

## Comparison of MY09/11 consensus PCR and type-specific PCRs in the detection of oncogenic HPV types

C. E. Depuydt <sup># a</sup>, G. A. V. Boulet <sup># b \*</sup>, C. A. J. Horvath <sup>b</sup>, I. H. Benoy <sup>a</sup>,  
A. J. Vereecken <sup>a</sup>, J. J. Bogers <sup>a, b</sup>

<sup>a</sup> Laboratory for Clinical Pathology (Labo Lokeren, campus RIATOL), Antwerp, Belgium

<sup>b</sup> Applied Molecular Biology Research (AMBIOR) group, Laboratory for Cell Biology and Histology, University of Antwerp, Antwerp, Belgium

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### Abstract

The causal relationship between persistent infection with high-risk HPV and cervical cancer has resulted in the development of HPV DNA detection systems. The widely used MY09/11 consensus PCR targets a 450bp conserved sequence in the HPV L1 gene, and can therefore amplify a broad spectrum of HPV types. However, limitations of these consensus primers are evident, particularly in regard to the variability in detection sensitivity among different HPV types. This study compared MY09/11 PCR with type-specific PCRs in the detection of oncogenic HPV types. The study population comprised 15,774 patients. Consensus PCR failed to detect 522 (10.9%) HPV infections indicated by type-specific PCRs. A significant correlation between failure of consensus PCR and HPV type was found. HPV types 51, 68 and 45 were missed most frequently. The clinical relevance of the HPV infections missed by MY09/11 PCR was reflected in the fraction of cases with cytological abnormalities and in follow-up, showing 104 (25.4%) CIN2+ cases. The MY09/11 false negativity could be the result of poor sensitivity, mismatch of MY09/11 primers or disruption of L1 target by HPV integration or DNA degradation. Furthermore, MY09/11 PCR lacked specificity for oncogenic HPVs. Diagnostic accuracy of the PCR systems, in terms of sensitivity (MY09/11 PCR: 87.9%; type-specific PCRs: 98.3%) and specificity (MY09/11 PCR: 38.7%; type-specific PCRs: 76.14%), and predictive values for histologically confirmed CIN2+, suggest that type-specific PCRs could be used in a clinical setting as a reliable screening tool.

**Keywords:** human papillomavirus • cervical cancer screening • Belgium

### Introduction

Cervical carcinoma is the second most common cancer in women worldwide. In Europe, it is only the 10<sup>th</sup> most common cause of cancer death in women as a

result of organized screening programs [1]. Since cervical cancer is the only cancer that is almost completely preventable through regular screening, further implementation of effectively organized screening programs and improvement of existing screening strategies and technologies would inevitably decrease the burden of this disease [2].

There is now overwhelming evidence that infection with certain types of the human papillomavirus (HPV) is the primary risk factor for cervical cancer

<sup>#</sup>Both authors contributed equally to this work.

\*Correspondence to: Gaëlle BOULET  
University of Antwerp (Campus Groenenborger)  
AMBIOR, Laboratory for Cell Biology and Histology  
Groenenborgerlaan 171, B-2020 Antwerp, Belgium.  
Tel.: +32 32 65 33 25; Fax.: +32 32 65 33 26  
E-mail: gaelle.boulet@ua.ac.be

and its precursor lesions [3, 4]. The strong causal relationship between persistent infection with high-risk HPV (HR-HPV) and cervical cancer has resulted in the development of HPV DNA detection systems. The idea that HPV testing could play a crucial role in cervical cancer screening programs becomes more universal [5]. Several applications for HPV DNA detection have been proposed: triage of women with equivocal cytological results [6], follow-up of women treated for cervical intraepithelial neoplasia (CIN) to evaluate treatment [7] or primary screening for oncogenic HPV types alone or in combination with cytology [2, 8–10]. Considering the rising importance of HPV testing, the performance of HPV tests should be carefully assessed and validated. Consensus polymerase chain reaction (PCR) assays have been devised to amplify the most relevant genital types in one reaction. The widely used MY09/11 consensus primer set targets a 450bp conserved sequence in the HPV L1 gene, and is therefore able to amplify a broad spectrum of genital HPV types [11, 12]. However, limitations of this consensus primer system are evident, particularly in regard to the variability of detection sensitivity among specific HPV types [13, 14]. The aim of the study is to compare the performance of the MY09/11 consensus PCR with that of type-specific PCRs for oncogenic HPV detection in a large number of clinical samples. The study includes an assessment of the sensitivity, specificity and predictive values (PV) of both PCR systems to detect biopsy-proven moderate and severe CIN (CIN2+) cases.

