Screening in low resource settings, towards a world without cervical cancer

Vet, J.N.

Chapter 5

Human papillomavirus clearance and persistence after cryotherapy

J.N.I. Vet, G. Purwoto, L. Nuranna, K.H. Nuryanto, G.J. Fleuren, V.T.H.B.M. Smit, J.B. Beltman

Submitted

Abstract

Objective: Cryotherapy is widely used in single visit "see and treat" screening programs for cervical cancer. We investigated type specific HPV clearance after treatment with cryotherapy in a population-based cervical cancer screening program in Indonesia, with special interest for types associated with glandular cell infection.

Methods: HPV testing was performed before and 6 months after cryotherapy in 367 women. Samples were tested for HPV presence and type by PCR and INNO-line probe assay.

Results: Sixty-six (18%) of the samples were HPV positive. The type specific clearance rate 6 months after cryotherapy was 80.3%. The most prevalent persistent high-risk types were 18, 51, and 52. There was no difference in risk of persistence of high-risk HPV types more associated with glandular cells, compared to those more associated with squamous cells, (relative risk 0.63, 95% confidence interval 0.12–3.29). The risk of persistence of low-risk HPV types was significantly higher compared to that of high-risk types (relative risk 3.3, 95% confidence interval 1.66–6.67).

Conclusion: The HPV clearance rate after cryotherapy was high and cryotherapy seems to effectively eradicate HPV, including types associated with glandular cells. The risk of persistence of low-risk HPV types after cryotherapy treatment is significantly higher than for high-risk types.

Keywords: Cryotherapy, HPV, HPV clearance, HPV persistence, See and Treat, Low- and Middle-income countries, pap smear, VIA

Introduction

Cervical cancer is the fourth leading cancer in women in incidence and mortality worldwide with over 600.000 new cases and over 341.000 deaths estimated in 2020, of which approximately 85% of the cases occurred in low-and middle-income countries (LIMC)(1, 2). In Indonesia cervical cancer is even the most common cancer in women, accounting for 25% of all female cancer cases reported from 2008-2012 based on Hospital Based Cancer Registry data (3, 4).

WHO recommendations for See and Treat screening programs for cervical cancer in LMIC countries include treatment of premalignant lesions with cryotherapy if the lesions are eligible (5). Cryotherapy is suitable for low-resource settings, because the treatment is easy to use, does not require electricity, is safe and cost effective. Direct treatment of identified premalignant lesions increases the effectiveness of screening programs, as it minimizes the loss of patients due to inadequate follow-up (5). It has proven to be effective with histology proven cure rates of 94.0% for CIN 1, 92.0% for CIN 2, and 85.0% for CIN 3, and complications and adverse effects are rare (6-8).

The persistence of a human papillomavirus infection (HPV) in the uterine cervix has been established as the cause of cervical cancer (9, 10). Screening with HPV testing and treatment with cryotherapy has been described to lead to a 73% reduced cumulative risk of CIN 2+ lesions 36 months after treatment with cryotherapy compared to a control arm in which further evaluation or treatment was delayed for 6 months (11). And a single round of HPV testing and treatment is associated with a significant reduction in numbers of advanced cervical cancers and associated deaths (HR .52 95% CI 0.33-0.83) (12).

Cryotherapy is an effective method for eradicating HPV infections, and treating premalignant lesions, but a percentage of HPV infections will be persistent after treatment (13). HPV types that infect the mucosal epithelium of the human genital tract are subdivided into various genera. HPV types 16, 31, 33, 35, 52, and 58 are phylogenetically related and grouped as clade a-9, while HPV types 18, 39, 45, 59, and 68 are phylogenetically related and grouped as clade a-7. The clades show differences in preferences with respect to the site of infection. The clade a-9 genotypes are more associated with squamous cell carcinomas (SCC), the clade a-7 genotypes are more commonly associated with adenocarcinomas (ADC) (14, 15). Although most HPV types in these clades can lead to both ADC and SCC, the difference in association may be caused by a greater tropism for infection and/or a better ability to “neoplastically” transform the glandular tissue or squamous cell tissue (16, 17). As some HPV types are more associated with squamous cell tissue and others are more associated with glandular cell tissue, there might be a difference in persistence of HPV after cryotherapy due to the location of the lesion. The

cryotherapy probe has been designed to effectively treat both ectocervical and endocervical lesions, but endocervical lesions higher up in the cervical canal might not be reached.

