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Treatment of first-void urine with Aptima Transfer Solution increases detection of high-risk HPV E6/E7 mRNA

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ABSTRACT

Because of its non-invasive nature urine testing may enable increased screening for HPV in women who avoid cervical sampling. Comparisons have shown fewer HPV positives in urine. The objectives were to compare first-void urine (FVU) treated with proteinase K (PK) to untreated FVU and cervical samples collected from women attending a colposcopy clinic using an Aptima HPV mRNA assay, and comparing the HPV rates to cytology and pathology results. Female FVU (n = 433) was treated with Aptima Transfer Solution (ATS) containing PK within 24 h or after months of storage. Untreated female FVU samples were HPV-positive in 20.8–27.6% compared to 34.4–45.6% of ATS-treated FVU and 44.9–48.4% of PreservCyt samples. Good overall agreement for HR-HPV detection between ATS-FVU and PreservCyt was observed (81.1%; k 0.63). Validation of ATS treatment was performed on 356 male FVU, detecting 6.7% HPV positive compared to 3.4% of untreated samples (p = 0.059). Although HPV presence in ATS FVU and PreservCyt samples were similar, significantly more women with abnormal cervical cytology and histopathology were HPV-positive in cervical specimens than in ATS-treated FVU.

1. Introduction

First-void urine (FVU) is a convenient and noninvasive sample which has been successfully employed for the diagnosis of sexually transmitted infections (Cook et al., 2005). Urine HPV-DNA detection for cervical cancer screening has shown promise and limitations in several studies (Sehgal et al., 2009; Kitchener and Owens, 2014). A meta-analysis of 14 studies comparing FVU to cervical samples for detection of HPV DNA reported high specificity and fair sensitivity, indicating that FVU might be an acceptable specimen type for the detection of cervical precancerous lesions or cancer without 100% sensitivity (Pathak et al., 2014). In men, a significant challenge in evaluating urine for HPV detection is the lack of a standardized comparator sample type (Enerly et al., 2013).

The Aptima HPV (AHPV) assay which detects E6 and E7 mRNA from 14 high risk human papillomavirus (HR-HPV) genotypes is a sensitive and specific transcription-mediated amplification (TMA) assay and is approved for use with genital samples for the diagnosis of cervical precancer and cancer in various regions of the world (Dockter et al., 2009a, b). Nucleic acid amplification tests (NAAT) often require prompt transfer of specimens into ethanol and formalin-based specimen transport media, but the latter formulation can cause formalin-induced cross-linking of nucleic acids and proteins which can have a negative effect on recovery and detection of HR-HPV (Moelans et al., 2011; Powell et al., 2006). Extraction of HPV RNA from SurePath samples after Proteinase K digestion has shown optimal recovery (Dixon et al., 2008; Murphy et al., 2009; Chernesky et al., 2017). Hologic has developed a proprietary solution of Proteinase K called Aptima Transfer Solution (ATS) for use with AHPV assays.

The objective of this study was to measure AHPV positivity in untreated and ATS-treated FVU compared to cervical samples collected into PreservCyt media from women with various cervical cytology and pathology scores. In males, AHPV detection in untreated FVU was compared to ATS-treated FVU to confirm the impact of ATS treatment of urine.

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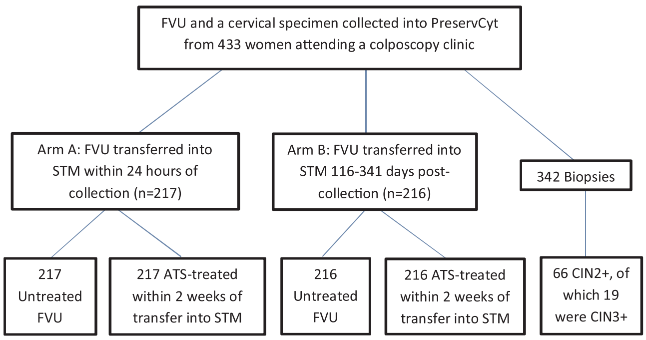

Fig. 1. Specimen collection and treatment scheme for female first-void urine (FVU).

