Detection by Polymerase Chain Reaction and Restriction Fragment Length Polymorphism of Human Papillomavirus in Archival Cervical Disease

A Padmanathan BSc*, M Yadav PhD*, A R A Gregory MPath**, *Department of Genetics & Cellular Biology, University of Malaya, 59100 Kuala Lumpur, **Department of Haematology, Institute for Medical Research, 50588 Kuala Lumpur

Summary

Human papillomavirus (HPV) infection has been strongly linked to the development of cervical carcinoma. The incidence of HPV was studied in 65 histologically confirmed cervical carcinoma cases using the polymerase chain reaction (PCR) with consensus primers. This was followed by restriction fragment length polymorphism (RFLP) analysis for HPV typing.

The overall incidence of HPV in the patients screened was 49/65 (75.4%). HPV 16 was found in 44/65 (67.7%) of the cases and HPV 18 in only 5/65 (7.7%). HPV 16 infection was seen to increase with age but no specific pattern was observed for HPV 18 infection. We found that the prevalence of HPV 16 and 18 were unevenly distributed in the various cervical carcinomas. Interestingly it was noted the Malays showed a marked lower prevalence of HPV infection of the cervix. Since such a large proportion of Malaysian cervical carcinomas are associated with HPV, we conclude that the early detection of HPV in cervical diseases could form an adjunct in clinical evaluation and approach to treatment.

Key Words: Human papillomavirus, HPV, Cervical carcinoma, Polymerase chain reaction, Restriction fragment length polymorphism

Introduction

Human papillomaviruses (HPV) are small, non-enveloped double stranded DNA viruses of the family Papovaviridae which include the Papilloma viruses and the Polyoma viruses[1]. HPVs usually infect the epithelial or fibroepithelial tissues[2] of the skin and mucous membranes causing proliferating lesions varying from warty condyloma to flat condyloma and low grade dysplasias, otherwise referred to as cervical intraepithelial neoplasias grade I (CIN I). Presently more than 70 HPV types have been molecularly identified and of which 30 were isolated from genital sites[3]. HPV types 6 and 11 are commonly associated with low grade (benign) squamous intraepithelial lesions and do not usually progress to carcinoma of the cervix. On the other hand, HPV types 16, 18, 31, 33 and 35 are frequently associated with high grade squamous intraepithelial lesions otherwise referred to as cervical intraepithelial neoplasia grades II and III (CIN II & III)[4]. The putative role of HPV in carcinogenesis has evoked much interest particularly in relation to the strategy used by the virus for progression of infected cells to malignancy[5].

Many laboratories use polymerase chain reaction (PCR) amplification with degenerate primers that are capable of recognizing a sequence of the L1 gene from a wide variety of genital papillomaviruses[6] as a first step to identify the HPV infection in the clinical material. The

PCR amplified product can subsequently be typed with specific oligonucleotide probes but this is cumbersome. Often a large number of amplicons remain untyped because probes for all the HPVs are not available easily. Recently, the use of restriction fragment length polymorphism (RFLP) analysis has been proposed to identify untyped PCR amplified products[7]. This RFLP technique is relatively simple and with availability of data base for identifying HPV restricted sequences, the majority of the HPVs including novel HPVs can be typed.

In Malaysia, little is known of the geographic distribution of oncogenic types of HPV in cancer of the cervix, oral and the other anogenital sites. Since the virus is transmitted sexually, the diagnosis of HPV associated lesions of the anogenital sites carries considerable clinical and social implications[8]. Traditionally, the diagnosis of HPV associated lesions has been through identifications of gross lesions clinically followed by routine histopathologic examination of a biopsy. The presence of koilocytes and dyskeratosis in the lesions have been taken as indicative of HPV infection. However, many lesions lack the characteristic histologic features and these cases can be especially difficult for the clinicians. In such cases other approaches may be desirable for the detection of HPV associated proteins or DNA[9]. In addition, viruses other than HPV may be found within the cells which may induce the koilocytic features. We have previously reported the common presence of HPV 16 and HPV 18 and the infrequent prevalence of HPV 31 and HPV 33 in cervical tumour tissues which were collected fresh at surgery[10]. In addition, using the *in situ* hybridisation technique, we have found that HPV 16 and 18 were frequently present in archival tissues of cervical carcinoma[11]. Here we report the detection and prevalence of HPV in archival tissues from various diseases of the cervix. With the method used, it was possible to extract DNA from formalin-fixed paraffin-embedded tissues and use it for the identification of HPV types by PCR and RFLP.