Data from a population-based single-visit screening program for cervical cancer in low resource settings in Indonesia were used (18) for this study. Research on HPV prevalence in this population showed that in Indonesia, the overall HPV prevalence was 11.4% and HPV types 52, 16, and 18 are mostly prevalent (15). In this study, we investigated the effectiveness of cryotherapy on the eradication of HPV infections, when treating premalignant lesions.

Materials and Methods

The approval for this study was given by the institutional review boards of the three collaborating University Hospitals (Cipto Mungunkusumo in Jakarta, Hasan Sadikin in Bandung, Sanglah Bali) and the regional hospital in Tasikmalaya.

Sample collection

Data from an earlier population based single visit cervical cancer screening program conducted in low resource areas in Jakarta, Tasikmalaya (Java) and on the island of Bali were used for this study, this has been described in detail earlier (14). In short, the aim of the project was to screen women in low resource areas with limited access to health care facilities by visiting them in their villages with mobile clinics. The teams consisted of two doctors, a cytologist, two nurses, two public health nurses and a driver, and the women were informed about the project by the 'Pembinaan Kesejahteraan Keluarga' (PKK), the national Indonesian family welfare organization. that has access to the smallest villages. After haven given informed consent, women were submitted to screening using visual inspection with acetic acid (VIA) and Papanicolaou test (Pap smear). For research purposes, a cell sample for HPV detection was collected from each participant. The Pap smear was examined by a trained cytologist on the spot, and were classified according to the Bethesda classification(19). In case of a positive VIA, a biopsy was taken of the lesion and examined by a pathologist in the hospital.
In case the VIA and/or cytology results were positive, cryotherapy was performed. Cryotherapy was provided in cycles of 3 minutes of freeze, 3 minutes of thaw, and 3 minutes of freeze with an Erbe cryogun (Germany) with a cone-shaped probe and CO2 (Jakarta and Tasikmalaya) or N2O (Bali). Women were not eligible for cryotherapy if the lesion was suspected of cervical cancer, extended over more than 75% of the cervix, extended into the cervical canal or vaginal wall, or could not be covered fully by the cryotherapy probe.

All of the women who were treated, were registered for follow-up after 6 months, which consisted of repeating cytology, VIA, and HPV sampling. Women were excluded from the screening programme if they had never been sexually active, were pregnant, had a history of treatment to the cervix, or had a history of cervical cancer.

Women from the screenings programme described above were included in this study investigating HPV persistence after cryotherapy, if they were screened in the screenings programme, treated with cryotherapy and if HPV results were available both prior to and 6 months after cryotherapy was performed.

Classification of results

For this study lesions were classified as high risk when histology results were ≥ CIN II or cytology results were ≥ HSIL, and classified as low risk when histology results were ≤ CIN II or cytology results were ≤HSIL. VIA results were classified negative, positive, or suspicious of cervical cancer.

Typing and analysis of HPV samples

Exfoliated cells remaining on the Pap-smear spatula and brush were suspended in 25 ml of phosphate-buffered saline in a 50 ml Falcon tube. The tubes were centrifuged at 3000 × g for 5 minutes. The supernatant was removed, and the cell pellet was resuspended in 1 ml of phosphate-buffered saline and transferred to a 1.5 ml Eppendorf tube with a safety lock. All of the tubes were directly frozen and stored in a −20°C freezer and shipped on dry ice to the Department of Pathology, Leiden University Medical Center, the Netherlands. To test the quality of DNA obtained from the exfoliated cells, PCR was performed with the human genomic •-globin gene as the control template. HPV DNA was amplified using the SPF10 primer set, and the presence of HPV amplimers was detected on agarose gels. Genotyping of positive products was performed with the INNO-LiPA line-probe assay (Innogenetics). This assay can detect the following 25 HPV types: 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74.

Data management and statistics

Data management was performed with Microsoft Access 2003. Statistical analysis was performed with SPSS software (SPSS, version 12, SPSS Inc., Chicago, IL). To compare proportions, we used chi-square and Fisher's exact tests.

Results

In total 367 women were included in this study. The mean age was 38.2 years (range 18–66 years). Reasons for cryotherapy at time of screening were: positive VIA (44.7%) test, abnormal cytology (6.0%) , and positive results for both tests 181 (49.3%) . Cryotherapy was administered to 315 women with CO2 (Jakarta and Tasikmalaya) and 52 women with N2O (Bali).

