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Dried cervical spots for Human Papillomaviruses identification

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Abstract

Financial and operational constraints limit low-resource countries in the screening of high-risk genital Human Papillomaviruses (HR-HPV), the etiological agents of cervical cancer. With its simple storage, conservation and shipping, dried cervical sample (DCS) could represent an efficient tool.

The aim of the study was to evaluate the reliability of HPV genotyping from DCS. Cervical samples were obtained from 50 women infected with HIV-1 in Côte d'Ivoire. After DNA extraction from both DCS and matched liquid cervical samples (LCS), HPV genotyping was performed and the concordance of genotyping results was evaluated.

HPV prevalence was 88 % in LCS and 78 % in DCS. kappa statistic was 0.51 for the presence of any genotype (95% confidence interval, 0.25-0.77) and 0.73 for HR-HPV (0.45-0.99). Out of 50 samples, 45 were HPV-positive for DCS and/or LCS, and HR-HPV were detected in 37 samples (74%) with 36 HR-HPV multiple infections. Any genotype and HR genotype identification was concordant/compatible in 86% (43/50) and 88% (44/50) of samples, respectively. In most instances, kappa statistics for detection of type-specific HPV was over 0.6 (including

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Competing interests

The authors declare that they have no conflict of interest.

Ethical approval

This study was approved by the national ethics committee for HIV research in Côte d'Ivoire, and all women gave their informed consent prior to participation.

HPV-16,-18,-31-33). An excellent agreement (κ statistic = 0.81) was evidenced for eight genotypes (HPV-6, -31, -35, -40, -56, -58, -66, -82).

In spite of interfering factors (multiple infections, different HPV loads, amplification competition, different inputs), DCS and LCS led to concordant/compatible results in most cases. DCS could represent an efficient tool for epidemiological field studies in resource-limited settings, and more importantly for improving the screening coverage and care management in women infected with HPV.

Keywords

Human Papillomavirus; cervical cancer screening; cervical sample; dried spot; HPV genotyping

Introduction

Cervical cancer is the second most common cancer among women worldwide [Ferlay et al., 2010]. Human Papillomaviruses (HPV) are responsible for carcinogenic and non carcinogenic cervical lesions. To date, more than 100 different HPV genotypes have been fully characterised and about one-third infect epithelial cells in the genital tract [de Villiers et al., 2004; IARC, 2007]. Year after year, molecular epidemiologic data yield sufficient evidence to classify genotypes according to their oncogenic risk. Indeed, high-risk genital human papillomaviruses (HR-HPV) are a necessary cause of high-grade cervical neoplasia. Following the last classification of the International Agency for Research on Cancer (IARC) [2011], HPV types are considered as carcinogenic to humans when belonging to Group I (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66) and as probably carcinogenic when in Group 2A (type 68).

Most women are infected shortly after the beginning of their sexual activity [Kjaer et al., 2001]. HPV multiple infections are frequent, likewise sequential detection of different HPV types. However, the majority of infections are transient, since a spontaneous viral clearance occurs within one to two years [Ho et al., 1998; Plummer et al., 2007]. The risk for cervical intraepithelial neoplasia grade 3 and cancer is increased when a HR-HPV persistence is observed [Koshiol et al., 2008].

For years, cervical cytology screening has been performed to detect equivocal cytology or neoplastic lesions. However, it is showing a limited sensitivity for the detection of high-grade cervical intraepithelial neoplasia [van den Akker-van Marle et al., 2002; Cuzick et al., 2006]. Recently, HPV molecular tests for detection and/or identification of HPV genotype(s) have been introduced as an adjunct to cytology. In sub-Saharan Africa, financial and operational constraints limit the access to HR-HPV infection detection. Many of these countries bear a high prevalence of human immunodeficiency virus (HIV) infection, and women living with HIV present an increased risk to be co-infected with HPV [Grulich et al., 2007]; cervical cancer has been classified as an AIDS-defining disease since 1993 [Control, 1992].