2. Materials and methods

2.1. Female colposcopy attendees

As part of a large ethics-approved cross-sectional study assessing cervical specimens tested with AHPV (Chernesky et al., 2017), 433 consecutive sexually active women referred to the Juravinski Cancer Center colposcopy clinic (Hamilton, ON, Canada) due to a previous abnormal Pap test, participated in the study (Fig. 1). Women self-collected a FVU (first 20 ml of any micturition), followed by physician collection of cervical samples with a Cervex broom into PreservCyt L-Pap. Cervical biopsies were performed when warranted as per standard practice, and histological assessment was done by a single study pathologist blinded to HPV results and clinical information. L-Pap cytology was performed on PreservCyt samples as per the local standard of care and scored as negative for intraepithelial lesions or malignancy (NILM), abnormal squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesions (LSIL), or high-grade squamous intraepithelial lesions (HSIL). Samples were then shipped to the Infections Research Laboratory (IRL) at St. Joseph's Healthcare in Hamilton ON, Canada on the same day of collection. PreservCyt cervical samples (1.0 ml) were transferred to tubes containing Specimen Transport media (STM) within 7 days of collection. To examine whether the time between FVU collection and transfer into urine STM influenced HPV results, female participants were randomized into either Arm A (n = 217) where 2.0 ml of FVU was transferred into urine STM within 24 h of collection, or Arm B (n = 216) where FVU was stored in urine jars at 4 °C and 1.0 ml was transferred into STM at a later date (116–341 days post-collection; median 202 days) (Fig. 1). Cervical biopsies were performed on 342 patients, stained with H and E and read by the institute's pathologists. Histology slides were scored negative, cervical intraepithelial neoplasia (CIN) grade 1, 2 or 3, or cancer. CIN2+ was categorized as disease positive and a sub-analysis of CIN3 was conducted. All cytology and histopathology slides were re-read in a blinded fashion by a second research pathologist.

2.2. Male street youth

Consecutive healthy men (n = 356) attending a clinic for street youth between May-July 2015 participated in the study. Following consent, participants collected a FVU. Specimens were shipped to the IRL on the same date of collection. For all males, 2.0 ml of FVU was transferred into urine STM within 24 h of collection.

2.3. ATS treatment and AHPV testing

Within 2 weeks of transfer into STM, all samples designated for ATS treatment were treated with 300 μl of ATS, followed immediately by a 15-minute incubation at 90 °C as per the manufacturer's instructions. Samples were then tested in the AHPV assay on a Panther automated instrument as per the manufacturer's package insert (Hologic Inc) on the same day of ATS treatment.

2.4. Statistical analysis

AHPV positivity was determined by specimen type and ATS treatment condition. Sub-analyses of AHPV positivity were performed relative to cytological and histological findings. Raw agreement and agreement beyond chance (kappa statistic) of AHPV results were calculated for untreated and ATS-treated FVU relative to PreservCyt samples. Two-tailed Fisher's exact test was used to calculate p-values between proportions, and results were considered statistically significant at $p < 0.05$.

3. Results

Women from the colposcopy clinic ranged in age from 21 to 74 (mean age 36). Table 1 summarizes AHPV positivity rates for FVU and PreservCyt samples. There were significantly more AHPV-positives in ATS-treated FVU than untreated FVU in study Arm A (45.6% versus 27.6%, $p < 0.001$) and in study Arm B (34.3% versus 20.8%, $p = 0.003$) as well as PreservCyt compared to untreated FVU (study Arm A: 48.4% versus 27.6%, $p < 0.001$ and in study Arm B 44.9% versus 20.8%, $p < 0.001$). AHPV positivity between ATS-FVU and PreservCyt was 45.6% versus 48.4% in Arm A, but was higher for PreservCyt than ATS-FVU in Arm B (44.9% versus 34.3%).

Table 2 reports positive, negative and overall percent agreements and agreement beyond chance between untreated and ATS-treated FVU compared to cervical samples for the detection of HR-HPV. ATS-FVU showed higher agreements with PreservCyt than did untreated FVU in both study arms. AHPV detection in FVU samples transferred into STM within 24 h (Arm A) and ATS-treated within 2 weeks showed good overall agreement of 81.1% (k 0.63, 95% CI: 0.52-0.73) compared to PreservCyt samples.