Before cryotherapy, 66 out of 367 samples (18.0%) were HPV positive; of these, 57 were single HPV infections and 9 were multiple HPV infections (8x 2 types, 1x 3 types). In total, 76 HPV infections were identified and genotyped; 60 were high risk, 12 were low risk types. In four samples, HPV was detected but could not be classified into the known types; these were referred to as "type X." The most prevalent high-risk HPV types were 52 (n = 15), 53 (n = 9), 18 (n = 8), and 16 (n = 7). See table 1 contains details HPV types.

Of all infections, 29 HPV infections were identified in high-risk lesions, and 33 infections were identified in low-risk lesions. There were 10 HPV infections identified in lesions with a positive VIA test but turned out to have normal histology and normal Pap smears. Another 4 HPV infections were found in lesions with a positive VIA test, normal Pap smear but with histology results indicating infection. See table 2 for details regarding the distribution of HPV types and lesions.

At the follow-up screening 6 months after cryotherapy, the clearance rate in the HPV positive patients was 80.3%; 61 of the 76 detected HPV infections were cleared, 15 infections (19.7%) persisted. In this group also 11 new HPV infections were detected. In the group of 301 samples that were HPV negative before cryotherapy, 25 samples (8.3%) were positive after 6 months; in these samples, the most prevalent HPV types were 54 (n = 7), 74 (n = 4), and 16 (n = 4) (Table 1).

Table 1. HPV types before and 6 months after cryotherapy.

HPV Before Cryotherapy		*n*	Cleared	Persistent	New	HPV after Cryotherapy
Multiple infections						
High risk	16/53	2	2	0		
	18/6	1	1	0		
	18/68	1	0	1		18
	18/70	1	0	1		18/70/45*/68*
	42/43	1	0	1		43
	53/68/74	1	0	1		74
	54/39	1	1	0		
	66/6	1	1	0		
	16/33	1			1	
	31/74	1			1	
	39/52/54/11	1			1	
Low risk	54/74	1			1	
Single infections						
High risk	16	5	4	1	3	16
	18	5	5	0	1	
				1		54*/74*
	31	2	2	0		
	45	1	1	0		
	51	6	4	1		51/53*
				1		51/54*/74*
				1		X*
	52	15	13	2	1	52
				1		54*/66*
	53	6	6	0	1	
	56	1	0	1		56
	58	1	1	0		
	66	4	4	0		
Low risk	6	1	1	1	1	6
	11	1	0	1		11
	44	1	0	1		44
	54	1	1	0	1	
	62	1	0	0		X*
	70	1	1	0	1	
	74				1	
	81	1	0	1	1	81
Unknown	X	4	4	0		

The risk of persistence of low-risk HPV types (6 out of 12 infections, 50.0%) was significantly higher compared to the high-risk HPV types (9 out of 60, 15.0%) (relative risk [RR] 3.3, 95% confidence interval (1.66–6.67). All HPV X types were cleared.

Of the most common high-risk HPV types 52, 53, 18, and 16; respectively, 13.3% (2/15), 0.0% (0/9), 25.0% (2/8), and 14.3% (1/7) persisted 6 months after cryotherapy. The groups were too small to allow us to calculate differences in the risk of persistence among the different HPV types.

Table 2. HPV distribution and persistence according to premalignant lesions

	Before cryotherapy		After cryotherapy	
			Persistent HPV(%)	HPV type
High grade lesions[1]	HR-HPV	27	5 (18.5)	16/18/18/52/70
	LR-HPV	2	1 (50.0)	11
total		29	6 (20.7)	
Low grade lesions[2]	HR-HPV	24	2 (8.3)	51/52
	LR-HPV	7	3 (42.8)	43/44/74
	HPV X	2	0	
total		33	5 (15.2)	
Histology negative	HR-HPV	7	2 (28.6)	51/56
	LR-HPV	3	2 (66.7)	6/81
total		10	4 (40.0)	
VIA + cyt - Histology?	HR-HPV	2	0	
	LR-HPV	0	0	
	HPV X	2	0	
total		4	0	

There was no significant difference in the persistence of clade a-9 HPV types (more associated with squamous cell tissue) compared to the clade a-7 HPV types (more associated with glandular cell tissue): 3/26 (11.5%) for clade a-9 and 2/11 (18.2%) for clade a-7 (Fisher's exact test p = 0.6; RR 0.63, 95% CI 0.12–3.29).

There were no differences in the risk of persistence between the following groups: patients treated with CO2 and those treated with N20, VIA-negative and VIA-positive groups, cytology-negative and cytology-positive groups, cytology-positive and VIA-positive groups, and single-infection and multiple-infection groups, for statistic details see table 3.

