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Comparison of Oncogenic HPV Type-Specific Viral DNA Load and E6/E7 mRNA Detection in Cervical Samples: Results From a Multicenter Study

Francesco Broccolo,[1]* Lisa Fusetti,[2] Sandra Rosini,[3] Donatella Caraceni,[4] Roberta Zappacosta,[3] Lucia Ciccocioppo,[4] Barbara Matteoli,[2] Philippe Halfon,[5] Mauro S. Malnati,[6] and Luca Ceccherini-Nelli[2]

[1]*Department of Health Sciences, University of Milano-Bicocca, Milan, Italy*
[2]*Retrovirus Center and Virology Section, Department of Experimental Pathology, University of Pisa, Pisa, Italy*
[3]*Oncology and Neurosciences Department, Cytopathology Section, G. D'Annunzio University of Chieti, Pescara, Italy*
[4]*Cytopathology Unit, F. Renzetti Hospital, Lanciano, Chieti, Italy*
[5]*Hôpital[Q2] Ambroise Paré, Marseille, France*
[6]*Unit of Human Virology, DIBIT, San Raffaele Scientific Institute, Milan, Italy*

High-risk human papillomavirus (HR-HPV) genotype viral load and E6/E7 mRNA detection are proposed as surrogate markers of malignant cervical lesion progression. Currently, the use of commercially available DNA-based or mRNA-based tests is under investigation. In this study, the viral DNA load and E6/E7 mRNA detection of the five most common HR-HPV types detected in cervical cancer worldwide were compared in 308 cervical samples by using in-house type-specific quantitative real-time PCR assays and PreTect HPV-Proofer test, respectively. Sensitivity and negative predictive values were higher for the HPV-DNA assays combined (95.0% and 96.0%, respectively) than the RNA assays (77.0% and 88.0%, respectively); conversely, the mRNA test showed a higher specificity and higher positive predictive value (81.7% and 66.9%, respectively) than the DNA test (58.6% and 52.5%, respectively) for detecting histology-confirmed high-grade cervical intraepithelial neoplasia. A significantly higher association between viral DNA load and severity of disease was observed for HPV 16 and 31 ($\gamma = 0.62$ and $\gamma = 0.40$, respectively) than for the other HPV types screened. A good degree of association between the two assays was found for detection of HPV 16 ($k = 0.83$), HPV 18 ($k = 0.72$), HPV 33 ($k = 0.66$), and HPV 45 ($k = 0.60$) but not for HPV 31 ($k = 0.24$). Sequence analysis in L1 and E6-LCR regions of HPV 31 genotypes showed a high level of intratype variation. HR-HPV viral DNA load was significantly higher in E6/E7 mRNA positive than negative samples ($P < 0.001$), except for HPV 31. These findings suggest that transcriptional and replicative activities can coexist within the same sample. ***J. Med. Virol. 9999:1–11, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: high-risk HPV-genotypes; viral load; E6/E7 mRNA; cervical cytological samples

INTRODUCTION

Infection with oncogenic human papillomavirus (HPV) is the main etiological factor that predisposes the development of cervical cancer. HPV 16, 18, 45, 31, and 33 are the most frequently identified high-risk (HR) HPV types in cervical cancer [Munoz et al., 2000[Q3]]. Type specific persistence of infection with HR-HPV is a prerequisite for the development of high-grade squamous intraepithelial lesions or cervical cancer, but fortunately most HPV infections are transient and resolve within 2 years [Ylitalo et al., 2000; Kjaer et al., 2002; Zur Hausen, 2002].

In recent years, HR-HPV viral load [Ho et al., 1998; Swan et al., 1999; Sun et al., 2002] and E6/E7 mRNA detection [Cuschieri et al., 2004] have been proposed as surrogate markers of persistent infection and high-grade cervical intraepithelial neoplasia; a correlation between the quantity of viral DNA [Josefsson et al., 2000; Moberg et al., 2003, 2004, 2005; Flores et al.,

*Correspondence to: Francesco Broccolo, Laboratory of Molecular Microbiology and Virology, Department of Health Sciences, Via Cadore, 48; 20900 Monza (MB), Italy.
E-mail: francesco.broccolo@unimib.it

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2006; Broccolo et al., 2009] or viral mRNA [Kraus et al., 2004, 2006] and the severity of cervical dysplasia has been described [Trope, 2010[Q4]; Koliopoulos et al., 2012]. HR-HPV viral load has also been suggested as a candidate marker for predicting progression of precancerous lesions because high viral load may result from an active replication that could support viral persistence [Ylitalo et al., 2000]. Currently, the prevailing consensus is that up-regulated expression of E6/E7 is necessary for the initiation and progression of cervical neoplasia. The E6 and E7 oncoproteins have significant roles in malignant transformation, and are consistently expressed in malignant tissue. Their mechanism of action is centered on inactivation of the p53 and pRb tumor suppressor proteins [Zur Hausen, 2002]. The detection of mRNA transcripts may therefore be better than HPV DNA detection as an indicator of HPV infection associated with increased risk of progression to neoplasia.