## Materials and methods

### Sample population

Between January 2000 and December 2005 the Laboratory for Clinical Pathology (Labo Lokeren, campus RIATOL, Antwerp, Belgium) received 524,000 cervical samples for cytological evaluation, taken by general practitioners and gynecologists in Flanders (Belgium) during routine screening or gynecological examination. During this period, all samples with cytological abnormalities were automatically tested by a series of PCR analyses according to an algorithm based on MY09/11 triage for the detection and typing of HPV [15]. Samples with normal cytology were only tested in view of quality control or at specific

request of the clinician. In this study, only samples subjected to HPV detection by both MY09/11 PCR and HPV typing with all type-specific PCRs were included, yielding a study population of 15,774 patients.

Retrospective follow-up of these patients was done regardless of the PCR outcome. As histological examination of biopsy material disclosed the true disease status, the database of the Laboratory for Clinical Pathology was searched for available histological results, submitting women to gold standard verification. A CIN2<sup>+</sup> histological outcome was considered as a positive follow-up result. Cytological follow-up was only included when at least two consecutive negative smears were available, which was considered as absence of CIN2<sup>+</sup>.

The study was performed in accordance with the guidelines of the local ethical committee. Informed consent was not obtained because the study posed no risks. Study-specific patient identification codes were assigned and transmitted in accordance with patient confidentiality standards. All investigations were conducted in the Laboratory for Clinical Pathology, a private laboratory member of the AML-RIATOL group, which has been using liquid-based cytology (LBC) in combination with the Cervex-Brush<sup>®</sup> (Rovers, Oss, the Netherlands) since 1998.

### Sample processing and cytological procedure

Cervical cells were collected using the Cervex-Brush<sup>®</sup> and transferred to ethanol-based preservative (SurePath, Tripath Imaging Inc., Burlington, NC, USA). Thin-layer LBC preparations were made with the fully robotic Autocyte PREP system (Tripath Imaging Inc., Burlington, NC, USA). The cytological results were classified according to the Bethesda system 2001, using the classes negative for intraepithelial lesions, 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), high-grade squamous intraepithelial lesions (H-SIL) and atypical glandular cells (AGC) [16]. Cytology was performed without knowledge of the patient's HPV DNA status.

### DNA extraction from cervical cells

DNA extraction was performed as previously described [15, 17]. Briefly, for the remainder of each liquid-based preparation, the height of the cell pellet was measured. After vortexing, 400 µl of the remaining cell suspension was transferred to an Eppendorf tube and cells were pelleted

by centrifugation. The pellet was resuspended in 50- $\mu$ l digestion solution (10 mM Tris, 1 mM EDTA, 200  $\mu$ g/ml Proteinase K) for pellets <3 mm, 100  $\mu$ l for pellets >3 and >6 mm and 150  $\mu$ l for pellets >6 mm and digested for 3 hrs at 55°C. Digestion was followed by 10-min incubation at 95°C to inactivate Proteinase K. The DNA extracts were stored at -20°C until PCR was performed.

## HPV detection and typing by PCR

Sensitivity of the MY09/11 consensus PCR and type-specific PCRs was determined by using plasmids containing the entire genome of the different HPV types, together with 30 ng of female human DNA (Promega, Madison, USA), to mimic the complex nucleic acid environment present when amplifying genomic DNA. Dilution series were made from 10<sup>6</sup> to 1 HPV copies and standard curves were constructed for each of the 16 type-specific PCRs. Specificity of the type-specific PCRs was tested by determining the ability to discriminate against plasmids of different HPV types.

Plasmids containing HPV 16 and 18 were commercially available (Clonit, Milan, Italy). Plasmids containing HPV 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67 and 68 were kindly supplied by T. Matsukara (National Institute of Infectious Diseases, Tokyo, Japan), A. Lőrincz (Digene Corp., Gaithersburg, USA), E.-M. de Villiers (DKFZ, Heidelberg, Germany) and G. Orth (Institut Pasteur, Paris, France) or prepared by cloning from PCR products of clinical samples, estimating the copy numbers for individual plasmid preparations by spectrophotometry [17].

