samples (kappa=NC-1.00).

Strengths and Limitations

The main strength of this study is that we used paired urine samples, vaginal samples and cervical samples that were all collected and stored at the same time and under the same circumstances. The collection of first-void urine was standardized using a device that was developed to collect only the first fraction of urine. Samples were all stored in a preservative and stored at -80°C shortly after collection, optimizing storage conditions. Additionally, both a cytological and histological sample were taken, providing a histological endpoint for all women.

A limitation of our study is that it was conducted in a small population of women referred to one colposcopy clinic after an abnormal Pap-smear. In this selected population, the (hr)HPV positivity rate and CIN2+ rate are high (73.6% and 20.8%, resp.) and these results cannot be directly extrapolated to a screening population. The confidence intervals around both sensitivity and specificity are quite wide and a larger study of a screening population is needed.

Interpretation

Several studies focused on the role of self-sampling in screening have been published. Bosgraaf et al. [23] showed that the Evalyn-brush is also suitable for screening purposes and Burroni et al. [24] found that urine is suitable for HPV detection and has high concordance with HPV detected in physician-taken smears. No histological diagnoses were available for that study. A study by Stanczuk et al. found the detection of CIN3+ was >90% when testing urine samples with the for cervical smears clinically validated Cobas 4800 assay. [25] There is need for a study focusing on the detection of CIN2+ and CIN3+ lesions in a screening population, including those who do not attend for a PTS, that should include validated clinical hrHPV testing and sensitive HPV detection systems such as SPF10 to assess the utility of urine sampling in cervical cancer prevention through screening.

Our results match with results previously found by Stanczuk et al. [25] and are higher than sensitivities previously found in other studies comparing HPV detection in SS to PTS. [26], [27] The specificity with the GP5+/6+ was higher than with the SPF10 assay (42% and 32% in U1 and 42% and 29% in U2, respectively), but no significant differences were found between the different types of samples. On genotype level a strong to near perfect agreement was found between all samples, for both SPF10 and GP5+6+ with discordant results not showing any particular pattern with regard to type of sample.

CONCLUSION

In this study, first-void urine samples appeared suitable for CIN2+ detection through HPV testing with a test with a high analytical sensitivity and a test with a high clinical sensitivity, validated for physician taken samples. Analyses performed on genotype concordance imply that the HPV found in urine is representative of the HPV in the cervix, with the high concordance between the two urine samples demonstrating reproducibility of results. When comparing morning first-void urine to first-void urine from later in the day, 5.5% of the patients showed discordant results with SPF10. All five patients had a HPV negative U1 and HPV positive U2, with histological diagnoses negative in four patients and CIN2 in one. With the GP5+/6+, 8.8% of the patients had discordant HPV results in U1 and U2, with U2 being the only sample positive in half of the cases. Our results suggest that there is no advantage in testing morning first-void urine over a portion of first-void urine that was collected at a later time during the day. The fraction of the urine appears to be more important than the timing, which is in line with results from a recent study by Senkomago et al., comparing different fractions and collecting times. [28] A similar amount of human DNA was collected in both samples but the propor-

tion of cells originating from the urinary tract or the genital tract and cervix remains to be determined.

Both the SS and the urine sampling were rated as excellent overall by most of the women. Instructions were clear and the devices were easy to use. A great advantage of both samples is that they can be sent by mail for collecting samples at home.

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