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Successful retrieval of human papillomavirus DNA after a 4.5 year storage on FTA elute cards

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Abstract

Efficient primary (vaccination) and secondary (screening) prevention strategies have the potential to eliminate cervical cancer worldwide. In this context, surveillance of HPV infections remains mandatory to assess the efficacy and the impact of screening and vaccination policies. Therefore there is a need to safely store cervical samples to conduct long-term studies in vaccinated and non-vaccinated subjects. Up-dated data on cervical specimen preservation on FTA[®] cards indicate that HPV DNA can be safely retrieved after 54 months of storage. A concordance of 97% was achieved between HPV genotypes detected in initial cervical samples and on FTA[®] cards 4.5 years later. Even if a drop in HPV viral loads was observed in some cases at 4.5 years, using FTA[®] cards for safe and long-term storage of cervical samples represents an interesting option.

Keywords: DNA preservation, FTA, Papillomavirus, HPV, biobanking

1. Introduction

Using the latest available incidence estimates, C de Martel *et al.* reported attributable fractions (AF) of different papillomaviruses (HPV) types by cancer site. Thus the HPV AF is 100% for cervix uteri, 88% for anus, 78% for vagina, 24.9% for vulva, 50% for penis, 30.8% for oropharynx (de Martel *et al.*, 2017). HPV-based screening of cervical cancer as a secondary prevention and vaccination against HPV as a primary prevention have proven their efficacy and their implementation worldwide has the potential to eliminate cervical cancer as a public health problem by the end of the century (Bosch *et al.*, 2016; Simms *et al.*, 2019). In this line the World Health Organization recently called for the elimination of cervical cancer thanks to vaccination, screening and treatment (Das, 2020). However continued surveillance of HPV infections and related-precancerous and cancerous lesions of the cervix is still required to assess, at the population level, the effectiveness of both prevention strategies. Regarding HPV infections, there is a particular interest to monitor HPV genotypes among vaccinated and unvaccinated females to track potential HPV vaccine failure and/or HPV type replacement (Covert *et al.*, 2019; Gray *et al.*, 2018). Therefore, there is a need to safely archive and store cervical samples at ambient temperature while maintaining a long-term stability of HPV DNA to carry out molecular diagnosis. In this context, we conducted a previous study demonstrating the feasibility of cervical sample storage on Flinders Technology Associates (FTA) matrix cards and an efficient retrieval by successfully detecting HPV DNA after 1-month, 6-month and 12-month periods (Barth *et al.*, 2016). In the present study, updated data on HPV DNA stability on FTA[®] cards stored in sealed plastic bags after a 4.5 year storage of cervical cells are presented.

2. Material and methods

2.1. Preparation of FTA® cards with cervical specimens

The preparation of FTA® cards with cervico-vaginal samples is described in details elsewhere (Barth et al., 2016). Briefly, 4 mL of cervical cell samples collected in liquid-based cytology medium were transferred onto Prep-stain density gradient reagent and centrifuged at 200g for 2 min. After mucus, blood and cell debris trapping in the gradient, the remaining fluid was harvested and centrifuged at 800g for 10 min. The cell pellet was then suspended in 100 µL PBS and deposited onto FTA® cards. After drying, FTA® cards were stored in the dark at room temperature. For the present work, punches were preferentially taken at the periphery of the deposit area because several punches had already been harvested in the central part for the previous study.

2.2. Preparation of FTA® cards with HPV positive cells

SiHa cells (1-2 copies of integrated HPV16 genome), HeLa cells (20-40 copies of integrated HPV18 genome) and C33-A cells (HPV negative cells) were cultured at 37°C in Eagle's minimum essential medium supplemented with 10% (v/v) fetal bovine serum (Lonza, Basel, Switzerland) and 1% penicillin/streptomycin. In order to assess heterogeneity on FTA® cards, several SiHa and HeLa cell dilutions (from 100% to 0.01% of HPV positive cells) were prepared with C-33A cells. The equivalent of $8 \cdot 10^5$ cells were centrifuged, suspended in 100 µL PBS and deposited on FTA® cards. Five punches (A-E; 3 mm diameter) were then taken along a line corresponding to the diameter of the sample deposit area (Figure 1). An additional punch (F) was taken outside the sample deposit area as a negative control.

