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RESEARCH ARTICLE

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Detection of human papillomavirus in fresh and dried urine through an automated system for cervical cancer screening in low- and middle-income countries

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Abstract

The majority of cervical cancer cases and associated deaths occur in low- and middle-income countries (LMICs), where sociocultural barriers, poor access to prevention and care, and technical and practical difficulties hinder screening coverage improvement. Using urine specimens for human papillomaviruses (HPV) molecular screening through automated testing platforms can help to overcome these problems. We evaluated the high-risk (HR) HPV detection performance of the Xpert® HPV test on GeneXpert® System (Cepheid), on fresh and dried urine (Dried Urine Spot [DUS]) samples as compared to an in-house polymerase chain reaction (PCR) genotyping assay. Forty-five concentrated urine samples collected from women with known cytological and HPV infection status, determined through in-house PCR and genotyping assays, were tested "as is" and as DUS with the Xpert® HPV test. This system detected HR-HPV in 86.4% of fresh and in 77.3% of dried urine samples collected from HPV+ women, correctly identifying HR-HPV infection in 100% of women with low- and high-grade lesions. High concordance (91.4%, $k = 0.82$) was found between PCR test and Xpert® HPV Test from urine. Urine-based Xpert® HPV test seems to be a suitable screening test for detection of HR-HPV infections associated with low- and high-grade lesions requiring follow-up monitoring or treatment. This methodology, relying on noninvasively collected samples and on available rapid testing platforms, could facilitate large, at-scale screening programs, particularly in LMICs and rural areas, thus reducing adverse outcomes of HPV infection and facilitating achievement of the WHO cervical cancer elimination goal.

KEYWORDS
Dried Urine Spot, HPV, low- and middle-income countries, molecular screening, urine

1 | INTRODUCTION

Human papillomaviruses (HPV) are small circular double-stranded DNA viruses responsible for an infection that is the necessary condition for the development of cervical cancer. Most HPV infections are asymptomatic and clear up within a few months—about 90% of them within 2 years—and only a small proportion of those caused by oncogenic types (high-risk HPV [HR-HPV]) have the potential to persist and progress to cervical or other types of cancers.[1] Cervical cancer is the fourth most common cancer among women worldwide, with an estimated 604 127 new cases and 341 831 deaths in 2020.[2] Because of its viral aetiology, cervical cancer is potentially one of the easiest female cancers to prevent and available strategies can be classified into primary (vaccination, health education), secondary (screening), and tertiary (treatment). According to World Health Organization (WHO), on the path towards cervical cancer elimination, it is essential to achieve three goals by the year 2030: 90% of girls fully vaccinated with HPV vaccine by the age of 15; 70% of women screened with a high-performance test by the age of 35 and again by the age of 45; 90% of women with cervical disease receiving treatment.[3]

Nearly 70% of cervical cancer cases and 90% of associated deaths occur in low- and middle-income countries (LMICs),[2] reflecting global inequalities in access to vaccination, screening programs, health services, and high-quality treatment opportunities. Specifically, the cost of equipment, the need for trained staff, and the limited availability of community services have been identified as the main reasons for the difficult implementation of cytology-based screening programs and, consequently, low screening rates in LMICs.[4] In an effort to overcome these obstacles, the "WHO Global Strategy to eliminate cervical cancer," launched in May 2018, suggested high-performance HPV screening tests for all women as one of the leading strategy to achieve its goals.[3] Although these tests can be costly, they can still be carried out in resource-limited settings by adapting existing diagnostic platforms, simultaneously minimizing the stringent conditions that molecular methods impose and making them applicable virtually everywhere. The GeneXpert® System (Cepheid) is one such platform. This system is already widely used in several LMICs for the diagnosis and management of tuberculosis and human immunodeficiency virus (HIV) infections and, using cervical cytological samples as input, it can also be adopted for the rapid detection of HPV infection and the genotyping of the most common carcinogenic types (i.e., 16, 18/45, and 11 other HR-HPV types).