Two commercial tests exist for detecting HPV E6/E7 mRNA expression from the five most prevalent HPV types in cervical cancer (16, 18, 31, 33, 45): the PreTect HPV-Proofer (NorChip) and the NucliSENS Easy Q HPV (bioMérieux[Q5]), marketed under different brand names in different countries but based on the same nucleic acid sequence-based amplification (NASBA) technology. A previous systematic report on the performance of HPV mRNA E6/E7 expression detection revealed higher clinical specificity for detecting high-grade histological diagnoses than DNA-based tests, but a lower clinical sensitivity [Burger et al., 2011]. However, it is not clear whether the increased specificity of mRNA testing by the PreTect HPV-Proofer or NucliSENS EasyQ HPV can be attributed to the specific detection of their targeted transcripts or, to the more limited range of HPV types recognized [Keegan et al., 2009; Burger et al., 2011], all involved selectively in malignant transformation, but a positive HPV mRNA test has shown higher specificity and higher PPV for high-grade histological diagnoses than a positive HPV DNA test for the same five genotypes [Koliopoulos et al., 2012].

Numerous studies have compared commercial DNA-based and mRNA-based tests with important limitations regarding the different spectrum of detectable HPV genotypes [Molden et al., 2005; Halfon et al., 2010; Ratnam et al., 2010; Benevolo et al., 2011; Burger et al., 2011; Szarewski et al., 2012]. Only few studies have compared the presence of HR-HPV E6/E7 mRNA and the viral DNA load [Andersson et al., 2006; de Boer et al., 2007]; finally, several quantitative PCR (qPCR) assays have also not been standardized for number of cells present in the samples [Dalstein et al., 2003; Castle et al., 2005], thus making quantitation of HR-HPV viral load unreliable.

In the present study, the HPV E6/E7 mRNA was detected in a cohort of 308 cytological specimens using PreTect HPV-Proofer or NucliSENS EasyQ HPV kits and was directly compared with the HPV-DNA levels viral load measured with an in-house developed quantitative real-time PCR (qPCR) assay covering the same spectrum of HPV genotypes (HPV 16, 18, 31, 33, and 45). This study hypothesized that E6/E7 mRNA detection and viral DNA load correlate with each other and with the degree of cervical dysplasia. It also suggests that the transcriptional and replicative activities of each HR-HPV type may be different.

MATERIAL AND METHODS

Collection and Preparation of Patients Samples

A retrospective study was conducted on cervical cytology specimens from 308 women (age range, 20–65 years) undergoing tests for HPV E6/E7 mRNA at three Hospital Research Laboratories: the first a medical school hospital: Cytopathology Section, G. D'Annunzio University, Chieti (148 cervical samples), the second from a cervical carcinoma screening center, the F. Renzetti Hospital Lanciano, Vasto (100 cervical samples), and the third from Virological Departement Laboratoire Alphabio Hôpital Ambroise Paré, Marseille (60 cervical samples). Specimens were collected in the period from May 2008 to September 2010. Patients were grouped based on cytological findings as follow: 70 women showed normal cytology, 59 with atypical squamous cells of undetermined significance, 100 with low-grade squamous intraepithelial lesions, and 79 with high-grade squamous intraepithelial lesions. The Medical Ethical Committee of the medical school approved all of the described studies. The study was conducted according to the Declaration of Helsinki principles. All patients or the patients' guardians gave their written informed consent. Cervical cytological material was scraped from the endocervix using a rotary motion with a Cytobrusch (Digene Cervical sampler, Digene Corp., Gaithersburg, MD) and transferred to collection devices containing liquid based cytology medium preservation solution (PreservCyt; Cytyc Corporation[Q6]).

Cytological and histological diagnoses, HPV mRNA results, and cytology specimens were collected from each of the centers. After collection, 5 ml aliquots of cytological samples archived in PreservCyt was removed and sent to Retrovirus Center and Virology Section, University of Pisa for DNA extraction, amplification of the L1 and E6-LCR regions and sequencing analysis. Viral DNA load was determined by qPCR assays in the Unit of Human Virology, DIBIT-HSR.

DNA and RNA Extraction

PreservCyt medium was removed from cell pellets by centrifugation for 10 min at 2,800*g*. DNA was extracted from cell pellets using the QIAamp DNA Blood Mini Kit (Qiagen, Italy[Q7]) according to manufacturer's instructions. Extracted DNA was eluted in 200 ml AE buffer (10 mM Tris, pH 8.5) and stored at −20°C until analysis. RNA was extracted from a second cell pellet via the RNeasy Mini Kit (Qiagen) according to manufacturer's instruction. The residual

DNA was removed by optional on column DNase digestion using the RNase-Free DNase Set (Qiagen) and the DNase was efficiently removed in subsequent wash steps. Extracted RNA was eluted with 200 μl RNase-free water and stored at −70°C until analysis.