Table 3 shows AHPV positivity in untreated FVU, ATS-FVU and PreservCyt specimens stratified according to the cytology and histopathology results. A total of 135 were NILM and 298 were classified as abnormal (124 ASCUS, 120 were LSIL and 54 were HSIL). AHPV rates were the same (24.4%) for ATS-FVU and PreservCyt in patients with NILM, and were significantly higher in the abnormal cytology group compared to NILM for both sample types. Among those with abnormal cytology, AHPV positivity was higher in PreservCyt samples compared to ATS-FVU (56.4% versus 47.0%, $p = 0.027$). Sub-analysis by specific category of abnormal cytology revealed no significant difference between ATS-FVU and PreservCyt for ASCUS cytology (36.3% versus 30.6%, $p = 0.423$), but higher positivity in PreservCyt compared to ATS-FVU for LSIL ($p = 0.048$) and HSIL ($p < 0.001$) cytology. Of 342

Table 1
AHPV positivity and 95% CI by sample type and treatment conditions among females.

Sample Type	Arm A [95% CI]	p-value*	Arm B [95% CI]	p-value*
Untreated FVU	27.6% (60/217);[22.0-33.8]		20.8% (45/216);[15.3-26.1]	
ATS-FVU	45.6% (99/217);[39.5-52.7]	< 0.001	34.3% (74/216);[27.8-40.4]	0.003
PreservCyt	48.4% (105/217);[42.3-55.5]	< 0.001	44.9% (97/216); [37.6-50.8]	< 0.001

* Relative to untreated FVU.

Table 2
Positive, negative and overall % agreements of untreated FVU and ATS-treated FVU to PreservCyt L-Pap specimens for the detection of HR-HPV E6/E7 mRNA.

		PreservCyt			
Study Group	Sample Type	Positive % Agreement	Negative % Agreement	Overall % agreement	Kappa (95% CI)
Arm A (n = 217)	Untreated FVU	42.9% (45/105)	85.7% (96/112)	65.0% (141/217)	0.29 (0.16-0.42)
	ATS-FVU	78.1% (82/105)	83.9% (94/112)	81.1% (176/217)	0.63 (0.52-0.73)
Arm B (n = 216)	Untreated FVU	35.0% (34/97)	90.8% (108/119)	65.7% (142/216)	0.27 (0.14-0.41)
	ATS-FVU	56.7% (55/97)	84.0% (100/119)	71.8% (155/216)	0.42 (0.30-0.54)

cervical biopsies performed, 66 were CIN2+, of which 19 were CIN3. Among CIN2+ patients, ATS treatment of FVU resulted in an increased AHPV positivity rate from 36.4% (24/66 untreated) to 50% (33/66 ATS-treated). AHPV positivity in the corresponding PreservCyt samples was substantially higher at 87.9%, $p < 0.001$ (58/66). Similar observations were seen for the 19 CIN3 cases.

Males in the street youth population ranged in age from 16 to 25 (mean age 22) and 28.4% (101/356) reported a previous history of STI; of which 85% (86/101) had prior *Chlamydia trachomatis* infection, 13% (13/101) reported a prior *Neisseria gonorrhoeae* infection, and 11% (11/101) had history of another STI. ATS-FVU detected twice as many HR-HPV infections compared to untreated FVU (6.7% versus 3.4%, $p = 0.059$, data not shown). AHPV positivity in ATS-FVU was not found to be influenced by a previous history of STI ($p = 0.062$) or the presence of symptoms ($p = 0.454$).