Materials and Methods

Source of clinical material

Sixty-five histologically confirmed tissue of cervical carcinoma were selected for analysis of HPV sequences. These patients were clinically staged according to the Federation of International Gynaecology and Obstetrics (FIGO) classification, stage I to stage IV and varied in histological grading, including, well differentiated keratinizing, moderately differentiated partially keratinizing, poorly differentiated nonkeratinizing large cell and small cell type squamous carcinomas and adenocarcinomas[12,13]. The cases were diagnosed during regular diagnosis of surgical biopsies for cervical carcinoma from 1986 to 1988 at the General Hospital of Seremban, Negeri Sembilan. The tissues were fixed in 10% buffered formaldehyde solution for less than 48 hours. Three 4 µm thick sections were cut from the paraffin blocks and placed in 1.5 ml Eppendorf tubes for DNA extraction. A fresh disposable scapel blade was used for each block to avoid cross contamination between samples. At least three sections obtained at the beginning and towards the end of the block were stained with hematoxylin and eosinofil (H & E) for histological examination. Only tissues that contained tumour cells were included in the study. All the biopsy specimens were assessed histologically by one of us (A.R.A.G.) without knowledge of the PCR results.

DNA extraction from archival tissue

The sections were deparaffinized first by heating at 56°C for 20 minutes and then by adding 1.0 ml of xylene, and centrifuging for 5 minutes (MicroSpin 24S, Sorvall Instruments). The xylene was decanted and its residue removed using 95% ethanol, centrifuging and decanting again. The tissue pellets were vacuum dried (Savant, USA.) and 50 µl of sterile distilled water added then frozen at -20°C. Subsequently the samples were thawed after 45 minutes and digested with 50(l of Proteinase K Mix (10mM Tris.Cl pH 7.5, 1.5mM $MgCl_2$, 0.45% Tween 20 and 60 µg/ml Proteinase K) for an hour at 55°C in a waterbath. The samples were then centrifuged (Sigma 2K15) at 12000 rpm for 15 minutes at 4°C and stored for subsequent PCR analysis.

Polymerase chain reaction

The technique has been previously described[16]. Briefly, ten microlitres of each sample was used for the PCR. Each reaction of 50 µl contained sterile distilled water,

10mM Tris - HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$ and 0.001% w/v gelatin, 0.2mM each deoxynucleotide triphosphate (dNTP); 2.5 units Taq Polymerase (Boehringer Mannheim, Germany); 10 pM of each of the L1 Consensus Primers MY09 (5'CGTCCMMARRGGAWACTGATC3') and MY11 (5'GCMCAGGGWCATAAYAATGG3'), (M: A+C, R: A+G, W: A+T, Y: C+T) (Perkin-Elmer Cetus, USA.) The mixture was overlaid with 40 µl of heavy mineral oil (Sigma) and subjected to 40 cycles of amplification using a DNA Thermal Cycler (Perkin-Elmer Cetus, USA.). Each cycle consisted of a denaturation step at 95°C for 1 minute, an annealing step at 55°C for 1 minute, and an elongation step at 72°C for 2 minutes. There was a final elongation step at 72°C for 5 minutes[14]. All experiments were performed in parallel with positive and negative controls. Negative controls included reaction mixtures lacking DNA template. As positive controls for HPVs, 1 pg of each HPV plasmid and CaSki (for HPV 16) and HeLa (for HPV 18) were employed during PCR amplification. Eight microlitre aliquots of the PCR products were subjected to 2.0% agarose gel electrophoresis and then stained with ethidium bromide and photographed under ultraviolet transillumination.