Cytological and Histological Diagnoses

A PAP test was performed on all cohort participants during clinical investigation. The cytological specimens were reported using the 2001 Bethesda Reporting System. All recruited women were also examined by colposcopy with biopsies taken from abnormal areas and followed by local surgical treatment if necessary. The classification of cervical lesions was based on histological findings according to the World Health Organisation's classification. Women with normal colposcopy/biopsy had follow up cytology performed at 3- to 6-month intervals. For the purpose of our study, women with normal colposcopy/biopsy who had normal cytology results throughout the subsequent 12-month follow-up period were classified as normal. The few atypical squamous cells results reported were included in the "high-grade squamous intraepithelial lesions" group, while the atypical glandular cells reports were grouped together with the "atypical squamous cells of undetermined significance" group. When there were discrepancies between the histological findings of colposcopic biopsies and excised tissues, the worst result was regarded as the final diagnosis. Atypical squamous cells of undetermined significance are referred to equivocal cervical lesions. Diagnoses of moderate dysplasia or worse (including severe dysplasia, carcinoma in situ, and invasive squamous cell carcinoma) are referred to here as high-grade histological diagnoses. High-grade histological diagnoses was detected in a total of 100 women of which 8 were atypical squamous cells of undetermined significance, 22 were low-grade squamous intraepithelial lesions, and 70 were high-grade squamous intraepithelial lesions.

Normalized Quantitative Real-Time PCR (qPCR) Assays

The extracted DNA was quantified and HR-HPV typed (16, 18, 31, 33, 45) by five independent real-time quantitative TaqMan PCR assays. qPCR assays specific for HPV 16, 18, 31, 45 were performed as described in detail [Broccolo and Cocuzza, 2008; Broccolo et al., 2009]. Briefly, the target sequences for HPV 16 was chosen within E1 open reading frame (ORF), for HPV 31 these were within the E2 ORF while for HPV 18 and HPV 45, were chosen within E6 ORF. An additional qPCR assay specific for HPV 33 was performed as recently described by Keegan et al. [2009]; primers and TaqMan probe for this assay were localized in E6 ORF. Cervical samples differ widely in the amount of DNA present. A CCR5 quantitative detection system was also used to quantify human genomic DNA in each sample and to normalize the viral load [Broccolo and Cocuzza, 2008; Broccolo et al., 2009]. DNA from cervical samples was considered suitable for HPV viral load determination if the human CCR5 copy number for reaction was higher than 2×10^3 (corresponding to 10^3 cells for reaction). The viral load is expressed as copy number per 10^4 cells. Amplification was performed using TaqMan technology and an ABI Prism device (7500 fast real-time PCR; Applied Biosystems, Forster City, CA). HPV viral DNA load values obtained from the duplicate tests were averaged for calculations. Normalization of HPV type-specific viral load was calculated as:

$$\mathrm{VL} = \frac{\mathrm{Cn_{HPV}}}{(\mathrm{Cn_{CCR5}}/2)} \times 10^4 \text{ cells}$$

where VL is the number of HPV genomes per 10^4 cells (corresponding to 2×10^4 CCR5 copies), $\mathrm{Cn_{HPV}}$ is the number of HPV genomes, and $\mathrm{Cn_{CCR5}}/2$ is the number of cells.

Amplification of the L1 and E6-LCR Regions/ Sequencing Analysis

A set of 16 samples resulted positive for only HPV 31 with high DNA levels (≥10,000 copies/10^4 cells) and negative mRNA were amplified and sequenced in the L1 and E6-LCR regions. A 523-bp segment between the positions 7527 and 137 was amplified with primers LCR 31-F (5′-AGTAGTTCTGCGGTTTTTGG TTTC-3′) and LCR 31-R (5′-CCGAGGTCTTTCTG-CAGGATTTTT-3′). The genomic sequence was established for 503 bp of the 523-bp fragment. In order, to exclude possible PCR artifacts, all samples were amplified twice and both strands were sequenced twice. Amplification with E6-LCR and L1 consensus primers/sequencing analysis were used. The region E6-LCR was amplified with primers E6 31-F (5′-AAAAG-TAGGGAGTGACCGAAAGTGG-3′) and E6 31-R (5′-TCGGGTAATTGCTCATAACAGTGGA-3′), resulting in a 625-bp fragment. A PCR fragment (450 bp) of the L1 gene was amplified with the consensus primers MY09/MY11 (MY-PCR) and the genomic sequences were established for 351 bp of these 450 bp; the conditions of the GP+-PCR and MY-PCR systems were performed as described previously [Jacobs[Q8] et al., 1995; de Roda Husman et al., 1995; Qu et al., 1997]. The sequences generated were compared to HPV sequences at GeneBank using the Fasta program (program manual for the Wisconsin package; Genetics Computer Group, Madison, WI). The sequences of the PCR product were obtained using a fluorescently labeled dideoxy terminator kit (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

RNA-Based Genotyping Assay

PreservCyt solution (5 mL) was used analyzed for the presence of E6/E7 mRNA from HPV types 16, 18, 31, 33, and 45, within 14 days of sample collection. HR-HPV E6/E7 mRNA was analyzed by PreTect HPV-Proofer (NorChip) also called NucliSENS EasyQ

(bioMérieux) according to the manufacturer's instructions. The PreTect HPV-Proofer utilizes an isothermal NASBA that amplifies mRNA from specific HPV genotypes in a DNA background, detecting and genotyping HPV transcripts in the same reaction. The amplified products were detected in real time using fluorescence-labeled molecular beacon probes directed against full-length E6/E7 mRNA. Accumulated mRNA fluorescent profiles were analyzed and assigned a positive or negative status by the supplied PreTect analysis software. Human U1 small ribonucleoprotein (U1A mRNA) was used as an RNA integrity/adequacy internal control. When the U1A amplification was not detected, the test result was deemed invalid.

