

Cervical sampling with the Rovers[®] Cervex-brush[®] Combi results in significantly more endocervical cells and a higher detection rate of HPV 18 in the second half of the menstrual cycle

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Acknowledgements:

The Dutch Organisation for Health Research and Development ZonMw supported this study; grant 2200.0147

Abstract

BACKGROUND: The last decades an increase in the incidence of cervical adenocarcinoma has been observed, especially in the younger age-groups. As these lesions are etiologically related to high risk HPV and in particular to HPV 18, it has been suggested that the endocervix is the preference site for HPV 18. Effective detection of glandular lesions as well as HPV 18 depends partly on a collection device that adequately samples the endocervical canal. A new cell collection device, the Rovers® Cervex-Brush® Combi has been developed to yield higher quantities of endocervical cells.

OBJECTIVES: To test the hypothesis that the new sampling device is superior to the traditional Rovers® Cervex-Brush® in endocervical cell collection and furthermore results in higher detection rate of HPV 18.

METHODS: Cervical samples were taken consecutively with both the traditional Rovers® Cervex-Brush® as well as the new Rovers® Cervex-Brush® Combi in one session from 49 healthy women on two different moments during a single menstrual cycle. All 196 samples were processed using the Thinprep® Pap test™. The amount of endocervical glandular cells, squamous cells and the presence of HPV 18 were assessed.

RESULTS: Samples taken in the second half of the cycle showed a higher cellular density for both endocervical as well as squamous cells. The Rovers® Cervex-Brush® Combi yields significantly more endocervical cells per sample and in addition results in higher detection rate of HPV 18 when used in the second half of the cycle.

CONCLUSIONS: The Rovers® Cervex-Brush® Combi is superior in endocervical cell collection and detection of HPV 18 in the second half of the menstrual cycle and may therefore improve the sensitivity for detection of glandular lesions in cervical screening programs.

Keywords: endocervical, HPV 18, menstrual cycle

Introduction

Cancer of the uterine cervix is the third most common cancer in women accounting for 9.8% of all new cancer cases worldwide (D. Maxwell Parkin, Paola Pisani, Jacques Ferlay, International Journal of Cancer, 1999: 80(6), 827-841). Although population-based screening programmes for the prevention of cervical cancer have contributed to a significant decline in incidence and mortality of *squamous* cervical cancer (Bergstrom M 1999, Parkin D 1993), the rate of cervical **adenocarcinomas** has not yet been decreased (Miller Be 1993, Bulk s 2005, Vizcaino a 1998). The purpose of cervical screening is to identify women at risk for cervical cancer by detecting precursor lesions in order to treat them in time before they can develop into invasive cervical cancer. Adenocarcinoma in situ (AIS) is more difficult to detect both cytologically and colposcopically than are squamous intra-epithelial lesions (SIL) and, therefore, might not be detected before the development of invasive adenocarcinomas. Although the presence or absence of endocervical cells is a widely used indicator of the quality of a pap test, it is controversial among pathologist as to whether the presence or absence of identifiable columnar cells has a major bearing on the adequacy of a cervical sample (Mitchell H, 1992, Acta Cyt 36:875-880, Bos A. 2001 Am J Clin Path 115(6): 851-855, Spires S et al, Acta Cytol 1993 37:778, Szarewski A, 1993, Acta Cyt 37: 457-460, Boon et al 1986 Acta Cyt 30-246-270). Within the scope of the increasing incidence of glandular lesions, the issue of adequate sampling of the endocervical canal is of increasing importance. It is obvious that a qualitatively optimal collection device, focussing on the ectocervix as well as on the endocervix, is a condition sine qua non for effective cytological detection of glandular lesions.