In this study HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67 and 68 were considered high risk. Therefore, all samples were tested with MY09/11 consensus PCR in combination with type-specific real-time PCRs with primers and probes for the detection of HPV 16 E7, 18 E7, 31 E6, 33 E6, 35 E6, 39 E7, 45 E7, 51 E7, 52 E7, 53 E6, 56 E7, 58 E6, 59 E7, 66 E6, 67 L1, 68 E7 and  $\beta$ -globin as described previously [11, 15, 17, 18].

Several precautions were taken to prevent false positive results. Different steps, such as DNA extraction, sample preparation, amplification and post-PCR, were performed in strictly separated rooms. The negative PCR control included all PCR components without template DNA. A positive control sample was used for each HPV type.

## Statistical analysis

Data were analyzed using SPSS 14.0 for Windows 2000 (SPSS Inc., Chicago, IL, USA) by descriptive statistics and contingency table analysis. Chi-square statistics were performed to assess independence of two variables. To examine the influence of the number of primer mismatches on

the efficiency of PCR amplification of the different HPV types one-way ANOVA was performed.

To assess the clinical use of MY09/11 PCR and type-specific PCRs in predicting biopsy-proven CIN2+, their sensitivity, specificity and PVs were determined.

## Results

### Study population

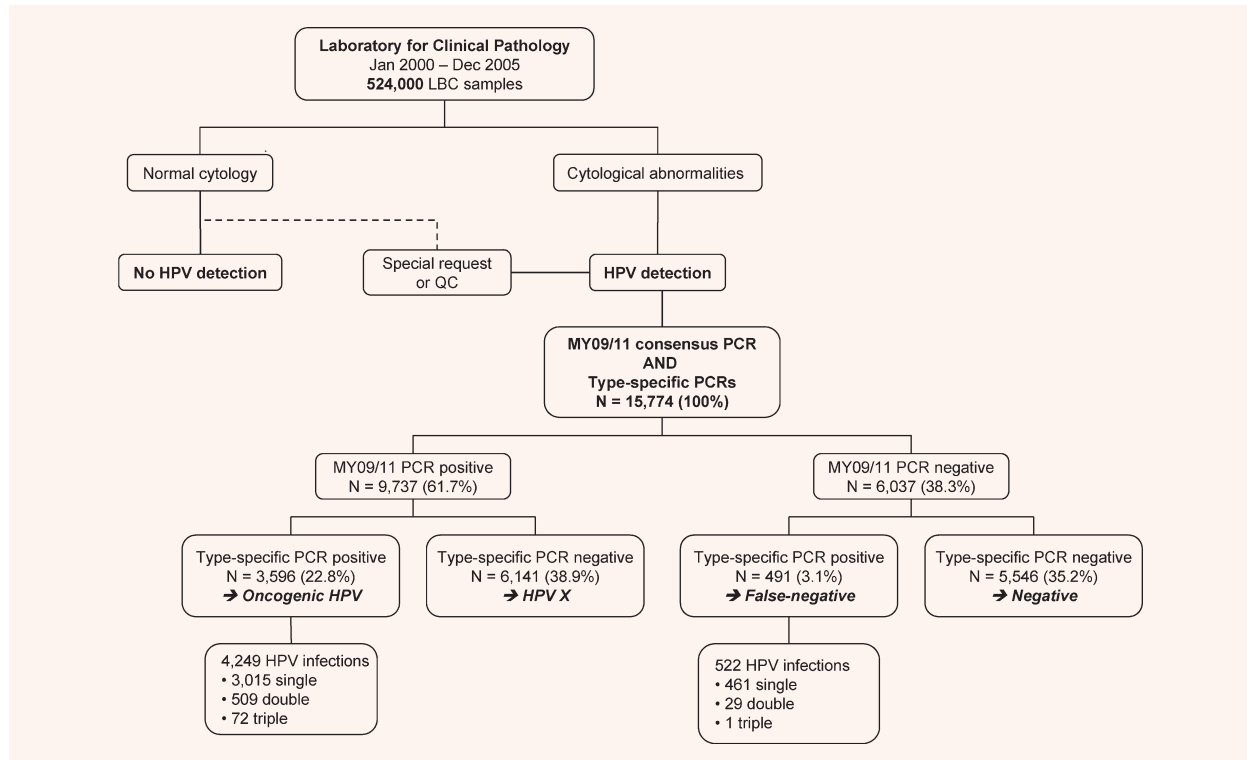
Between January 2000 and December 2005, 524,000 liquid-based cytology samples were processed in the Laboratory for Clinical Pathology. This study comprised 15,774 samples that were subjected to both MY09/11 consensus PCR and HPV typing with type-specific real-time PCRs for HPV 16 E7, 18 E7, 31 E6, 33 E6, 35 E6, 39 E7, 45 E7, 51 E7, 52 E7, 53 E6, 56 E7, 58 E6, 59 E7, 66 E6, 67 L1 and 68 E7 (Fig. 1).