For a successful screening campaign, it is crucial to gain a solid understanding of the social, cultural, and societal barriers that may hinder adherence to existing screening schedules, especially by women living in low-income countries. Even when access to health care facilities is granted, women may be reluctant and avoid cervical cancer screening tests due to socio-cultural barriers or to a lack of knowledge about the benefits of early detection. More importantly, issues linked to sample collection methods constitute an impediment. The perceived invasion of privacy and fear of possible pain during the invasive pelvic examination and sampling by cervical brush, as well as the need in some settings to obtain spousal consent for such procedures, may prevent some women undergoing HPV screening.[5] However, concerns related to cervical sample collection method can be overcome by using fresh urine as an alternative material for HPV molecular screening. In 2013, we developed and validated an in-house polymerase chain reaction (PCR) assay for the detection and genotyping of HPV DNA in urine samples by testing paired cervical and urine samples from HIV-positive women, demonstrating excellent result concordance.[6] Other studies also showed that the HPV profile detectable in urine can reflect the one found at the level of the cervix.[7–9] Therefore, although urine-based HR-HPV assays seem to be less sensitive, they may be an acceptable alternative to help increasing screening coverage in difficult-to-reach women[7–9] given the less invasiveness of the sample collection method.[10,11]

A major but easily avertible disadvantage of urine samples is that they require stringent conditions for storage and transport, especially for long distances in hot conditions, which are common in low-income countries where clinics or reference laboratories are often far from rural areas. The Dried Urine Spot (DUS), which is a urine sample collected and dried on filter paper, can help overcome these challenges, offering significant logistical advantages. In fact, DUS are easily transportable and can be stored at room temperature (RT), while also reducing the potential biological risk associated with urine leakage. A previous study conducted by our group showed high sensitivity (98.2% and 96.4% at 1 and 4 weeks' storage, respectively) and specificity (100% at both storage times) and a high concordance rate ($k \geq 0.81$, "almost perfect") for HPV testing from DUS compared with fresh urine samples.[12]

Urine/DUS collection could easily help overcome both socio-cultural barriers and technical-practical difficulties associated with HPV testing, and automated testing systems already in-use may facilitate sample processing in resource-limited settings. Therefore, we combined a urine sampling strategy with the automated platform GeneXpert® System for HR-HPV detection. We evaluated this system by testing samples collected from women attending an outpatient clinic in Milan, Italy, and provide here the proof-of-concept for the development of a screening method that could aid cervical cancer prevention, especially in high-burden LMICs, by increasing the acceptance of, and adherence to, HPV screening in high-risk populations.

2 | MATERIALS AND METHODS

Urine samples (at least 30 mL) were collected in sterile containers from 60 women (age range: 23–50 years, median age: 31 years; interquartile range: 28–37) attending the Infectious Diseases outpatient clinic of ASST Santi Paolo e Carlo (Milan, Italy) as part of their follow-up visits for HPV or other sexually transmitted infections/diseases and were used, both in liquid and dry form, for Xpert® HPV testing (Cepheid). All women provided informed consent approved by the Ethics Committee of the Institution (Comitato Etico, ASST Santi Paolo e Carlo, Milan, Italy) for further anonymous

research testing on the residual samples after routine analyses. Samples were stored at 4°C until processing, performed within 36 h. A corresponding cytological or histological diagnosis performed on a brush sample collected during the same visit was available for each patient: 16 (26.7%) had normal cytological results, 17 (28.3%) showed atypical squamous cells of undetermined significance (ASCUS), 22 (36.7%) low-grade squamous intraepithelial lesion (L-SIL), and 5 (8.3%) high-grade squamous intraepithelial lesion (H-SIL), CIN3 (Cervical Intraepithelial Neoplasia of grade 3), or AGC (Atypical Glandular Cells).[13]