Since, infections with high-risk human papillomavirus (hr-HPV) play an essential role in the development of premalignant cervical lesions and cervical cancer (Bosch 2003 J Natl Cancer Inst Monogr 3-13, Bosch 2002 J Clin Path 55; 244-65, Schiffman 1993, Chicareon 1998, Ngelangel 1998, Walboomers 1999, Andersson 2001), HPV testing has been postulated as an adjunct or even a substitute to cytological screening programmes for the prevention of

cervical cancer (Bulkmans N 2004, Nobbenhuis M 1999, Solomon D 2001). Indeed, in the United States, the American College of Obstetrics and Gynecology (ACOG) & the American Cancer Society (ACS) both approved that women over 30 years old should be screened with a cytological examination combined with an HPV DNA test.

Up till now, over 120 distinct genotypes of HPV have been identified. About 30 of the known HPVs have been detected in genital mucosa. Fifteen HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, 82) are classified as high-risk and another 3 HPV types (26, 53, 66) as probably high-risk (Munoz N *New Engl J Med* 2003).

Interestingly, although almost 100% of the cervical carcinomas are hr-HPV DNA positive (Walboomers 1999), the prevalence of specific hr-HPV genotypes detected in squamous cell carcinomas and adenocarcinomas of the cervix clearly differ indicating a potential malignant tropism of different hr-genotypes. For example, HPV 16 is found in over 60% of cervical squamous cell carcinomas and in about 20% of the cervical adenocarcinomas, while, on the other hand, HPV 18 is detected in only 10% of the cervical squamous cell carcinomas but in about 50% of the cervical adenocarcinomas (6;16-19). Adenocarcinomas in situ (AIS) are associated with HPV 18 as opposed to HPV 16 for SIL. HPV 18 was detected in 89% of AIS and the ratio of HPV 18 to HPV 16 in positive cases was 2:1 (Farnsworth A et al *Int J Gynec Path* 1989, 8:321-330).

Therefore, HPV 18 seems to play a more prominent role in the development of cervical adenocarcinomas than in squamous tumours of the cervix (Andersson S 2001, Bosch X *J Clin Path* 2002, Altekruze S 2003, Pirog e 2000) suggesting the existence of a local preference site for specific hr-HPV genotypes (Andersson 2001, van Ham 2002, Brink A 2006)

Several sampling devices are especially developed to reach these upper layers of the cervical canal (and are usually used in combination with another device, which collects cells from the ectocervical surface) in order to get a specimen that is "satisfactory for cytological evaluation". Recently the Rovers® Cervex-Brush® Combi (Rovers Medical devices, Oss, The Netherlands, picture 1) was developed to combine collection of ectocervical cells as well as endocervical cells in a single sampling device. The Combi device differs from the

conventional Rovers® Cervex-brush® (picture 2) in that the longer middle hairs have been replaced by a complete endocervical sampler which can make it easier to reach the upper layers of the endocervical canal.

The purpose of this study was to test the hypothesis that the new sampling device is superior to the traditional Cervex-Brush in endocervical cell collection and results in higher detection rate of HPV 18. Furthermore, the influence of the menstrual cycle on this phenomenon will be studied.

MATERIALS AND METHODS

After approval of the local ethical committee 49 participants were recruited from a database of volunteers from a commercial research company (Dinox, Nijmegen, The Netherlands). Eligible candidates were not allowed to take any hormonal medication and menstrual cycles had to be on a regular 4-weeks basis.

After written informed consent, cervical smears were prospectively taken twice during one spontaneous menstrual cycle. The first smears were taken between 12th and 15th day of the cycle and the second smears 1 week later. Per visit 2 cervical scrapes were taken of each participant with two different sampling devices. In order to avoid contamination of HPV from the upper layers of the endocervical canal to the ectocervical surface, cells were scraped first from the ectocervix with a Cervex-brush, followed by a second smear with the Cervex-brush-Combi. Specimens were then processed using a liquid-based (ThinPrep®, Cytoc Corporation, Marlborough, MA, USA) approach that provides monolayer distributions for cytological assessment and the possibility of isolating DNA for HPV detection assays. This method has received approval for clinical use from the US FDA (Lee 1997, Sherman 1997). HPV detection was performed on the liquid-based cervical scrapes, using a broad-spectrum short fragment polymerase chain reaction (SPF₁₀ PCR) assay as described by Kleter et al (Kleter et al 1998). In case of a positive HPV test, subsequent HPV genotyping was performed via a reverse hybridization line probe assay (LiPA), allowing for simultaneous