### Cytology

Liquid-based cytology was performed on all 15,774 samples: 4,361 (27.6%) showed normal cytology, 7,229 (45.8%) were classified as ASC-US, 319 (2.0%) as ASC-H, 2,834 (18.0%) as L-SIL and 605 (3.8%) as H-SIL and 426 (2.7%) as AGC. In total 11,413 samples were classified as abnormal. HPV detection with both PCR methods was predominantly performed on clinically suspicious or abnormal samples, resulting in a study population enriched with clinically abnormal cases.

### MY09/11 consensus PCR versus type-specific PCRs

Table 1 shows the characteristics of the different PCR assays. Type-specific PCRs were more sensitive than MY09/11 consensus PCR for all HPV types. In our hands, MY09/11 PCR was able to detect 10 copies of HPV 31, but the detection limit was higher than 10<sup>2</sup> for all other oncogenic HPV types. For each type-specific PCR a highly significant linear regression was seen between HPV copy number and threshold cycle (CT), defined as the point at which



**Fig. 1** Study overview.

the fluorescence rises appreciably above the background fluorescence. The type-specificity of each PCR was validated. Each PCR exclusively amplified a single HPV genotype.

Using MY09/11 consensus PCR, 9,737 (61.7%) of a total of 15,774 samples turned out to be positive for HPV DNA. In 6,037 (38.3%) cases, consensus PCR failed to detect HPV DNA.

Type-specific PCRs for 16 oncogenic HPV types were performed on both consensus PCR-positive and -negative samples. 11,687 (74.1%) samples did not reveal any oncogenic HPV type; a single HPV type was detected in 3,476 (22.0%) samples; 538 (3.4%) samples showed double infections and 73 (0.5%) showed triple infections. Multiple infections were separated in constituent types, thus type-specific prevalence represents HPV types present in either single or multiple infections. HPV DNA typing identified 4,771 infections: 771 (16.2%) HPV 16, 431 (2.1%) HPV 18, 338 (7.1%) HPV 31, 359 (7.5%) HPV 33, 456 (9.5%) HPV 35, 361 (7.6%) HPV 39, 129 (2.7%) HPV 45, 563 (11.8%) HPV 51, 258 (5.4%) HPV 52, 79 (1.7%) HPV 53, 366 (7.7%) HPV

56, 66 (1.4%) HPV 58, 88 (1.8%) HPV 59, 368 (7.7%) HPV 66, 37 (0.8%) HPV 67 and 101 (2.1%) HPV 68. A total of 6,141 samples negative for all type-specific PCRs, but positive for consensus PCR, were considered to contain unidentified HPV genotypes of unknown malignant potential (HPV X).

For each HPV type the fraction of MY09/11 consensus PCR-positive and -negative cases were assessed and are summarized in Table 2.

Overall, MY09/11 consensus PCR failed to detect 522 (10.9%) of all HPV infections. A significant correlation between failure of consensus PCR and HPV type was found ( $P < 0.001$ ). HPV 51, HPV 68 and HPV 45 were missed most frequently, in 188 (34.3%), 27 (26.7%) and 20 (15.5%) cases, respectively. False negativity of consensus PCR also occurred for all other HPV types: 4.8% HPV 16, 9.0% HPV 18, 5.3% HPV 31, 2.8% HPV 33, 8.3% HPV 35, 12.7% HPV 39, 9.3% HPV 52, 3.8% HPV 53, 9.3% HPV 56, 7.6% HPV 58, 10.2% HPV 59, 6.3% HPV 66 and 2.7% HPV 67.

