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Application of a multiplex PCR to cervical cells collected by a paper smear for the simultaneous detection of all mucosal human papillomaviruses (HPVs) and typing of high-risk HPV types 16 and 18

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A simple paper smear (PS) method for dry collection and storage of cervical specimens was employed to develop an easy multiplex (MPX) PCR for simultaneous detection of generic human papillomaviruses (HPVs) as well as typing of the high-risk HPV-16 and -18, the two clinically most important HPV genotypes, which are responsible for more than 80 % of cervical cancers. Multiplexing was performed with a small amount of DNA eluted by boiling from a single PS punch in a single tube and using a mixture of four pairs of primers specific for the HPV L1 consensus sequence, HPV-16, HPV-18 and the β-globin gene. Sixty HPV-positive biopsies and corresponding PS specimens from cervical cancer patients as well as cervical smears from 100 healthy women with or without abnormal cytology were collected both as PSs and in PBS. Detection of HPV DNA from cervical biopsies collected in PBS and corresponding cervical scrapes on a PS or in PBS by conventional and MPX-PCR showed a concordance of 100 % and adequacy of 93 %. A similar comparative study in cervical scrapes from normal women also revealed 100 % concordance. The technique was validated in a multicentric study at four different national laboratories. PSs collected by different centres showed variable adequacy (73–82 %) but the use of multiple PS discs for DNA extraction significantly increased the adequacy. Integration of PSs with MPX-PCR for the detection and typing of HPVs is a highly convenient, efficient, simple and cost-effective method for large-scale clinico-epidemiological studies and is also suitable for HPV vaccine monitoring programmes in resource-poor settings.

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INTRODUCTION

With an annual incidence of 493 000 cases and the death of about 273 000 women, cervical cancer has become the second most common malignancy in women worldwide (Ferlay *et al.*, 2004). More than three-quarters of these deaths are from developing countries. In India, about 132 000 women get cervical cancer every year, contributing approximately 27 % of the global annual incidence (Parkin *et al.*, 2005). Numerous epidemiological, clinical and experimental studies have established the involvement of human papillomaviruses (HPVs), particularly the 'high-risk' (HR) HPV types, as indispensable carcinogenic agents for the development of cervical cancer, and almost all cases of cervical cancers show the presence of HR-HPV infection (Muñoz *et al.*, 2006). Of more than 110 HPV types described so far, about 30 are mucosotropic and are linked

Abbreviations: HPV, human papillomavirus; HR, high risk; MPX, multiplex; PS, paper smear.

to cervical and other anogenital cancers (Bosch *et al.*, 2002; Burd, 2003; Cuzick *et al.*, 1992; zur Hausen & de Villiers, 1994). Fifteen of these are well characterized as HR types, and of these HPV-16 and -18 are detected in 60–90 % of invasive cancers from almost all parts of the world (Bosch & de Sanjosé, 2003). In India, almost all cases of cervical cancer show the presence of HPV infection and HPV-16 and -18 contribute to >90 % of them (Das *et al.*, 2008). Recently, two prophylactic HPV vaccines, Gardasil (Villa *et al.*, 2006) and Cervarix (Harper *et al.*, 2006), have been developed against these two most prevalent and clinically important HPV genotypes.

HPV infections are often present in the general population as clinically latent and asymptomatic infections. It has also been established that women with dysplastic lesions and carrying HR-HPV infections show a high rate of progression to invasive cancer (Wallin *et al.*, 1999). However, cervical cancer is preventable, as well as curable, if detected early through screening. The currently available method for mass screening is the cytology-based Pap test; however, this is not very reliable and does not detect or type the HPV infection that causes the cervical cancer. Several authors have shown the potential relevance of HPV DNA testing in cervical cancer screening programmes for the management and control of cervical cancer. Recently, Sankaranarayanan *et al.* (2009) demonstrated that once-in-a-lifetime screening for HPV can cause a substantial reduction in the incidence and mortality of cervical cancer (Sankaranarayanan *et al.*, 2009).