4. Discussion

To our knowledge, this is the first comparative study of HPV E6/E7 mRNA detection in ATS-treated FVU compared to PreservCyt LPap samples in women using the Aptima HPV assay. ATS treatment of female FVU increased HR-HPV detection to rates comparable to PreservCyt. When FVU was transferred into STM within 24 h of collection (Arm A) or after months of storage at 4 °C (Arm B) then treated with ATS within two weeks (Table 1), there was no statistically significant difference in detection rates between ATS-FVU and PreservCyt. Although there was a significant increase in positivity for ATS-FVU compared to untreated FVU in Arm B, significantly lower rates compared to PreservCyt point to the importance of prompt stabilization of nucleic acids in STM. Confirmation of enhanced AHPV positivity by ATS treatment of FVU was also evident in the male group, where ATS-treated FVU identified twice as many HR-HPV infections as untreated FVU (6.7% versus 3.4%, $p = 0.059$).

Our findings showed markedly higher positive, negative and overall percent agreements for ATS-FVU over untreated FVU when compared to PreservCyt (Table 2). In Arm A, the overall percent agreement for ATS-FVU was 81.1% (k 0.63). Slightly lower overall agreement of 71.8% (k 0.42) for ATS-FVU in Arm B can likely be attributed to delayed nucleic acid stabilization into STM. These results support findings from a meta-analysis of 14 studies comparing detection of HR-HPV DNA in paired urine and cervical samples, which reported a pooled sensitivity of 77% (95% CI: 68%–84%) and pooled specificity of 88% (95% CI: 58%–97%) (Pathak et al., 2014). HR-HPV positivity in cervical samples and urine in our study can be compared to previous publications from referral colposcopy clinics. Using PCR, (Forslund et al. (1993)) reported HR-HPV DNA in 49% of cervical samples compared to 38% in urine and Sellors et al. reported a comparison of 62.5% in cervical swabs to 34.5% in urines (Sellors et al., 2000) regardless of cytological status. (Daponte et al. (2006)) found higher HR-HPV DNA rates related to cervical cytology severity and higher infection rates in cervical samples than in urine for LSIL (28.2% vs 12.8%), HSIL (58.6% vs 44.8%) and cancer (100% vs 88.9%). Using the Aptima mRNA assay showed similar trends between cervical samples and untreated urine related to cervical abnormalities (Table 3). ATS treatment increased concordance but the differences were not significant. The lower rates in FVU compared to cervical samples among patients with LSIL or HSIL cytology are consistent with previous observations (Payan et al., 2007; Jacobson et al., 2000; Asciutto et al., 2018). Using the Aptima HPV assay to compare FVU, self-collected vaginal swabs and cervical PreservCyt L-Pap samples, (Asciutto et al. (2018)) showed that untreated FVU had a sensitivity of 44.8% and specificity of 61.9% compared to PreservCyt and correlated poorly with abnormal cytology and histology. (Payan et al., 2007) quantified HR-HPV DNA, showing a 50 fold lower mean viral load in urine compared to cervical samples, which might partially explain differences between urine and swabs.

When measuring HPV test performance on different types of specimens the ultimate comparative endpoint is pre-cancer (CIN2+, CIN3) or cancer, measured from biopsy. Testing the PreservCyt samples with

Table 3
AHPV positivity in ATS-FVU and PreservCyt samples from 433 female colposcopy participants stratified by L-Pap cytology and histopathology results.

PreservCyt L-Pap Cytology Result	n (% of total)	AHPV Positivity (%)			p-value[7]
		Untreated FVU	ATS-FVU	PreservCyt	
NILM[1]	135 (31.2)	18/135 (13.3)	33/135 (24.4)	33/135 (24.4)	1.000
Abnormal[2]	298 (68.8)	87/298 (29.2)	140/298 (47.0)	168/298 (56.4)	0.027
ASCUS[3]	124 (28.6)	25/124 (20.2)	45/124 (36.3)	38/124 (30.6)	0.423
LSIL[4]	120 (27.7)	40/120 (33.3)	64/120 (53.3)	80/120 (66.7)	0.048
HSIL[5]	54 (12.5)	22/54 (40.7)	31/54 (57.4)	50/54 (92.6)	< 0.001
Histopathology					
CIN[6] 2+	66	24/66 (36.4)	33/66 (50.0)	58/66 (87.9)	< 0.001
CIN 3	19	10/19 (52.6)	9/19 (47.4)	18/19 (94.7)	< 0.001