Since the development of dried blood spots (DBS) in 1963 by Guthrie, DBS have been used in the postnatal screening of congenital disorders and metabolic diseases. These solid carriers show many advantages: no bulky storage, conservation at room temperature, easy and low cost shipping of samples with a regular mailing at ambient temperature. Consequently, DBS are particularly attractive for developing countries and have been adapted for viral detection, quantitation or genotyping of viruses such as HIV, hepatitis B and C viruses [Mwaba et al., 2003; Reigadas et al., 2009; Komas et al., 2010; Tuaillon et al., 2010]. More recently, dried spots have been exploited for other biological fluids.

To facilitate studies in low-resource countries, blotting paper was evaluated in this work for conservation and secondary detection of HPV: dried cervical spots (DCS) were performed from cervical samples of women living with HIV in Côte d'Ivoire and screened for HPV infection and genotype identification. The aim was to compare the results of HPV genotyping performed from liquid cervical samples (LCS) and paired DCS.

Materials and Methods

Patients

A cervical cancer screening program based on visual inspection methods was proposed in Abidjan, Côte d'Ivoire, to women of child-bearing age attending three HIV clinics for their routine HIV follow-up within the IeDEA West Africa Collaboration, as described elsewhere [Horo et al., 2012]. All women gave their informed consent. Prior to the application of coloration (acetic acid and Lugol's iodine) for visual inspection of the cervix, 510 women also underwent a cervical sample collection stored at -80°C and shipped to the Bordeaux University for HPV genotype identification. A subset of 50 cervical samples was then consecutively selected to perform HPV genotyping from both dried spots and liquid cervical samples.

Cervical samples and extraction procedures

Study design is described in Figure I. Briefly, cervical swabs were collected by midwives and discharged in viral transport medium containing antibiotics (Penicillin/Streptomycin (3.125 $\mu\text{g/mL}$), Gentamycin (0.05 $\mu\text{g/mL}$)), and antifungal agent (Fungizone (0.625 $\mu\text{g/mL}$)). Samples were kept at -80°C , until frozen shipment to the Laboratory of Virology of the University Hospital of Bordeaux, France, where they were stored at -80°C .

Before use, samples were thawed and vortexed during 20 seconds. Total nucleic acid extraction was performed from 200 μl added to 2 ml of NucliSENS Lysis Buffer (bioMérieux, Marcy l'Etoile, France). Additional 50 μl were dispensed on filter paper (two spots with 25 $\mu\text{l/spot}$) (Whatman 903, GE Healthcare Bio-Sciences Corp., Westborough, MA); after an overnight drying, spots were stored at ambient temperature for one to two weeks into an auto-sealed storage bag with both desiccant and humidity marker; then, DNA extraction was performed from two cut-out spots (two circles of 13 mm diameter) transferred into 2 ml of NucliSENS Lysis Buffer, and filter papers were removed from the buffer after a 30 minutes incubation with rotation. The following steps were identical for both extractions performed with NucliSENS Magnetic Extraction Reagents using

NucliSENS MiniMAG (bioMérieux, Marcy l'Etoile, France) according to manufacturer's instructions. Nucleic acids were eluted with 60µl of the NucliSENS elution buffer. DNA was stored at -80°C until use.

HPV Genotyping

After thawing, genotyping was performed in the same run for paired LCS and DCS with INNO-LiPA HPV Genotyping *Extra* kit (INNOGENETICS, Les Ulis, France). This assay was based on the amplification of a 65 bp fragment within L1 with SPF10 primers from an input of 10µl (10 min at 37°C, 9 min at 94°C, and 40 amplification cycles: 94°C, 30 sec; 52°C, 45 sec; 72°C, 45 sec). Then, 10µl of the biotinylated amplicons were denatured and hybridized with 28 specific oligonucleotide probes immobilized as parallel lines on a single membrane strip along with four control lines (conjugate control, human DNA (hDNA) control and two HPV controls (pools of HPV probes)).

The primers pair for the amplification of hDNA, targeting the human HLA-DPB1 gene, was used to monitor sample quality and extraction. In the absence of HPV positive signal, a result was validated as HPV-negative if the hDNA was successfully amplified.

HPV types for which the type-specific line pattern was a subset of the full line pattern of another genotype observed in the strip were scored as possibly present.

