Improved endocervical sampling and HPV viral load detection by Cervex-Brush® Combi

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Objective: Liquid-based cytology (LBC) for cervical screening is becoming increasingly used. Together with SurePath® LBC, various collecting devices can be utilized, among which the Cervex-Brush® is the most widely used. The new Rovers® Cervex-Brush® Combi combines the advantages of the Cervex-Brush® with the EndoCervex-Brush® increasing sampling of the endocervical canal. The objective of this study was to analyse and to compare the Cervex-Brush® Combi with the Cervex-Brush® for the collection of squamous and endocervical cells, human papillomavirus (HPV) typing/quantification and disease detection in SurePath® LBC.

Methods: Using either the Cervex-Brush® or the Cervex-Brush® Combi 100 consecutive SurePath® LBC samples were collected using each brush type. All 200 slides were read by the FocalPoint™ and screened by guided screening using slide wizards. The viral load of HPV type 16 E7, 18 E7, 31 E6, 33 L1, 33 E6, 35 E4, 39 E7, 45 E7, 51 E6, 52 L1, 52 E7, 53 E6, 56 E7, 58 L1, 58 E6, 59 E7, 66 E6 and 68 E7 was determined using a *Taq*Man-based real-time quantitative PCR analysis.

Results: The mean number of sampled squamous cells did not differ between the two brush types (54 963 versus 54 595 cells). The use of the Cervex-Brush® Combi, however, resulted in a two- to threefold increase in the number of sampled endocervical cells ($P < 0.00001$). Using the Cervex-Brush® Combi slightly more lesions were detected (three versus two low-grade squamous intraepithelial lesions), and resulted in the detection of more atypical squamous cells of undetermined significance (six versus three). In the Cervex-Brush® group, 60% (3/5) of abnormal smears were positive for oncogenic HPV types, whereas 66.7% (6/9) of abnormal smears in the Cervex-Brush® Combi group tested positive. The median HPV viral load for samples taken with the Cervex-Brush® Combi was 0.1825 copies/cell and was significantly higher than in samples taken with the Cervex-Brush® (0.0042 copies/cell) ($P = 0.02$).

Conclusion: Sampling with the Cervex-Brush® Combi resulted in the collection of the same amount of squamous cells, but in a two to threefold harvest of endocervical cells. This led to the detection of a higher viral load for oncogenic HPV and an increase in the number of detected abnormal smears.

Keywords: cervical cytology, screening programmes, sampling devices, endocervical cell component, Cervex-Brush® Combi, cytodiagnosis, diagnosis, cytological techniques, laboratory diagnosis, cervical screening

Introduction

Cervical cancer is still an important public health issue in Europe. It is the 10th most common cause of cancer death in women,[1] and while it is currently not among the most frequent cancers, a very important distinction with cervical cancer is that it is the only cancer that is almost completely preventable through regular screening.[2] There is now an overwhelming body of evidence demonstrating that infection with certain types of the human papillomavirus (HPV) is the primary risk factor for the development of cervical cancer and its precursor lesions.[3,4] Irrespective of the primary screening method used, cervical smear or

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HPV testing, the collection technique is critical in the performance of the liquid-based cervical smears and/or HPV testing as an important screening tool for cervical cancer.[5] Although superior devices are available, less effective devices continue to be used for both conventional and liquid-based smears.[6] The Cervex-Brush® Combi is a new high-tech sampling device that combines the Cervex-Brush® and endocervical brush. It is easy to use and only two rotations are sufficient for sampling. Our aim was to compare the performance of the Cervex-Brush® Combi with the performance of the Cervex-Brush® collection devices currently used in obtaining liquid-based Pap smears and to evaluate whether the Cervex-Brush® Combi is at least as good as the Cervex-Brush® when comparing cellularity (for both squamous and endocervical cells), HPV typing/quantification and disease detection. We present our experience of sampling with the Cervex-Brush® Combi and discuss its relative merits.