2.1 | Sample pretreatment

A total of 10 mL of fresh urine was centrifuged at 4500 rpm for 15 min to obtain the cellular component (opaque phase), while the clear upper phase (8.5 mL) was eliminated. The remaining urine was centrifuged again at 3500 rpm for 5 min and the upper phase (0.5 mL) was eliminated to obtain 1 mL of concentrated urine. For each urine sample, pretreatment was simultaneously performed three times, and 1 mL was used as input for the Xpert® HPV test (Cepheid), 1 mL was dried on filter paper (DUS preparation), and 1 mL was used for in-house detection and genotyping. The conditions of DUS use (number of spots, volume, and type of elution buffer) were adapted for the Xpert® HPV Test by modifying the protocol previously described by Frati et al.[12] Specifically, 100 µL aliquots of concentrated urine were spotted on five preprinted circles of two filter paper cards (Euroimmun, PerkinElmer Health Sciences), which were left to dry for at least 2 h and then stored in paper bags in a dry location at RT (25°C–30°C). At the moment of testing (within few days),[12] 8 circles were cut out from the DUS filter paper cards using a sterile scissor, broken into small pieces, submerged in 1.7 mL of Dulbecco's phosphate buffered saline (PromoCell), and incubated overnight at 4°C. The following day, the whole recovered liquid (about 1.2 mL) was quickly centrifuged to remove any paper residue and used as input for the Xpert® HPV test on the Cepheid GeneXpert® System.

2.2 | In-house HPV detection and genotyping

Concentrated urine was tested following a quality-controlled protocol for HPV detection and genotyping previously validated to be used with urine samples.[6] Briefly, HPV DNA was extracted from 1 mL of each sample with the NucliSENS® easyMAG™ automated platform (bioMérieux bv) and eluted in 100 µL of elution buffer. The concentration and purity of extracted DNA were evaluated with the NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and DNA quality and integrity was assessed by amplifying a 268 bp (base pair) segment of the ubiquitous β-globin gene using the GH20 and PCO4 primer pair.[14] HPV DNA was detected through an in-house PCR amplification of a 450 bp L1 fragment using 10 µL of isolated DNA and the degenerated primer pair ELSI-f and ELSI-r.[6] Amplified products were genotyped with the restriction fragment length polymorphism (RFLP) method, able to identify all HR- and low risk (LR-) HPV types of the genus *Alphapapillomavirus* according to the latest International Agency for Research on Cancer (IARC) classification system (HR types, group 1: HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59; group 2A: HPV-68; group 2B: HPV-26, 30, 34, 53, 66, 67, 69, 70, 73, 82, 85; LR types: HPV-6, 11, 28, 32, 40, 42, 43, 44, 54, 55, 57, 61, 62, 71, 72, 74, 81, 83, 84, 86, 87, 89).[15] Samples that for two consecutive times could not be assigned with confidence to a certain type were classified as "untyped".

Samples that tested negative (NEG) or weakly positive (W) as well as "untyped" samples were subjected to a second amplification step (nested-PCR) with primer pair Gp5+ and Gp6+[16,17] (amplified product: 150 bp). The nested-PCR assay was performed using 10 µL amplified DNA, GoTaq® DNA Polymerase (Promega), and following these conditions: 5 min at 94°C, 25 cycles of 30 s at 94°C, 30 s at 40°C, and 30 s at 72°C, followed by a 7 min step at 72°C. Amplicons from positive samples were purified with the NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel GmbH & Co. KG) and, if suitable (DNA concentration ≥15 ng/µL), outsourced for Sanger sequencing at Microsynth AG. HPV genotypes were identified by comparing obtained nucleotide sequences to reference strains in the GenBank database through BLAST.[18]

2.3 | Xpert® HPV test

The protocol for using the Xpert® HPV Test from urine samples has been optimized in our laboratory by testing HPV-16 DNA-positive urine samples at known concentration and subjected to serial dilutions (from 5×10^6 genomic copies/mL to 5×10^3 genomic copies/mL), all above the limit of detection of the test for HPV-16 (2903 IU/mL equal to 2.7×10^3 genome equivalent/mL)[19,20] (data not shown).

