A paired comparison of dot blot hybridization and PCR amplification for HPV testing of cervical scrapes interpreted as CIN 1

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Summary: The rate of Human Papillomavirus (HPV) detection in CIN 1 lesions is quite variable for several reasons. Amongst these, the sensitivity level of the HPV detection system probably ranks supreme. The prevalence of HPV DNA in cervical scrape samples from 234 patients referred for colposcopic investigation of a CIN 1 lesion was compared using dot blot hybridization (DBH) and polymerase chain reaction (PCR) amplification. Both methods were performed on the same patient sample so that determinants of HPV positivity other than the detection system could be controlled. Probes and primers to HPV 6, 11, 16, 18, 31, 33, and 35, and consensus HPV primers were used. The overall HPV positivity rate was 24% using DBH and 70% using PCR. Identeified types by DBH and PCR respectively were; HPV 6/11: 1% and 2%, HPV 16/18: 16% and 41%, and HPV 31/33/35: 7% and 14%. PCR detected unidentified types in 13%.

Since PCR resulted in a 2.9 times higher HPV DNA detection rate, the choice of detection system has a major impact on the HPV status of cervical smears interpreted as CIN 1.

Key words: CIN 1; HPV - detection; Dot blot versus PCR.

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INTRODUCTION

Pre-invasive squamous cell carcinoma of the cervix is a spectrum of intraepithelial changes progressing over time to an invasive carcinoma, and variably referred to as squamous dysplasia or cervical intraepithelial neoplasia (CIN) ([1]). CIN I is the earliest and CIN III the most advanced form of pre-invasive squamous cell carcinoma. Accumulating evidence suggests that specific types of the human papillomavirus (HPV) are associated with the development of CIN and invasive cervical carcinoma, and that CIN I and CIN III have different HPV profiles ([2]).

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The rate of HPV detection in CIN I lesions is quite variable ranging from 25% to 100% ([3–9]), and is possibly influenced by a variety of clinical, pathologic, and methodologic determinants. They include the size and demographics of the patient group, the accuracy of the pathologic diagnosis, the method of specimen collection and type of specimen used for HPV testing, the number of HPV probes or primers used for testing, and the sensitivity level of the HPV detection system.

The HPV detection capacity of polymerase chain reaction (PCR) amplification, particularly when the products are confirmed with a hybridization technique greatly exceeds such non PCR detection systems as dot blot hybridization (DBH) and in situ hybridization ([10]). This increased detection capacity is partly a function of PCRS increased sensitivity level as well as the use of consensus primers which can detect unidentified HPV types. Consequently, the type of HPV detection system selected may have a significant impact on the HPV positivity rate of CIN I lesions.

Whereas there are a number of descriptive studies on the HPV status of CIN I lesions using either PCR ([4, 5, 8, 9]) or non PCR methods ([3, 6, 7, 9]), they are not comparable because of a variety of design and technical inconsistencies. Only the small study of Melchers *et al.* ([9]), involving 68 patients with CIN I lesions used paired clinical samples to directly compare positivity rates obtained by non PCR and PCR methodologies. Although the PCR products were not confirmed by hybridization, still the authors reported a 24% increased HPV detection rate with this technique. To determine the increase in HPV detection using PCR combined with DBH of the products (PCR/DBH) compared to DBH alone, a large study of 234 patients referred for colposcopic examination of a cervical smear abnormality interpreted as CIN I was designed. The same patient sample was tested by each method so that determinants of the positivity rate other than the detection system were controlled.

The results were analyzed to determine the sensitivity and specificity of PCR/DBH relative to DBH.

MATERIALS AND METHODS

Patients referred to the colposcopic clinics of the Tom Baker Cancer Centre, the Peter Lougheed Medical Centre, and the Cross Cancer Institute for investigation of a cervical smear abnormality of CIN I were approached to participate in a study of the natural history of CIN I lesions. This report relates to a portion of this larger study group. The referral smears were interpreted in a large number of cytology laboratories, and no attempt was made to review them for accuracy of diagnosis so as to mimic the usual practice of cytology in the province. Using a previously published protocol, all patients first had a cervical scrape for routine cytology ([11]). A second scrape for HPV typing purposes was next taken with a prewashed Ayre wooden spatula. The spatula tip was immersed in a cell lysis solution and transported to the laboratory for HPV testing. Following DNA extraction, HPV testing by DBH and then by PCR/DBH was performed.

Mixed HPV infections were grouped by the level of oncogenic risk associated with the different HPV types ([2]). HPV infections detected by PCR amplification with the consensus primer were referred to as unidentified types, and arbitrarily ascribed an unknown oncogenic risk category. In cases of samples having types from more than one risk group, known risk group categories took precedence over unknown risk groups, and final categorization was based on the most oncogenic risk group.

Dot blot hybridization

Cloned HPV 6, 11, 16, and 18 were obtained from Dr. L. Gissmann, Cancer Research Center, Heidelberg, Germany, cloned HPV 33 from Dr. G. Orth, Pasteur Institute, Paris, France, and cloned HPV 31, and 35 from Dr. A. Lorincz, Bethesda Research Laboratories, Maryland, USA. Storage and culture of the bacteria, plasmid amplification, and purification of the DNA were performed as

described by Maniatis *et al.*, 1982 ([12]). The purified DNA was subsequently labelled with biotin 16-dUTP by nick translation ([13]).