2.3. HPV genotyping

For the study, FTA[®] cards with cervical specimen were sent to the French Papillomavirus National Reference Center that routinely uses the INNO-LiPA[®] HPV Genotyping Extra II assay (Fujirebio, Courtaboeuf, France) for HPV genotyping. For DNA extraction, a 3 mm diameter punch was removed from the FTA[®] cards and treated as previously described in detail (Barth et al., 2016). Then 10 µL of eluted DNA was tested with the INNO-LiPA[®] HPV Genotyping Extra II assay according to the manufacturer's instructions. This technique, based on PCR amplification with SPF10 primers followed by a reverse line blot hybridization with specific probes, allows the identification of 32 different alpha human papillomaviruses: 13 high risk HPV (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68), 6 probably high risk HPV (HPV26, 53, 66, 70, 73, 82) and 13 low risk HPV or not classified HPV (HPV6, 11, 40, 42, 43, 44, 54, 61, 62, 67, 81, 83, 89). Results of HPV genotyping from 30 punches were compared to those obtained from the initial liquid-based cytology media (LM) used to prepare the FTA[®] cards and tested with the CLART[®] HPV2 assay (Genomica, Madrid, Spain). The CLART[®] HPV2 assay is based on genotype amplicon-specific hybridization on a microarray and detecting 35 genotypes: 13 high risk HPV as INNO-LiPA and 22 HPV considered as low risk (6, 11, 26, 40, 42, 43, 44, 53, 54, 61, 62, 66, 70, 71, 72, 73, 81, 82, 83, 84, 85, 89) (Barth et al., 2016). When available, some LM have been retested with the INNO-LiPA[®] assay.

2.4. HPV16/18 DNA and albumin gene copy number quantification

To provide further information on the use of FTA[®] cards, HPV16/18 DNA and albumin gene copy number was assessed after 54 months of specimen storage and compared to those determined from FTA[®] cards stored for 1, 6 and 12 months (Barth et al., 2016). To study sample heterogeneity, HPV16/18 DNA copy number was also measured from punches harvested from FTA[®] cards prepared with SiHa and HeLa cells. HPV16/18 DNA and albumin

gene copy number were measured by real-time PCR on 5 μ L of DNA as described previously (Jacquin et al., 2013). Viral and cellular DNA copy numbers were expressed per PCR reaction.

3. Results

3.1. HPV genotype detection

As shown in Table 1, a total agreement in detecting HPV infections (same HPV genotypes detected with INNO-LiPA[®] on FTA[®] cards and with CLART[®] HPV2 on LM at M0) was observed for 19/30 (63%) punches. Sample #17 was considered concordant because the INNO-LiPA[®] assay is not able to detect HPV84. A partial agreement (at least a shared HPV genotype) was observed for 10/30 (33%) punches and only one sample was discordant. Regarding partially concordant samples, some genotypes were missed when DNA extracted from FTA[®] cards at month 54 (#20, #21, #24 and #26) was analyzed with the INNO-LiPA[®] assay. It has to be noted that HPV42 (#20, #24) and HPV18 (#21) have been identified in LM samples with the CLART[®] HPV2 assay. In contrast, additional HPV types (HPV44, 45, 51, 68, 70, 73, 89) were detected with the INNO-LiPA[®] assay from FTA[®] cards, namely in samples #22, #23, #28 and #29 compared with HPV types identified with the CLART[®] HPV2 assay in LM at M0. When available the LM samples (#22 and #23) were retested with the INNO-LiPA[®] kit and the same additional HPV types were retrieved. Samples #25 and #27 presented some discrepant HPV genotypes belonging to the group 2B of carcinogens or not classified by IARC, namely HPV81, HPV89, HPV70, HPV73 (with the exception for HPV45). Finally, sample #30 gave clearly discrepant results between LM at M0 and FTA[®] cards at 54 months. While LM was HPV negative with the CLART[®] HPV2 test, the INNO-LiPA[®] HR

HPV Extra II assay detected 3 different HPVs from three different punches harvested at 54 months (once HPV16, once HPV51 and once HPVX).

3.2. HPV16 DNA and albumin gene copy number in FTA[®] samples

Sufficient material was available for 9 cervical samples stored on FTA[®] cards. As shown in figure 2, the absolute HPV16 genome copy number per reaction and the number of albumin gene copy number remained very stable up to 54 months for 5 out of 9 samples tested.

Interestingly, these samples were also those with the highest copy number of HPV16 DNA and albumin gene. Sample #9 presented a regular decrease in viral and cellular DNA copy numbers during the 54 months of storage. A decrease was observed only at month 54 for samples #7, #8 and #16. Notably, samples #7 and #16 had the lowest initial viral and cellular DNA copy numbers.