Cytological diagnosis was evaluated to assess the clinical importance of the HPV infections missed by

**Table 1** Sensitivity of PCR assays and characteristics of standard curves for 16 type-specific PCRs constructed with plasmids containing the entire genome of the different HPV types

Gene	Sensitivity (HPV copies)		Standard curves		
	MY09/11 PCR	Type-specific PCRs	Slope	Efficiency	Value of fit ( $R^2$ )
HPV 16 E7	10 <sup>2</sup>	10	-3.4836	1.940	0.9991
HPV 18 E7	10 <sup>2</sup>	10	-3.3632	1.928	0.9982
HPV 31 E6	10	1	-3.4508	1.977	0.9998
HPV 33 E6	10 <sup>2</sup>	10	-3.4632	1.912	0.9954
HPV 35 E6	10 <sup>4</sup>	10	-3.3551	1.974	0.9995
HPV 39 E7	10 <sup>2</sup>	10	-3.5730	1.932	0.9965
HPV 45 E7	10 <sup>2</sup>	1	-3.1596	1.835	0.9755
HPV 51 E7	10 <sup>5</sup>	1	-3.3482	2.000	0.9979
HPV 52 E7	10 <sup>4</sup>	10	-3.3143	1.995	0.9990
HPV 53 E6	10 <sup>4</sup>	10	-3.4650	1.918	0.9999
HPV 56 E7	10 <sup>4</sup>	10	-3.1775	1.961	0.9953
HPV 58 E6	10 <sup>2</sup>	10	-3.5450	1.919	0.9962
HPV 59 E7	10 <sup>2</sup>	10	-3.6836	1.860	0.9995
HPV 66 E6	10 <sup>2</sup>	10	-3.5661	1.927	0.9977
HPV 67 L1	10 <sup>3</sup>	10 <sup>2</sup>	-3.4973	1.835	0.9968
HPV 68 E7	10 <sup>3</sup>	10	-3.2000	2.044	0.9998

MY09/11 consensus PCR. Forty-two (8.6%) showed normal cytology, 186 (37.9%) were diagnosed ASC-US, 25 (5.1%) ASC-H, 170 (34.6%) L-SIL, 54 (11.0%) H-SIL and 14 (2.9%) AGC as summarized in Table 3. Focusing on squamous cell abnormalities, MY09/11 consensus PCR was unable to detect the oncogenic HPV type present in 79 (16.1%) cases with severe cervical dysplasia (ASC-H and H-SIL).

The cytological results of cases with HPV X were compared with those of cases with oncogenic HPV types. The latter showed significantly more squamous cell abnormalities (n = 4,087; 2.9% ASC-H + 38.9% L-SIL + 11.8% H-SIL = 53.6%) than cases with HPV X (n = 6,141; 2.3% ASC-H + 16.2% L-SIL + 1.7% H-SIL = 20.2%) (P < 0.001) (Table 3).

Analysis of the primer-target sequence homology for the MY09/11 primers showed a significant (P < 0.05) association between inefficient amplification of HPV

types and the number and position of mismatches, as shown in Table 4 and Figure 2.

### Follow-up

Histological (n = 3,124; mean follow-up period = 256 ± 245 days; median follow-up period = 127 days; range = 0–1978 days) or cytological (n = 9,426; mean follow-up period = 709 ± 399 days; median follow-up period = 602 days; range = 25–2133 days) follow-up was available for 12,550 (79.6%) of the 15,774 cases. Cytological follow-up was partially based on a reliable estimation of the prevalence of at least two consecutive smears within the different HPV groups. Table 3 shows the follow-up results according to the initial cytological diagnosis. Biopsy showed 771 (23.7%) CIN2+ cases in the MY09/11

**Table 2** Fraction of MY09/11 consensus PCR-negative and -positive cases, according to HPV type

HPV type	MY09/11 consensus PCR				Total	
	Negative		Positive		n	%
	n	%	n	%		
16	37	4.8%	734	95.2%	771	16.2%
18	39	9.0%	392	91.0%	431	9.0%
31	18	5.3%	320	94.7%	338	7.1%
33	10	2.8%	349	97.2%	359	7.5%
35	38	8.3%	418	91.7%	456	9.6%
39	46	12.7%	315	87.3%	361	7.6%
45	20	15.5%	109	84.5%	129	2.7%
51	188	33.4%	375	66.6%	563	11.8%
52	24	9.3%	234	90.7%	258	5.4%
53	3	3.8%	76	96.2%	79	1.7%
56	34	9.3%	332	90.7%	366	7.7%
58	5	7.6%	61	92.4%	66	1.4%
59	9	10.2%	79	89.8%	88	1.8%
66	23	6.3%	345	93.8%	368	7.7%
67	1	2.7%	36	97.3%	37	0.8%
68	27	26.7%	74	73.3%	101	2.1%
<b>Total</b>	<b>522</b>	<b>11%</b>	<b>4,249</b>	<b>89%</b>	<b>4,771</b>	<b>100%</b>