Of the 29 HPV infections found in high grade lesions, 6 HPV infections (20.7%) persisted (5x HR-HPV, 1x LR-HPV). Of the 33 HPV infections found in low grade lesions, 5 infections (15.1%) persisted (2xHR-HPV, 3x LR-HPV). Of the 10 infections in the VIA positive, histology negative group, 4 HPV infections (40%) persisted (2xHR-

HPV, 2xLR-HPV). In the VIA positive, histology negative, cytology negative group (4 infections), nil HPV types persisted, for details see table 2.

The overall cure rate after cryotherapy in the 66 women in whom these 76 HPV infections were identified, was 85.7%; 54 women had a negative pap smear and VIA test in follow up, 2 still had a positive VIA, 5 still had positive cytology and 2 had both a positive VIA and Pap smear, for 1 woman the results were incomplete, for 2 the results were missing.

In the 9 women with persisting disease after cryotherapy, HPV 52 was found in one follow up HSIL pap smear, HPV X and HPV 11 were found in two follow up LSIL pap smears, and HPV 18 was found in one follow up VIA positive patient. No HPV was identified in the samples of the other 6 patients with 4 LSIL, 1 ASCUS follow up Pap smears and one positive VIA test.

Table 3. Risk of persistence

Variable	A	B	Risk of persistence
HPV	Low risk-HPV (6/12, 50%)	High risk-HPV (9/60, 15%)	RR 3.3, 95% CI 1.66-6.67
Clade	Clade a-9 (3/26, 11.5%)	Clade a-7 (2/11, 18.2%)	RR 0.63, 95% CI 0.12-3.29
Cryotherapy	CO2 (11/65, 16.9%)	NO2 (4/11, 36.4%)	RR 0.47, 95%CI 0.18-1.20
VIA	VIA negative (4/16, 25%)	VIA positive (11/60, 18.3%)	RR1.36, 95% CI 0.50-3.72
Cytology	Cytology negative (5/26, 19.2%)	Cytology positive (10/50, 20%)	RR 0.96, 95% CI 0.36-2.52
VIA	Cytology positive (10/50, 20%)	VIA positive (11/60, 18.3%)	RR 0.92, 95% CI 0.42-1.98
HPV infections	Single infections (10/55, 18.2%)	Multiple infections (4/9, 44.4%)	RR 0.98, 95% CI 0.60-1.57

Discussion

In this study, we investigated HPV persistence in women treated with cryotherapy in a single-visit screening program for cervical cancer in a rural Indonesian setting. We found that the overall HPV infection clearance rate was 80.3% at the follow-up screening 6 months after cryotherapy, which means a persistence rate of 19.7%. In our study, from the persisting HPV types, the low-risk HPV types were significantly more persistent than high risk HPV types and we did not find any difference in persistence of HPV types more associated with the endocervical glandular cells, the HPV clade-a7.

According to a systematic review of patterns of HPV persistence by Hoffman et al, the median HPV persistence after any kind of cervical treatment tends to decrease with increasing follow up time, from 27% at 3 months after treatment, 21% at 6 months, 15% at 12 months and 10% after 24 months (13). The same pattern of ongoing gradual clearance has been observed after treatment with cryotherapy. In a study by Aerssens et al. the type specific HPV clearance rate of proven CIN 1 lesion was 62.4% at 6 months after cryotherapy and had progressed to 82.3% 24 months after cryotherapy (20). Another study by Elfgren et al showed a clearance rate of 64% at 6 months and 76% at 12 months after treating CIN I and II lesions with cryotherapy (21). Our study showed a 80.3% type specific HPV clearance rate at 6 months. The initial rates of the first studies are lower at 6 months compared to our study, this might be due to the a single-freeze technique they used in cryotherapy, compared to the double-freeze technique used in this study (22). Moreover, their rate of proven pathology was higher than in our population, which might have influenced the clearance. Most of the HPV infections are cleared within 2 years, the 10% that persists longer are highly associated with premalignant lesions. Although many HPV infections are cleared spontaneously within 2 years (23), we can conclude that cryotherapy does effectively increase the clearance rate.