Apart from Hybrid Capture II, which is a generic test for HPV infection, HPV detection and typing is most commonly carried out by PCR-based assays, which are well established and constantly being improved to enhance the sensitivity and specificity for detecting HPV infection. Detection of the HPV genome by PCR relies on an assay of cellular DNA extracted from cervical scrapes or tumour biopsy specimens. However, these methods are cumbersome and suffer from several technical limitations associated with the collection, transport and storage of biological specimens. Scraped cervical cells or tumour biopsies are generally collected in cold PBS or kit-specific solutions and transported on ice, requiring constant refrigeration, or may be stored in a liquid-based preservation medium (Cytyc or ThinPrep solution) that allows storage at room temperature. These methods are, however, hazardous and cost-intensive for developing countries like India. The procedures of DNA isolation by standard proteinase K digestion and chloroform/phenol extraction or using commercially available DNA extraction kits are complex, hazardous, time-consuming and neither cost-effective nor suitable for screening large numbers of specimens at a time. To overcome these difficulties, we previously developed an easy 'paper smear' (PS) method for the dry collection of cervical scrapes and biopsy imprints from field stations or clinics for screening of HPV infection (Kailash *et al.*, 2002). This method has been used by several other authors to collect a variety of biological specimens to detect various infectious agents (Beebe & Briggs, 1990; Gupta *et al.*, 1992; Kain & Lanar, 1991), gene sequences and metabolic or genetic diseases (Garrick *et al.*, 1973; Maeda *et al.*, 1985). This is an extremely sought-after method for easy sample collection, transport and storage without liquid medium or refrigeration, specifically in resource-poor settings. However, the yield of DNA per disc obtained from PSs was found to be relatively low compared with that in liquid-based collection (Banura *et al.*, 2008) and was a limiting factor for doing multiple reactions and experiments. Moreover, conventional uniplex PCR is time-consuming, laborious and not suited to large-scale screening programmes.

In order to make HPV testing simple and cost-effective with cervical scrapes and applicable to a robust mass screening, an HPV test needs to detect all mucosal and genital HPV genotypes and to identify at least the two most common and clinically important HR-HPV types, HPV-16 and -18, which are responsible for more than 80 % of cervical cancers. Therefore, we developed a simple multiplex PCR (MPX-PCR) using a single PS punch to produce an easy and cost-effective method for screening a broad spectrum of genital HPV types and for typing of HPV-16 and -18, using β-globin gene detection as an internal control in clinical specimens, all in a single reaction tube. The efficacy of this test was validated in clinical settings by collecting PS samples at four different national centres located in different regions of the country.

METHODS

Sample collection. A total of 100 cervical cancer tissue biopsies and corresponding cervical scrape samples on PSs from women diagnosed at different clinical stages (stages III and IV) were obtained from patients attending the Cancer Clinic of Gynaecology Outpatient Department of Lok Nayak Hospital, New Delhi, and of Jawaharlal Nehru Medical College and Hospital, Aligarh, India. Each biopsy specimen was bisected and one half was subjected to histopathological examination and the other was immediately frozen in liquid nitrogen for molecular investigation. The cervical scrapes from these cancer patients were collected as PSs before taking the punch biopsies, with the help of an Ayre's spatula by rotating it 360° in the os of the endocervix and smearing it within a 0.5–1.0 cm diameter on a piece of 3MM Whatman filter paper cut to the size of a microscope glass slide. Informed consent was obtained from each patient prior to collection of the specimens. The study was approved by the Institutional Ethical Committee. In addition, 100 cervical scrapes were collected from women with or without abnormal cytology attending the Cancer Clinic of Gynaecology Outpatient Department of Lok Nayak Hospital for a routine check-up or for problems other than malignant lesions. Sixty histopathologically proven cancer biopsies with high-quality extracted DNA and HPV L1 region consensus primers were selected for standardization of the MPX-PCR and for a comparison of the performance of the MPX-PCR technique on DNA extracted by boiling using corresponding PSs. For multicentric evaluation of PS-based sample collection and the influence on the rate of detection of HPV DNA sequences, a total of 248 samples was collected from the Tata Memorial Cancer Hospital, Mumbai (n=55), Kidwai Memorial Hospital, Bangalore (n=35), Regional Cancer Centre, Trivandrum (n=58), Lok Nayak Hospital, New Delhi, and Jawaharlal Nehru Medical College Hospital, Aligarh (n=100). The PS samples were collected, dried and transported to the Institute of Cytology & Preventive Oncology at room temperature for subsequent HPV analysis.

