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Evaluation of clinical performance of a novel urine-based HPV detection assay among women attending a colposcopy clinic

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Ethical Approval: The study was approved by the Institutional Review Board of the University of Oklahoma Health Sciences Center in Oklahoma City, OK and the Special Studies Institutional Review Board of the National Cancer Institute in Bethesda, MD.

Conflicts of Interests:
S. Terence Dunn serves on the editorial board of Journal of Clinical Virology.
Philip E. Castle has served as a consultant to BD, Roche, Cepheid, Gen-Probe/Hologic, and GE, Healthcare. He has been compensated as a member of a data and safety monitoring board for HPV vaccines for Merck. He has received HPV tests and reagents for research at a reduced or no cost from Qiagen, Roche, Norchip, and MTM.
Mark Schiffman has received HPV testing at no cost from Roche for an NCI study.
David Robbins was employed by Trovagene at the time of the conduct of assays and owns stocks in the company.

Role of Authors:
Conception and design of the study: VVS, PEG, STD, DR, DB, RAA, YJE, KMS, REZ, RRZ, MAG, MS, JLW, PEC, NW
Acquisition of specimens/data: PEG, STD, DR, DB, RAA, YJE, KMS, REZ, RRZ, MAG, JLW
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Abstract

Background—Human papillomavirus (HPV) testing in urine offers a convenient approach for cervical cancer screening but has previously suffered from limited clinical sensitivity.

Objectives—We evaluated clinical performance of the prototype Trovagene HPV test, a novel polymerase chain reaction assay that targets the *E1* region of the HPV genome and detects and amplifies short fragments of cell-free HPV DNA in urine.

Study design—We conducted a pilot study among seventy two women referred to colposcopy following abnormal screening. Participants provided a urine sample prior to clinician-collected cervical sampling and colposcopically-directed punch biopsy. Trovagene HPV test results on urine samples were compared with cervical and urine testing by Linear Array HPV Genotyping Test (LA-HPV) for detection of histologically-confirmed cervical precancerous lesions.

Results—There was high concordance between urine samples tested by the Trovagene HPV test and corresponding cervical (87.5%) and urine (81.9%) samples tested by LA-HPV. The Trovagene HPV test had high sensitivity (92.3% for detecting CIN2/3, and 100% for CIN3), comparable to LA-HPV testing on cervical samples (96.0% and 100%, respectively), and higher than LA-HPV testing on urine samples (80.8% and 90.0%, respectively). In this referral population, the specificity of the Trovagene urine HPV test was non-significantly lower (29% f CIN2/3 and 25% for CIN3) than corresponding estimates of LA-HPV testing on cervical (36% and 28%, respectively) and urine (42% and 38%, respectively) samples.

Conclusions—This pilot study suggests that the Trovagene HPV test has high sensitivity for urine-based detection of cervical precancer and merits evaluation in larger studies.

Keywords

human papillomavirus; cervical cancer; urine; screening

Background

Urine-based testing for human papillomavirus (HPV) has been explored as an alternative approach for cervical cancer screening[1, 2]. Yet, existing approaches for urine HPV testing have less than optimal analytical and clinical sensitivity, likely due to factors such as low levels of HPV DNA in urine, inappropriate extraction or amplification protocols, and the presence of contaminants or inhibitors[3]. It is important to evaluate new approaches that may improve the sensitivity of urine-based HPV screening since this approach to screening for cervical cancer would offer significant advantages, especially related to user convenience and reducing number of clinic visits.

Objectives

We conducted a pilot study in a colposcopy referral population to evaluate the clinical performance of the prototype Trovagene HPV test, a novel urine-based screening assay that relies on the detection of specific sequences of the HPV *E1* gene in small DNA fragments in urine.