The risk of persistence for low-risk HPV types (50.0%) was significantly higher compared to high-risk HPV types (15.0%) (RR 3.3, 95%CI 1.66–6.67). In two studies by Castle et al, carcinogenic HPV types appeared to have a similar affinity for vaginal and cervical epithelium, but noncarcinogenic HPV types may have a tropism for vaginal epithelium (24, 25). They described this for the α-3, α-4, and α-15 clades (HPV types 2, 27, 57, 61,71, 72, 81, 83, and 84), which were the prevalent HPV types in their study. The same phenomenon was observed for the prevalent noncarcinogenic HPV types in our study, which were largely from clade α-10 (HPV types 6, 11, 13, 44, and 74). A possible explanation for the observed higher risk of persistence of low-risk HPV types is that after destroying the squamous columnar junction (SCJ) with cryotherapy, relatively more low-risk HPV types remain in the vagina and can be detected. The same patterns were observed in two studies after treatment with LEEP, performed by

Lidroth et al, and by Kreimer et al. Their persistency rates were respectively 14% and 18 % for high-risk HPV types compared to 34% and 41% for the low-risk HPV types (26, 27).

The groups were too small to calculate differences in the risk of persistence among the different HPV types. In our study, of the most common high-risk HPV types, HPV 52, 53, 18 and 16, respectively 13.3% (2/15), 0% (0/9), 25.0% (2/8), and 14.3% (1/7) persisted 6 months after cryotherapy. HPV 51 persisted in 33.3% (2/6) of the cases. Data on specific HPV persistence after cryotherapy are limited, in Elgrens study HPV 16 and HPV 18 or a combination were most likely to persist after cryotherapy (21). Women with a persistent high risk HPV infection after treatment have a higher risk for residual/ recurrent disease and require close surveillance (28). Women with a compromised immune status like HIV seropositive women, are at a higher risk for HPV infections to persist and to progress to cervical lesions (29, 30). In addition, lesions in HIV positive women are more likely to recur after treatment therefore it would be important to know the HIV status (31, 32). At the time of this study HIV testing was not feasible due to resistance of the population because of the sensitivity of the subject.

In our study, there was no difference in the risk of persistence after cryotherapy between the HPV types that are associated with glandular cells (clade a-7) compared to those associated with squamous cells (clade a-9) (Fisher's exact test p = 0.6; RR 0.63, 95% CI 0.12–3.29). In an American HPV persistence study using LEEP as treatment modality, there was no difference in rates of persistence between clade a-9 (HPV 16 and related types) and clade a-7 (HPV 18 and related types) 17.6% and 17.9% respectively (27). In cryotherapy the concern of persistence of clade a-7 HPV types is somewhat higher because of the characteristics of the cryotherapy device and its ability to reach into the cervical canal. Earlier studies have shown that the effectivity of the cryotherapy device and the different shapes of the tip (flat or shallow curved) do not influence the effectivity of the treatment (33, 34). Although the numbers in our study are small, we do think the risk of undertreatment by cryotherapy of HPV clade a-7 infections is relatively small, Fisher's exact test p = 0.6; RR 0.63, 95% CI 0.12–3.29 (table 3).

Six months after cryotherapy, the rate of newly diagnosed infections was 8.61% (n = 25) in the 301 samples that were HPV negative before cryotherapy. Of the 30 newly detected HPV infections, 40.0% were high-risk types. Cryotherapy is associated with a significant reduction in newly detected high-risk HPV infections. A South African RCT study shows that after cryotherapy women were 55% (95% CI 0.28–0.71) less likely to have a newly detected high-risk HPV infection compared to women in the control group (no treatment) (35). The authors' explanation lies in the destruction of the SCJ on the surface of the cervix and the cascade of immunological

responses that cryotherapy probably induces. Moreover, re-infection might become more difficult after cryotherapy since the new SCJ migrates deeper into the endocervix (33, 34).

Our study has a few limitations. The follow-up was limited to 6 months; we do not have information on HPV infections over a longer period since this research was part of an existing screening program with a set endpoint. The number of samples available before and after cryotherapy was too small to draw definite conclusions on persistence between the different HPV types; investigations with larger numbers of samples are warranted.

In conclusion, among the small percentage of persisting HPV types, the low-risk types are significantly more likely to persist in the genital tract after cryotherapy compared to high-risk types, and there was no difference in the persistence of HPV types more associated with glandular cells compared to those more associated with squamous cells. Overall the HPV clearance rate after cryotherapy was high, and therefore cure rates of cryotherapy are excellent for treating precancerous cervical lesions these settings if patients are eligible.

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Conflict of interest

There was no conflict of interest in this study for any of the other authors contributed.

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