Human papillomavirus genotyping by multiplex pyrosequencing in cervical cancer patients from India

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Cervical cancer is a leading cause of cancer-related deaths among women in India. Human papillomavirus (HPV) infection is the causative agent of cervical cancer; and infection with the high-risk genotypes, predominantly HPV16 and 18, is the biggest risk factor. Vaccines targeting HPV16 and 18 have been found to confer protection in large-scale clinical trials. HPV genotyping has traditionally been carried out to screen the population "at risk" using indirect methods based on polymerase chain reaction (PCR) using consensus primers combined with various DNA hybridization techniques, and often followed by the sequencing of candidate products. Recently, a high-throughput and direct method based on DNA sequencing has been described for HPV genotyping using multiplex pyrosequencing. We present a pilot study on HPV genotyping of cervical cancer and non-malignant cervical samples using multiplex pyrosequencing. Using genomic DNA from cell lines, cervical biopsies, surgical tissues or formalin-fixed, paraffin-embedded tissue samples, we could successfully resolve 6 different HPV types out of the 7 tested, with their prevalence found to be in agreement with earlier reports. We also resolved coinfections with two different HPV types in several samples. An HPV16 genotype with a specific and recurrent sequence variation was observed in 8 cancer samples and one non-malignant sample. We find this technique eminently suited for high-throughput applications, which can be easily extended to large sample cohorts to determine a robust benchmark for HPV genotypes prevalent in India.

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1. Introduction

Cervical cancer is a major cause of cancer-related deaths among women from poor socioeconomic strata worldwide, with a global annual incidence of over 500, 000 (GLOBOCAN 2002). The fact that cervical cancer is caused by the human papillomavirus (HPV) has led to the development of a number of vaccines targeting HPV for the prevention of the disease. About 15 different carcinogenic (high-risk) HPV types have been described, those most important for cervical cancer being 16, 18, 45, 31, 33, 52, 58 and 35. Of these, HPV16 and 18 are the most prevalent (Bosch *et al* 2002) and primary targets of all the vaccines under development (Roden and Wu 2006).

HPV detection and genotyping has been conventionally carried out using consensus polymerase chain reactions (PCRs) combined with various hybridization-based techniques as well as some non-PCR methods. PCR-based methods include outdated dot blots (Jacobs *et al* 1995; Ylitalo *et al* 1995) and T-ladder generation (Nelson *et al* 2000), as well as the popular reverse hybridization line-probe assays (Coutlee *et al* 2002; van den Brule *et al* 2002) and, more

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recently, DNA microarrays (Klaassen *et al* 2004; Choi *et al* 2005). Hybridization techniques are prone to false signals due to cross-hybridization of closely related genotypes as well as non-specific hybridization and often require subsequent sequencing of PCR products for reliability. The non-PCR techniques include Hybrid Capture II system (HCII, Digene Corp., USA) (Smits *et al* 1995; Cope *et al* 1997) and the less commonly used *in situ* hybridization (Unger 2000) and other RNA-based hybridization techniques (Lie *et al* 2005). HCII has been the most popular among the non-PCR methods but it is less sensitive than PCR methods and only distinguishes low- and high-risk HPV genotypes without identifying the actual genotype. Recently, a new sequencing-based technique called pyrosequencing (Ronaghi 2001; Elahi and Ronaghi 2004) has been described for HPV genotyping; this technique is amenable to multiplexing and high-throughput applications (Gharizadeh *et al* 2001, 2005).

HPV testing studies in India have largely focused on "detection" for screening purposes (Legood *et al* 2005; Sankaranarayanan *et al* 2004, 2005; Shastri *et al* 2005) . Menon *et al* (1995) reported HPV 16/18 in 76.4% of 72 cervical malignancy cases, less than half of 24 cervical intraepithelial neoplasia cases and in none of 4 normal cervical tissues tested. Munirajan *et al* (1998) detected HPV in 70% of 43 stage IIB/IIIB tumours, with HPV16 in 23 cases (53%), HPV18 in 4 cases (13.3%) and 1 case each of HPV33, 35(?) and 58(?). In a hospital-based, case–control study from Chennai, HPV was detected by PCR in all but one of the 205 invasive cervical carcinoma cases and in 27.7% of the 213 age-matched controls. Twenty-three different HPV types were reported, with HPV16 being the most common type, followed by types 18 and 33 (Franceschi *et al* 2003).