Statistical Analysis

The Cochran-Armitage Trend test was used to detect an increasing trend in the proportion of HPV positive samples from women with normal cytology to those with H-SIL. χ^2-test was used to analyze the significance of the different HR-HPV prevalence in cervical samples from patients and controls. The two-tailed Student's t-test was used to evaluate the significance of differences in HPV type-specific DNA levels in HPV 16, 18, 31, 33, and 45 E6/E7 mRNA positive and negative samples. Agreement between tests was assessed by Kappa statistics. Sensitivity, specificity and positive and negative predictive values (PPVs and NPVs) for detecting histology-confirmed high-grade intraepithelial neoplasia or worse (CIN2+), were calculated for both tests (qPCR and PreTect HPV-Proofer). Sensitivity and specificity were calculated using the following formula whereby:

	Test +	Test −	Sum
Gold standard +	a	c	g
Gold standard −	b	d	h
Sum	e	f	n

Population size = n; sensitivity = a/g; specificity = d/h; PPV = a/e; NPV = d/f.

An index of co-graduation (γ Goodman and Kruskall's index) was used to ordinal variables. This test was also applied to measure the association grade (co-graduation) between levels of DNA for each genotype and the severity of lesions. Goodman and Kruskall's index (γ) less than 0.3 represents fair to poor association, values of more than 0.3 represents good association. A $P \leq 0.05$ was regarded as statistically significant. All statistical analyses were performed using SPSS software (version 19.0, Chicago, IL).

RESULTS

Prevalence of DNA and E6/E7 mRNA From HPV 16, 18, 31, 33, and 45 in Cervical Samples

A total of 308 cervical specimens obtained from patients with abnormal cytology (n = 238) and women with normal cytology (n = 70) were analyzed for viral DNA load and mRNA from the five most common HR-HPV genotypes (16, 18, 31, 33, 45) in cervical cancer. Overall, the HR-HPV DNA detection rate was higher than the E6/E7 mRNA detection rate (181/308 [58.7%] vs. 115/308 [37.3%]) regardless of their cytology results and HPV genotypes present (Table I). As expected, the prevalence of HPV types studied was markedly higher in pathological samples with respect to normal samples as detected by both the DNA and RNA tests (DNA, 161/238 [67.6%] vs. 20/70 [28.6%], $P < 0.0001$; E6/E7 mRNA, 105/238 [44.1%] vs. 10/70 [7.0%], $P < 0.0001$) (Table I). The prevalence of DNA and E6/E7 mRNA of one or more genotypes increased with the severity of lesions (HPV-DNA, P-trend = 0.006; HPV-E6/E7 mRNA, P-trend < 0.0001), with a higher proportion in cytological H-SIL (88.6% [70/79] for and 70.9% [56/79] prevalence of DNA and mRNA, respectively). This was especially true for HPV16. Multiple infections were detected in a total of 28 (9.1%) of 308 samples analyzed. In particular, the co-presence of the DNA of two or more oncogenic genotypes was demonstrated in 26 cases (8.4%); conversely, multiplex infections were detected by PreTect HPV-Proofer test in only 7 (2.3%) of 308 samples analyzed.

HPV Genotypes Distribution in Cervical Cytological Specimens

Overall, within the 308 specimens tested, there were 210 positive HR-HPV DNA tests and 122 positive mRNA tests. The most frequent HR-HPV genotypes revealed by RNA and DNA testing were HPV 16 (49.2% and 36%) and HPV 31 (15% and 37.6%), respectively. The different distribution of HR-HPV genotypes detected by qPCR and PreTect HPV-Proofer is shown in Figure 1.

Agreement Between Oncogenic HPV-DNA and HPV-E6/E7 mRNA Test

Overall, the concordance between the two assays was 92.5% with a moderate degree of association ($k = 0.61 \pm 0.03$) independently detected regardless of the cytological findings. The degree of association between the two assays for each genotype was calculated. A good degree of association was found for HPV 16 ($k = 0.83$), HPV 18 ($k = 0.72$), HPV 33 ($k = 0.66$), and HPV 45 ($k = 0.60$) testing but not for HPV 31 ($k = 0.24$). The vast majority (108/122; 88.5%) of the E6/E7 mRNA positive samples were also positive for HR-HPV DNA. In addition, HR-HPV DNA was detected in 102 cases in which the E6/E7 mRNA detection system was either negative or detected a different genotype; of these discordant results, 64 were associated with HPV 31 genotype. Conversely, in 14 cases in which the qPCR assay detected no DNA from HR-HPV, the E6/E7 mRNA assay yielded positive results: six for HPV 45, three for HPV 31, three for HPV 18, one for HPV 16, and one for HPV 33. Detailed results are shown in Table II.