Isolation of genomic DNA from tumour biopsies and PSs. Isolation of genomic DNA from PSs was performed as described previously (Kailash *et al.*, 2002) with minor modifications. Briefly, a 4 mm diameter disc of the dried smear was punched out with a sterile paper-punching machine and transferred to a 0.5 ml microcentrifuge tube containing 100 µl double-distilled water. For individual PS samples, a separate sterile punching machine was used each time to prevent cross-contamination. The tubes were incubated for 30 min at 65 °C in a water bath, boiled for 5 min in a microwave oven (350 W) and immediately chilled on ice. To improve extraction, the boiling and snap-chilling steps were repeated twice, and the extracted DNA was digested with 20 ng RNase prior to PCR. After reduction of the extracted DNA volume to 10 µl in a Speed-Vac Concentrator (Labconco), PCR master mix was added directly to the tube and PCR amplification was carried out using a DNA Engine Tetrad (MJ Research/Bio-Rad). To assess the adequacy of PS samples, an increased number of PS discs (from two to five) of 4 mm diameter were punched out and DNA isolation was performed as described above and the adequacy was examined by PCR for β-globin amplification.

Standard proteinase K digestion and phenol/chloroform methodology routinely employed in our laboratory were used as a reference method for the extraction of DNA from cervical scrapes and tumour biopsy specimens collected in PBS and stored at −70 °C (Das *et al.*, 1992; Gopalkrishna *et al.*, 1992; Sambrook *et al.*, 1989). All other reagents used were molecular biology grade and were from Sigma Aldrich.

Development and standardization of an MPX-PCR assay. The MPX-PCR assay for the simultaneous amplification of the HPV consensus L1 region and HPV-16 and -18, along with the β-globin gene as an internal control, was developed by using an equimolar mix of HPV-16 and -18 plasmids in a background of human genomic DNA. The developed assay was then applied to genomic DNA of tumour tissues. The primers used were MY09/MY11 targeting the HPV L1 consensus sequence, HPV-16 primers for the upstream regulatory region (URR), HPV-18 primers for the E6 gene and primers for the β-globin gene. The primers were custom-synthesized and HPLC-purified by Microsynth GmbH. The primer sequences along with the final concentration used and other characteristics are given in Table 1. A common annealing temperature for all four primer pairs was standardized to 55 °C by performing gradient uniplex PCR for each primer set. PCR was carried out in a 25 µl reaction volume containing either ~100 ng genomic DNA from biopsies or known copy numbers of HPV-16/-18 plasmid DNA, 10 mM Tris/HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each dNTP, the indicated concentration of each oligonucleotide primer (Table 1) and 0.5 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems). PCR was performed in a DNA Engine Tetrad with an initial denaturation at 95 °C for 9 min, followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, with a final extension for 7 min at 72 °C. Ten microlitres of PCR product was electrophoresed in a 3 % agarose gel and stained with ethidium bromide (0.5 µg ml^{-1}). Transilluminated gel images were digitized and analysed using an AlphaDigiDoc RT Gel Documentation System (Alpha Innotech). The sensitivity of the MPX-PCR was determined using a tenfold dilution series of HPV-16 and -18 plasmid mixture in a background of C33a cell DNA and the assay was calibrated using WHO international standards for HPV-16 and -18 developed under the WHO HPV LabNet programme (Wilkinson, 2008).

RESULTS AND DISCUSSION

In the present study, an MPX-PCR method was integrated with the PS method (Kailash *et al.*, 2002) (PS-MPX-PCR). This was specifically designed to detect the two major carcinogenic HR-HPV types, HPV-16 and -18, along with a generic screen for all mucosal HPVs based on the MY09/MY11 primers targeting the consensus sequences in the HPV L1 region and an internal-control β-globin gene to overcome the issue of conventional DNA extraction and multiple uniplex PCRs, which are labour-intensive and expensive. The MPX-PCR assay was developed and tested on DNA extracted using a standard phenol/chloroform protocol from cervical biopsies collected in PBS to analyse its performance on clinical samples. As shown in Fig. 1, the biopsy samples showed discrete bands indicating co-amplification of the HPV L1 consensus (450 bp), HPV-16 (217 bp), HPV-18 (100 bp) and β-globin (268 bp) sequences. To determine the sensitivity of the MPX-PCR, a tenfold dilution series ranging from 10^4 to one copy of HPV-16 and -18 international standards was used as a reference against a background of genomic DNA from an HPV-negative cervical cancer cell line (C33a). The sensitivity of the MPX-PCR assay as determined by amplification of the HPV-16/-18 international standards revealed an assay detection limit as low as 100 copies for both HPV-16 and -18, which is similar to the WHO HPV LabNet recommendations of a detection limit up to 50 copies of genome equivalents (Wilkinson, 2008). Inclusion

Table 1. Oligonucleotide primers used for establishing MPX-PCR

M=A or C; W=A or T; Y=C or T; R=A or G.