More recently, with the emergence of HPV vaccines and large-scale clinical trials proposed and under way, an interest in the distribution of HPV genotypes prevalent in the Indian population has gained importance. In an HPV genotyping effort reported recently from Andhra Pradesh, India, primary screening for high-risk HPV was performed using the Digene Hybrid Capture 2 assay followed by line blot (Sowjanya *et al* 2005). High-risk HPV types were found in 87.8% of the 41 squamous cell carcinomas, with HPV16 (66.7%) and HPV18 (19.4%), being the most prevalent. The relatively small number of samples and the indirect methods of genotyping involving PCRs followed by hybridization-based type determination are a serious limitation.

We describe here the HPV genotyping of cervical samples from women in India using a recently described adaptation of the pyrosequencing technique that combines the advantage of high-throughput multiplex PCR as well as the specificity of DNA sequencing, to simultaneously identify multiple HPV types in a sample, in a 96-well plate format (Gharizadeh *et al* 2003, 2005). This methodology was found to be very convenient and appears suitable for scale up to facilitate large-scale, sequence-based accurate determination of HPV genotypes prevalent in India. We surmise that, if sequencing-based HPV genotyping could be extended over a larger cohort of patients on cervical cancer vaccines, it could help in following the impact of the HPV vaccines on the diversity and potential alteration in the prevalence of HPV genotypes in the population.

2. Materials and methods

2.1 *Sample collection*

A total of 86 clinical samples were used in this study. They were obtained after getting written informed consent from patients at the Tata Memorial Hospital (TMH), Mumbai and King Edward Memorial (KEM) Hospital, Mumbai. These samples included 64 cervical punch biopsy tissues, obtained from the outpatient department of TMH, 7 surgical samples of cervical tissues from the TMH Tumor Tissue Repository and 15 samples of non-malignant cervical tissues from KEM Hospital. Non-malignant/benign cervical tissues were obtained from patients undergoing hysterectomy for reasons other than cervical cancer, such as prolapse, fibroids, cyst, etc. After obtaining these samples, a portion of the tumour was sent for histopathological analysis and tissues free from cervical dysplasia or preneoplastic lesions were used in our study. Since these samples were not from "healthy controls" as the patients were undergoing surgery for uterine/cervical-related diseases, we have referred to them as "non-malignant/benign" samples.

All samples were snap frozen in liquid nitrogen and stored at –80°C until use. Paraffin sections of all the samples were histopathologically examined at TMH. In addition, cervical cancer cell lines Caski, HeLa and SiHa, and a normal immortalized keratinocyte cell line HaCaT were used as controls.

2.2 *DNA extraction*

DNA was extracted from the punch biopsies, surgical samples and the cell lines using the QIAmp system (Qiagen Inc., Valencia, CA,USA) according to standard procedures as described in the kit. The quality and quantity of the DNA were ascertained by agarose gel electrophoresis and spectrophotometry, respectively, and suitability for PCR was assessed by performing PCR with the *GAPDH* housekeeping gene (forward primer, 5′ tgaaggtcggagtcaac ggatttggt3′, reverse primer, 5′ catgtgggccatgaggtccaccac3′, 983 bp product).

For the formalin-fixed, paraffin-embedded (FFPE) samples, 10 x 10 μm tissue sections were deparaffinized in xylene at 55°C for 5 h, dehydrated in ethanol and dried overnight, followed by incubation in digestion buffer (proteinase K, 10 mM Tris.Cl [pH 8.0], 1 mM EDTA [pH 8.0], 1% SDS) at 55°C for 3 h and overnight at room temperature. The DNA was phenol extracted and precipitated with 100% alcohol in the presence of 0.3 M sodium acetate (pH 5.2).

2.3 *Consensus PCR*

GP5+/6+ consensus primers (de Roda Husman *et al* 1995) that are known to recognize more than 30 different HPV types were used to amplify a region of the L1 major capsid protein of HPV from the samples. The reverse primer GP6+ used in the reactions was biotinylated. The PCR reaction mixture (1X Taq PCR buffer [Invitrogen], 2.5 mM $MgCl_2$ [Invitrogen], 200 nM of each dNTP, 1 μM of each primer and 5 U of Taq polymerase) was subjected to denaturation at 94°C for 1 min, 40 cycles at 94°C, 1 min; 40°C, 2 min and 72°C, 1.5 min, followed by final extension at 72°C, 5 min. The 140 bp biotinylated PCR product was checked on an 8% polyacrylamide gel.

