

Editorial

Genital papillomaviruses, polymerase chain reaction and cervical cancer

The epidemiological profile of cervical cancer suggests strongly that this is a sexually transmitted disease and that the aetiological agent is a genital pathogen. At one time or another almost every sexually transmitted pathogen has been implicated as the oncogen (often without any substantial evidence), but at the present time the genital papillomaviruses (HPVs) are the favoured candidates¹ and their claim is supported by two principal lines of evidence. The first is the demonstration that 80–90% (using Southern blotting) of invasive cancers of the cervix contain HPV DNA sequences of the other so-called oncogenic types, predominantly HPV 16 (which is present in about 50% of lesions) HPV 18 or 31, 33, 35.¹⁻⁵ Viral sequences are present in the malignant cells; these sequences are transcribed and viral proteins are expressed.^{6,7} Cervical intra-epithelial lesions also contain HPV DNA with the oncogenic types occurring in high frequency in high grade CIN's.^{8,9} The evidence that these viral DNAs may be more than passengers comes from *in vitro* studies which show that HPV 16 or HPV 18 DNA when transfected into normal human genital keratinocytes can immortalise or transform these cells,¹⁰⁻¹⁴ a phenomenon observed hitherto in keratinocytes only with the experimental DNA tumour virus SV 40.

There is however no clear epidemiological evidence supporting an aetiological role for HPV in cervical cancer and this relates in part to our ignorance of the natural history of HPVs and the difficulties encountered in determining the prevalence of HPV infection. These viruses cannot be grown in tissue culture, and thus the identity of authentic viral antigens is uncertain, prohibiting both serological classification and the use of serology in epidemiological studies. The viruses are classified on the basis of DNA sequence homology and hitherto "infection" has been determined by the detection in clinical samples of viral DNA using hybridisation technologies such as Southern blot, dot blot, filter *in situ* (FISH) or tissue *in situ* (for review see reference 15). Southern hybridisation is accepted as the "gold standard" methodology capable of quantitating viral copy number and providing, under conditions of high stringency, type specificity and a sensitivity

such that 0.1–1 viral genome per cell can be identified. It is, none the less, unsuitable for epidemiological studies since it is expensive, labour intensive and relatively large tissue volumes are required for analysis. Studies on prevalence have used less laborious technologies such as FISH or dot blot but these have problems of specificity and sensitivity and almost certainly have underestimated the prevalence of HPV DNA.¹⁵

The polymerase chain reaction (PCR)¹⁶ has been welcomed enthusiastically since this technology seems to be ideal for large population surveys providing an assay of high sensitivity and specificity for the detection of HPV DNA in cervical smears or swabs.¹⁷ Furthermore the technique can be used on paraffin embedded tissue allowing retrospective studies on the huge amounts of archival material.¹⁸ PCR is a gene amplification technology capable of amplifying specific DNA sequences in an impure sample *in vitro* within hours. This ability to generate millions of copies of a specific nucleotide sequence gives PCR enormous sensitivity and one copy of HPV DNA can be detected in 10⁵ cells, a sensitivity at least 10⁴ greater than Southern blotting.¹⁹ The PCR is based upon the repetitive cycling of three simple reactions all of which occur in the same test-tube. The first step is the denaturation of native DNA, at high temperature, into single strands which can then re-anneal with any other complementary DNA sequence under the appropriate conditions. In the second step synthetic oligonucleotide primers complementary to the 5' and 3' ends of the target DNA sequence are annealed to these complementary sequences. The specificity of the PCR derives from the precision of this DNA-DNA annealing reaction. Primer design is crucial; the primers must not anneal to one another and their sites of annealing must be significantly distant to allow the subsequent synthesis of new products. In the third step the synthesis of a complementary second strand of new DNA occurs by the extension of each annealed primer by DNA polymerase. This cycle of events is then repeated and in each ensuing cycle all previously synthesised products act as templates for new primer extension leading to massive amplification of the original target

sequences. Clearly any errors in sequence introduced during in vitro synthesis will be amplified and in vitro mutation is a potential hazard in this technique. Ironically, however, the cause of most of the problems encountered in using this technique is the extraordinary sensitivity of the PCR. Since the PCR can detect one molecule of "foreign" DNA in 10^5 cells then the procedure is very vulnerable to accidental contamination and this is particularly so when, for example, cloned HPV DNA is being manipulated in the same working environment as clinical samples are being assayed by PCR for HPV DNA. To avoid these problems laboratories must implement special working procedures;^{20,21} primer design and the selection of appropriate positive and negative controls are crucial and validation of results by sensitive hybridisation techniques is essential.