Materials and methods

Study design

All samples in this prospective, blinded, cohort study were taken by the same gynaecologist (EB) and all samples were sent to the same clinical pathology laboratory. We used consecutive samples collected during opportunistic routine gynaecological health checks from women in Flanders (Belgium), which were taken by the gynaecologist (EB). Study-specific patient identification codes were assigned and transmitted in such a manner that patient confidentiality was preserved. Two cytology sampling devices were examined, the currently used Cervex-Brush® and the Cervex-Brush® Combi (Rovers Medical Devices B.V., Oss, the Netherlands). Both brush types have Food and Drug Administration (FDA) approval for use in cervical cancer screening. One hundred consecutive smears were taken with each sampling device (Cervex, $n = 100$; Combi, $n = 100$) and assessed for an even distribution of cells, the percentage of slide surface covered with cells and the presence and number of squamous/endocervical cells. Using the liquid-based cytology (LBC) leftover, we measured the cell pellet, the amount of DNA after DNA extraction, HPV type prevalence and oncogenic HPV viral load. All 200 samples were tested for 15 oncogenic HPV types by real-time quantitative PCR. The screening results were blinded from the HPV results.

As an endpoint, we used disease detection, i.e. abnormal cytology positive for oncogenic HPV. All investigations were conducted in the Laboratory Clinical Pathology, a private laboratory member of the AML-Riatol group. Riatol has used LBC in combination with the Cervex-Brush® since 1998, analysing approximately 100 000 slides per year.

Cervical sample processing and cytological procedure

Cervical cells were collected using the Cervex-Brush® ($5 \times 360°$ rotations) or Cervex-Brush® Combi ($2 \times 360°$ rotations). After collection, brush heads were transferred directly into alcohol-based preservative (SurePath®; Tripath Imaging Inc., Burlington, NC, USA), and the vials were transported to the laboratory. Thin-layer slide preparations were made with the fully robotic AutoCyte® PREP System[7] (AutoCyte®; Tripath Imaging Inc.), and were prepared as described elsewhere.[8]

The cytological results were classified according to the Bethesda system 2001,[9] using the classes negative for intraepithelial lesions (NEG), atypical squamous cells of undetermined significance (ASC-US), atypical squamous cells of undetermined significance cannot exclude high-grade squamous intraepithelial lesions (ASC-H), low-grade squamous intraepithelial lesions (L-SIL) and high-grade squamous intraepithelial lesions (H-SIL). On each slide ($n = 200$) the squamous and endocervical cells were counted manually using a 40× magnification with a conventional light microscope, by one of the trained cytotechnologists. For each slide, four fields (40× magnification) were counted, and the total number of cells was calculated.[10] The cytotechnologist was unaware of the cytological or HPV status of the slide, or which brush type was used.

FocalPoint-guided screening procedure

The FocalPoint (previously AutoPap) is a computerized scanning system for the primary screening of cervical smears produced by Tripath. The system classifies smears into three different categories: (i) no further review (NFR), these being smears that can be stored with confidence as within normal limits without being evaluated by a cytologist, (ii) guided review or guided screening and (iii) process review, where smears need to be reviewed manually because of technical problems. The system is set to provide a fixed proportion of 25% classed as NFR smears. This is

the standard percentage recommended by the manufacturer and was approved by the FDA.

All slides were scanned by the FocalPoint system with categorization as noted earlier, based on the slide score. Slides classified as NFR immediately were designated as 'negative for intraepithelial lesions' with no manual review performed except for slides from patients with a history of an abnormal smear, which were always manually reviewed. Slides with a clinical history were excluded from the study population. 'Review' slides were screened using slide wizards with guided screening by cytotechnologists with knowledge of the relative score ranking of each slide, given as quintiles within the 'review' category, using the 15 PapMaps. After looking at the 15 PapMaps, each cytotechnician rapidly reviewed the entire slide (15 seconds). The FocalPoint and slide wizards were used according to the manufacturer's instruction (Tripath). In addition, as a positive control, a known slide previously diagnosed as H-SIL cervical intraepithelial neoplasia (CIN) 3 biopsy confirmed and positive for HPV 16 was loaded in between the routine samples each day onto the FocalPoint. This control slide was always classified in the first quintile of the review category.