When a genotype was not identified (no type-specific line with at least one positive HPV control line) or when its lines pattern was not complete, HPV was untypeable (HPV X).

HPV strips were scanned and analysed thanks to the Line Reader and Analysis Software (LiRAS) for LiPA HPV software. The interpretation followed the classification of Munoz N. et al. for the IARC [2003]: 15 HPV were classified as HR (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82), three HPV as probable HR (26, 53, 66), and seven HPV as LR (6, 11, 40, 43, 44, 54, 70). Finally, three additional types were considered as undetermined risk genotypes (69, 71, 74).

Statistical analysis

The proportion of agreement between paired LCS and DCS was calculated by dividing the number of samples tested positive or negative for HPV with both the LCS and DCS by the total number of samples testing with both LCS and DCS. To determine the proportion of agreement beyond that expected by chance, the kappa (κ) statistic was calculated by dividing the difference between the observed proportion of agreement and the expected proportion agreement by 1 minus the expected proportion of agreement. Proportions of agreement and κ were calculated considering several endpoints: the presence or absence of any HPV infection and the presence or absence of each HPV genotype considered separately.

For comparison of HPV genotyping from LCS and DBS, genotype results were considered as concordant when identical HPV type specific profile was obtained from both extracts, compatible when one or more genotypes were identified with both methods and discordant when no common genotype was found after both extractions.

Results

A total of 50 women infected with HIV-1 were screened; median age (interquartile range, IQR) was 38 years (32-46) and median CD4+ T cells, 440 cell/mm³ (IQR: 295-593). Seven women presented with a positive cervical visual inspection (14%): two samples were HPV negative with both methods, five samples were multiple HR-HPV infected. Six women went through a cervical biopsy, all with a result of condyloma or low-grade cervical intraepithelial neoplasia.

Out of 50 samples, the prevalence of any HPV type was 88 % in LCS and 78 % in DCS: 144 infections with 24 different genotypes, and 117 infections with 23 genotypes detected, respectively. Two or more HPV were found in 37 (74%) LCS and 30 (60%) DCS. Except for one case with only HPV-66, HR-HPV positive samples showed the presence of two or more HR genotypes.

The most frequently detected HR-HPV in LCS and DCS were genotype 51 (20% and 14%, respectively), genotype 52 (14% and 20%, respectively, reaching 32 and 24%, respectively, when this genotype was considered as possibly present), and genotypes 31 and 53. For LR-HPV, genotype 44 was the most often identified (Table I).

Among the 50 samples, five were HPV negative with both methods and 45 were HPV positive: 44 samples were LCS positive of which 38 (86.4%) were also DCS positive, and one sample was positive only in DCS. κ statistic for any type of HPV was 0.51 (95% CI, 0.25 to 0.77). HR-HPV were detected in 37 (74%) samples: 31 LCS positive /DCS positive, five LCS positive /DCS negative and one LCS negative /DCS positive. κ statistic for HR-HPV was 0.73 (95% CI, 0.45 to 0.99). The majority of κ statistics for detection of specific genotype was over 0.6, which represents a good agreement (including HPV-16, HPV-18, HPV-31 and HPV-33). For eight genotypes (6, 31, 35, 40, 56, 58, 66 and 82), an excellent agreement (κ statistics = 0.81) was evidenced (Table I).

Finally, results of any genotype and HR genotype identification after both extractions were concordant in 20/50 (40%) and 27/50 (54%) of samples, respectively; results were compatible in 23/50 (46%) and 17/50 (34%) of samples, respectively. A total of nine samples showed discordant results, of which one LCS negative /DCS positive (sample 19), with no HR-HPV (Table II). Six paired samples were LCS positive /DCS negative, of which two without presence of HR-HPV.

Discussion

Following a single sampling of cervical exfoliated cells in a liquid transport medium, HPV genotyping was analysed after two different extractions. Other groups have demonstrated that dry collection was suitable for reliable genotyping [Kailash et al., 2002; Gustavsson et al., 2009; Feng et al., 2010]. However, they compared genotyping from different materials (for example, two successive Dacron swabs; brushed cells applied on filter paper card compared to the remaining material). In the present work, a single sample was analysed twice, and a software for strips reading, enabling objective interpretation and comparison, was used. Thus, these results should allow a (more) accurate technical comparison.