TABLE I. Prevalence of HPV-DNA and Oncogenic Transcripts of Each Genotype in Cervical Samples According to Cytological Diagnosis

Cytological diagnosis (no. of samples)[aQ9]	HPV-DNA detected by QPCR (no. of cases positive for (%))						HPV-E6/E7 mRNA detected by PreTect HPV-Proofer (no. of cases positive for (%))					
	16	18	31	33	45	One or more genotype (%)	16	18	31	33	45	One or more genotype (%)
Normal (70)	8 (11.4)	2 (2.8)	10 (14.3)	2 (2.9)	2 (2.9)	20 (28.6)	4 (5.6)	2 (2.8)	1 (1.4)	1 (1.4)	4 (5.6)	10 (7.0)
ASC-US (59)	8 (13.6)	5 (8.4)	14 (23.7)	1 (3.4)	3 (5.1)	27 (45.7)	3 (5.1)	4 (6.8)	4 (6.8)	—	2 (3.4)	12 (20.3)
L-SIL (100)	23 (23.0)	9 (9.0)	30 (30.0)	3 (3.0)	4 (4.0)	64 (64.0)	20 (20.0)	6 (6.0)	7 (7.0)	1 (1.0)	5 (5.0)	37 (37.0)
H-SIL (79)	37 (46.8)	10 (12.6)	25 (31.6)	7 (8.9)	4 (5.1)	70 (88.6)	33 (41.7)	8 (10.1)	6 (7.6)	6 (7.6)	5 (6.3)	56 (70.9)

ASC-US, atypical squamous cells of undetermined significance cells; L or H-SIL, low- or high-grade squamous intraepithelial lesions.

Fig. 1. Distribution HR HPV genotypes detected by qPCR and PreTect HPV-Proofer.

Sensitivity, Specificity, and Predictive Values

We used the histological findings to estimate sensitivity, specificity, PPVs, and NPVs of a positive DNA or RNA test result as summarized in Table III; the target condition defining disease was histology-confirmed CIN2+. Sensitivity and NPV results were significantly higher for HPV-DNA (95.0% and 96.0%, respectively) than the RNA assay (77.0% and 88.0%, respectively); conversely, the mRNA test showed a higher specificity and higher positive predictive value (81.7% and 66.9%, respectively) than the DNA test (58.6% and 52.5%, respectively) for detection of histology-confirmed CIN2+. Detailed results about the sensitivity and specificity are shown in Figure 2 and in Table III.

HR-HPV Viral Load and E6/E7 mRNA

Except for HPV 31, HR-HPV DNA levels for all the remaining genotypes were significantly higher in E6/E7 mRNA positive samples than in E6/E7 mRNA negative samples (unpaired t test, $P < 0.001$) (Fig. 3). The comparison of each individual HPV genotype viral load with the histological grading demonstrated a significantly higher DNA load for HPV 16 and 31 genotypes in patients with high-grade squamous intraepithelial lesions as compared to women with normal cytology ($P < 0.005$); by contrast, no statistically significant difference was found for HPV 18, 33, and 45. In addition, a linear increase of the median values of HPV 16 and 31 viral loads with disease progression

TABLE II. Concordance Between HR-HPV Types in Real-Time PCR and E6/E7 mRNA Assay

	HPV-DNA (QPCR)									
	HPV-16		HPV-18		HPV-31		HPV-33		HPV-45	
Assay (HPV mRNA E6/E7)	+	−	+	−	+	−	+	−	+	−
Pos	59	1	17	3	15	3	7	1	10	6
Neg	17	231	9	279	64	226	6	294	6	286
Kappa value	0.83 ± 0.04		0.72 ± 0.08		0.24 ± 0.06		0.66 ± 0.12		0.60 ± 0.10	

TABLE III. Sensitivity, Specificity, PPV, and NPV of DNA and mRNA Tests for Prediction of High-Grade Histological Diagnosis

	Sensitivity		Specificity		PPV		NPV	
Assay	%	95% CI	%	95% CI	%	95% CI	%	95% CI
HPV-DNA	95.0	88.1–98.1	58.6	52.2–65.3	52.5	45.0–59.9	96.0	90.1–98.5
HPV-mRNA	77.0	67.3–84.5	81.7	75.6–86.6	66.9	57.5–75.2	88.0	82.5–92.1

95% CI, 95% confidence interval; PPV, positive predictive value, NPV, negative predictive value.

was found (Fig. 4). Noteworthy, a significantly higher association between viral DNA load and severity of disease was observed for HPV 16 and HPV 31 ($\gamma = 0.62$ and $\gamma = 0.40$, respectively) and less significant association was found in the case of HPV 18, 33 and 45 ($\gamma = 0.25$, $\gamma = 0.13$ and $\gamma = 0.19$, respectively) (Fig. 4). No significant differences in the mean viral DNA load values of the different HPV types considered were observed (data not shown).