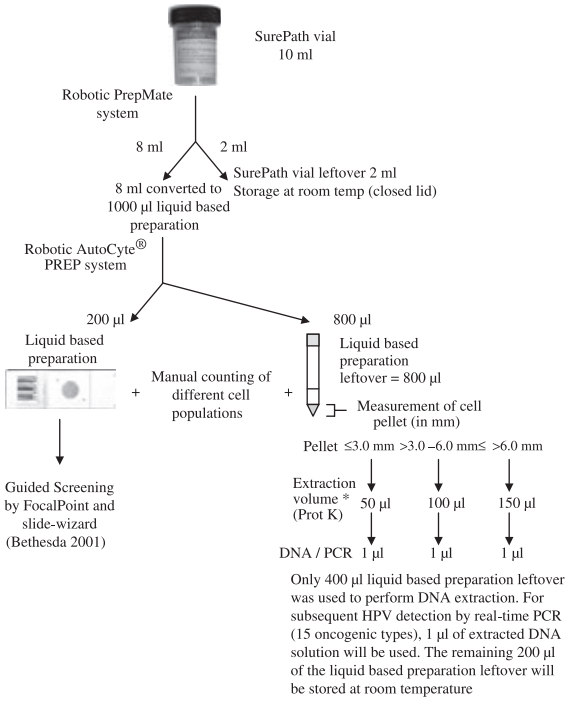

Figure 1. Schematic overview of SurePath vial processing into liquid-based cytology preparations and DNA extraction.

Isolation of DNA from cervical cells

DNA isolation from LBC was performed as previously described.[11] A detailed overview of the processing of SurePath® vials to liquid-based preparation and DNA extraction is given in Figure 1. Starting from the liquid-based preparation leftover, the cell pellet was measured with a ruler. For each sample, the height of the cell pellet (mm) and appearance was recorded. The appearance can either be normal (white or pink) or abnormal (red or brown) because of contamination with blood. After measurement of the cell pellet, the tube containing the liquid preparation leftover was vortexed, and 400 µl was transferred to an eppendorf tube. After centrifugation of the Eppendorf tube, the supernatant was discarded, and the remaining cell pellet was extracted by adding a corrected volume of proteinase K buffer, namely, 50 µl for pellets <3.0 mm, 100 µl for pellets >3.0 and <6.0 mm and 150 µl for pellets >6.0 mm. Only half of the 800 µl of the liquid-based preparation leftover was used for DNA extraction, to ensure that there was still enough material left to prepare an extra slide or to do a second DNA extraction. The DNA extracts were stored at −20 °C until PCR was performed.

Real-time qPCR analysis of HPV DNA

All samples were subjected to 19 different quantitative PCRs, including quantitative amplification for the detection of β-globin. The viral load of HPV type 16 E7, 18 E7, 31 E6, 33 L1, 33 E6, 35 E4, 39 E7, 45 E7, 51 E6, 52 L1, 52 E7, 53 E6, 56 E7, 58 L1, 58 E6, 59 E7, 66 E6 and 68 E7 was determined using a *Taq*Man-based real-time qPCR analysis. Oligonucleotide primers and probes for PCR detection and quantification of HPV 16 E7, 31 E6, 33 L1, 35 E4, 39 E7, 51 E6, 52 L1, 58 L1, 58 E6 and 67 L1 were selected as previously reported .[12,13] For HPV types 18 E7, 45 E7, 52 E7, 53 E6, 56 E7, 59 E7, 66 E6 and 68 E7, the sequences of the selected primers and probes are shown in Table 1. A multiplex real-time qPCR for HPV 33 E6, 52 E7 and 58 E6 was performed to identify the exact HPV type when samples were positive for the Moberg HPV 33, 52, 58, 67 L1 qPCR.[12]