Restriction enzyme digestion and RFLP analysis

Restriction enzyme digestion was carried out on PCR amplified products using three enzymes, namely, *Hinf* I, *Pst* I and *Rsa* I to determine the HPV types present. Aliquots of the PCR product that were sufficient for visualisation on a gel were used for each digestion. The PCR product varied in samples from 25 to 50% of the total reaction mix. The digestion mixture consisted of sterile distilled water, a 10X Buffer (50mM NaCl, 10mM Tris-HCl , 10mM $MgCl_2$, 1mM DTT); PCR amplified DNA and the respective restriction enzymes. This mixture was digested overnight at 37°C. Digested products were electrophoretically separated on 2.0% agarose gels in the presence of standard DNA size markers. The gel was then stained with ethidium bromide and patterns visualised using ultraviolet transillumination.

Statistical Test

The Chi-squared test (2x2 contingency tables) was performed to assess the significant correlation between HPV DNA prevalence and the racial descent of the patients analysed. The 'Statistics Graphics System Version 4.0' was used to conduct the correlation test. The level of significance used during this statistical test was 1% (p < 0.01).

Results

PCR detection of HPV in cervical diseases

The DNA extracted from cervical tissues obtained from 65 women were assayed by PCR for the presence of HPV. The L1 consensus primers were used for amplification of a conserved region of the virus L1 gene. As a negative control, sterile distilled water replaced the DNA template in the reaction mixture and was always found to be negative. Forty-nine of the sixty-five (75.4%) women examined, were found to be positive for HPV infection. Figure 1 shows a representative pattern of ethidium bromide stained and agarose gel electrophoresis separated PCR amplified
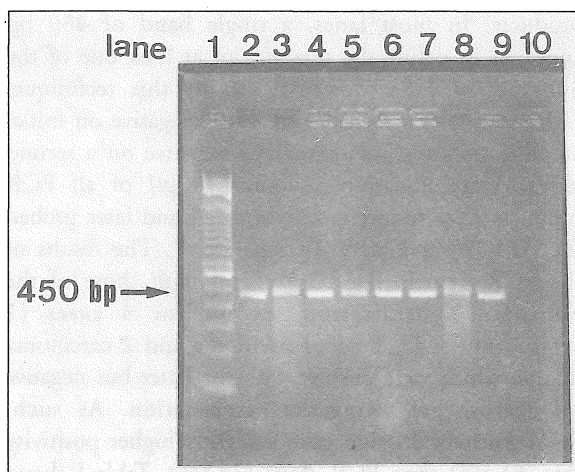

Fig. 1:   The agarose gel electrophoretic pattern of the 450 basepair PCR amplified HPV DNA sequence (arrow) derived from target genomic DNA extracted from cervical diseases. Lane 1 contains 100 bp molecular weight ladder standard, Lane 2 to 8 contain PCR amplified HPV sequence derived from cancer tissue samples. Lane 9 and 10 contain positive controls and negative controls respectively.

Table I
Detection by PCR of HPV in cervical diseases

Cervical Diseases	No. of cases analysed	PCR Negative No. (%)	PCR Positive No. (%)	*Southern Hybridisation No. (%)
Invasive keratinizing squamous cell carcinoma	13	4 (30.8)	9 (69.2)	10 (77.0)
Invasive nonkeratinizing squamous cell carcinoma (large cell)	22	4 (18.2)	18 (81.8)	18 (81.8)
Invasive nonkeratinizing squamous cell carcinoma (small cell)	4	1 (25.0)	3 (75.0)	3 (75.0)
Adenocarcinoma	5	2 (40.0)	3 (60.0)	4 (80.0)
Carcinoma in situ	21	5 (23.8)	16 (76.2)	18 (85.7)
Total	65	16 (24.6)	49 (75.4)	53 (81.50)