Primer	Nucleotide position	Amplicon size (bp)	Sequence (5′–3′)	Final concentration (µM)	Reference
MY09 (forward)	L1	450	CGTCCMARRGGAWACTGATC	20	Manos *et al.* (1989)
MY11 (reverse)	L1		GCMCAGGGWCATAAYAATGC		
HPV-16 forward	7763–7781	217	AAGGCCAACTAAATGTCAC	20	Das *et al.* (1992)
HPV-16 reverse	57–75		CTGCTTTTATACAACCGG		
HPV-18 forward	463–482	100	ACCTTAATGAAAAACCACGA	20	Das *et al.* (1992)
HPV-18 reverse	534–562		CGTCGTTTAGAGTCGTTCCTG		
β-Globin forward		268	GAAGAGCCAAGGACAGGTAC	10	Das *et al.* (1992)
β-Globin reverse			CAACTTCATCCACGTTACACC		

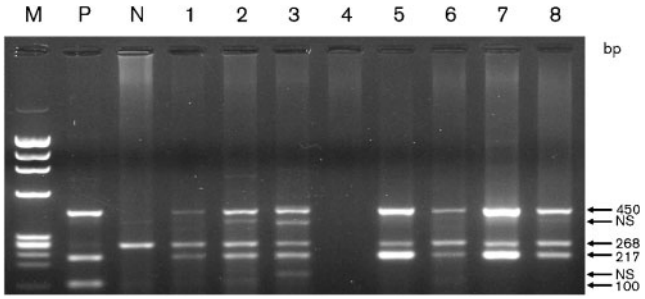

Fig. 1. Representative MPX-PCR showing amplification of the HPV L1 consensus (450 bp), HPV-16 (217 bp), HPV-18 (100 bp) and β-globin internal control (268 bp) sequences in genomic DNA from cervical cancer biopsies. Lanes: 1–3 and 5–8, genomic DNA from cervical cancer biopsies; M, molecular marker; P, cocktail of HPV-16 and -18 plasmids; N, negative control with genomic DNA from an HPV-negative human cervical cancer cell line, C33a; 4, negative control with no DNA. NS, Non-specific amplification.

of the β-globin primers in the reaction cocktail allowed a simultaneous assessment of the integrity and quality of the genomic DNA eluted from the PSs. As several authors have demonstrated that co-amplification of β-globin primers with HPV could reduce the sensitivity of HPV detection (Coutlée *et al.*, 1999; Vernon *et al.*, 2000), in the present study, we reduced the concentration of β-globin primers to the extent that they did not interfere with amplification of HPV DNA or β-globin itself. Furthermore, the amplification parameters were optimized in such a way that amplification of all four pairs of primers occurred in a single reaction without any interference among them.

To evaluate the test performance of cervical scrapes using PSs, 60 histopathologically confirmed cervical cancer cases with known HPV positivity were tested by MPX-PCR on the DNA extracted from PSs by a boiling method and the results obtained were compared with the results of uniplex PCR and also with MPX-PCR amplification of DNA extracted from corresponding punch biopsies using a standard protocol (Fig. 2). Out of 60 PS samples, 55 were positive for HPV-16, four for HPV-18 and two had co-infection of HPV-16 and -18 as revealed by MPX-PCR. As indicated in Table 2, DNA extracted from punch biopsies by a standard procedure also revealed similar positivity in cases by uniplex PCR, which matched the MPX-PCR results, except for five PS cases where the β-globin amplification also failed, thus indicating sample inadequacy. Therefore, overall adequacy using a single 4 mm disc PS was approximately 92 %. PS-MPX-PCR was also found to detect the co-infection, as detected by uniplex PCR, in the corresponding tissue biopsy DNA. Therefore, similar to a standard protocol, the PS technique can effectively detect HPV-16 and -18 individually as well as in co-infections along with other HPVs. The use of β-globin detection in the MPX-PCR allowed the distinction