2.4 *Pyrosequencing*

The biotinylated PCR products were purified from the reaction mixture by immobilizing onto streptavidin-coated sepharose beads on the vacuum-based workstation provided with the BIOTAGE PSQ 96MA instrument in a 96-well plate format as described (Gharizadeh *et al* 2005). Briefly, the DNA was denatured in 100 mM NaOH, mixed with 15 pmol of the seven-HPV primers mix and incubated at 80°C for 3 min followed by slow cooling to room temperature, in the presence of annealing buffer (10 mmol/l Tris-acetate [pH 7.75], 5 mmol/l Mg acetate). The primer pool used for the detection of high-risk HPV types 16, 18, 31, 33, 35 and low-risk types 6 and 11 contained the oligonucleotides as previously described (Gharizadeh *et al* 2005). The sequencing reactions were set up in 96-well plates with 6 cycles of sequential dispensation of dATPαS, dCTP, dGTP and dTTP.

3. Results

3.1 *Prevalence of the different HPV types in the cervical samples*

We selected 7 different HPV genotypes – HPV16, 18, 33, 31, 45, 11 and 6 for multiplex genotyping in the cervical samples. HPV16 and 18 are the most prevalent genotypes known, together accounting for about 90% of all cervical cancer cases worldwide. HPV 31, 33 and 45 account for a high proportion of HPV16/18-negative cervical cancer cases (Pretet *et al* 2007a, b) and to complete the representative spectrum of HPV genotypes, two low-risk HPV types, namely HPV6 and 11, known to be prevalent in benign genital warts, were also included in the study.

Genomic DNA from cervical samples that amplified PCR product(s) with the cervical cancer-specific general primers, GP5+/GP6+, were subjected to genotyping (figure 1). Out of a total of 86 clinical samples analysed (comprising 71 snap-frozen tissue and 15 FFPE samples, 80 could be assigned specific HPV genotype(s) using pyrosequencing (figure 2). These included 65 cervical cancer samples and

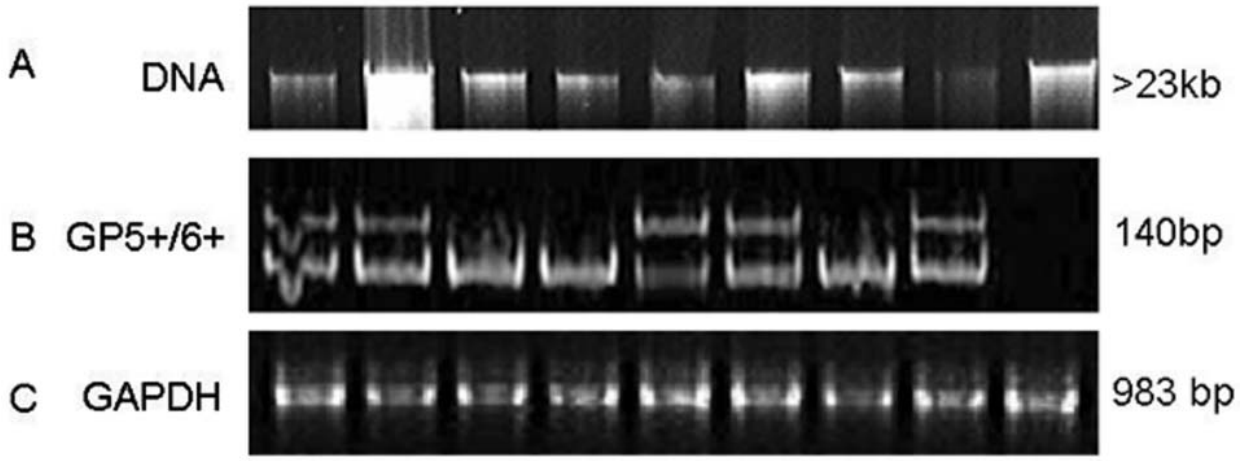

Figure 1. Representative DNA analysis prior to pyrosequencing. (**A**) DNA extraction from biopsies/surgically obtained samples. (**B**) 140 bp biotinylated GP5+/6+ PCR product with accompanying non-specific amplification. (**C**) 983 bp product from housekeeping gene glyceraldehyde-3-phosphate dehydrogenase.