A total of 1 mL of concentrated urine or DUS preparation was loaded in the single-use GeneXpert® cartridge that includes reagents for the detection of HPV DNA of 14 HPV-types (detected in different channels), a human reference gene (HMBS—hydroxymethylbilane-synthase), and an internal Probe Check Control that verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability. The 14 targeted HPV types are detected in five fluorescent channels:

- fluorescent channel 1 (16) detects HPV-16
- fluorescent channel 2 (18/45) detects HPV-18 and/or -45
- fluorescent channel 3 (P3) detects HPV-31 and/or -33, -35, -52, -58
- fluorescent channel 4 (P4) detects HPV-51 and/or −59
- fluorescent channel 5 (P5) detects HPV-39, and/or -56, -66, -68.

The Xpert® HPV Test was run on GeneXpert IV (Cepheid). Each test was considered "valid" if sufficient signal was detected for the human reference gene. The assay results were reported as an overall "positive" if any type of targeted HPV was detected, and specific

positive and negative results were collected for each type or type group identified by one channel.

2.4 | Statistical analysis

Concordance between detection methods was assessed using the Kappa statistic (Cohen's unweighted Kappa, *k*) and defined as "poor" ($k = 0$), "slight" ($0.01 < k < 0.20$), "fair" ($0.21 < k < 0.40$), "moderate" ($0.41 < k < 0.60$), "substantial" ($0.61 < k < 0.80$), "almost perfect" ($0.81 < k < 1.00$), or "perfect" ($k = 1$).

3 | RESULTS

3.1 | HPV detection and genotyping

The β-globin gene was amplified from all 60 urine samples and 78.3% (47/60) of the samples tested positive for HPV-DNA. Genotyping by RFLP was successful for 30 fresh urine samples, while 17 samples resulted positive by nested-PCR, and 16 of these were genotyped by sequencing. One urine sample was "untyped." A total of 37 single infections and 9 infections sustained by two or more types were found in the 46 samples.

Figure 1 shows the distribution of HPV types detected in urine samples by in-house PCR and genotyping divided according to whether they can be detected by the five channels (16, 18/45, P3-P5) of the Xpert® HPV test.

A total of 35 out of the 46 successfully typed samples (76.1%) matched a genotype detectable by Xpert® HPV test. Table 1 shows the genotypes detected in HPV-DNA positive samples according to the cytological/histological status of the respective women and detectability by the Xpert® HPV test.

3.2 | Xpert® HPV testing

Xpert® HPV testing on paired fresh urine and DUS samples was carried out on a total of 45 urine samples (Table 2) selected as follows: 28 HPV-DNA positive specimens (groups A1 and A2), 22 of which positive for HPV types detectable by Xpert® HPV Test (group A1) and 6 positive for HPV types not detectable by Xpert® HPV Test (group A2); 13 HPV-DNA negative specimens (group B); 4 HPV-DNA positive specimens characterized by low load (as they were only positive after nested-PCR) of HPV types detectable by Xpert® HPV Test (group C). Paired urine and DUS samples were tested simultaneously by Xpert® HPV Test.

The Xpert® HPV Test was able to detect HPV infection in 19/22 (86.4%, 95% confidence interval: 66.7%–95.3%) nonweakly positive samples containing detectable genotypes (group A1). Specifically, HPV infection was detected in 71.4% (5/7) fresh urine samples collected from women with normal cytology, 75.0% (3/4) of those collected from women with ASCUS lesions, and in 100% of those with L-SIL (8/8) and H-SIL/CIN-3 (3/3) lesions (Table 3). No tests were considered "invalid". In the same group (A1), discordant results (3/22) occurred for the P3 channel in one case (HR-HPV-31, IARC