3.3. Assessment of heterogeneity of sample deposit

Table 2 shows the absolute HPV16/18 genome copy number and absolute albumin gene copy number quantified in each PCR reaction. HPV16/18 DNA and albumin gene were consistently detected in punches B, C and D (at least up to 1% dilution of HPV+ cells for HPV DNA). Seemingly, the number of albumin gene copies and the number of HPV16/18 DNA copies were, in most cases, lower in punches B and D compared to punch C. Moreover, substantial differences were also noted when comparing punches B and D. Most of the time neither HPV16/18 DNA nor albumin gene was detected in punches A and E. As expected, punches F were systematically negative for both viral and cellular genes.

4. Discussion

In this study, we provide evidence for a good preservation of HPV DNA on FTA[®] cards after 54 months of specimen storage. Indeed, for most samples tested (2/3), a perfect concordance in HPV genotype was observed after this long period of storage. As for the remaining samples, at least one common HPV type was retrieved.

Most discrepant results for the detection of HPV DNA from the initial LM and the archived samples on FTA[®] cards during 54 months concern samples with multiple infections. These discrepancies may probably depend on different analytical performances of both INNO-LiPA[®] and CLART[®] HPV assays. This is illustrated by the fact that additional HPV types were identified with the INNO-LiPA[®] assay confirming the high sensitivity of this test to detect more genotypes compared to the CLART[®] HPV2 assay, especially in the case of multiple infections as we previously observed (Prétet et al., 2008). Notably these additional genotypes were also detected from the initial samples (#22 and #23) with the INNO-LiPA[®] assay confirming their presence in these samples. On the contrary, some HPV genotypes were missed (samples #20 and #21). It has to be noted that in our previous study these missed genotypes (HPV18 and HPV45) had not been detected from FTA[®] matrix cards even if they were present in the initial LM sample (Barth et al., 2016). In these cases, a simple explanation could be a heterogeneous distribution of cells/HPV DNA on FTA[®] cards, especially if some HPV types were present at low titers. The hypothesis of heterogeneous distribution of cells/HPV DNA appears also plausible to explain the loss of HPV58, HPV82 and HPV42 on the FTA[®] cards at 54 months for sample #26 and #24.

The patterns of HPV detection are also questionable for samples #25 and #27. The discordant HPV genotypes are rarely detected in precancerous and cancerous lesions of the cervix (e.g. HPV81, HPV89, HPV70, HPV73) and their potential for carcinogenicity is very limited or not established (Bouvard et al., 2009). Differences in intrinsic analytical performances of each

test might explain these data. Unfortunately we did not have the possibility to retest DNA extracted at 54 months with the CLART[®] HPV test. As for sample #30, no assumptions can be formulated to explain the discrepancies and a sampling and/or a technical error cannot be totally ruled out.

To demonstrate the preservation of the DNA the evolution of the absolute copy numbers for HPV and albumin DNA were used. Whether 54 months of storage consistently alters DNA detection from FTA[®] cards is unlikely because a decrease in HPV16 copy number and in albumin gene copy number should have been observed for all samples which is not the case. While HPV16 DNA and albumin gene were systematically detected in the central part of the spot, viral or cellular DNA were absent or detected at very low frequency in punches localized at the periphery (A and E) confirming that cells are probably not uniformly spotted in the overall area. This is also probably why the absolute number of viral and cellular DNA copies appeared generally lower in punches B/D compared to punch C in the heterogeneity study. Moreover, heterogeneity was also documented between punches B and D. In this study punches from cervical samples were preferentially taken at the periphery of the deposit area. This may explain the decreased HPV DNA and albumin gene copy numbers observed after 4.5 years of storage.

It should have been interesting to test DNA stability for HPV other than HPV16. However, only few samples were available and it would have been hard to draw firm conclusion.

Furthermore, because no dramatic loss of human DNA was noticed during the 54 months of storage, there is no reason for the DNA from HPV other than HPV16 to be specifically altered.

As a whole and when all discrepant results were analyzed, only one sample was readily discordant leading to a final concordance of 97% for the detection of HR HPV DNA. These results are in line with previous studies showing the stability of nucleic acids stored on FTA[®]

cards up to 22 years after the deposit of the primary sample (Dong et al., 2017; Gonzalez et al., 2012; Guan et al., 2013; Gustavsson et al., 2009; Li et al., 2004; Rajendram et al., 2006; Wang et al., 2015).