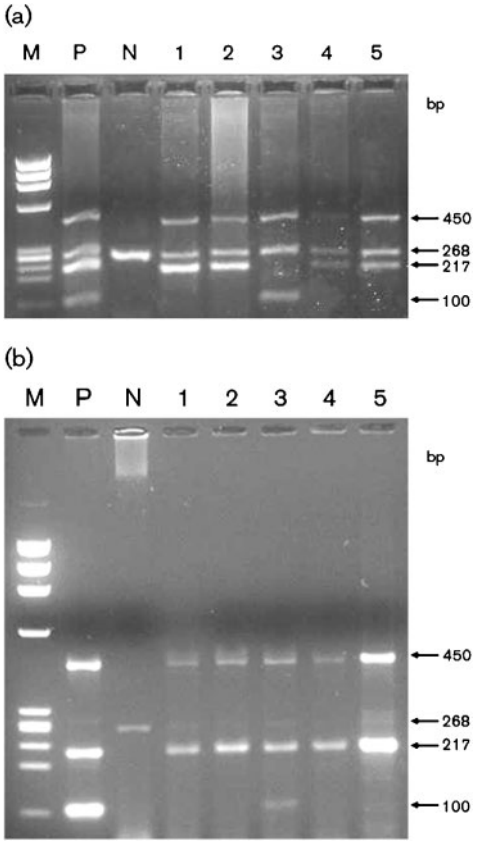

Fig. 2. Consensus and type-specific identification of HPV by MPX-PCR using genomic DNA from cervical cancer biopsies collected in PBS and extracted using a standard chloroform/phenol method (a) and genomic DNA from the corresponding cervical scrapes collected on PSs and isolated by a rapid boiling method (b). Lanes: 1–5, genomic DNA from cervical cancer biopsies; P, cocktail of HPV-16 and -18 plasmids; N, negative control with genomic DNA from C33a cells.

to be made between an HPV-negative sample and an inadequate sample. Therefore, despite smearing of the specimen on filter paper, which is a highly variable step where it is difficult to control the spot size and where there may be an uneven distribution of cells, the technique was able to distinguish between true negatives and samples that failed to perform effectively. A recent study by Banura *et al.* (2008), which showed poor performance of the PS technique, had an obvious shortcoming, as sample adequacy was not taken into account or controlled by using an internal control. Most importantly, the use of a cotton swab instead of an Ayre's spatula or cytobrush for collecting the cervical smears for preparation of the PS is certainly not effective and hence not recommended. Generally, the collection of a cervical smear involves gentle scraping of the ectocervix and endocervical os to remove epithelial cells, which mostly get attached to the cotton swab, and it is difficult, if not impossible, to detach and smear them onto filter paper as most of the cells remain

Table 2. Comparison of HPV detection in cervical biopsies by uniplex PCR with the corresponding PS by MPX-PCR (n=60)

HSIL, High-grade squamous intraepithelial lesion; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma; WDSCC, well-differentiated squamous cell carcinoma.

Histopathological grade	HPV positivity (n) in biopsy DNA				HPV positivity (n) in PS DNA			
	Total	HPV-16	HPV-18	Co-infection	Total	HPV-16	HPV-18	Co-infection
HSIL (n=7)	7	7	0	0	5	5 (2*)	0	0
WDSCC (n=18)	18	17	1	0	17	16 (1*)	1	0
MDSCC (n=22)	22	21	1	0	21	20 (1*)	1	0
PDSCC (n=10)	10	10	1	1	10	10	1	1
Adenocarcinoma (n=3)	3	3	1	1	2	2 (1*)	1	1
Total	60	58 (97 %)	4 (7 %)	2 (3 %)	55 (92 %)	53 (88 %)	4 (7 %)	2 (3 %)

*Number of PS specimens that were inadequate (i.e. negative for β-globin amplification).

adsorbed to the cotton swab. The results obtained showed a good correlation of PS-MPX-PCR with that of conventional methods used for sample collection, DNA extraction and uniplex/MPX-PCR for the detection of HPV infection. The use of a single-tube MPX-PCR not only enabled the detection of HPV infection with the same sensitivity and specificity as uniplex PCR but also minimizes the carryover of PCR products, thus reducing the chances of cross-contamination.