HPV 31 L1 and E6-LCR Sequences

On the basis of the discordant results obtained from HPV 31, a total of 20 samples with high DNA levels ($\geq$10,000 copies/10^4 cells) and negative mRNA for genotype 31 were sequenced in the L1 and E6-LCR regions. Presence of substitutions and/or nucleotide deletions compared to the reference sequence published in GeneBank were detected in E6-LCR (15 [75%]) and/or L1 11 [55%] regions, respectively; of note, substitutions and/or nucleotide deletions were detected in both regions in 10 of the 20 samples analyzed. The total number of nucleotide substitutions within the L1 and E6-LCR and regions were 38 (7.6%) of 503 bp and 26 (7.4%) of 351 bp, respectively. The nucleotide mismatch sites are displayed in Figure 5; no evidence of premature stop codon or nucleotide deletions was found (data not shown).

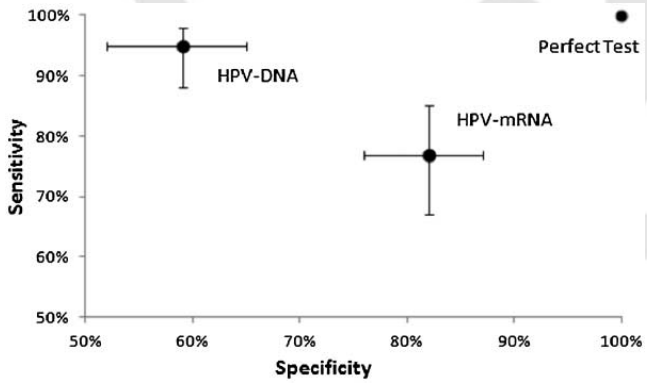

Fig. 2. Sensitivity and specificity. The lines that extend from each circle indicate the extremes of values (whiskers represent the extreme values). The "Perfect Test" is identified as a test with a sensitivity and a specificity of 100%. Performances of HPV-mRNA test are closer to the "Perfect Test" than performance of HPV-DNA test are.

DISCUSSION

Viral persistence is required for neoplastic progression [Kjaer et al., 2002], and increased risk has been associated with early high viral loads [Beskow and Gyllensten, 2002; Dalstein et al., 2003]. HR-HPV may be integrated into host cell genomes, and this event is regarded as critical for subsequent malignant transformation. However, only a minimal fraction of the integrated HPV DNA is non-methylated thus causing high and stable expression of E6 full-length proteins [Kalantari et al., 2004]. HPV integration often results in breakage and deletions in the E2 reading frame; since an E2 gene product exerts a repressing effect on E6 and E7 expression, such integration results in increasing levels of these oncogenes.

Therefore, emphasis has been placed on HPV viral load and the E6/E7 mRNA detection as surrogate markers of persistent HPV infections, although few studies have investigated the relationship between type-specific HR-HPV-DNA levels and mRNA detection [Andersson et al., 2006; de Boer et al., 2007].

Several studies compared qualitative data obtained by DNA and mRNA-based tests (with important limitations) regarding the different spectrum of detectable HPV genotypes [Molden et al., 2005; Halfon et al., 2010; Benevolo et al., 2011; Burger et al., 2011;

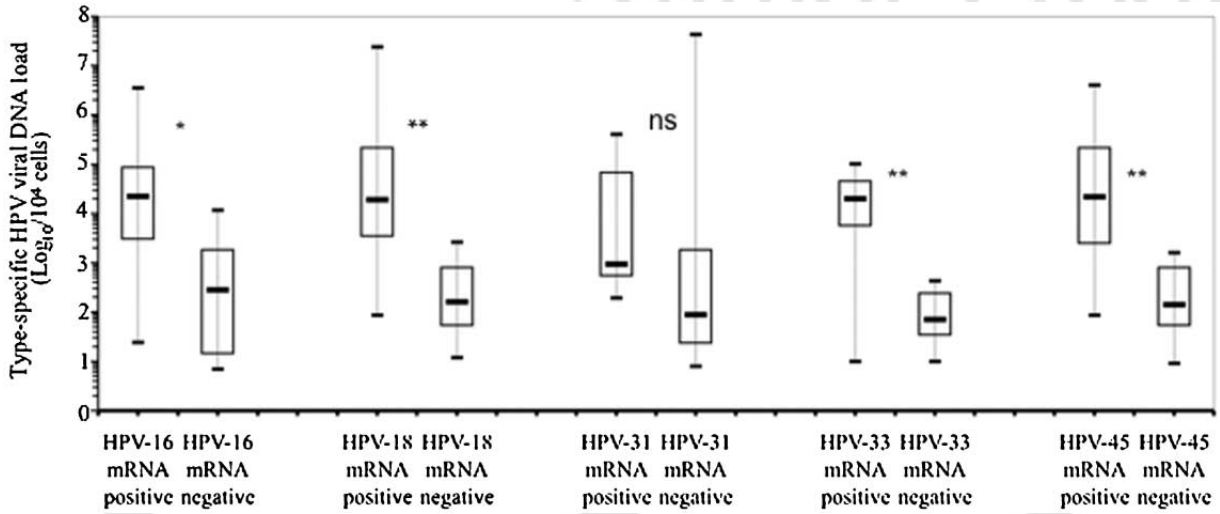

Fig. 3. HR-HPV DNA levels in E6/E7 mRNA positive and negative samples. Each box indicates the interquartile range. The lines that extend from each box indicate the extremes of values (whiskers represent the extreme values), and the line across each box indicates the median. The viral load was expressed in a log scale. Note: ns, not significant difference; $^{*}P < 0.001$; $^{**}P < 0.05$.