Among the 50 samples, 45 were HPV-positive after one or both extractions (88 % of any HPV-positivity in LCS and 78 % in DCS). This high prevalence was certainly due to the HIV-positivity of this population, which is known to show frequent HPV co-infections, as already reported in African countries [Didelot-Rousseau et al., 2006; Safaeian et al., 2008; Luchters et al., 2010]. However, the present study was neither focusing on HPV epidemiology nor on women with invasive cervical cancer; indeed, only seven women showed a positive visual examination. These 50 samples cannot be considered as a representative sampling of the women infected with HIV attending the National Blood Transfusion Center in Abidjan. Since HPV infection may be detected with normal cervical cytology [Bruni et al., 2010], paper samples could allow the prospective detection of HR-HPV in women without cervical lesions or without gynecological follow-up.

Fewer infections were detected from DCS than from LCS (117 and 144 infections, respectively). Even though this assay does not provide a quantitative approach, genotypes not detected from DCS could be present with a low viral load. The most frequently detected HR-HPV were types 31, 51, 52 and 53 (Table I). The predominance of other types than HPV-16 has been already described in women living with HIV [Clifford et al., 2006]. In addition, these women are more frequently multi-infected with two or more genotypes; the present work underlines that in all patients but one infected with HR-HPV, several HR genotypes were detected. Amplification competition could hamper detection of one or the other genotype or lead to a variable recovery of HPV genotypes, thus with a compatible or discordant result between paired extracts [Dunn et al., 2007; Dona et al., 2011]. Consequently, in this study, the classification of concordance/compatibility/discordance was strict and rigorous according to this multi-infected population. Nevertheless, genotyping results showed concordance/compatibility in 86% (43/50) of samples for any HPV genotype and in 88% (44/50) for HR-HPV.

Banura et al. [2008] compared DCS and LCS for detection of cervical HPV, resulting in a poor agreement with a best performance for LCS (κ statistics, 0.18). This may be due to the difference of inputs for their two extractions: a punch of 4 mm of Whatmann paper on which was smeared a cervical swab, and 200 μ l of LCS obtained from the remaining cervical material. In the present study, a higher global agreement was reached between the two assays (κ statistics, 0.51), and even higher for 18/24 specific genotypes (κ statistics 0.61), starting from 200 μ l for LCS and 50 μ l for DCS. It was not possible to dispense a larger volume on DCS because liquid would have spread over and too much filter paper would have been embarked for extraction, impairing its efficiency. However, when cells were concentrated by sample centrifugation before depositing $2 \times 25 \mu$ l on filter paper, no additional genotypes were detected in the six samples assayed (data not shown). Together with other studies [Kailash et al., 2002; Gustavsson et al., 2009; Feng et al., 2010], this work further supports dry and cheap sample storage for HPV testing.

Out of nine discordances between LCS and DCS results (Table II), six samples were LCS positive /DCS negative for any type. Five were LCS positive /DCS negative for HR-HPV but one showed a positive result in DCS for HPV X, so that identification of four HR-HPV infections was missed by DCS. One may conclude that extraction from DCS should be improved, but it also can be hypothesized that DNA degradation has occurred,

predominantly in dried clinical samples, as already described by Feng et al. [2010]. However, more genotypes were detected from two DCS (# 29 and 19). Even though detection of both LR and HR genotypes is interesting from an epidemiological point of view, the sole persistence of HR-HPV infection is necessary for the development, maintenance and progression of high-grade cervical lesions [Ho et al., 1998; Nobbenhuis et al., 1999]. Consequently, reliability in HR-HPV detection is essential, with potential clinical consequences.

Different HPV type specific profiles may be observed when using different extraction procedures even from liquid samples. Dona et al. compared Linear Array (Roche) and INNO-LiPA *Extra* (Innogenetics) after three different procedures to purify DNA from cervicovaginal samples: results (different HPV type-specific profiles) were affected by the method of DNA extraction [Dona et al., 2011]; in the present study, the same extraction was used after two different sample preparations. Studies on HPV prevalence in different populations are comparable only when extraction and genotyping methods are properly evaluated, particularly in the context of HPV infections, characterized by co-infections and/or viral clearance over time.