5. Conclusions

One major advantage of using FTA[®] cards lies in the fact that cervical samples could be safely stored at room temperature without the need of large infrastructures and resource costs. Dry collection of cervical samples on FTA matrix paper could then be a reliable and affordable approach to constitute large biobanks. Nevertheless, due to some limitations (different genotyping tests used at different time points, evidence that the sample deposit is heterogeneous) further validation and optimization are required.

Declaration of interest

Authors declare no competing interest

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Author contributions

Conceptualization: GA, MF, CM, JJB

Formal analysis: AB, CS, JR, JLP

Funding acquisition: JJB, MF

Methodology: JLP, GA, CM, JJB

Project administration: JJB

Supervision: JLP, JJB

Validation: JLP, JJB, CS

Writing original draft: CS, JLP

Writing - review & editing: all authors

Figure 1: FTA[®] card used to assess heterogeneity of sample deposit

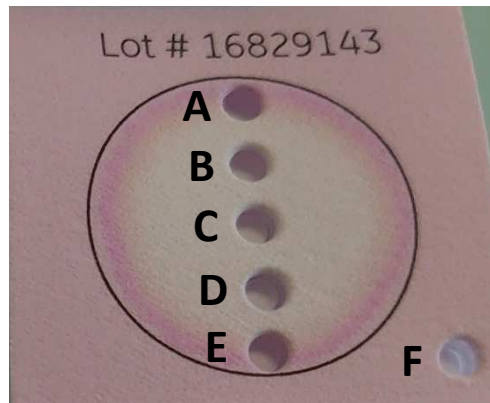


Figure 2: Absolute HPV16 DNA copy number and albumin gene copy number per reaction after long term storage of FTA cards.