typing of 25 different genotypes of HPV (including 15 hr-HPV genotypes) as described previously (Kleter et al 1998). The SPF₁₀ INNO-LiPA assay has proven to be sensitive, specific, simple, and reproducible in the assessment of HPV and has been clinically validated (Kleter et al 1998, 1999; Melchers et al 1999).

Thinlayer specimens were processed with the Thinprep T3000 (CYTYC; MA, ASA) and stained with the papanicolaou method. Cytological assessment was done by an experienced pathologist according to the Dutch KOPAC-B system.

The total number of endocervical cells in every cytological specimen was counted. Since most endocervical cells were grouped in clusters, the total number of endocervical cells in a specimen was estimated, using four categories: clusters of 1-25 cells; 25-50 cells; 50-100 cells and more than 100 endocervical cells. The total number of squamous cells in the specimen was estimated by counting squamous cells in 10 consecutive fields of view (FN20x eyepiece;10x objective) with an image analysis system (Zeiss KS400, Kontron Elektronik GmbH; Munich, Germany). Using the method of moving average, counting of the number of squamous cells in 10 fields of view was found to result in a reliable estimation of the total number of squamous cells in the Thinprep specimen. The total covered area of the Thinprep specimen was 314.2 mm² which is equivalent to 100 microscopic fields of view (FN20 eyepiece;10x objective). The area of the digital field of view of the camera was 1.5 mm². The following formula describes the estimation of the total number of squamous cells in the Thinprep slide, where n is the number of squamous cells counted in 10 consecutive fields of view:

$$\left(\frac{\frac{n \times 3.142}{1.5} \times 100}{10} \right) = n \times 20.9466667$$

Women were asked to fill in a questionnaire about pregnancy rate, parity, sexarche, and frequency of intercourse with current sexual partner(s), total amount of different sexual partners, time of current relationship, condom use, and use of oral contraceptives or other

contraceptive drugs or devices, smoking habits, pelvic infections and cytological cervical disorders in the past.

All statistical analyses were performed with SPSS software (version 12.0.1). Statistical analysis was done by Chi-square, McNemar's or Wilcoxon's sign rank test were appropriate.

RESULTS

A total of 196 cervical specimens were taken from 49 women with a mean age of $26.8 \pm$ SD 6.4 years (range 18-44 yrs). Only 7 women were multiparous (range 1-7) and the remaining 42 were nulliparous. Thirty-three were non-smokers while the remaining 16 smoked 1 to 25 cigarettes a day. The age of sexarche ranged from 14 to 25 yrs (mean 17.6 yrs) while seven women had never had a sexual relationship with men. The number of sexual partners varied from 0 to over 10 male partners. Of 7 women who had never had sexual intercourse with a male partner, five were virgins and two had had only sexual contact with women. The use of condoms was consequently carried out in only one case and five women had never used oral contraceptives.

Cytological classification was made for the smears obtained in the first half of the cycle and none of them showed any abnormality. The total number of endocervical cells in a Thinprep slide was significantly higher in samples taken with the new Rovers® Cervex-Brush® Combi device at the second half of the menstrual cycle, as is shown in table 1. Overall, samples which were taken with the new device, had 563 endocervical cells more on average in the Thinprep slide as compared to samples taken with the traditional device. This is especially true when samples were taken in the second half of the cycle, with mean difference of more than 1,000 endocervical cells on average. Results of sampling in the first half of the cycle showed the same trend, however significance could not be shown here.