Primers and probes were synthesized by Applied Biosystems (Cheshire, UK) or Eurogentec (Liège, Belgium). The PCR amplifications were performed in a 25-µl volume containing 2x *Taq*Man Universal PCR master mix (Applied Biosystems), 200-nM concentration of each primer and probe and 1 µl of DNA. The

Table 1. Primers and probes used for real-time detection of oncogenic human papillomavirus (HPV) and β-globin

Gene	Primer-probe	Sequence (5′ → 3′)
HPV 18 E7	HPV18–153F	CCGACGAGCCGAACCA
	HPV18-219R	CTCAATTCTGGCTTCACACTTACAA
	HPV18-170T	AACGTCACACAATGTT
HPV 33 E6	HPV33E6-FP	TGTGCGGCGTGTTGGA
	HPV33E6-RP	TGGCGTTTTTACACGTCACAGT
	Probe 33E6	CCCGACGTAGAGAAA
HPV 45 E7	HPV45-161F	CGTCGGGCTGGTAGTTGTG
	HPV45-286R	ATTGCATTTGGAACCTCAGAATG
	HPV45-181T	ATGACTAACTCCATCTGC
HPV 52 E7	HPV52-116F	GTGTGGACCGGCCAGATG
	HPV52-228R	CGTCGCAGTGCTATGAATGC
	HPV52-135T	ACAAGCAGAACAAGCC
HPV 53 E6	HPV53 FP	AACGGTTTCACAAAATTTCACATATG
	HPV53 RP	TGATTCAGTTGCTGTTGTGTGTCT
	HPV53 Probe	ACCGGGTCGTGCCTGACATGC
HPV 56 E7	HPV56seq 807F	CCAAAGAGGACCTGCGTGTT
	HPV56seq 884R	TACTTGATGCGCAGAGTGGG
	HPV56seq 828T	TACAACAGCTGCTTATGG
HPV 59 E7	HPV59E7-FP	TGTGCTACGAGCAATTACCTGACT
	HPV59E7-RP	TGATTAACTCCATCTGGTTCATCTTT
	HPV59E7-Probe	CGACTCCGAGAATGA
HPV 66 E6	HPV66 433 F	GTCCGTTAACACCGGAGGAA
	HPV66 495 R	CCCGGTCCATGCATATGC
	HPV66 454 Probe	AACAATTGCACTGTGAACATAAAAGACGATTTCATT
HPV 68 E7	HPV68-FP	ACAACAGCGTCACACAATTCAGT
	HPV68-RP	CAGTTCTACGTTCCGCAGGTT
	HPV68 Probe	ACTGCAACTAGTAGTAGAAGCGTCGCGGG
β-Globin	B-globine-143F	TGCATTTGACTCCTGAGGAGAA
	B-globine-223R	GGGCCTCACCACCAACTTC
	B-globine-167T	CTGCCGTTACTGCCCT

amount of DNA added (1 μl) to the PCR mixture represents 0.21–0.64% of the DNA obtained from a SurePath® LBC (Figure 1). Amplification and detection was performed using an ABI Prism 7000 and 7500 sequence detection system (Applied Biosystems). The amplification conditions were 2 minutes at 50 °C to activate the uracil *N*′-glycosylase followed by 10 minutes at 95 °C to inactivate the uracil *N*′-glycosylase and release the activity of the DNA polymerase, and a two-step cycle of 95 °C for 15 seconds and 60 °C for 60 seconds for a total of 45 cycles.