Szarewski et al., 2012]. Moreover, there is evidence to suggest that there is no correlation between HPV viral load and expression of HPV16 or HPV18/45 E6/E7 mRNA in cases with abnormal cytology [Andersson et al., 2006; de Boer et al., 2007]. This may be explained by viral integration into the host genome and the observed loss of viral replication upon integration [Doorbar, 2006]. It is therefore plausible that in patients with high-grade disease, the HPV viral DNA load is relatively low compared to mRNA expression, as indicated by a higher RNA/DNA copy number in patients with low- and high-grade squamous intraepithelial lesions, than in patients with normal cytology or atypical squamous cells of undetermined significance [Cattani et al., 2009].

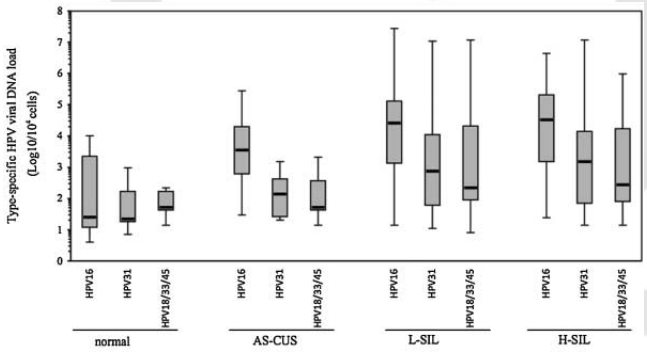

Fig. 4. Distribution of HPV viral load, according to the degree of cervical lesion or cytological findings: normal, atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesions (L-SIL) and high-grade squamous intraepithelial lesions (H-SIL). Each box indicates the interquartile range. The lines that extend from each box indicate the extremes of values (whiskers represent the extreme values), and the line across each box indicates the median. The viral load was expressed in a log scale. A good association was shown between HPV 16 ($\gamma = 0.62$) and HPV 31 ($\gamma = 0.40$) and lesion degree; by contrast, poor or no association was found for HPV18/33/45 ($\gamma = 0.18$).

In this study, HPV DNA viral load (measured by normalized qPCR assays [Broccolo and Cocuzza, 2008; Broccolo et al., 2009]) and mRNA detection (by PreTect HPV-Proofer and NucliSENS EasyQ assays) from five common HR-HPV types were compared.

The prevalence of DNA and E6/E7 mRNA of one or more genotypes progressively increased with the severity of lesions, with a higher prevalence detected in high-grade squamous intraepithelial lesions (88.6% and 71%, DNA and mRNA, respectively) corresponding to figures from other authors [Burger et al., 2011]. As expected, the mRNA test showed a higher specificity than the DNA test for detecting severe dysplasia or cancer (81.7% and 58.6%) and higher PPV (66.9% and 52.5%, respectively). These data taken together with published data [Benevolo et al., 2011; Koliopoulos et al., 2012], suggest that the highly specific HPV mRNA test may serve as a better triage test than HPV DNA to reduce colposcopy referral; nevertheless, its low sensitivity demands strict follow-up of HPV DNA positive-mRNA negative cases.

E6/E7 mRNA was detected in only a subset of HR-HPV-positive cases; in fact, only (51.4%, 108/210) of the specimens that were HR-HPV DNA positive were also positive by mRNA analysis. This was to be expected, since not all of the HR-HPV infected cases will be transcriptionally active for E6/E7 expression. The lack of RNA transcripts possibly reflects an episomal state of the virus in which regulation of the transcription process is still effective, creating a higher probability that the infection will be cleared spontaneously. Negative mRNA test results were common for women with normal cytology; in contrast,

A

| HPV-31 | E6- LCR | | |

abortive HPV infections in which the life cycle of the virus is altered [Middleton et al., 2003]. This is demonstrated by an increased expression of E6 and E7 oncogenes throughout the entire thickness of the epithelium [Snijders et al., 2006]. Therefore, the detection of E6 and E7 mRNA in exfoliated cervical epithelial cells rather reflects a high-grade cervical lesions more likely to persist or progress. Nevertheless, the mRNA test in the present study was negative in 29% of high-grade cervical lesions cytology, resulting in 71% sensitivity for detecting cytological high-grade cervical lesions. In the Predictor 2 study, the sensitivity of the HPV mRNA test with five genotypes was 74.1% for moderate dysplasia (95% CI: 69.1–78.6) and 80.3% for severe dysplasia (95% CI: 74.4–85.3) [Szarewski et al., 2012]. Although the HPV mRNA test has a lower sensitivity for detecting moderate or severe dysplasia, it is probable that it still identifies the lesions that are destined for cancer progression. Furthermore, several studies have shown that the sensitivity of the HPV mRNA test for high-grade histological diagnoses is the same as for the HPV DNA tests [Lie et al., 2005; Kraus et al., 2006; Basu et al., 2009; Hovland et al., 2010]. Conversely, only a minority (0.9%) of discrepant cases were HPV-DNA negative and mRNA positive; this could be due to deleted sequences during viral integration. In this study, except for HPV 31, location of primers and probe were selected so as not to recognize the E2 hinge region, which is the part of the E2 ORF found to be frequently deleted upon HPV 16 viral integration in patients with cervical carcinoma. In addition, short targets of $<$100 bp were chosen for real-time amplification in order to avoid the detection of disrupted regions. However, there is no irrevocable proof that the chosen sequences allow the detection of both episomal and integrated forms.