**Table 3** Results of MY09/11 consensus PCR and type-specific PCRs and overview of follow-up for different cytological groups

MY09/11 PCR	+				-				+				-			
	+				+				-				-			
Type-specific PCRs	HR-HPV				HR-HPV				HPV X				Negative			
HPV	HR-HPV				HR-HPV				HPV X				Negative			
Initial diagnosis	Follow-up		CIN2+	Follow-up		CIN2+	Follow-up		CIN2+	Follow-up		CIN2+	Follow-up		CIN2+	
	N	%	n	n	n	%	n	n	n	%	n	n	N	%	n	n
<b>Squamous lesions</b>																
<b>Negative</b>	401	11.2	367	80	42	8.6	34	6	1,776	28.9	1,349	0	2,142	38.6	1,626	1
<b>ASC-US</b>	1,217	33.8	1,069	173	186	37.9	142	24	2,965	48.3	2,253	2	2,861	51.6	2,166	0
<b>ASC-H</b>	93	2.6	81	17	25	5.1	23	8	139	2.3	107	0	62	1.1	49	1
<b>L-SIL</b>	1,418	39.4	1,357	265	170	34.6	142	20	995	16.2	762	2	251	4.5	193	1
<b>H-SIL</b>	427	11.9	356	236	54	11.0	54	40	107	1.7	86	7	17	0.3	16	1
<b>Glandular lesions</b>																
<b>AGC</b>	40	1.1	18	0	14	2.9	14	6	159	2.6	122	0	213	3.8	164	0
<b>Total</b>	3,596	100	3,248	771	491	100	409	104	6,141	100	4,679	11	5,546	100	4,214	4
<b>Follow-up</b>		%		90.3		%		83.3		%		76.2		%		76.0
<b>CIN2+</b>		%		23.7		%		25.4		%		0.2		%		0.1



and type-specific PCR-positive HR-HPV group, 104 (25.4%) in the MY09/11-negative, type-specific PCR-positive HR-HPV group, 11 (0.2%) in the HPV X group and 4 (0.1%) in the HPV-negative group.

## **Diagnostic accuracy and predictive values**

Type-specific PCRs showed a sensitivity of 98.3% and a specificity of 76.1% to detect biopsy-proven CIN2+. Positive and negative PVs were 23.9% and 99.8%, respectively.

MY09/11 consensus PCR showed a sensitivity of 87.9% and a specificity of 38.7% to detect biopsy-proven CIN2+. Positive and negative PVs were 9.9% and 97.7%, respectively.

## **Discussion**

Establishment of the critical role of HPV in the carcinogenesis of cervical carcinoma has led to the development of new applications to identify cancer precursors. Currently, PCR amplification is considered as the most sensitive method for detection of HR-HPV DNA and is highly reproducible, easily monitored, provides an objective test outcome and can easily be automated. Consensus PCR assays have been devised to amplify the most relevant genital HPV types in one reaction. When the MY09/11 PCR was designed, only HPV 6, 11, 16, 18 and 33 were known [12]. The MY09/11 primers were designed in a conserved region of the L1 open reading frame (ORF) with the intent to amplify these five genotypes and any other genital HPVs with shared sequence homology in a single reaction [11]. Because the targets were not entirely homologues, positions with nucleotide base heterogeneity were accommodated by inclusion of degenerate base sites. A mixture of 24 oligonucleotides forms a degenerated pool of primers amplifying a broad spectrum of HPV genotypes with various levels of sensitivity. Insertion of nucleotide bases at positions of degeneracy is a random process, and hence does not ensure an equivalent representation of all primers. Lot-to-lot variations among separate syntheses of primers could result in differences in type-specific amplification efficiencies [13]. In this study, only two different lots of

MY09/11 primers were used and lot-to-lot variations in type-specific amplification efficiencies were monitored and considered absent.

This study compared the performance of MY09/11 consensus PCR with that of type-specific PCRs, mainly directed at HPV E6 and E7 genes, which encode oncogenic products.