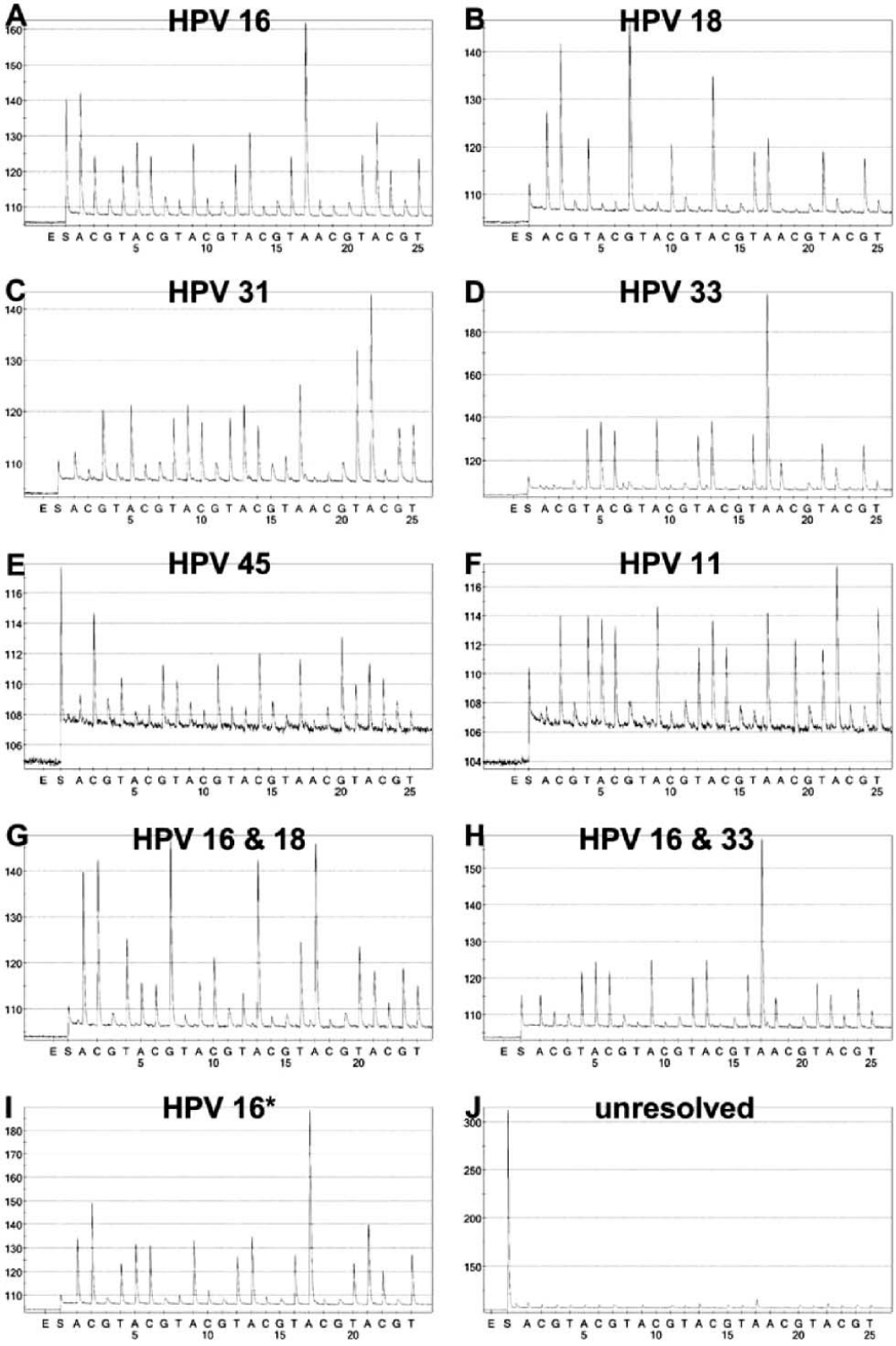

Figure 2. Representative pyrograms of the various HPV types detected. (**A**) HPV16 (AACTACATATAAAAATACT). (**B**) HPV18 (ACCTGGGCAATATG). **(C)** HPV31 (GATACTACATTTAAAAGT). (**D**) HPV33 (TACATATAAAAATG). (**E**) HPV45 (CCTGTGCCAAGTAC). (**F**) HPV11 (CTACATACACTAATT). (**G**) Coinfection of HPV types 16 and 18. (**H**) Coinfection of HPV types 16 and 33. (**I**) HPV 16* with the A to C substitution at the second base (ACCTACATATAAAAATACT). (**J**) Unresolved pyrogram to which no HPV type was assigned.

21 non-malignant cervical tissue samples. Besides these clinical samples, three cervical cancer cell lines were genotyped for standardization of the protocols and to serve as positive controls for known genotypes. As expected, among the cell lines analysed, HeLa cells tested positive for HPV18, Caski and SiHa for HPV16 and the immortalized normal keratinocyte cell line HaCaT did not show the presence of any of the HPV types tested.

Among the cervical cancer samples, 63/65 (96.9%) showed the presence of high-risk HPVs, including HPV16 (48/65; 73.8%), HPV18 (7/65; 10.77%), HPV33 (2/65; 3.07%), HPV31 (1/65; 1.53%) and HPV45 (1/65; 1.53%) (table 1, figure 3). Coinfections of HPV16 and 18 (3/65; 4.61%) and 16 and 33 (1/65; 1.53%) were also observed. Interestingly, one of the cancer samples tested positive for a low-risk HPV type 11 and one sample did not show the presence of any of the 7 HPVs tested. Clearly, HPV16 and/or 18 were present in an overwhelming 90.76% of cancers with the other high-risk HPVs accounting for only 7.69% of the samples. The low-risk HPV6 was not detected in any of our samples.

Interestingly, among the non-cancer samples, 16 out of 21 cases (76.19%) tested positive for HPVs and a large proportion of these (11/21; 52.38%) were found to be coinfected with HPV16 and 18. Among the HPV-positive samples, 4 harboured HPV16 and 1 HPV 18; no other HPV types were detected among the non-malignant samples.