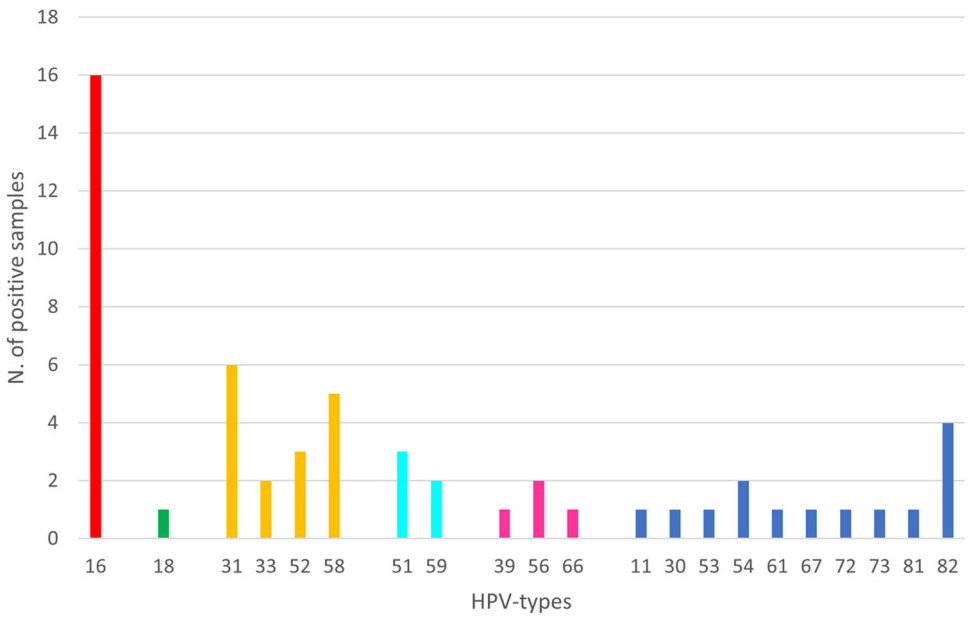

FIGURE 1 Distribution of HPV types detected through in-house PCR and genotyping in 46 urine samples. Red: genotype detected by Xpert® HPV test HPV-16 channel; green: genotypes detected by Xpert® HPV test HPV-18, 45 channel; orange: genotypes detected by Xpert® HPV test P3 channel; light blue: genotypes detected by Xpert® HPV test P4 channel; pink: genotypes detected by Xpert® HPV test P5 channel; blue: HPV genotypes not detectable with the Xpert® HPV test. HPV, human papillomaviruses.

TABLE 1 HPV genotyping results stratified according to genotype and cytology of the respective women.

Cytology (No. of women)	HPV-16	HPV-18, 45	P3[a]	P4[a]	P5[a]	Other types[a]	Untyped	Negative
Normal (16)	3	0	5	1	2	1	0	4
ASCUS (17)	3	1	1	2	1	5	1	5
L-SIL (22)	7	0	8	0	1	5	0	3
H-SIL/CIN3/AGC (5)	3	0	1	1	0	0	0	1
Regardless of cytology	16	1	15	4	4	11	1	13

Abbreviations: AGC, atypical glandular cells; ASCUS, atypical squamous cells of undetermined significance; CIN3, cervical intraepithelial neoplasia of grade 3; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion.

[a]P3 channel detects HPV-31 and/or 33, 35, 52, 58; P4 channel detects HPV-51 and/or 59; P5 channel detects HPV-39 and/or 56, 66, 68; other types are those not detected by the Xpert® HPV Test.

group 1) and for the P5 channel in two cases (HR-HPV-39, IARC group 1, and HR-HPV-66, IARC group 2B). Both HPV-DNA negative (13/13, 100%) and HPV-DNA positive samples undetectable by Xpert® HPV test (6/6, 100%) yielded the expected result (Table 2). Considering groups A1 and B, the percentage agreement between the Xpert® HPV test and the in-house PCR from urine was 91.4%, with a Cohen's kappa = 0.82 (almost perfect).

Finally, 17 out of 19 (89.5%) samples in group A that tested positive with the Xpert® HPV on concentrated urine were also positive when tested in DUS format. Most importantly, HPV infection was detected in all samples collected from women with L-SIL (8/8) and H-SIL/CIN-3 (3/3) lesions (Table 3). All of the 13 DUS from HPV-DNA negative samples were negative.