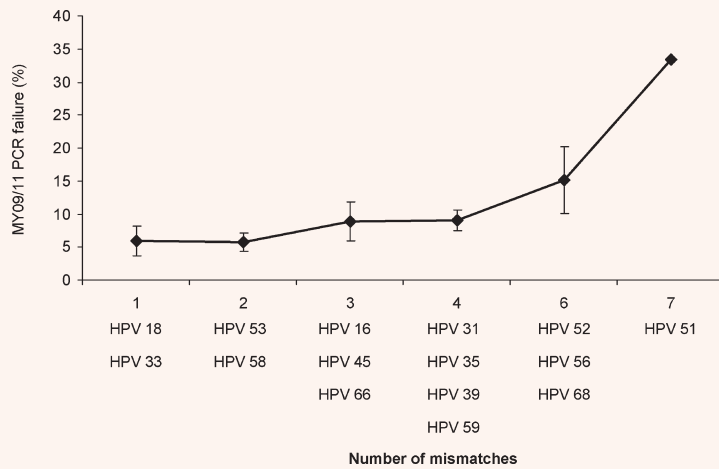
The set of HR-HPV types varies between studies and here HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67 and 68 were detected. These include the 13 HPV types detected by the high-risk probe cocktail of the Hybrid Capture II system (HC2, Digene Corp., Gaithersburg, USA), the only HPV test that has been FDA-approved for cervical cancer screening in combination with cytology after the age of 30 [5, 19]. However, this test shows a number of disadvantages such as the inability to identify HPV types and the risk of cross-hybridization of additional HPV types with the probe mix [20, 21].

MY09/11 consensus PCR failed to detect 522 HPV infections indicated by type-specific PCRs (Fig. 1). False negativity of MY09/11 PCR occurred in 10.9% of all HPV infections detected with type-specific PCRs ( $n = 4,771$ ). Statistical analysis showed a significant correlation between failure of consensus PCR and HPV type. HPV types 51 (33.4%), 68 (26.7%) and 45 (15.5%) were missed most frequently (Table 2). Type-specific prevalence and risk of cervical cancer associated with different HPV types were assessed to evaluate the clinical importance of PCR false negativity. HPV 45 is the third most common type worldwide with an odds ratio for cervical cancer of 197.6. The prevalence of HPV 51 and 68 is lower, but odds ratios for cervical cancer confirm the high-risk nature of these HPV types [22, 23].

The clinical relevance of the HPV infections missed by MY09/11 PCR is also reflected in the fraction of cases with cytological abnormalities ASC-H (5.1%) and H-SIL (11.0%) (Table 3). Moreover, follow-up of 409 (83.3%) of the 491 cases with MY09/11 false negativity resulted in 104 (25.4%) CIN2+ cases (Table 3).

The sensitivity of MY09/11 PCR was shown to be significantly lower than that of the type-specific PCRs for all HPV types, leading to false negative results due to a viral load beneath the detection limit of the MY09/11 PCR (Table 1).

PCR failure may be caused by mismatches between MY09/11 primers and the L1 target (Table 4, Fig. 2). A significant association between inefficient



**Fig. 2** Association between inefficient amplification of different oncogenic HPV types with MY09/11 consensus PCR and the number of mismatches between MY09/11 primers and target sequences. MY09/11 primers showed five mismatches with HPV 67, which was only missed once (2.7%). This result, however, was not included in the analysis because of the low prevalence of HPV 67 and because this was the only type for which the type-specific PCR was directed against L1, showing a lower sensitivity than the other type-specific PCRs.

**Table 4** Positions and number of mismatches between MY09/11 primers and different oncogenic HPV types

HPV type	Primer mismatch		n mismatches
	MY09 Forward primer 5'-CGT CCM ARR GGA WAC TGA TC-3'	MY11 Reversed primer 5'-GCM CAG GGW CAT AAY AAT GG-3'	
16	...T ... ..	... ..C ..C ... ..	3
18	... ..T ... ..	... ..	1
31	..A ... ..T ... ..	..T ... ..C ... ..	4
33	... ..	... ..A ... ..	1
35	..G ... ..C ... ..	... ..A ..C ... ..	4
39	... ..G ..T ... ..	... ..C ..C ... ..	4
45	..A ... ..T ... ..	... ..C ... ..	3
51	..A ... ..T ..C ..T ..G ..	..G ... ..C ... ..	7
52	..TA ..T ... ..	..G ... ..C ..C ... ..	6
53	..TG ... ..	... ..	2
56	..TA ... ..T ... ..T ... ..	... ..A ..C ... ..	6
58	... ..	... ..A ..C ... ..	2
59	... ..	..T ... .. TTA ... ..	4
66	..TA ... ..	... ..C ... ..	3
67	..TA ..T ... ..T ... ..	... ..A ... ..	5
68	... ..T ..T ..G ..T ..G ..	... ..C ... ..	6