3.2 *Detection of an HPV16 sequence variant*

Among the 48 HPV16-positive samples of cancer tissue, 40 gave unambiguous sequencing pyrograms corresponding to the expected sequence – 5′AACTACATATAAAAATAC3′; however, 8 of the cancer samples (represented as HPV16* in figure 2) as well as 1 of the non-malignant cervical tissues, yielded a different readout from the expected pyrogram sequence, viz. 5′ACCTACATATAAAAATAC3′, with the second nucleotide base changed from A to C, thus changing the amino acid coded for from Thr (ACT) to Pro (CCT) and putatively changing the protein sequence from TTYKN to PTYKN. This alternative sequence was consistently obtained for those samples upon repeated testing (data not shown). Considering that HPV vaccines are made of L1 major capsid protein, any variation in its coding sequence is of interest, although sequence variations in HPV genotypes *per se* are not uncommon. Interestingly, in an earlier report on HPV typing by pyrosequencing (Gharizadeh *et al* 2005) it was proposed that for all the 7 HPV genotypes tested here, three bases may be sufficient to distinguish each type. A later report by the same group proposed identification of different HPV types with the help of a single base "sentinel sequence" (Gharizadeh *et al* 2006). Our observations here suggest that it may be necessary to sequence a bigger stretch in order to not miss out on likely variations.

Table 1. HPV distribution in the 86 samples genotyped

HPV type	Number (percent)	
	Cancer, *n*= 65	Non malignant, *n*= 21
HPV 16	48 (73.84)	04 (19.04)
HPV 18	07 (10.76)	01 (4.76)
HPV 33	02 (3.07)	-
HPV 31	01 (1.53)	-
HPV 45	01 (1.53)	-
HPV 6	-	-
HPV 11	01 (1.53)	-
HPV 16 & 18	03 (4.61)	11 (52.38)
HPV 16 & 33	01 (1.53)	-
Unresolved	01 (1.53)	05 (23.8)