For the mean number of squamous cells in the samples we found opposite results as compared to endocervical cells. Overall, as well as in the two cycle-halves separately, we

found significantly more squamous cells when the cervix was sampled with the traditional brush. The difference in yield of squamous cells is more marked in the second half of the cycle as it is in the first half and amounts over 13,000 squamous cells on average. (table 2) Only 11 (22%) women were overall HPV negative while 38 (78%) women were at least once HPV positive of which 37 were at least once hr-HPV positive. Only one virgin was overall HPV negative while the remaining four virgins were at least once hr-HPV positive as was the “virgin” with only female sexual partners (table 2).

The prevalence of all HPV genotypes in cervical material sampled by the Cervex-brush-Combi in the first half of the menstrual cycle was 43% (hr-HPV prevalence 37%) and this was 45% (hr-HPV prevalence 41%) for the Cervex- brush. In the second half of the menstrual cycle the Cervex-brush-Combi showed a HPV prevalence of 47% (hr-HPV prevalence 45%) and the Cervex-brush of 43% (hr-HPV prevalence 41%). Overall, the prevalence of all HPV genotypes assessed during a single menstrual cycle on two different occasions is not significantly different for the two sampling techniques ($p > 0.01$). At the first half of the cycle 28 (57%) women and at the second half of the cycle 29 (59%) women were HPV positive either in one or in both specimens. However, the concordance of both sampling techniques in the first half of the cycle was 57% and in the second half 63%.

In the second half of the cycle detection of HPV 18 was higher than in the first half of the cycle (Table 3). The Cervex-brush detected HPV 18 in cervical samples of four women during the second half of the menstrual cycle, while the Combi-brush detected HPV 18 in cervical material of the same 4 women as well in an additional 5 women. The differences were not significant. There were no significant differences in prevalence of any one of the other hr-HPV genotypes. (table 4)

A total number of 12 women were at least once HPV 18 positive. Overall, 48 (2x2x12) cervical samples were taken of these HPV 18 positive women. Thirty-five specimens contained endocervical cells (ECC+) whereas 13 scrapes yield no endocervical cells ECC-). In the ECC+ group 16 samples (45.7%) were HPV 18 positive in contrast with 2 (15.4%) HPV

18 positive samples in the EEC- group. (Table 4) The difference was not significant $p=0.054$ (Chi-square test) .

DISCUSSION

The aim of the study was to assess differences in HPV genotype prevalence and the amount of endocervical cells in cervical specimens in women with normal cervical cytology, collected by a conventional sampling device and a recently developed device that can collect not only material from the ectocervical surface and transformation zone (like the conventional sampler) as well as from the upper layers of the endocervical canal in the first and latter half of the menstrual cycle. The overall cumulative hr-HPV prevalence was 75%, which is in accordance with another study on short-term fluctuations of HPV during a single spontaneous menstrual cycle (van Ham et al 2002). In that particular study, the cumulative hr-HPV prevalence was 70% after 4 samples obtained on weekly-based visits. Other studies have reported cumulative hr-HPV prevalence after repeated smears 34.7% to 66.7% (Schneider 1992, Wheeler 1996, de Villiers 1992, Ho 1998, Woodman 2001) in women with normal cervical cytology after different sampling periods.

The mean age of women from the present study was 26.8 years and since It has been well established that prevalence of HPV shows an age-related pattern with the highest incidence in women in their early twenties (de Villiers 1992, Melkert 1993), a high prevalence of HPV was expected. Moreover, the detection rate of HPV is highly depended on the method used and PCR-based tests are known to be highly sensitive because they “need” only a small amount of viral load to give a positive test result. We used the highly sensitive PCR-based SPF10-LiPA which has contributed to the high detection rate of HPV.