Nucleotide homology is indicated with a period and mismatches are indicated with the nucleotide change in the corresponding sequence. The degenerate base code is as follows: M = A or C, W = A or T, Y = C or T, and R = A or G.



amplification of HPV types and the number and position of mismatches was found. For HPV 51 and 68, which were missed most frequently, MY09/11 PCR clearly malfunctions because of the lack of primer specificity (Table 4, Fig. 2). Addition of an extra sequence-specific oligonucleotide (HMB01) directed to the minus strand of HPV may facilitate HPV 51 amplification [24]. Moreover, PGMY09/11 primers were designed to eliminate degeneracies and improve sensitivity across the type spectrum with increased detection of multiple infections and improved reproducibility and specificity [13, 25]. Surprisingly, one report shows similar analytical sensitivities for MY09/11 and PGMY09/11, with better detection of several important HPV types, including HPV 16, by MY09/11. As such, caution should be exerted and reproducibility should be monitored when comparing performances of different primer systems [26].

MY09/11 false negativity could also result from loss of the L1 ORF during integration of the viral DNA into the host genome. Integration is one of the critical contributing factors to malignant transformation and often occurs in the L1 region [4]. This could particularly be the case for HPV 18, which is more often disrupted in the L1 region than other HPV types (Table 2) [27]. PCR primers targeting L1 can therefore be considered less reliable than PCRs directed against the E6 or E7 genes, which encode oncogenic products and always remain intact [28]. Besides the risk of integration, the extensive length of the MY09/11 L1 amplicon promotes false negative results because the efficiency of a PCR reaction generally decreases with increasing amplicon size. Subjecting clinical samples to treatments, such as fixation, is known to degrade DNA. Consequently, the efficiency of PCR primers generating a small product is considerably higher than primers yielding larger amplicons [29]. Overall, the choice of the region used for PCR has important implications for successful HPV detection in clinical diagnosis and management of cervical cancer.

Furthermore, MY09/11 PCR lacks specificity for oncogenic HPVs. A total of 6,141 samples tested positive for MY09/11 consensus PCR, but negative for all PCRs targeting oncogenic HPV types (Fig. 1). Since MY09/11 PCR can produce nonspecific DNA fragments in the same size range as the HPV L1

product, and since the MY09/11 results are based on gel analysis and not on hybridization with HPV L1 probes, a number of samples could have been falsely diagnosed as HPV-positive. Considering the overall higher sensitivity of the type-specific PCRs, it seems very unlikely that consensus PCR would detect true oncogenic HPVs missed by type-specific PCRs. As such, the samples likely contain unidentified HPV genotypes of unknown malignant potential (HPV X), such as low-risk HPV 6 and 11, which are not likely to induce the carcinogenic process, or such as HPV 26 and 73, which are probably oncogenic but not targeted by this study's type-specific PCRs [19]. This is reflected in the cytology results of cases with HPV X-infection and in their follow-up, yielding a minor fraction of CIN2+ cases (0.2%).

The performance characteristics of both PCR systems in the prediction of CIN2+ lesions further strengthen the clinical importance of our findings. The difference in sensitivity for the detection of CIN2+, 98.3% for the type-specific PCRs *versus* 87.9% for MY09/11 PCR, is a direct consequence of the difference in detection sensitivity for oncogenic HPVs. The poor specificity of MY09/11 PCR for the detection of CIN2+, 38.7% *versus* 76.1% for the type-specific PCRs, is a result of its lack of specificity for oncogenic HPV types.

In conclusion, type-specific PCRs for a defined range of HR-HPV types may constitute a more suitable HPV screening test, when compared with a consensus approach, provided that practical issues related to the performance and analysis of multiple PCRs are dealt with. Our current laboratory set-up allows high-throughput, type-specific HPV detection with a turnaround time of less than 72 hrs and better cost-effectiveness than commercial alternatives. Nevertheless, type-specific HPV testing is valuable to address the burden of HPV infections epidemiologically and to gain more insights into the natural history and dynamics of HPV infections. Therefore, it can be considered as an indispensable tool to monitor the impact of vaccination on the risk of acquisition of individual HPV types. Since type-specific PCRs enable the determination of viral load and integration, which have been suggested as type-dependent risk markers for high-grade cervical lesions and carcinoma, their niche in clinical practice seems undisputable [30–34].

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