Positive and negative controls for PCR analysis

In all runs, tubes that contained all PCR components but without template DNA were used to ensure that the reagents were free of contamination. As positive control samples, HPV-containing cell lines Hela, Caski or Siha containing HPV 16 or 18 was used in each run. Standard curves were obtained by the amplification of a dilution series of five million to five copies of a full-length plasmid of HPV 16/18 (Clonit, Milano, Italy) and a dilution series of 150–0.15 ng of female human DNA (Promega, Madison, WI, USA), or made from plasmids containing HPV 31, 33, 35, 39, 45, 51, 56, 58, 59 which were either kindly supplied by T. Matsukura (National Institute of Health in Japan, Tokyo), A. Lörincz (Digene Corp., Gaithersburg, MD, USA), and G. Orth (Institut Pasteur, Paris, France) or else prepared by cloning from PCR products of clinical samples and used to estimate the sensitivity of the assay.

Calculations

The amount of β-globin DNA (ng) present in each sample was divided by the weight of 1 genome equivalent (i.e. 6.6 pg/cell) and a factor of 2 (because there are two copies of β-globin DNA/genome

equivalent) to obtain the number of genome equivalents (cell) in the sample. Viral loads in each specimen were expressed as the number of HPV copies/cell.

Statistical analysis

Comparisons of means were studied by analysis of variance (ANOVA), followed by Student–Newman–Keuls test for all pairwise comparisons. The chi-square statistics for trend was used to verify the existence of a trend across ordered groups (such as an increase in HPV positivity according to the degree of cytological abnormality). Statistical tests were considered significant at $P < 0.05$. Statistical analysis was performed using the MedCalc® programme (MedCalc Software, Mariakerke, Belgium).[14]

Results

Between 1 June and 1 September 2005, 200 samples were included in the study, 100 consecutive samples collected using the Cervex-Brush® (Cervex) and 100 consecutive samples collected with the Cervex-Brush® Combi (Combi). The mean age of the patients in the Cervex group was 40.6 years (SD 12.6 years) and was not different from the mean age of patients in the Combi group (41.7 years; SD 13.3 years).

Cytology

For all samples, an LBC slide was prepared (SurePath®) and all slides were read by the FocalPoint. FocalPoint reading implies that there was an even distribution of cells and that at least 90% of the slide surface was covered with cells. After guided screening with slide wizards, 14 slides were diagnosed as abnormal, three ASC-US and two L-SIL cases in the Cervex group, and three L-SIL and six ASC-US cases in the Combi group. On this limited series ($n = 200$), no ASC-H or H-SIL were detected.

For each of the SurePath® slides of both groups, the number of endocervical and squamous cells was counted manually. An overview of the cell populations for each brush type is given in Table 2. The mean number of squamous cells per slide did not differ between the two brush types (Cervex = 54 963 versus Combi = 54 595 cells). The use of the Cervex-Brush® Combi, however, resulted in a significant two- to threefold ($t = 4.483$, d.f. = 197, $P = 0.00001$) increase in the number of endocervical cells per slide (Cervex = 371 versus Combi = 986 cells).

Table 2. Mean number of sampled squamous and endocervical cells for each brush type

	Brush type	
	Cervex-Brush®	Cervex-Brush® Combi
Number of slides	100	100
Squamous cells (per slide)	54 963	54 595
95% CI	50 805–59 120	49 861–59 328
Endocervical cells (per slide)	371	981*
95% CI	241–501	740–1221
Total cells per slide	55 334	55 575
95% CI	51 188–59 479	50 820–60 331

*$P = 0.00001$, $t = 4.483$, d.f. = 197.

DNA extraction and HPV testing

The pellet height of samples in the Cervex group was 4.2 mm (SD 1.8 mm) and was not different from that measured in samples collected with the Cervex-Brush® Combi (4.0 mm; SD 1.8 mm). Also the DNA concentration after extraction was not different between the two brush types, Cervex-Brush® (36.6 ng/μl) and Cervex-Brush® Combi (31.2 ng/μl). In all samples, β-globin could be amplified by PCR.

HPV testing by PCR resulted in 73 positive samples, of which 53 were positive for oncogenic HPV types. The combined cytology and HPV results for each brush type are given in Table 3. In the Cervex-Brush® group, 60% of abnormal smears were positive for oncogenic HPV types, whereas 66.7% of abnormal smears in the Cervex-Brush® Combi group tested positive.