Even though the final aim was to enable detection of HPV in developing countries where women are frequently multi-infected, this technical comparison would also be interesting in monoinfected samples; this could help to determine the sensitivity of HPV detection with DCS in clinical samples. It will also be useful to test different time limits between spotting and HPV detection.

Although obtained from a limited number of samples, these results show that DCS could represent an efficient tool for epidemiological field studies in resource-limited settings. Besides the management of HPV- infected women, the improvement of HPV screening is also a challenge to evaluate the impact of a possible prevention through vaccination.

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Figure I.
Study design.

Table I

HPV genotypes detected in liquid and dried cervicals samples (n=50).

		Negative *	positive						kappa
			LCS		DCS		Both		statistic
		n	n	%	n	%	n	%	
Any type		5	44	88	39	78	38	76	0.51
HR-HPV	Any type	13	36	72	32	64	31	62	0.73
	56	49	1	2	1	2	1	2	1.00
	66	42	7	14	8	16	7	14	0.92
	58	46	4	8	3	6	3	6	0.85
	82	46	4	8	3	6	3	6	0.85
	31	42	8	16	6	12	6	12	0.83
	35	43	5	10	7	14	5	10	0.81
	39	47	2	4	3	6	2	4	0.79
	53	42	8	16	5	10	5	10	0.74
	33	45	4	8	4	8	3	6	0.73
	68	48	2	4	1	2	1	2	0.66
	16	46	4	8	2	4	2	4	0.65
	51	39	10	20	7	14	6	12	0.65
	18	44	6	12	3	6	3	6	0.64
	52	38	7	14	10	20	5	10	0.50
	45	49	1	2	0	0	0	0	0.00
	56/74	49	1	2	0	0	0	0	0.00
pHR-HPV	31	49	1	2	1	2	1	2	1.00
	52	34	16	32	12	24	12	24	0.80
	39	42	8	16	4	8	4	8	0.63
LR-HPV	40	49	1	2	1	2	1	2	1.00
	6	44	5	10	6	12	5	10	0.89
	70	47	3	6	2	4	2	4	0.79
	44	40	8	16	7	14	5	10	0.60
	54	49	0	0	1	2	0	0	0.00
pLR-HPV	54	41	9	18	6	12	6	12	0.77
other HPV	74	40	9	18	4	8	3	6	0.39
	69/71	47	3	6	2	4	2	4	0.79
	X	41	7	14	8	16	6	12	0.76

* negative with both LCS and DCS

DCS, dried cervical sample; LCS, liquid cervical sample; CI, confidence interval; HR-HPV, high-risk HPV; LR-HPV, low-risk HPV; pHR-HPV and pLR-HPV, possible presence of HR-HPV and possible presence of low-risk HPV: the presence of a HPV genotype can be uncertain when its line pattern is a subset of the full line pattern of another genotype observed in the same strip.

Table II

Description of the 9 discordant results.

ID sample	LCS			DCS		
	HR-HPV	LR-HPV	Ind-HPV	HR-HPV	LR-HPV	Ind-HPV
1 ^a	51,52,68,(p39)		HPV X			HPV X
29 ^a		70		51,66	70	
7 ^a	18,(p39)					
18 ^a	51,82					
8 ^{a,b}	16,18,(p39)	70				
41 ^{a,b}	31,(p52)	(54)				
26 ^b			74			
14 ^b			74			
19 ^b					6	74

Ind, indeterminate (untypeable HPV-X or HPV-69, 71, 74, see Materials and Methods); LCS, liquid cervical sample; DCS, dried cervical sample; HR, high risk; LR, low risk

(p), possible presence of the indicated genotype: the presence of a HPV genotype can be uncertain when its line pattern is a subset of the full line pattern of another genotype observed in the same strip.

^a, discordance of HR-HPV results between the 2 extractions from the same sample

^b, discordance of LR-HPV results between the 2 extractions from the same sample