4 | DISCUSSION

Cervical cancer is a preventable disease. Yet it remains one of the most common cancers and a leading cause of cancer death in women worldwide. The implementation of cost-effective preventive measures that take into account not only women's needs, but also personal, cultural, social, structural, and economic barriers hindering their access to health services, are critical to reduce the impact of cervical cancer and pursue its elimination.[3] To date, different types of cervical cancer screening tests are available and include conventional cytology test, liquid-based cytology, visual inspection with acetic acid (VIA), and HPV testing from cervical brush. However, to be appropriately performed, any of these screening test types requires adequate financial resources, developed infrastructure, and trained personnel. In addition, monitoring the outcomes of screening, treatment, and follow-up measures must be enforced to ensure adequacy and effectiveness of the prevention interventions.[21,22] Using existing local expertise and infrastructure may be a good approach to ensure that screening needs are met with low investment. To accelerate towards the 70% screening goal, the WHO strategy for cervical cancer elimination encourages, in fact, the use of integrated technological platforms that are already widely employed to test for HIV, tuberculosis, and several other infections.[3] The GeneXpert® System (Cepheid) is one of these platforms that is already widely used for HPV testing in high-income countries. While the unit cost of the Xpert® HPV test is not negligible for LMICs, this is a high-performance test (as required by the WHO) that has the advantage of providing same-day screening results, allowing for immediate and early treatment beginning.

Xpert® HPV test has been developed and approved for testing cytological samples collected using cervical swabs. However, the introduction of less invasive and more acceptable sampling methods is pivotal to improve screening coverage in every setting, and particularly in LMICs, since they allow overcoming social and cultural barriers, misconceptions, and prejudices. These impediments negatively impact the number of target women that can be successfully reached and, therefore, population interventions at-scale. Urine, in addition to being a more acceptable and less expensive sample, can be easily collected bypassing medical examination. Urine in dried form (DUS) can be a possible additional alternative because it has the added advantage of overcoming difficulties related to storage and transportation conditions, allowing to facilitate HPV screening of women living in rural areas and/or far from clinics. Urine samples were used in this study in combination with the Xpert® HPV Test and results were compared to a validated HPV testing method based on PCR and molecular typing.[6] A high concordance (91.4%, $k = 0.82$) was found between the PCR test and the Xpert® HPV Test from urine.

Several authors[10,11] have reported lower sensitivity (up to 9-fold lower[23]) when HPV testing is performed on urine samples compared with cytological specimens. Recently, Marcus et al.[24] described the application of the Xpert® HPV test on the GeneXpert® platform using urine samples as feasible and reproducible, although they concluded that the analytical sensitivity needed to be improved. Our experience[6] suggests that some methodological details related to sample collection and processing before testing may be the key elements leading to improved test sensitivity. In fact, our protocol includes an initial concentration step, performed through a medium-speed centrifugation of the urine sample, to concentrate cell-associated viral DNA and obtain viral loads comparable to those found in cervical brushes. Although still a challenge in extremely rural areas, this step can be easily carried out with basic facilities and by

TABLE 2 HPV-DNA detection and genotyping in paired urine and DUS samples using in-house PCR and Xpert® HPV test.