To validate the PS protocol for its utility for large-scale screening programmes or vaccine monitoring, the performance of the PS-MPX-PCR was also determined on specimens collected from the normal population. A total of 100 samples was collected from normal asymptomatic women for cytological diagnosis and HPV detection by a standard protocol as well as by the PS method. The standardized MPX protocol was employed to detect HPV in the genomic DNA extracted from samples collected in PBS by the conventional method or from PS samples by the boiling method. As described in Table 3, using the standard protocol on cervical scrapes collected in PBS, the adequacy as measured by β-globin amplification was 92 % whereas use of a single 4 mm disc from PS for MPX-PCR analysis demonstrated an adequacy of 83 %. However, the HPV positivity as determined by PS-MPX-PCR was comparable to the positivity determined by the standard procedure. Therefore, the technique is also suitable for screening of women with pre-cancerous lesions or normal cytology where the number of scraped cervical epithelial cells as well as the amount of DNA per sample is less compared with that in cancer cases.

Sample adequacy plays a critical role in the success of the PS-MPX-PCR methodology. Considering the non-standardized procedure of cervical scrape collection, the variability in the expertise of sample collectors and the preparation of PSs by healthcare professionals of variable expertise, the possible variations in test results are obvious. We therefore determined the magnitude of this variation in a national multicentric evaluation in clinical settings of four different cancer hospitals having no prior exposure to collection of cervical scrapes on PSs. For this multicentric validation study, each centre collected cervical scrapes on PSs from women diagnosed with cervical pre-cancer or cancer and transported the PS samples to our laboratory where the PS-MPX-PCR was performed on all samples. Table 4 describes

Table 3. Detection of HPV infection by MPX-PCR from PS specimens collected from a normal asymptomatic population (n=100)

ASCUS, Atypical squamous cells of undetermined significance; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion.

Cytological diagnosis	Samples analysed (n)	HPV detection by conventional in-house methods				HPV detection by PS-MPX-PCR			
		Adequate samples (n)	HPV-positive samples (n)	HPV-16 (n)	HPV-18 (n)	Adequate samples (n)	HPV-positive samples (n)	HPV-16 (n)	HPV-18 (n)
Normal	58	**55**	6	6	0	**50**	6	6	0
Inflammation	32	**28**	3	3	0	**25**	3	3	0
ASCUS	3	**3**	1	1	0	**2**	1	1	0
LSIL	5	**4**	2	2	0	**4**	2	2	0
HSIL	2	**2**	2	2	0	**2**	2	2	0
Total	100	**92**	14	14	0	**83**	14	14	0

Table 4. Adequacy and rate of HPV detection in a single 4 mm punch from a PS of cervical scrapes with cervical pre-cancer and cancer collected from four different centres

LH, Lok Nayak Hospital; JNMCH, Jawaharlal Nehru Medical College and Hospital; RCC, Regional Cancer Centre; TMH, Tata Memorial Hospital; NCBS, National Centre for Biological Sciences. Inadequate samples are those for which there was no amplification of the β-globin internal control.

Sample attribute	Sample collection site				Total
	LH, New Delhi/JNMCH, Aligarh	RCC, Trivandrum	TMH, Mumbai	NCBS, Bangalore	
Samples analysed (*n*)	100	58	55	35	248
Inadequate samples (*n*)	18	13	15	8	54
Adequacy (%)	82	78	73	77	78
Samples analysable for HPV infection (*n*)	82	45	40	27	194
HPV-positive samples [*n* (%)]	70 (85)	38 (84)	34 (85)	22 (81)	164 (85)
HPV-16 positive [*n* (%)]	57 (81)	28 (74)	26 (76)	16 (73)	127 (77)
HPV-18 positive [*n* (%)]	5 (7)	2 (5%)	2 (6)	1 (5)	10 (6)
HPV-16/HPV-18 co-infection [*n* (%)]	1 (1.5)	1 (3)	0	0	2 (1)