* Probed for HPV 16 and HPV 18 only

products. In most lanes, a single band of 450 bp appeared to define the presence of at least one of the 30 types of HPV detected also by this technique. Sixteen samples screened were HPV negative on initial amplification and they remained negative on a second repeated amplification. Aliquots (8 μl) of all PCR products were transferred, hybridized and later probed for HPV 16 and HPV 18 separately[15]. The results of the PCR were in good agreement with those of the Southern hybridisation, except for 4 cases (1 keratinizing SCC, 1 adenocarcinoma and 2 carcinoma in situ) which were positive with the latter but negative on agarose gel ultraviolet visualisation. As such, Southern hybridisation gave a slightly higher positivity rate (81.5%) than PCR alone (75.4%). Table I shows the prevalence of HPV infection in various types of cervical carcinoma.

The histologic types of cervical carcinomas were correlated with HPV infection. Thirty (76.9%) of the 39 invasive squamous cell carcinoma (SCC) cases analysed contained HPV DNA. The highest infection rates were found in the large cell nonkeratinizing SCC tumours (81.8%), followed by 75.0% positivity rate found in the small nonkeratinizing SCC tumours and 69.2% in the keratinizing SCC tumours. Of the
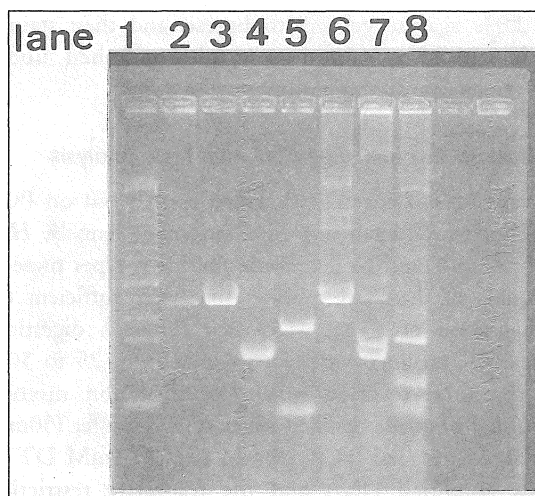

Fig. 2: The agarose electrophoretic pattern of restriction enzyme digested PCR amplified HPV DNA. Lane 1 contains 100 bp molecular weight ladder standard, Lane 2 contains undigested PCR amplified HPV DNA, Lanes 3 to 5 contain HPV 16 DNA digested with Hin fI, Pst I, Rsa I respectively and lanes 6 to 8 contain HPV 18 DNA digested with Hin fI, Pst I and Rsa I respectively.

adenocarcinoma cases analysed, a relatively low percentage (60.0%) were found to harbour HPV DNA. Prevalence of HPV DNA did not differ significantly in the carcinoma *in situ* cases (76.2%) in comparison to the invasive carcinoma cases (75.0%).

Restriction enzyme digestion and RFLP analysis

The PCR amplified product (450 bp) was subjected to restriction endonuclease digestion with *Hinf* I, *Pst* I, and *Rsa* I. Restriction enzyme digestion gave fragments of the expected size, demonstrating that the amplified DNA was specific for different HPV types. The use of one or more of these enzymes lead to an accurate identification of the PCR product. Our results of HPV typing were based on independent digestion of the no-cut enzyme *Hinf* I, and multi-cut enzyme digestion with *Pst* I and *Rsa* I for HPV 16 and 18 and also the comparison of HPV patterns with that of undigested HPV DNA (~450 bp). Representative results are shown in Figure 2 for the digestion patterns of HPV 16 and HPV 18 and Table II summarises the various cleavage patterns obtained with the restriction enzymes.