3.3 *High prevalence of HPV16/18 coinfections among non-malignant/benign cervical tissue*

Among the non-malignant samples, a high number of samples (11/21; 52.38%) showed the presence of both HPV16 and 18, while 4 of the 21 cases (19.04%) harboured only HPV16 (figure 3). There was one case of HPV18 while the other 5 samples were negative for the 7 HPVs tested. One of the non-malignant cervical sample set (1/21; 4.76%) showed the presence of HPV18 while the remaining (5/21; 23.8%) samples did not show the presence of any of the HPVs tested. The high HPV prevalence observed among the non-malignant samples could be due to a sampling bias since the women from whom these samples were obtained were from the lower socioeconomic group, known to be at high risk for cervical cancer because of high parity, young age at childbirth, poor sexual hygiene and irregular health check-ups. In another interesting observation, two samples from the same patient – one from a tumour and the other from normal tissue – were found to harbour different HPVs. The normal tissue showed HPV18 while the tumour contained HPV16. Other matched tumour and normal samples from the same patient were positive for the same HPV types.

4. Discussion

In our study, the HPV prevalence statistics broadly matched the existing literature, with HPV16 and 18 accounting for the bulk of HPV infections followed by HPV33 (Clifford *et al* 2003; Franceschi *et al* 2003). HPV types 31, 45 and low-risk type 11 were also detected. The pyrosequencing platform also facilitated detection of mixed infections including HPV16 and 18, and HPV16 and 33. A considerably large

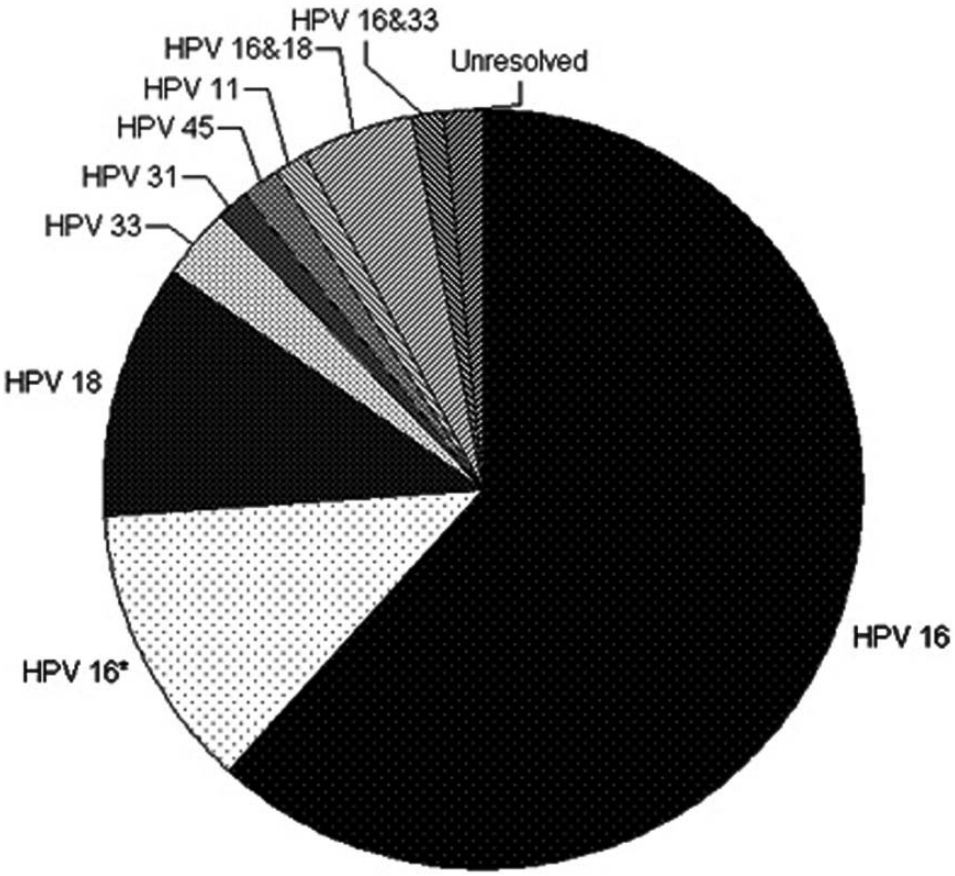

Figure 3. Pie chart representing the relative prevalence of the different HPV types in the cervical cancer samples.