Table 4 gives an overview of the different HPV types detected according to cytological diagnosis and brush type. The percentage of samples positive for oncogenic HPV types increased for both brush types from NEG Cervex-Brush® 27.4%; Cervex-Brush® Combi 19.8%) to ASC-US (Cervex-Brush® 33.3%; Cervex-Brush® Combi 50.0%) to L-SIL (Cervex-Brush® 100%; Cervex-Brush® Combi 100%).

HPV viral load

There was no difference in viral load between the different oncogenic HPV types ($P > 0.05$). Therefore, all oncogenic HPV-positive samples were pooled in the following analysis. The median viral load for samples collected with the Cervex-Brush® Combi was 0.1825

Table 3. Cytology versus human papillomavirus (HPV)results

	Cervex-Brush®				Cervex-Brush® Combi			
		HPV				HPV		
Bethesda	Cytology	Onc+	X+	Negative	Cytology	Onc+	X+	Negative
Normal	95	26	9	60	91	18	6	67
ASC-US	3	1	2	0	6	3	3	0
L-SIL	2	2	0	0	3	3	0	0
H-SIL	0				0			
Total	100	29	11	60	100	24	9	67
Abnormal cytology	5	3	2	0	9	6	3	0

Onc, samples positive for one of the 16 tested oncogenic HPV types; X, samples positive for HPV consensus PCR but negative for oncogenic HPV types; ASC-US, atypical squamous cells of undetermined significance; L-SIL, low-grade squamous intraepithelial lesions; H-SIL, high-grade squamous intraepithelial lesions; abnormal cytology, samples with ASC-US, L-SIL or H-SIL.

copies/cell and was significantly higher than in samples sampled with the Cervex-Brush® group (0.0042 copies/cell) ($P = 0.02$). For infections with multiple HPV types, the type with the highest viral load was used (Figure 2).

The use of the Cervex-Brush® Combi resulted in the detection of a higher viral load (for HPV detection of oncogenic types) than when using the Cervex-Brush®.

Discussion

All LBC cytology slides, sampled either with the Cervex-Brush® or with the Cervex-Brush® Combi, were judged as adequate by the automated screening device FocalPoint. Similarly, in all 200 samples, the isolated DNA was of excellent quality and could be amplified by real-time β-globin quantitative PCR. Therefore, both brush types in combination with SurePath® fixative provide excellent preservation and sampling, allowing simple DNA isolation. Counting the total amount of collected cells for each brush type did not reveal a difference between brushes. Because collecting for both brush types was done by the same gynaecologist, the variation in the number of collected cells was very low. An advantage of the Cervex-Brush® Combi is that only two 360° rotations have to be performed instead of five to obtain the same amount of cellular material. Although the total number of collected cells was the same for the different brush types, there was a significant increase in the number of endocervical cells collected with the Cervex-Brush® Combi. A more targeted sampling of the transition zone with the Cervex-Brush® Combi resulted in a threefold increase in harvest of endocervical cells. Because the total number of collected cells remained the same for the two brush types, but the viral load for oncogenic HPV types per collected cell was significantly higher for sampling with the Cervex-Brush® Combi, we assume that by using the Cervex-Brush® Combi more HPV infected cells were sampled. These HPV-infected cells are most likely to be present at the transformation zone and to be cytologically abnormal because of virus infection. Hypothetically, one could also consider endocervical cell numbers to be a surrogate marker of the transformation zone, and/or sampling of relevant HPV-infected metaplastic or reserve cells. Therefore, using the Cervex-Brush® Combi will result in the increased collection of HPV-infected cells present at the transformation zone. Increased sampling of HPV-infected cells probably leads to an increased detection of abnormal cells.

When only abnormal smears positive for oncogenic HPV types are considered as true positive cytology, sampling with the Cervex-Brush® Combi resulted in the detection of more abnormal smears (six onc+ ASC+ versus three onc+ ASC+). This means that for cytology the Cervex-Brush® Combi is at least as good as the Cervex-Brush®, while for HPV detection the Cervex-Brush® Combi proved to be superior.