Data from studies using PCR to determine the prevalence of HPV in normal and neoplastic cervical tissue have been published recently^{19,22-30} and at the latest International Papillomavirus workshop (Heidelberg, May 1990) more than 60 presentations concerned the use of PCR in HPV detection in the cervix. Basically three messages can be culled from the mass of information. HPV DNA sequences are present in 80–100% of cervical cancers, HPV 16 is the type most frequently present and there is little disagreement between the data obtained from cancer biopsies using PCR and that using hybridisation. HPV DNA sequences are present in 60–90% of high grade CIN; HPV 16 is the type most frequently present but "unknown" HPVs are detected by PCR in a proportion (maximum 10–15%) of cases. There is wide disparity between the results from several groups using PCR to determine the prevalence of HPV infection in women with normal cervical cytology and this is particularly so with respect to the prevalence of HPV 16. Very high rates of cervical infection with HPV 16, 60–80%, in women with normal cervical cytology have been reported in the literature.^{19,22-24} Similar estimates were given in Heidelberg by several groups from India, Australia, USA and UK. In contrast Manos and colleagues²⁶ have published much lower and widely differing prevalence rates for HPV 16 in three separate populations, 0% in Californians attending Planned Parenthood, 11% in women presenting for annual routine gynaecological examination at the University of Mexico health clinic and 22% in women presenting to the University of Washington student health clinic. Van den Brule *et al* (1990)²⁸ in a Dutch study found only 8% of normal women with HPV 16 DNA in cervical swabs; similarly low estimates were reported by many groups from Scandinavia, France and the USA at the Heidelberg meeting.

There are several possible explanations for these wide disparities. PCR is vulnerable to accidental contamination and high numbers of false positives

are a hazard when using this technique.³¹ The design of the detection primer sets has differed in different studies and this may be important. The incorporation of anti-contamination primers into the assays has been shown to reduce the number of false positives significantly²⁷⁻²⁹ bringing the rates for detection found by PCR to the same range as those determined by hybridisation. Anti-contamination primers²⁷ are mixtures of primers of the common HPVs 6, 11, 16, 18, 33 with the oligonucleotide sequences designed to flank the plasmid cloning sites to prevent amplification of contaminating cloned HPV types. In the best PCR studies which have been reported consensus primers, those located in the highly conserved regions of the HPV genome, L1 and E1 are used to detect HPV DNA sequences. Type specific PCR is then used to type the HPVs in these samples and it is here that anti-contamination primers are so useful in eliminating or reducing contamination from cloned HPV used in that laboratory. Of course any lab using consensus primers to do large scale epidemiological studies may then run the risk of contamination from novel HPV DNA amplified from one sample round in the next set of samples and contamination primers cannot be designed unless the sequence of the contaminating DNA is known. This is a possible explanation for the rather high detection rate by PCR of novel uncharacterised HPVs. Those studies which reported high prevalence of HPV 16 DNA and did not use anti-contamination primers should be re-evaluated. The prevalence of infection with HPV 16 and other HPV types may be, of course, genuinely different in different geographic locations, in differing social classes and in different age groups all of which would be of crucial importance in determining the role of HPV infection in cervical neoplasia. However, this cannot be evaluated until reliable data are available on the prevalence of HPV, and in particular HPV 16, in the genital tract. At the present time the PCR is the best technique with which to determine the prevalence of HPV DNA in large scale population studies but standardised and agreed methodologies which are also quantitative will have to be employed in well designed and controlled studies if the results are to be meaningful.

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- zur Hausen H. Papillomaviruses as carcinomaviruses. *Advances in Viral Oncology* 1989;8:1–26.
- Durst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci USA* 1983;80:3812–5.
- Boshart M, Gissmann L, Ikenberg H, Kleinheinz A, Scheurlen W, zur Hausen H. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO J* 1984;3:1151–7.
- Lorincz AT, Temple GF, Kurman RJ, Jenson AB, Lancaster