Overall, 67.7% of the women tested were found to be infected with HPV 16 and 7.7% with HPV 18. In all the carcinoma types analysed using restriction enzyme digestion, HPV 16 was predominant over HPV 18. The large cell nonkeratinizing tumours gave the highest HPV 16 infection rate (77.3%) and HPV 16 was the only subtype identified in all the cases of small cell nonkeratinizing SCC (75.0%). In keratinizing SCC, HPV 16 was found in only 61.5% of the cases. HPV 18 was found most frequently in adenocarcinomas than the other carcinoma types, and none at all in the small cell nonkeratinizing SCC. Distribution of the HPV types according to histopathology is presented in Table III.

HPV types in relation to race and age

The cervical tissue samples used were obtained from

Table II
Restriction enzyme characterisation of polymorphism in PCR amplified products

Restriction Enzyme	HPV 16	HPV 18
Hinf I	452 bp	455 bp
Pst I	216, 210, 26 bp	242, 213 bp
Rsa I	310, 72, 70 bp	135, 125, 85, 72, 38 bp

Table III
Distribution of HPV types according to histopathology

Carcinoma Types	No. of cases analysed	HPV 16 (%)	HPV 18 (%)	HPV Positive (%)	HPV Negative (%)
Invasive keratinizing squamous cell carcinoma	13	8 (61.5)	1 (7.7)	9 (69.2)	4 (30.8)
Invasive nonkeratinizing squamous cell carcinoma (large cell)	22	17 (77.3)	1 (4.5)	18 (81.8)	4 (18.2)
Invasive nonkeratinizing squamous cell carcinoma (small cell)	4	3 (75.0)	0	3 (75.0)	1 (25.0)
Adenocarcinoma	5	2 (40.0)	1 (20.0)	3 (60.0)	2 (40.0)
Carcinoma *in situ*	21	14 (66.7)	2 (9.5)	16 (76.2)	5 (23.8)
Total	65	44 (67.7)	5 (7.7)	49 (75.4)	16 (24.6)

Table IV
Frequency of positivity of HPV types among various Malaysian races

Race	No. Analysed	Virus Types Positive		
		HPV 16 (%)	HPV 18 (%)	All HPVs (%)
Malay	15	8 (53.3)	1 (6.7)	9 (60.0)
Chinese	36	26 (72.2)	3 (8.3)	29 (80.5)
Indian	14	10 (71.4)	1 (7.1)	11 (78.6)
Total	65	44 (67.7)	5 (7.7)	49 (75.4)

Table V
HPV DNA prevalence in 65 patients with cervical carcinoma analysed by racial descent

Race	HPV DNA Prevalence		Chi^2 Test
	Positive (%) (n = 49)	Negative (%) (n = 16)	
Malay	9 (60.0)	6 (40.0)	P 2.24 x 10^{-4}
Chinese	29 (80.5)	7 (19.4)	(p < 0.01, highly significant)
Indian	11 (78.6)	3 (21.4)	

Table VI
Prevalence of HPV types in relation to age of patients with cervical carcinoma

Age	HPV 16 (%)	HPV 18 (%)	HPV Positive (%)	HPV Negative (%)	Total analysed
<30-39	5 (55.5)	0	5 (55.5)	4 (44.4)	9
40-49	17 (65.4)	4 (15.3)	21 (80.8)	5 (19.2)	26
50-59	13 (72.2)	1 (5.5)	14 (77.8)	4 (22.2)	18
60-69	4 (66.7)	0	4 (66.7)	2 (33.3)	6
70-85	5 (83.3)	0	5 (83.3)	1 (16.7)	6

patients of the 3 major ethnic groups residing in Malaysia. Of these individuals, 36 were of ethnic Chinese descent, 15 were Malays and 14 were Indians. HPV DNA was found most frequently in the Chinese (80.0%) and Indians (78.6%) with a marked lower prevalence in the Malays (60.0%). Among all the races, HPV 16 was most prevalent with a positivity rate of 53.3% to 72.2% and HPV 18 found in only 6 to 8% of the cases. Table IV shows the frequency of HPV types in various Malaysian races.