In this study, the prevalence of women with normal cytology who were positive for oncogenic HPV types was 23.6% and is in agreement with a previous study where the same type-specific HPV real-time PCR technique was used.[15]

The use of the Cervex-Brush® Combi resulted in the detection of a higher viral load (for HPV detection of oncogenic types) and is better than the Cervex-Brush®. This probably also explains why more

Table 4. Human papillomavirus (HPV) prevalence and type distribution according to cytological diagnosis and brush type

	Bethesda					
	Negative		ASC-US		L-SIL	
HPV type	Cervex	Combi	Cervex	Combi	Cervex	Combi
16	1	1				
16,31		1				
16,51,52,59	1					
16,51,56,58					1	
16,53,67						1
18	1					
18,31,51,56,66,67	1					
18,51	1					
31	3	3		1		
31,33,52	1					
31,35,(52)	1(1)					
31,51,59		1				
31,53		1				
31,67	1					
33,(56)	1(1)					
35	1	1				
35,51	1					
35,52,(59)	1(1)					
35,59	1					
39	1	1				
39,66,68		1				
45		1				
51	4	1			1	
51,52		1				
51,66						1
52	1	1				
53,(67)	1	(1)		1		
56,(58)		(1)				1
58		2				
59				1		
66			1			
X	9	6	2	3		
HPV negative	60	67	0	0	0	0
Total HPV+	35	24	3	6	2	3
HR HPV+	26	18	1	3	2	3
HR (%)	27.4	19.8	33.3	50.0	100	100
Total tested	95	91	3	6	2	3

X denotes HPV types different from high risk (HR) types: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67 and 68. ASC-US, atypical squamous cells of undetermined significance; L-SIL, low-grade squamous intraepithelial lesions.

abnormal cytology was detected in the Cervex-Brush® Combi group. The cut-off for visual detection of abnormal cytology is 6.45 copies/cell (Depuydt CE, Arbyn M, Benoy IH, *et al.*, unpublished data) and more samples from the Cervex-Brush® Combi group are above this threshold when compared with samples from the Cervex-Brush® group.

The present data clearly illustrate that the use of the Cervex-Brush® Combi results in the detection of more abnormal cases, which is probably due to the combination of a more targeted sampling of the transformation zone and the sampling of more endocervical cells, which results in the detection of a higher viral load per cell, than the use of the Cervex-Brush®.

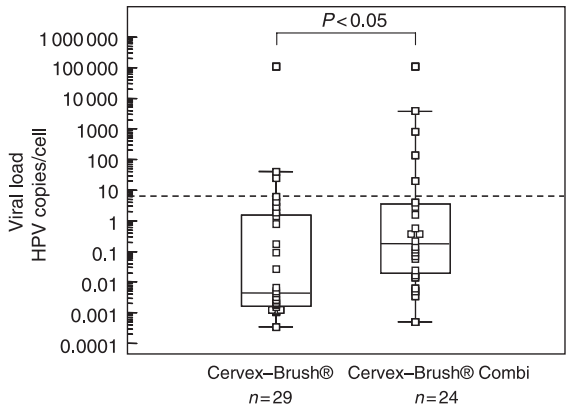

Figure 2. Box and whisker representation of the median viral load of the samples positive for oncogenic human papillomavirus (HPV) types for each of the brush types (Cervex-Brush® = 0.0042 copies/cell versus Cervex-Brush Combi® = 0.1825 copies/cell; $P < 0.05$). For infections with multiple HPV types, only the type with the highest viral load was used. The dashed line represents the 'cut-off' threshold (6.4508 copies/cell) for the visual detection of HPV-associated cytological abnormality.

Therefore, this study has shown that the Cervex-Brush® Combi is superior to the Cervex-Brush® for the collection of endocervical cells and for HPV typing/quantification, and is at least as good for the collection of squamous cells and disease detection.

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