Sample_ID	Cytology	HPV-DNA	HPV typing	Expected Xpert® HPV	Xpert® HPV urine	Xpert® HPV DUS
Group A1. HPV-DNA positive by *in-house* PCR and detectable by Xpert® HPV test						
1	Normal	POS	31	P3	NEG	INVALID
7	Normal	POS	31	P3	P3	P3
9	Normal	POS	31	P3	P3	P3
11	Normal	POS	66	P5	NEG	NEG
14	Normal	POS	59	P4	P4	NEG
16	Normal	POS	52, 58	P3	P3	P3
22	Normal	POS	56	P5	P5	P5
10	ASCUS	POS	39	P5	NEG	INVALID
12	ASCUS	POS	16, 18	16/18/45	16/18/45	16/18/45
20	ASCUS	POS	51	P4	P2/P4	NEG
21	ASCUS	POS	31, 51	P3/P4	P3/P4	P3/P4
3	L-SIL	POS	16	16	16/P3	16/P3
4	L-SIL	POS	16, 52	16/P3	16	16/P3
5	L-SIL	POS	16	16	16	16
8	L-SIL	POS	16, 52	16/P3	16/P3	16/P3
13	L-SIL	POS	16	16	16	16
15	L-SIL	POS	16	16	16/18/45/P3/P5	16
18	L-SIL	POS	33	P3	P3	P3
19	L-SIL	POS	58	P3	P3	P3
2	CIN3	POS	16	16	16	16
6	H-SIL	POS	16, 58	16/P3	16/P3	16/P3
17	H-SIL	POS	51, 59	P4	P3/P4	P3/P4
Group A2. HPV-DNA positive by *in-house* PCR but not detectable by Xpert® HPV test						
23	ASCUS	POS	72	NEG	NEG	NEG
27	ASCUS	POS	54, 61, 81	NEG	NEG	NEG
24	L-SIL	POS	54	NEG	NEG	NEG
25	L-SIL	POS	82	NEG	NEG	NEG
26	L-SIL	POS	11, 82	NEG	NEG	NEG
28	L-SIL	POS	53	NEG	NEG	NEG
Group B. HPV-DNA negative by *in-house* PCR (*nested*-PCR result)						
33	Normal	NEG (NEG)	–	NEG	NEG	NEG
35	Normal	NEG (NEG)	–	NEG	NEG	NEG
36	Normal	NEG (NEG)	–	NEG	NEG	NEG
40	Normal	NEG (NEG)	–	NEG	NEG	NEG
29	ASCUS	NEG (NEG)	–	NEG	NEG	NEG
32	ASCUS	NEG (NEG)	–	NEG	NEG	NEG
34	ASCUS	NEG (NEG)	–	NEG	NEG	NEG
37	ASCUS	NEG (NEG)	–	NEG	NEG	NEG

TABLE 2 (Continued)

Sample_ID	Cytology	HPV-DNA	HPV typing	Expected Xpert® HPV	Xpert® HPV urine	Xpert® HPV DUS
38	ASCUS	NEG (NEG)	–	NEG	NEG	NEG
31	L-SIL	NEG (NEG)	–	NEG	NEG	NEG
39	L-SIL	NEG (NEG)	–	NEG	NEG	NEG
41	L-SIL	NEG (NEG)	–	NEG	NEG	NEG
30	AGC	NEG (NEG)	–	NEG	NEG	NEG
Group C. HPV-DNA weakly POSITIVE by *in-house* PCR (*nested*-PCR result)						
43	Normal	W[a] (POS)	16	16	NEG	NEG
44	Normal	W[a] (POS)	16	16	16	NEG
45	Normal	W[a] (POS)	16	16	NEG	NEG
42	L-SIL	W[a] (POS)	58	P3	NEG	NEG

Abbreviations: DUS, Dried Urine Spot; HPV, human papillomaviruses; L-SIL, low-grade squamous intraepithelial lesion; PCR, polymerase chain reaction.
[a]W = weakly positive in the first PCR step (ELSI).

TABLE 3 Percentage of PCR-positive women from group A1 that tested positive with the Xpert® HPV test performed on urine and DUS stratified according to their cytological status.

	% Positive urine	% Positive DUS
Total	86.4	77.3
Normal cytology	71.4	57.1
ASCUS	75.0	50.0
LSIL	**100**	**100**
HSIL/CIN3	**100**	**100**

Abbreviations: ASCUS, atypical squamous cells of undetermined significance; CIN3, cervical intraepithelial neoplasia of grade 3; DUS, Dried Urine Spot; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion.

most local health centers. Additionally, since the purpose of this pretreatment is to concentrate the cellular component, it can be substituted by letting the sample spontaneously precipitate until the opaque phase (cells in solution) is visible. This can even be facilitated by the use of inexpensive and commercially available hand centrifuges, further reducing the reliance on specialized instruments or experienced personnel that may be lacking in remote rural areas where samples are collected. Finally, the addition of specific buffers containing nuclease inhibitors to fresh urine can be implemented to enable the detection of cell-free HPV,[25] avoiding the centrifugation and reconstitution steps but increasing pretreatment costs and adding difficulties linked to reagent obtainability.

Regarding DUS, this sample requires additional processing and an overnight reconstitution step, which increase complexity and processing time at the receiving laboratory. However, the use of commercially available prepunched discs could help reduce processing times and chances of contamination.