The prevalence of HPV DNA was found to correlate with high significance statistically p =0.0002, p <<0.01); to the various races as shown in Table V.

This further confirmed the low prevalence of HPV in the Malays in comparison to the other races.

Prevalence of HPV infection in relation to age was highest (80-83%) in the 70-85 and 40-49 year category. A significantly low infection rate (55.5%) was seen in patients below the age of 39 years. Although HPV 16 was found in all age ranges, HPV 18 was found in only the 40-59 year olds and at a low percentage of 15% or lower. Prevalence of the HPV types in relation to age is shown in Table VI.

Discussion

Compared to other techniques, such as Southern hybridisation, dot blot, immunohistochemistry and *in situ* hybridisation, PCR is the most sensitive technique for the detection of viral infections. The PCR technique has been recently adapted for the analysis of virus DNA in formalin-fixed and paraffin-embedded biological material[16]. In our study, we used the consensus sequence primers described by Manos *et al.,* 1989[14] to amplify a 450 base pair HPV segment of the L1 region which is common to more than 30 types of HPV that infect the human oral and anogenital tract[7]. The use of consensus sequence primers for detecting HPV DNA has several advantages over type specific primers. The main advantages are that with a single amplification reaction many different HPV types including non-sequenced novel ones can be detected. Moreover, PCR enables the laboratory to screen rapidly a large number of samples for HPV infection. Unlike previous reports[6,17,18,19], which used Southern blot hybridisation to determine the specificity of amplified products, we selected sequence recognition by restriction endonucleases.

The use of Southern blotting, followed by hybridisation using type specific probes for HPV is a very effective method of HPV typing. However, although it is more sensitive than visualisation via ethidium bromide staining of amplicons, it is not practical for routine screening of large numbers of sample. Restriction enzyme digestion as described in this study, has proved to be more specific than hybridisation which depend greatly on the conditions of stringency during detection. False positive hybridisation signals enhanced by partial recognition of the probe to amplified DNA may also occur. This is especially common when dealing with HPVs because of the high degree of the homology shared by many HPV genotypes.

In this series of cervical carcinoma, we have demonstrated that HPV is present in 75.4% (49/65) of the tumours. This figure is very much in agreement with another study from our group[11], using *in situ* hybridisation on paraffin-embedded cervical tissues. However, earlier we have reported HPV infection as high as 86.9% in fresh frozen tissues derived from cervical carcinoma[10]. The dissimilarities in the proportion positive for HPV in cervical carcinoma may be due largely to the starting material in each case. It is possible that the HPV sequence detection was inhibited due to quality and length of formalin fixation and paraffin-embedding[20]. For instance, PCR amplification of HPV sequences was undetectable in some cases by ethidium bromide visualisation following gel electrophoresis but the presence of HPV DNA was detected in these cases when analysed by Southern hybridisation. We found that HPV 16 was detected in a high proportion of the cases (67.7%) while only five cases (7.7%) were infected with HPV 18. These results are in agreement with other studies carried out in various parts of the world[21,22]. In addition we have previously noted that the predominant HPV type was HPV 16 in cervical squamous cell carcinomas and HPV 18 in cervical adenocarcinomas. Here, we report HPV 16 as dominant in both squamous cell and adenocarcinomas. HPV 18 was found present in only 20% of the adenocarcinomas screened. This finding is in agreement with the observations of the prevalence of HPV in adenocarcinomas using the *in situ* hybridisation technique conducted in India[23] and PCR based study in United Kingdom[24]. The incidence of HPV 18 infection found in our study though lower than reports from Europe coincide with studies from Korea[25] and Japan[26].

Among the various types of squamous cell carcinoma of the uterine cervix, the large cell nonkeratinizing carcinomas are the most common type, followed by the keratinizing type. The small cell nonkeratinizing carcinomas are rare in comparison. This series of cervical carcinomas screened from 1986 to 1988 seem to follow the above classification of incidence. This

also has some prognostic significance since small cell carcinomas carry the worst prognosis while the large cell type has a more favourable outcome[27].