Study Design

The study, described previously[1, 4], was nested within the National Cancer Institute (NCI) Biopsy Study, a population-based study of women referred to colposcopy for abnormal cervical cancer screening results, conducted at the University of Oklahoma Health Sciences Center (OUHSC), Oklahoma City, OK. Eligible and consenting participants provided first-void urine samples, prior to undergoing pelvic examination. Immediately post-collection, 10 mL of a colored 0.5M EDTA preservative liquid was added to the urine. In the lab, urine was shaken gently, then 20 ml aliquots were transferred to sterile Falcon tubes, and stored at room temperature for <3 weeks before either freezing (at -70°C) or conducting HPV DNA testing. A study clinician collected each cervical sample using a Wallach broom, which was placed in a ThinPrep® vial (Hologic, Inc., Marlborough, MA) for liquid cytology and HPV genotyping. Each patient then underwent colposcopy and biopsy, as previously described[1, 4].

Urine HPV testing from one of the aliquots was conducted using the prototype Trovagene HPV test (Trovagene Inc., San Diego, CA). For this test, DNA was isolated from a 1 ml volume of urine using the Trovagene DNA isolation protocol[5], and then amplified by PCR. Reaction products were subjected to capillary electrophoresis (Genewiz, South Plainfield, NJ). HPV positivity by the Trovagene test implies detection of size-specific PCR fragments of any of 15 high-risk HPV genotypes (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, and 70), with the result read as detected/not detected[6].

The Linear Array HPV Genotyping Test (LA-HPV; Roche Molecular Systems, Pleasanton, CA) was conducted on cervical specimens at the Molecular Pathology Laboratory at OUHSC, and on the 2nd urine aliquot at Johns Hopkins University, as previously described[4]. The LA-HPV assay is able to detect and individually differentiate 37 HPV genotypes, including all 15 targeted by the Trovagene assay. For the purposes of this study, specimens demonstrating any of the 15 HPV genotypes represented in the Trovagene assay were considered positive by LA-HPV.

We compared agreement between paired test results of Trovagene urine HPV test and LA-HPV performed on the cervical and urine samples, respectively. We evaluated sensitivity, specificity, positive and negative predictive values, and Youden's Index (sensitivity + specificity -1), with 95% confidence intervals (95%CI) of Trovagene urine HPV testing and LA-HPV testing on cervical and urine samples, against two histologically-confirmed endpoints: cervical intraepithelial neoplasia grades 2 and 3 (CIN2/3), and CIN3 (cervical precancer). Fisher's exact test was used to explore whether HPV positivity by Trovagene or LA-HPV testing of urine was higher among women with corresponding higher cervical LA-HPV signal strengths (at the strong and moderate signal intensity thresholds) than those with lower LA-HPV signal strengths.

Results

Cervical and urine samples were able to be evaluated among all 72 women (median age: 28 years, interquartile range, 24-34 years) participating in this pilot study. The Trovagene urine HPV test was positive in 56/72 (77.8%) participants. At least one of the same 15

carcinogenic HPV genotypes was present in 47/72 (65.3%) of urine samples, and 55/72 (76.4%) of cervical samples by LA-HPV.

The overall agreement and Kappa between urine samples tested by Trovagene HPV test and LA-HPV testing on cervical specimens was 87.5% and 0.65, respectively, and on urine specimens was 81.9% and 0.57, respectively (Table 1). There were only four cases of discordance where the Trovagene HPV test did not detect high-risk HPV in urine but high-risk HPV was detected in corresponding cervical samples using LA-HPV; two had single high-risk HPV genotype infections as determined by LA-HPV but were negative by the Trovagene HPV test, whereas the other two cases had multiple (2+) high-risk HPV infections in cervical samples by LA-HPV but the corresponding urine samples were negative by both LA-HPV and Trovagene HPV tests.

The Trovagene HPV test for urine-based detection of high-risk HPV had a sensitivity of 92.0% (95%CI=74.9%-99.1%) for the detection of CIN2/3 (Table 2). The corresponding sensitivity estimates for the LA-HPV test on cervical and urine samples were 96.2% (95%CI=80.4%-99.9%) and 80.8% (95%CI=60.6%-93.4%), respectively. The specificity of the Trovagene urine HPV test was 28.9% (95%CI=16.4%-44.3%) and those for the LA-HPV test for cervical and urine samples were 35.6% (95%CI=21.9%-51.2%) and 42.2% (95%CI=27.7%-57.8%), respectively. The sensitivity estimates for CIN3 were higher and the specificity estimates were correspondingly lower than the respective estimates for CIN2/3 for all testing approaches (Table 2). None of the differences were statistically significant at the given sample size.