In our study, the Xpert® urine HPV Test detected 71%–75% of positive samples collected from women with normal/ASCUS cytology. More importantly, the presence of HR-HPV infection was detected in 100% of the nonweakly PCR positive samples collected from women with low- and high-grade lesions, the most at risk of developing cancer. Notably, also 100% of DUS from women with L-SIL and H-SIL were correctly identified.

Overall, fresh urine should be used as the sample of choice whenever proper storage and transport conditions are possible. However, testing from DUS could still allow detection of clinically significant HR-HPV infections, at least for women living far from clinics and when fresh urine transportation is problematic.

It is known that contamination of urine with infected exfoliated cervical cells increases in the presence of progressively higher-grade cervical lesions. Therefore, failure to detect positivity in cases of low viral loads, typically observed in women with normal cytology or ASCUS,[26] could be acceptable for an HPV screening method, provided that strategies are put in place to ensure adequate recalls.

Furthermore, although whether viral DNA load can be used as a reliable marker to predict cervical lesions remains controversial, several studies described a direct correlation between increasing viral load and the probability of a CIN2/CIN3 in ASCUS cases,[26–28] suggesting a higher probability of HPV infection clearance when viral loads are low.

As the main requirement for a test to be considered a valid screening tool is the ability to detect persistent HPV infections, either already associated with cervical lesions or with the potential to evolve into cervical disease (early detection), HPV detection in urine could be considered a valid screening test, as it can successfully identify high-risk conditions.

Finally, since the Xpert® HPV Test cannot detect infections sustained by some of the HR types less frequently identified in women with cervical lesions, and since epidemiological data on HPV

infection (e.g., prevalence of infection, distribution of infecting types, association with different degrees of disease) are not always known, especially in many LMICs, targeted epidemiological studies are needed to fill these knowledge gaps and inform test upgrades to improve diagnosis and screening capabilities when needed.

In conclusion, the Xpert® HPV test seems to perform well when adapted to detect HPV infections in urine and, as a second choice, in DUS. These preliminary findings suggest that urine could reliably be used in this system as an alternative to samples requiring more invasive collection methods. This is highly relevant in LMICs and in rural areas where cytological brush collection and testing are today unfeasible.

Based on these positive observations, we have recently started a field application of Xpert®-based HPV testing using urine samples in eSwatini (South Africa), a country with a high urgency for intervention against HPV infection and cervical cancer given that it suffers from the highest incidence and mortality rate for cervical cancer worldwide. This operational research project will be critical in assessing feasibility, acceptability, and cost-effectiveness/benefits of the program. In addition, it will allow the evaluation of clinical sensitivity and specificity of the Xpert HPV® test on urine by collecting data from an appropriate number of women and according to cytological and disease status.

The more women are reached and diagnosed through screening strategies, the fewer will suffer the negative consequences of HPV infection, and the easier and faster will be the achievement of the WHO goal of global cervical cancer elimination.

AUTHOR CONTRIBUTIONS

Elisabetta Tanzi, Silvia Bianchi, and Antonella Amendola: developed the idea, designed the study, and were responsible for the accuracy of the data analysis. **Mario Raviglione**: helped realize the idea and make the study feasible. **Silvia Bianchi, Clara Fappani, Maria Gori, Daniela Colzani, and Ilaria Passera**: conducted experiments, collected and analysed data, and created graphs and tables. **Elisabetta Tanzi and Clara Fappani**: wrote the manuscript, and **Antonella Amendola** critically revised the manuscript. **Camilla Tincati**: collected clinical specimens and revised the manuscript. **Mario Raviglione and Marta Canuti**: provided advice and suggestion for improving the manuscript. All authors reviewed and approved the final version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data relevant to the study are included in the article or uploaded as supplementary information.

ETHICS STATEMENT

All participants signed an informed consent giving permission for anonymized data and samples use for research purposes. Informed consent was approved by the Ethics Committee of ASST Santi Paolo e Carlo, Milan, Italy. All methods were carried out in accordance with relevant guidelines and regulations. The authors assert that all procedures contributing to this work comply with ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

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