Although very few studies on HPV include the age of patients, it has been reported by Meanwell et al[28] that HPV 16 DNA positivity frequently increased with age and an editorial in the Lancet[29], supports this view. In this study, we find that the above statement holds true except for a slight decrease in the 60-69 age group. It was more difficult to suggest a trend for HPV 18 infection as it was only found in the 40-59 age group.

Among all the races screened, the Malays showed a significant lower prevalence of HPV infection of the uterine cervix. Since HPV is sexually transmitted, it is perhaps interesting to note that Kjaer et al[30] reported that wives of circumcised men were at a significantly low relative risk (0.3) of HPV infection. The lower prevalence among the Malays in our series could be explained by this religious requirement but further studies are needed to resolve this aspect.

Cervical cancer develops because of multifactorial causes and co-factors such as smoking, coexisting viral infection, natural and contraceptive hormones, host immune status, oncogene activation, dietary deficiencies and radiation exposure may play some part in the transformation process[31]. It is also important to take into consideration the socio-economic status and sexual behaviour of patients with carcinoma of the cervix.

We find that the prevalence of HPV 16 and 18 are unevenly distributed in the various cervical diseases. It has been reported[32] that HPV 18 is more aggressive and its presence in adenocarcinomas forbodes bad prognosis[33]. The early detection of HPV in cervical diseases could form an adjunct in clinical evaluation and approach to treatment. The technique described can be easily linked to the histopathology report and form part of the clinical evaluation of the patient.

Acknowledgements

References

1. Melnick JL, Allison AC, Butel JS. Papovaviridae. Intervirol 1974;3 : 106-20.

2. Howley PM. Papillomaviridae and their replication. In : Fields BN, Knipe DM et al (eds.) Virology Second Edition, New York: Raven Press, 1990 : 1625-50.

3. de Villiers EM. Heterogeneity of the HPV group. J Virol 1989;63 : 4898-903.

4. Lorinez AT, Temple GF, Kurman RJ, Jenson AB, Lancaster WD. Oncogenic association of the specific HPV types with cervical neoplasia. J Natl Cancer Inst 1987;79 : 671-7.

5. zur Hausen H. Papillomaviruses as carcinoma viruses. Advances Viral Oncol 1989;8 : 1-26.

6. Schiffman MH, Bauer HM, Lorinez AT, et al. Comparison of Southern blot hybridisation and PCR methods for the detection of HPV DNA. J Clin Microbiol 1991;29 : 573-7.

7. Bernard HU, Chan S, Manos MM, et al. Identification and assessment of known and novel HPVs by PCR amplification, RFLP, nucleotide sequence and phylogenetic algorithms J Inf Diseases 1994;170 : 1077-85.

8. Campion MJ, Brown JR, McCance DJ et al. Psychosexual trauma of an abnormal cervical smear. Br J Obstet Gynaecol 1988;95 : 175-81.

9. Meisels A, Morin C. HPV-induced changes. In : Cytopathology of the uterine cervix. American Society of Clinical Pathologists (ASCP) Press, Chicago, 1991 : 73-117.

10. Yadav M, Nurhayati ZA, Padmanathan A, Abdul Aziz Y, Norhanom AW. Polymerase chain reaction detection and typing of human papillomaviruses in cervical carcinoma. Med J Mal 1995;50 : 100-7.

11. Nurhayati ZA, Yadav M, Daniel JM, Norhanom AW. Association of HPV with preinvasive and invasive cervical carcinoma. Mal J Sci 1994;15A : 49-57.

12. Broders AC. Carcinoma : Grading and practical application. Arch Pathol Lab Med 1926;2 : 376-81.

13. Riotton G, Christopherson WM. Cytology of the female genital tract. In : International Histological Classification of Tumours. 1973; No. 8 Geneva, World Health Organisation.

14. Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. The use of PCR amplification for the detection of genital HPVs. Cancer Cells 1989;7 : 209-14.

15. Boehringer Mannheim. DNA Labelling and Nonradioactive Detection. A Manual. GmBH Biochemica, Germany, 1983.

16. Shibata DK, Arnheim N, Martin WJ. Detecton of HPV in paraffin-embedded tissue using the PCR. J Exp Med 1988; 167 : 225-30.

17. Ngan HY, Stanley M, Liu SS, Ma HK. HPV and p53 in cervical cancer. Genitourin-Med 1994;70 : 167-70.

18. Morrison EAB, Goldberg GL, Kadish AS, Burk RD. PCR detection of HPV : Quantitation may improve clinical utility. J Clin Microbiol 1992;30 : 2539-43.

19. Williamson A, Brink NS, Dehaeck MC, Ovens S, Soeters R, Rybicki EP. Typing of HPVs in cervical carcinoma biopsies from Cape Town. J Med Vir 1994;43 : 231-7.

20. An SF, Fleming KA. Removal of inhibitor(s) of the PCR from formalin-fixed, paraffin wax-embedded tissues. J Clin Pathol 1991;44 : 924-7.

21. Hagmar B, Johansson B, Kalantari M, Petersson Z, Skyldberg B, Walaas L. The incidence of HPV in a Swedish series of invasive cervical carcinoma. Med Oncol Pharmacother 1992; 9 : 113-7.

22. Chen SL, Han CP, Tsao YP, Lee JW, Yin CS. Identification and typing of HPV in cervical cancers in Taiwan. Cancer 1993; 729 : 1939-45.

23. Das BC, Gopalkrishna V, Das DK, Sharma JK, Singh V, Luthra UK. HPV DNA sequences in adenocarcinoma of the uterine cervix in Indian women. Cancer 1993;72 : 147-53.

24. Griffin NR, Dockey D, Lewis FA, Wells M. Demonstration of low frequency of HPV DNA in cervical adenocarcinoma and in situ hybridisation. Int J Gynecol Pathol 1991;10 : 36-43.

25. Park JS, Chee YH, Namkoong SE, et al. HPV detection in cervical carcinoma tissues and paraaortic lymph nodes by the PCR. Gynecol Oncol 1994;53 : 344-51.

26. Yoshikawa H, Matsukura T, Yamamoto E, et al. Occurrence of HPV type 16 and 18 DNA in cervical carcinomas from Japan. Jpn Cancer Res 1985;76 : 667-71.

27. Meisels A, Morin C. Invasive squamous carcinoma of the cervix. In : Cytopathology of the uterine cervix. American Society of Clinical Pathologists (ASCP) Press, Chicago, 1991; 165-78.

28. Meanwell CA, Cox MF, Blackledge G, Maitland NJ. HPV 16 DNA in normal and malignant cervical epithelium : Implications for the aetiology and behaviour of cervical neoplasia. Lancet 1987;i : 703.

29. Editorial. HPVs and cervical cancer : a fresh look at the evidence. Lancet 1987;i : 725-6.

30. Kjaer SK, de Villiers EM, Dahl C, et al. Case control study of risk factors for cervical neoplasia in Denmark. Int J Cancer 1991;48 : 39-44.

31. Meisels A, Morin C. HPV and cancer of the uterine cervix. In : Cytopathology of the uterine cervix. American Society of Clinical Pathologists (ASCP) Press, Chicago, 1991 : 119-45.

32. Arends MJ, Donaldson YK, Duvall E, Wyllie AH, Bird CC. HPV type 18 associated with more advanced cervical neoplasia than HPV 16. Hum Pathol 1988;24 : 432-7.

33. Wilezynski SP, Bergen S, Walker J. HPVs and cervical cancer : analysis of histopathology features associated with different viral types. Hum Pathol 1988;19 : 697-704.