the overall analysis of samples by PS-MPX-PCR. Sample adequacy as indicated by β-globin amplification was found to be variable with respect to each centre and ranged from 73% (40/55) to 82% (82/100). The majority of these samples were positive for HPV infection and demonstrated variable HPV-16 and -18 positivity that could be ascribed to the variable prevalence of HPV-16 and -18 infection in these populations. However, it is important to note that the present study focused mainly on validation of the PS-MPX-PCR assay for HPV genotyping rather than determining the prevalence of HPV infections in different populations in the Indian subcontinent; therefore, the prevalence data for HPV infection as indicated in Table 4 should be interpreted with caution. In contrast, the difference in adequacy could be attributed to sampling errors. The collection of cervical scrapes is a variable step and differs from clinic to clinic and from clinician to clinician. Van Doorn *et al.* (2002) observed the importance of sampling errors, as the aliquots from the same homogenized samples from two different laboratories gave different results when analysed for HPV infection. However, errors due to sampling can effectively be taken care of by assessing sample adequacy through β-globin amplification. Moreover, the adequacy could also be improved by using multiple punches from different sites of the PS samples. To test the effect of multiple numbers of PS discs on PCR adequacy, 60 PS samples collected from four different national laboratories that were found to be negative for β-globin amplification (see Table 4) were randomly assigned to four groups of 15 samples each. From each group, two to five PS discs were punched out and DNA was isolated as described for single PS discs and analysed for their amplification adequacy. As indicated in Fig. 3, by increasing the number of PS discs per case from two to five, the positivity for β-globin amplification increased from 26 to 74%. The positivity was found to be maximal (74%) when five discs were utilized simultaneously for analysis. No further increase in the detection rate could be achieved if the number of PS discs increased beyond five (not shown). Therefore, the variation due to non-standardized procedures of scrape collection could be decreased by increasing the number of scraped cervical cells by increasing the number of PS discs as starting material for DNA extraction.

With successful integration of the PS method with MPX-PCR technology, it appears that DNA extracted in this way would also be suitable for other MPX-based technologies for the detection and typing not only of HPV but also of other gene sequences that often use liquid-based conventional sample collection and standard phenol/chloroform DNA extraction (Gheit *et al.*, 2007; Han *et al.*, 2006; Lei *et al.*, 2008; Nishiwaki *et al.*, 2008; Sotlar *et al.*, 2004). When the PS is integrated with MPX-PCR, it makes the technique less time-consuming and it can be used for large screening studies with minimal infrastructure. Several different approaches have previously been utilized for the diagnosis of HPV infections and were based on the detection of

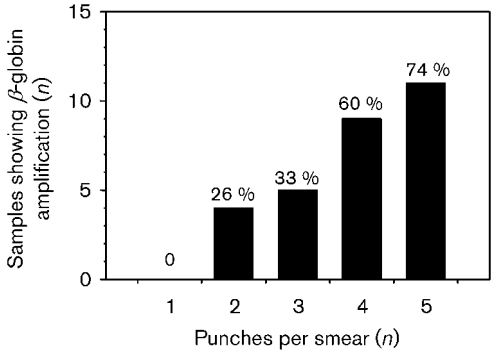

Fig. 3. Effect of increasing the number of paper punches for DNA extraction on sample adequacy. Sixty inadequate PS samples that failed to amplify β-globin using a single 4 mm disc were randomly assigned to four groups of 15 samples each and two to five punches of 4 mm were taken from each group per PS for DNA extraction and analysed by MPX-PCR.

conserved regions within the HPV genome by general or degenerate primer sets, such as MY09/MY11 (Manos *et al.*, 1989) or PGMY09/PGMY11 (Gravitt *et al.*, 2000), GP5+/GP6+ (Jacobs *et al.*, 1997; Schmitt *et al.*, 2008) and SPF_{10} (Quint *et al.*, 2001; van Doorn *et al.*, 2002). HPV typing with general or degenerate primers requires additional assays such as restriction fragment length polymorphisms, dot blots, Southern blot hybridization, direct sequencing and line-blot assays to analyse type-specific HPV infection but, when type-specific primers are used, multiple parallel amplifications are required, which are not suitable for resource-poor settings. The PS-MPX-PCR technique utilizes the advantages of both these approaches, without compromising the quality of results.

The study thus describes a simple single-tube MPX-PCR-based assay for the detection of genomic and clinically relevant HR-HPV genotypes, which utilizes a small amount of DNA extracted by boiling one or two discs punched from a PS that had been used for the collection of cytological samples in dry form, and for storage and transportation at room temperature. We validated its use in mass cervical screening programmes. In view of recent reports showing the immense importance of one-time HPV screening in the control of cervical cancer (Sankaranarayanan *et al.*, 2009), this technique offers a suitable, simple and economically viable alternative not only for HPV screening but possibly also as an effective solution for large epidemiological studies directed towards understanding the prevalence of the two most clinically relevant carcinogenic HPV types, HPV-16 and -18, as well as evaluating the impact of HPV vaccination programmes, particularly in remote areas and resource-poor countries such as India.

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