Concordance analysis by comparison of qualitative results was done for each genotype: a good degree of association was found for all genotypes analyzed except for HPV 31. Sequence analysis showed a high intra-type variation for HPV 31 in L1 and E6-LCR regions (55% and 75%, respectively). Furthermore, the prevalence of HPV 31 detected by our in house-qPCR assay was higher than that described in previous studies and in accordance with our previous publication [Broccolo et al., 2009]. Conversely, the prevalence of HR-HPV genotypes revealed by RNA test was in accordance with others studies [Halfon et al., 2010; Sørbye et al., 2010; Burger et al., 2011; Szarewski et al., 2012]. These findings suggest that standard diagnostic assays may fail to detect HPV 31 genotypic variants, thus causing an underestimation of this genotype. The poor concordance between DNA and mRNA detection for HPV 31 in particular may be partly explained by the high intra-type variation described in this study and in previous publications [Calleja-Macias et al., 2005], which may result in the failure to detect this genotype by consensus PCR based-standard assays and an underestimation of this genotype; nevertheless, it is possible that this genotype may be biologically differently to the others.

The most common HR-HPV genotype detected by RNA testing was HPV 16 (49%) whereas it was HPV 31 (38%) by DNA testing.

In accordance with our previous publication [Broccolo et al., 2009], a significantly higher association between viral load and severity of disease was observed for HPV 16 and HPV 31 ($\gamma = 0.62$ and $\gamma = 0.40$, respectively) while a less significant association was found for HPV 18, 33, and 45 ($\gamma = 0.25$, $\gamma = 0.13$, and $\gamma = 0.19$, respectively) indicating that viral load is a type-dependent risk marker for the development of high-grade cervical lesions [Broccolo et al., 2009]. The highest mean HPV viral loads were found for genotypes 16 and 31 (respectively 5.2×10^6 and 4.9×10^6 copies/10^4 cells equivalents). This may reflect differing biological behavior of the HPV genotypes studied and supports the finding that HPV load is a type-dependent risk marker for invasive carcinoma [Moberg et al., 2005]. However, studies about the association between HPV DNA levels and increasing severity of cervical lesions are conflicting. The discrepancy in the results obtained from previous studies could be due to several reasons: (1) variation in sampling techniques; (2) differences arising from the various methods used for its determination [Zerbini et al., 2001; Gravitt et al., 2003; Moberg et al., 2003; Castle et al., 2005; Broccolo and Cocuzza, 2008]; (3) presence of surrounding low-grade cervical lesions, strongly affecting cervical HPV viral load measurements in women with high-grade cervical lesions ("heterogeneity of the cervical lesions") [Sherman et al., 2003[Q10]]; (4) process of viral integration into the human genome, which commonly is associated with viral episome loss ("loss of the target sequence").

In conclusion, these findings demonstrate the existence of a high intra-type variation of the HPV 31 genotype, which could determine an underestimation of this genotype when commercial assays are used, and suggest that transcriptional and replicative activities, although two temporally distinct events, can coexist within the same sample.

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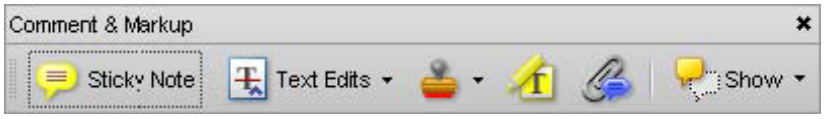

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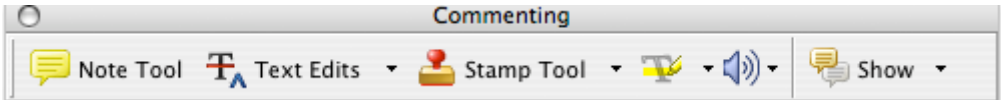

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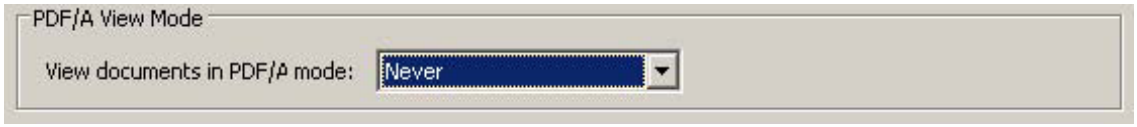

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