There were no statistical differences in prevalence of all (hr)-HPV genotypes, nor for the different samplers neither for the first or second half of the menstrual cycle. All 5 virgins were least once hr-HPV positive while 1 out of 2 lesbians tested also on one occasion hr-

HPV positive. Several studies have reported on HPV positive virgins and lesbians which points to possible, other than only sexual transmission routes of HPV (Baseman J, Koutsky L. 2005, and J Clin Vir 32S S16-24, Frega A Cancer 2003) like vertical transmission, skin-to-skin contact and fomites (Ferga A, Czedledy G).

Overall, the mean absolute number of endocervical cells per Thinprep slide was significantly higher in samples taken with the Cervex-brush®Combi device in comparison to the conventional device. However, if cervical scrapes were taken at the first half of the cycle the difference in mean absolute number of endocervical cells was not significant in contrast to scrapes taken at the second half of the cycle (table 1). A possible explanation for the difference in absolute number of endocervical cells between the first and the second half of the cycle could be that the cervical mucus production around the ovulation might not allow a collection of a large number of endocervical cells.

In general, the mean absolute number of squamous cells per Thinprep slide was significantly lower in specimens collected with the Cervex-Brush® Combi in comparison to the conventional device (table 2). This is also reflected in an increased proportion of inadequate smears, since a considerable number of smears showed less than the minimum required number of 5,000 squamous cells. So the superiority of the new sampling device with an increased yield of endocervical cells is compromised by a decrease in squamous cells in the present study. However, this finding, may not be accountable to the performance of the new sampling device but could be attributable to the study design which mandated sampling with the traditional device first in order to prevent possible spreading of HPV genotypes from the endocervical canal. It might be that the most superficial squamous layer had been removed by the first sampling, resulting in a residual squamous cell layer which is less efficacious for sampling. Furthermore, Depuydt and co-workers found also a two- to threefold significant increase in number of endocervical cells and no difference in the mean number of sampled squamous cells when comparing cervical samples of 100 women collected by the Cervex-brush® to cervical samples of another group of 100 women collected by the Cervex-brush® Combi (Depuydt C et al, Cytopathology 2006, in press).

The most remarkable finding of the present study is the higher detection rate of HPV 18 in samples taken at the second half of the menstrual cycle with the Cervex-brush-Combi in comparison to samples obtained by the Cervex-brush. The Cervex-brush assessed HPV 18 in 4 specimens but failed to detect HPV 18 in another 5, while the Cervex-brush-Combi assessed HPV 18 in collected specimens of nine women, including 4 who tested HPV 18 positive by the Cervex-brush. Although the difference was not significant, which is probably due to the low number of cases studied, these results support the hypothesis that a local preference site of HPV 18 in the upper layers of the cervical canal is plausible. Especially sustained by the fact that smears of HPV 18 positive women containing endocervical cells have a higher detection rate of HPV 18 in comparison to cervical scrapes of HPV 18 positive women without endocervical cells (table 5). The question why a higher detection rate of HPV 18 is found in the second half of the menstrual cycle remains to be elucidated. Michelin and co-workers showed that estrogen and progesterone have a positive effect on the replication of HPV 18 (Michelin D, 1997, Gyn Oncol). Indeed, in the second half of the cycle higher levels of endogenous progesterone are produced and may therefore contribute to a higher detection rate of HPV 18.

The above mentioned results are especially important considering the fact that, HPV testing has been postulated as an adjunct or even a substitute to cytological screening programmes for the prevention of cervical cancer (Bulkmans N 2004, Nobbenhuis M 1999, Solomon D 2001). Within the scope of the increasing incidence of glandular lesions, the issue of adequate sampling of the endocervical canal is of increasing importance. It is obvious that a qualitatively optimal collection device, focussing on the ectocervix as well as on the endocervix, is a condition sine qua non for effective cytological detection of glandular lesions. Accordingly, it is important to be aware of the local preference site of HPV 18 in order to focus attention to the most optimal collection method for HPV testing to increase the sensitivity and specificity of screening for cervical cancer to identify women at risk for the development of cervical cancer in general but in particular for cervical adenocarcinomas.