number of samples from our non-malignant/benign group were seen to harbour HPV16/18 coinfections. It is likely that they represent "at risk" women. KEM being a public hospital, the patients were apparently from the lower socioeconomic group with a likelihood of poor sexual hygiene. Low socioeconomic status and associated poor sexual hygiene are recognized as risk factors for HPV infection (Franceschi *et al* 2003, 2005; Sowjanya *et al* 2005). Moreover, these cases could represent transient infections that tend to resolve over time. As reviewed by Frazer *et al* (2004), more than 95% of incident HPV infections of the anogenital tract resolve over 3–5 years and cancer may develop in less than 5% of HPV16-infected individuals in their lifetime (Frazer 2004). Further, cervical cancer is typically a monoclonal event related to a single HPV type, but the surrounding cervical epithelium can still be infected with other types. Infection with many genotypes may be transient in nature, caused due to passenger viruses colonizing the malignant tissue or may largely represent benign infection in the presence of another high-risk type that is causally related to the given lesion (Clifford *et al* 2003; Moscicki *et al* 2006). In addition, there may be reactivation of latent infection acquired earlier in life due to a gradual loss of type-specific immunity, or these could represent acquisition of new infections due to sexual contacts with new partners later in life (Trottier and Franco 2006). However, since the women with non-malignant/benign cases underwent hysterectomy, the possibility of developing cervical cancer no longer arises. Further, the presence of HPV in healthy controls has been widely documented. Franceschi *et al* (2003) reported an HPV prevalence of 27.7% in healthy controls and Munoz *et al* (2003) reported an HPV prevalence of 13.4% among control women; of these, 86.1% were infected by a single HPV type and 13.9% were infected by multiple types. In genotyping with indirect methodologies, such as dot blot, hybrid capture, etc. sequence confirmation is limited to representative samples. Since the primary assay in our methodology is PCR, followed by sequencing, it is possible that we are missing fewer positive cases.

Besides HPV16 and 18, the less common genotypes, as expected, accounted for less than 10% of the cancer cases and it appears feasible to accurately determine their baseline prevalence using this multiplex pyrosequencing technique on larger sample cohorts.

The recombinant HPV vaccines Gardasil (Merck) and Cervarix (GSK) currently undergoing large clinical trials predominantly target HPV16 and 18. Cervarix is an HPV16/18 L1 virus-like particle vaccine that offers complete protection against HPV16- and 18-associated precancerous lesions as well as substantial protection against types 45 and 31 (Harper *et al* 2006). Gardasil is a quadrivalent vaccine made of the major capsid protein (L1) of high-risk HPV types 16 and 18, and low-risk types 6 and 11, which has been found to prevent 100% CIN2/3 and adenocarcinoma *in situ* associated with HPV16 and 18 infections (Schmiedeskamp and Kockler 2006). These vaccines may prove to be effective against a majority of cervical cancers, but may not benefit women infected with rarer HPV types. Given the high overall prevalence of cervical cancer in India, these numbers are likely to be at least 10,000–20,000 per year. Another area of concern is whether the use of these vaccines could alter the relative prevalence of the different HPV types in the population and whether that would make the rarer HPV genotypes more common. An accurate benchmark genotype profile of the population and follow-up studies could facilitate the answer to this question.

The HPV genotyping effort reported here could be further developed to include other HPV types, especially HPV52, 56 and 58, which have been shown to be prevalent in other parts of India and Asia (Clifford *et al* 2003; Franceschi *et al* 2005). Some of the samples that did not test positive for any of the 7 HPV types could technically be positive for the missing HPV types.

While multiplex pyrosequencing could detect 7 or more HPV genotypes simultaneously in a given sample – with 3 h required to sequence/genotype up to 96 samples – a similar analysis with the conventional Sanger sequencing method requires multiple, independent, primer-specific sequencing reactions to be set up, at a considerably higher cost, sample requirement, manpower and turnaround time.

The sequence-based, high-throughput HPV genotyping afforded by multiplex pyrosequencing provides a long-sought tool for accurate determination of the prevalence of HPV types and could become especially useful in the future for following the aetiological and epidemiological impact of HPV vaccines.

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