1 NILM negative for intraepithelial lesions or malignancy.
2 Combined ASCUS, LSIL and HSIL.
3 ASCUS abnormal squamous cells of undetermined significance.
4 LSIL low-grade squamous intraepithelial lesions.
5 HSIL high-grade squamous intraepithelial lesions.
6 CIN cervical intraepithelial neoplasia.
7 Two-tailed Fisher's exact test for ATS-FVU compared to PreservCyt.

the AHPV assay resulted in sensitivities of 87.9% (58/66) for CIN2+ and 94.7% (18/19) for CIN3 (Table 3). The comparative values for urine testing showed increases due to ATS treatment but the values were significantly lower. Few studies have compared HR-HPV testing of cervical samples to urine in patients with CIN2+ pathology. (Stanczuk et al. (2016)) used the cobas HPV DNA assay to test cervical samples collected into PreservCyt, self-collected vaginal swabs and random void urine samples, comparing each to CIN outcomes, and showed comparable high sensitivities of 95–98% for cervical and vaginal specimens compared to urine, which showed rates of 63% for CIN2+ and 51% for CIN3+; these lower rates are similar to ATS-treated urine rates in the current study. However, using the cobas HPV DNA assay, (Bernal et al. (2014)) found good agreement between cervical samples and FVU (k 0.76) and a clinical sensitivity of 95% for CIN2 + . Using the Trovagene assay, (Cuzick et al. (2017)) compared cervical samples to FVU and showed slightly but not significantly lower urine sensitivity estimates for CIN2+ and 3+ and the results for cervical samples were similar to other widely used HPV DNA assays. These data suggest that the outcomes of urine testing are dependent upon the HPV assay used, the concentration of analyte in urine and the susceptibility of the procedure to other substances in the sample. Rather than being a replacement for a physician collected sample, a high risk HPV-positive urine result could be used to send a woman to a clinic for cervical sampling, which may serve to increase screening uptake. A larger study with more CIN2+ and CIN3 cases would help to confirm or dispute our findings. More prospective studies assessing different assays on FVU and collection devices, in comparison to other self-collected sample types, such as vaginal swabs, using a CIN2+ primary endpoint are needed in referral and screening settings as discussed by (Arbyn et al. (2018)) for HPV DNA assays and should apply to HPV mRNA testing.

FVU has been suggested as a useful adjunct sample type to increase uptake in the context of cervical cancer screening (Pathak et al., 2014). Past reviews of HPV DNA testing of urine have suggested that the risk for false negatives during screening could be minimized by increasing the frequency of testing compared to existing screening guidelines. ATS treatment of FVU may be of some utility in low-resource settings and in regions with low compliance. Studies from Thailand (Nilyanimit et al., 2013, 2017) using PCR and sequencing compared to a DNA chip with loop mediated isothermal amplification reported similar lower rates of HPV infection for FVU compared to cervical samples in patients with normal and abnormal cytology scores. ATS treatment can be easily incorporated into a clinical diagnostics lab with minimal alterations to laboratory workflows.

This study has a few weaknesses. An internal control for assessing specimen adequacy, such as beta-globin or equivalent was not used in this investigation but a DNA adequacy control sample may not be truly indicative of mRNA in urine. The proportion of positives by ATS testing might be increased if inadequate AHPV-negative FVU samples (ie. beta-globin negative) were excluded from the analysis. Inadequacy of female urine samples has been previously estimated to be 10–20% (Kitchener and Owens, 2014), and although HPV may be present in the absence of beta-globin, the significance of this is unclear (Sellors et al., 2000). While our findings strongly suggest that prompt stabilization of urine into transport medium, followed by ATS treatment, yields favorable AHPV positivity relative to untreated urine and PreservCyt samples, further studies are needed to optimize and standardize sampling processing and handling procedures.

Competing interests

M. Chernesky has received travel expenses to present data related to the study. D. Getman and B. Weinbaum are employees of Hologic Inc. J. Dockter was an employee of Hologic Inc. at the time of the study but is presently an employee of Illumina (San Diego, CA, USA).

Ethical approval

Ethical approval was given by St. Joseph's Healthcare Research Ethics Board in 2012 (12-3758).

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