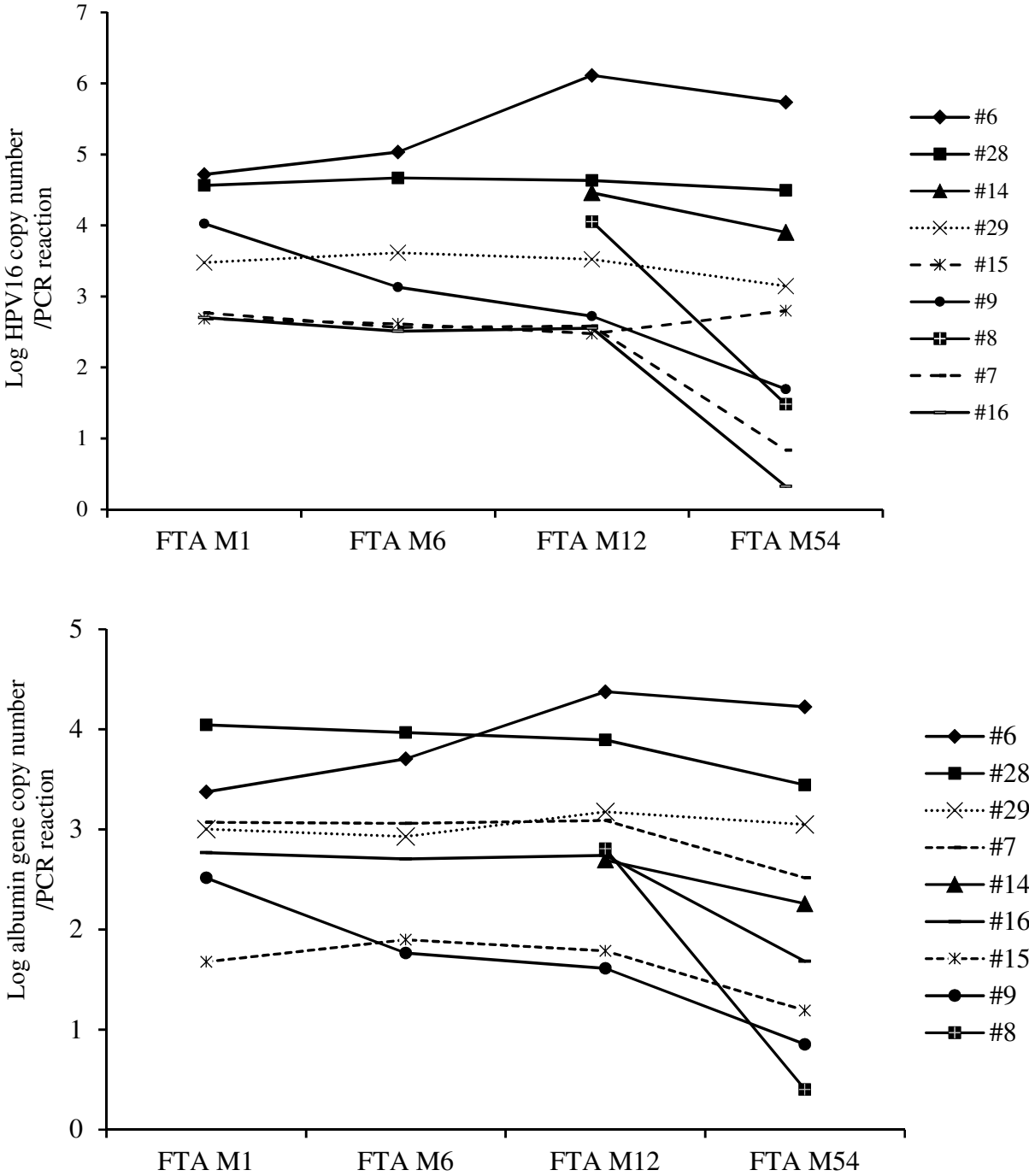


Table 1: Detection by INNO-LiPA[®] assay of individual HPV genotypes retrieved from 30 samples stored on FTA[®] cards for a 54 months period. Comparison with results obtained by CLART[®] HPV2 from the initial liquid-based cytology medium (LM) and by INNO-LiPA[®] when available.

Sample number	Sample ID	HPV genotypes		
		INNO-LiPA [®] FTA M54	CLART [®] HPV2 LM M0	INNO-LiPA [®] LM M0
#1	12390	NEG	NEG	nt
#2	12876	53	53	nt
#3	14130	58	58	nt
#4	44722	31	31	nt
#5	14894	16	16	nt
#6	45271	16	16	nt
#7	15487	16	16	nt
#8	222005	16	16	nt
#9	221983	16	16	nt
#10	221987	16	16	nt
#11	221995	16	16	nt
#12	222001	16	16	nt
#13	222007	16	16	nt
#14	14336	16	16	nt
#15	15613	16	16	nt
#16	14496	16, 59	16, 59	nt
#17	15508	16	16, 84^a	nt
#18	45523	18, 66	18, 66	nt
#19	41074	16, 52, 66	16, 52, 66	nt
#20	14122	45	42^b , 45	nt
#21	14697	16, 56	16, 18^b , 56	nt
#22	12024	51, 44	51	51, 44
#23	12045	31, 51, 68	31	31, 51, 68
#24	12715	53, 66	42^b , 53, 66	53, 66
#25	40673	56, 58, 81, 89	56, 58, 81	56, 58, 89
#26	40725	51	51, 82	51, 58, 82
#27	15804	45, 73, 83	42, 83, 84^a	45, 70, 73, 83
#28	43009	16, 51	16	na
#29	12393	16, 58, 89	16, 58	na
#30	44951	POS	NEG	na

Genotypes in bold appear discrepant with INNO-LiPA[®] and CLART[®] HPV genotyping tests.

^aHPV84 not detected by INNO-LiPA[®] HR HPV Extra II kit

^bindicates HPV genotypes never detected on FTA (either using CLART[®] HPV2 or INNO-LiPA[®] HR HPV Extra II)

nt: not tested; na: not available because no more LM sample to be tested with the INNO-LiPA[®] HR HPV Extra II

Table 2: Albumin and HPV16 DNA copy number quantification in punches

		Copy number detected in each PCR reaction											
Punch #	C33A	SiHa (Proportion in C33A cells)					HeLa (Proportion in C33A cells)						
		100%	10%	1%	0.1%	0.01%	100%	10%	1%	0.1%	0.01%		
Albumin	A	<1	<1	<1	<1	<1	<1	<1	2*	1	3*	4*	
DNA	B	2	10	40	6	1	6	117	33	5	35	19	
	C	2	263	64	20	14	13	31	55	7	74	32	
	D	1	59	35	3	1	8	14	1	4	24	3	
	E	<1	<1	1*	1	<1	<1	<1	<1	<1	<1	3	<1
	F	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
HPV	A	nd	<1	<1	<1	<1	<1	110	22	<1	31*	<1	
DNA	B	nd	270	221	4*	<1	<1	18600	1230	44	<1	<1	
	C	nd	3390	244	4	<1	<1	7060	2680	70	<1	24*	
	D	nd	1590	162	5	<1	<1	1870	45	43	25	<1	
	E	nd	<1	<1	<1	<1	<1	39	<1	<1	<1	<1	
	F	nd	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	

*only one duplicate positive

nd: not done