When restricting the LA-HPV positivity to 13 genotypes of group 1/group 2A carcinogenic genotypes (i.e., after excluding HPV53 and HPV70, as previously published[1]), the sensitivity estimates for the LA-HPV testing on cervical and urine samples were similar, although their specificity was slightly higher.

There was no correlation between positivity by Trovagene HPV test on urine and stronger hybridization signal strengths in the cervical LA-HPV test for cervical samples, at either the strong (p=0.43) or moderate (p=0.27) signal intensity threshold (Table 3). In contrast, positivity by LA-HPV testing on urine samples was significantly higher in women with stronger cervical signal strength at the moderate signal threshold (p=0.01), and also higher at the strong signal strength threshold, although with marginal statistical significance (p=0.09).

Discussion

We have demonstrated that the sensitivity of Trovagene HPV urine test is comparable to cervical HPV testing and higher than urine HPV testing conducted by LA-HPV, a widely-used and highly-sensitive assay[7]. The proprietary Trovagene HPV test targets cell-free DNA[6], an approach that has been utilized for the sensitive non-invasive detection of fetal DNA for prenatal monitoring, monitoring of tumor response, and monitoring in trauma and stroke cases[5, 8]. Its customized extraction process (binding of cell-free urinary nucleic acids or nucleoproteins to a Q-Sepharose anion-exchange resin, followed by elution with LiCl[5]) has been shown to be efficient in concentrating DNA in urine, and likely a major

contributing improvement over traditional extraction approaches. Moreover, targeting very short (50-150 base pairs) cell–free fragments of the *E1* gene[6] for PCR amplification likely contributes significantly to improved sensitivity.

The positivity of urine testing by LA-HPV was proportional to the cervical LA-HPV signal strength, while the Trovagene urine HPV positivity did not vary with that of cervical LA-HPV signal strength. Since HPV in urine in all likelihood represents secondary exfoliation from the cervix, this exploratory analysis further suggests that due to its cell-free, small-fragment HPV DNA detection approach, the Trovagene HPV test can provide robust results even among women with a lower degree of cervical exfoliation into the urine. The use of EDTA preservative to prevent nuclease inhibitors[9, 10], particularly those affecting friable cell-free DNA, also likely contributed to the improved detection seen with the Trovagene HPV test in this study.

Despite the small sample size, the study had well-characterized cervical precancerous outcomes. The high sensitivity of the urine testing observed in this pilot study may reflect the relatively controlled clinical research environment and might be the upper limit of what might be achievable in “real-world” field conditions where it might be influenced by several logistical (sampling and transportation) challenges. Furthermore, this being a referral population, the disease prevalence and positive predictive value estimates are higher and the specificity estimates are likely much lower in this population compared to a screening population. The lower specificity observed here could also be due to the inclusion of HPV53 and HPV70 in the prototype version of the Trovagene assay that was evaluated in this study. However, these genotypes are not included in the current commercial version of the Trovagene assay[11]. Future studies should evaluate the commercially available version in diverse clinical settings, especially to evaluate operational and practical aspects (e.g., sample processing turnaround) and its comparison to more established HPV testing assays.

In summary, the results of this pilot study add to the evidence for urine-based HPV detection for cervical cancer screening. The Trovagene HPV urine test should be evaluated in larger studies in the general population to assess its efficacy and effectiveness in clinical and field settings.

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Table 1

Comparison of test agreement between carcinogenic HPV detection by Linear Array HPV test (LA-HPV) (cervical and urine samples) and Trovagene HPV test

Sample types compared	N	No. of samples with indicated paired results[a]				Value (95%CI)			
		+/+	-/-	+/-	-/+	Overall % agreement (concordance)	% positive agreement	% negative agreement	Cohen's Kappa
Cervical LA-HPV/Urine Trovagene HPV	72	51	12	4	5	87.5 (77.6-94.1)	91.9 (86.6-97.2)	72.7 (55.6-89.9)	0.65 (0.44-0.86)
Urine LA-HPV/Urine Trovagene HPV	72	45	14	2	11	81.9 (71.1-90)	87.4 (80.6-94.2)	68.3 (51.9-84.6)	0.57 (0.36-0.77)

Footnote:

[a] +, presence of any of the 15 high-risk HPV genotypes (HPV16/18/31/33/35/39/45/51/52/53/56/58/59/68/70); -, absence of the aforementioned HPV genotypes. The result indicated on either side of each slash corresponds to the sample type listed in the same position in the first column.