- WD. Oncogenic association of specific human papillomavirus types with cervical neoplasia. *J Natl Cancer Inst* 1987;79:671-8.
- 5 Fuchs PG, Girardi F, Pfister H. Human papillomavirus DNA in normal, metaplastic, preneoplastic and neoplastic epithelia of the cervix uteri. *Int J Cancer* 1988;41:41-5.
 - 6 Smotkin D, Wettstein FO. Transcription of human papilloma virus type 16 early genes in a cervical cancer and cancer-derived cell line and identification of the E7 protein. *Proc Natl Acad Sci USA* 1986;83:4680-4.
 - 7 Shirasawa H, Tomita Y, Kubota K, et al. Transcriptional differences of the human papillomavirus type 16 genome between precancerous lesions and invasive carcinomas. *J Virol* 1988;62:1022-7.
 - 8 Durst M, Kleinheinz A, Hotz M, Gissmann L. The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. *J Gen Virol* 1985;66:1515-22.
 - 9 Syrjanen K, Mantyjarvi R, Parkkinen S, et al. Prospective follow-up in assessment of the biological behaviour of cervical HPV-associated dysplastic lesions. In: Peto R, zur Hausen H, eds. *Banbury Report 21: Viral Etiology of Cervical Cancer*, 1986, 167-78. USA: Cold Spring Harbor Laboratory.
 - 10 Pirisi L, Yasumoto S, Feller M, Doniger J, DiPaolo JA. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J Virol* 1987;61:1061-6.
 - 11 Durst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE, Gissmann L. Molecular and cytogenetic analysis of immortalised human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* 1987;1:251-6.
 - 12 Kaur P, McDougall JK. Characterisation of primary human keratinocytes transformed by human papillomavirus type 18. *J Virol* 1988;62:1917-24.
 - 13 McCance DJ, Kopan R, Fuchs E, Laimins LA. Human papillomavirus type 16 alters human cell differentiation *in vitro*. *Proc Natl Acad Sci USA* 1988;85:7169-73.
 - 14 Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT. HPV 16 E7 and E6 proteins cooperate to immortalise human foreskin keratinocytes. *EMBO J* 1989;8:3905-10.
 - 15 Koutsky LA, Galloway DA, Holmes KK. Epidemiology of genital human papillomavirus infection. *Epidemiologic Reviews* 1988;10:122-63.
 - 16 Saiki RK, Scharf SJ, Faloona F, et al. Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science* 1985;230:1350-4.
 - 17 Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* 1989;7:209-14.
 - 18 Shibata DK, Arnheim N, Martin WJ. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. *J Exp Med* 1988;167:225-30.
 - 19 Young LS, Bevan IS, Johnson MA, et al. The polymerase chain reaction: a new epidemiological tool for investigating cervical human papillomavirus infection. *Br Med J* 1989;298:14-8.
 - 20 Kwok S, Higuchi R. Avoiding false positives with PCR. *Nature* 1989;339:237-8.
 - 21 Kwok S. Procedures to minimise PCR-product carryover. In: Innis MA, Gelfand DH, Sninsky DJ, White TJ, eds. *PCR protocols: a guide to methods and applications*. San Diego: Academic Press 1990:142-5.
 - 22 Bevan IS, Blomfield PI, Johnson MA, Woodman CBJ, Young LS. Oncogenic viruses and cervical cancer. *Lancet* 1989;i:907-8.
 - 23 Tidy JA, Vousden KH, Farrell PJ. Relationship between infection with a sub-type of HPV 16 and cervical neoplasia. *Lancet* 1989;i:1225-7.
 - 24 Ward P, Parry GN, Yule R, Coleman DV, Malcolm ADB. Human papillomavirus subtype 16a. *Lancet* 1989;ii:170.
 - 25 Claas E, Melchers W, Quint W. Polymerase chain reaction for the detection of genital human papillomavirus infections. In: Howley PH, Broker TR, eds. *Papillomaviruses Proceedings of a Burroughs-Wellcome-UCLA Symposium Taos, New Mexico, 11-18 March 1989*, 45-54. New York: Wiley-Liss, Inc.
 - 26 Manos MM, Lee K, Greer C, et al. Looking for human papillomavirus type 16 by PCR. *Lancet* 1990;i:734.
 - 27 van den Brule AJC, Claas HCJ, Schrijnemakers HFJ, Snow G, Meijer CJLM, Walboomers JMM. Use of anti-contamination primers in the polymerase chain reaction for the detection of human papillomavirus genotypes in cervical scrapes and biopsies. *J Med Virol* 1989;29:20-7.
 - 28 van den Brule, Snijders PJF, Gordijn RLJ, Bleker OP, Meijer CJLM, Walboomers JMM. General primer-mediated polymerase chain reaction permits the detection of sequenced and still unsequenced human papillomavirus genotypes in cervical scrapes and carcinomas. *Int J Cancer* 1990;45:644-9.
 - 29 Beyer-Finkler E, Pfister H, Girardi F. Anti-contamination primers to improve specificity of polymerase chain reaction in human papillomavirus screening. *Lancet* 1990;i:1289-90.
 - 30 Nuovo GJ. Human papillomavirus DNA in genital tract lesions histologically negative for condylomata: analysis by in situ, Southern blot hybridisation and the polymerase chain reaction. *Am J Surg Pathol* 1990;14:643-51.
 - 31 Kitchen PA, Szotyori Z, Fromhole C, Almond N. Avoidance of false positives. *Nature* 1990;344:201.