Table 1: Absolute number of endocervical cells per Thinprep smear

Absolute number of endocervical cells					
	<i>number of cells</i>		mean difference	Z	p-value [#]
	mean	sd			
1st half of menstrual cycle					
(n=49)					
<i>Cervex-Brush®</i>	211	312			
<i>Cervex-Brush® Combi</i>	311	643			
			100	-1.055	0.291
2nd half of menstrual cycle					
(n=49)					
<i>Cervex-Brush®</i>	335	504			
<i>Cervex-Brush® Combi</i>	1,362	2,274			
			1,026	-3.320	0.001
Overall					
(n=98)					
<i>Cervex-Brush®</i>	273	421			
<i>Cervex-Brush® Combi</i>	837	1,744			
			563	-3.253	0.001

[#]Wilcoxon's sign rank test

Table 2: Absolute number of squamous cells per Thinprep smear

Absolute number of squamous cells					
	<i>number of cells</i>		mean difference	Z	p-value [#]
	mean	sd			
1st half of menstrual cycle					
(n=49)					
<i>Cervex-Brush@</i>	28,999	19,076			
<i>Cervex-Brush@ Combi</i>	18,933	19,393			
			-10,066	-2.939	0.003
2nd half of menstrual cycle					
(n=49)					
<i>Cervex-Brush@</i>	46,285	29,238			
<i>Cervex-Brush@ Combi</i>	29,037	31,028			
			-17,248	-4.362	0.000
Overall					
(n=98)					
<i>Cervex-Brush@</i>	37,342	26,049			
<i>Cervex-Brush@ Combi</i>	23,985	26,235			
			-13,657	-5.324	0.000

[#]Wilcoxon's sign rank test

Table 3. HPV prevalence in 7 virgins

case no.	1 st half of menstrual cycle		2 nd half of menstrual cycle	
	HPV type cervex	HPV type combi	HPV type cervex	HPV type combi
19	-	11	-	16
23	39	-	54	18 54
25	11 16	16	18	18
26	-	-	16	-
35	-	-	-	-
38	39 45 70	-	-	-
39	-	-	-	18

cervex = Cervex-brush ; combi = Cervex-bush-Combi

Note: case no. 19 and 35 have female sexual partners

Table 4. Overview of HPV genotype prevalence with two different devices during a single menstrual cycle

genotype	1 st half of menstrual cycle		2 nd half of menstrual cycle		p-value
	cervex	combi	cervex	combi	
HPV 16	5 (10%)	7 (14%)	6 (12%)	5 (10%)	NS
HPV 18	3 (6%)	2 (4%)	4 (8%)	9 (18%)	NS
HPV 31	2 (4%)	4 (8%)	6 (12%)	7 (14%)	NS
HPV 33	2 (4%)	2 (4%)	0	1 (2%)	NS
HPV 39	4 (8%)	1 (2%)	2 (4%)	1 (2%)	NS
HPV 45	4 (8%)	2 (4%)	0	1 (2%)	NS
HPV 51	1 (2%)	1 (2%)	2 (4%)	1 (2%)	NS
<i>all HPV</i>	22 (45%)	21 (43%)	21 (43%)	23 (47%)	NS
<i>all hr-HPV</i>	20 (41%)	18 (37%)	20 (41%)	22 (45%)	NS

cervex = Cervex-brush ; combi = Cervex-bush-Combi

Table 5. Percentage HPV18 positive smears in 48 smears of 12 HPV18 positive women according to the presence of endocervical cells

Presence of EEC	HPV 18		p-value [#]
	N	%	
ECC+ (n=35)	16	45.7%	0.054
ECC- (n=13)	2	15.4%	

ECC=endocervical cells

[#]Chi-square test



Image 1. Rovers®Cervex-brush®Combi



Image 2. Rovers®Cervex-brush®