Table 2

Comparison of accuracy of the urine Trovagene HPV test, and Linear Array HPV genotyping test (for 15 carcinogenic types) for cervical and urine samples for detection of histology-confirmed CIN2/3 lesions and CIN3 lesions

Sampling method	No. of samples					(%, 95 % CI)				Youden's Index (95%CI)
		Disease Present		Disease Absent		Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	
		CIN2/3=26		<CIN2=45						
	Total	*TP*	*FN*	*FP*	*TN*					
Urine Trovagene HPV test	71	24	2	32	13	92.3 (74.9-99.1)	28.9 (16.4-44.3)	42.9 (29.7-56.8)	86.7 (59.5-98.3)	0.21 (0.05-0.38)
Cervical LA-HPV test	71	25	1	29	16	96.2 (80.4-99.9)	35.6 (21.9-51.2)	46.3 (32.6-60.4)	94.1 (71.3-99.9)	0.32 (0.16-0.48)
Urine LA-HPV test	71	21	5	26	19	80.8 (60.6-93.4)	42.2 (27.7-57.8)	44.7 (30.2-59.9)	79.2 (57.8-92.9)	0.23 (0.02-0.44)
		CIN3=10		<CIN3=61						
	Total	*TP*	*FN*	*FP*	*TN*					
Urine Trovagene HPV test	71	10	0	46	15	100 (69.5-100)	24.6 (14.5-37.3)	17.9 (8.9-30.4)	100 (78.2-100)	0.25 (0.14-0.35)
Cervical LA-HPV test	71	10	0	44	17	100 (69.5-100)	27.9 (17.1-40.8)	18.5 (9.3-31.4)	100 (80.5-100)	0.28 (0.17-0.39)
Urine LA- HPV test	71	9	1	38	23	90 (55.6-99.7)	37.7 (25.6-51.0)	19.1 (9.1-33.3)	95.8 (78.9-99.9)	0.28 (0.06-0.5)

Footnote: HPV: human papillomavirus, LA-HPV: Linear Array HPV genotyping test, CIN: cervical intraepithelial neoplasia. This table uses histology-confirmed cervical diagnoses, which were available in only 71 out of 72 participants who participated in the study.

Table 3

Comparison of HPV test positivity by Trovagene urine HPV test and urine testing by Linear Array HPV test (LA-HPV) stratified by categories of positive signal strength thresholds of cervical HPV detection by LA-HPV.

Type of sample positivity	No. of positive urine HPV test samples / no. of positive cervical samples (%) by signal strength of LA-HPV			No. of positive urine HPV test samples / no. of positive cervical samples (%) by signal strength o f LA-HPV		
	Strong signal	≤Moderate signal	*P* value	≥Moderate signal	≤Weak signal	*P* value
Trovagene urine HPV test positive	45/48 (93.8)	6/7(85.7)	0.43	48/51 (94.1)	3/4 (75.0)	0.27
Urine LA-HPV test positive	41/48 (85.4)	4/7 (57.1)	0.09	43/51 (84.3)	2/4 (50.0)	0.01

Footnote: Hybridization signal strengths of the cervical samples presented in the Table is noted on a semi-quantitative scale as strong, moderate, weak, very weak and extremely weak signals. “≥Moderate signal” refers to strong or moderate signals, “≤moderate signal” refers to moderate, weak, very weak, or extremely weak signals, “≤weak signal” refers to weak, very weak, or extremely weak signals. The p-values for comparing differences in proportions of urine HPV positivity by corresponding categories of cervical signal strength positivity were computed by the Fisher's exact test.