Using a previously reported dot blot hybridization method which has a detection limit of 1 pg of viral DNA (0.1 copies per cell) in controlled experiments ([11]), the volume of extracted DNA was first quantitated using agarose gel electrophoresis and ethidium bromide staining. Thereafter, the DNA was reduced to 20 µL aliquots, from which 2 µL was directly spotted to the nitrocellulose membrane. The membranes were denatured, air dried, and baked for 60 minutes in an 80 °C vacuum oven. Hybridization was performed at 42 °C for 16 hours using a hybridization mixture containing 0.5 µg/ml of biotinylated probe. The membranes were washed at high stringency (Tm −5 °C), and incubated for 60 minutes at 63 °C with 3% (w/v) bovine serum albumin. The signal was generated with streptavidin-alkaline-phosphatase conjugate and the chromogen nitroblue tetrazolium /5-bromo-4-chloro-3-indolyl phosphate (Fig. 1). Positive controls composed of purified cloned HPV DNA 6, 11, 16, 18, 31, 33, and 35 (100 pg target DNA and 200 ng of salmon sperm DNA), and negative controls composed of 200 ng of salmon sperm DNA were run individually, and simultaneously with the test samples.

Polymerase chain reaction amplification

Following DBH, all patient samples were subjected to PCR amplification using a modification of the procedure described by Saiki *et al.* ([14]). The HPV target sequences were amplified in a 50 µL reaction mixture containing 0.05-0.1 µg of purified DNA sample, 10 mM Tris-HCl, pH 9 (at 25 °C), 50 mM KCl, 1.5 mM

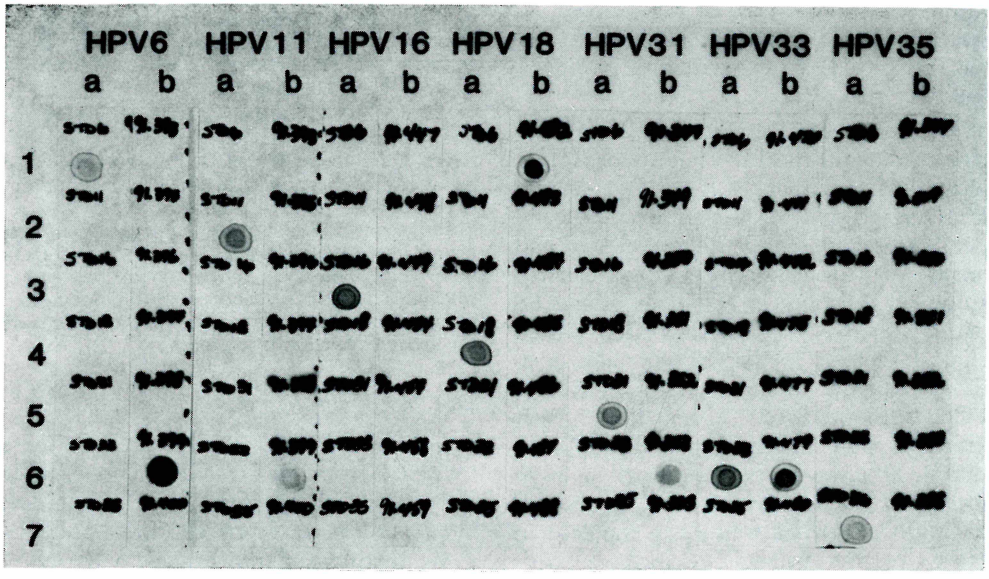

Fig. 1. — Dot blot hybridization of patient samples using biotinylated HPV probes. 100 pg of cloned HPV DNA standard are dotted in the a columns. a1 = HPV 6, a2 = HPV 11, a3 = HPV 16, a4 = HPV 18, a5 = HPV 31, a6 = HPV 33, a7 = HPV 35. Clinical samples are dotted in the b columns. Specimen number 91-399 is HPV 6 and 11 positive, 91-459 is HPV 16 positive, 91-482 is HPV 18 positive, 91-353 is HPV 31 positive, 91-499 is HPV 33 positive and 91-350 is HPV 35 (faint) positive.

$MgCl_2$, 80 μM each of dNTP's, 100 ng of salmon sperm DNA, 1.25 units of Taq DNA polymerase (Bio-Can Scientific, Ontario, Cdn), and 0.1-0.4 μM each of the primer sequences. Specimens were also subjected to β globin gene amplification. All primers were synthesized in the DNA Synthesis Laboratory at the University of Calgary (Table 1). Measures designed to minimize specimen contamination were strictly applied ([15]), and included carrying out specimen preparation and amplification in separate locations. Amplification was performed with a single primer pair.

The mixture was denatured initially for five minutes at 95 °C, followed by 30 cycles of amplification using a TR-96, Thermal Reactor (Tyler Research Instruments Company, Edmonton, AB, CAN). In the case of amplification with type specific primers, each cycle consisted of denaturation at 95 °C for 30 seconds, annealing at 56 °C for two minutes, and extension at 72 °C for one minute. In the case of amplification with consensus primers, annealing was carried out at 48 °C. Positive control samples consisting of 0.05 to 1.25 pg of purified HPV DNA and 0.1 ng of salmon sperm DNA, and a negative control consisting of 10. μg of salmon sperm DNA were run simultaneously with the test specimens. Following the last cycle of amplification, 8 μL of product was analyzed by agarose gel electrophoresis and ethidium bromide staining. The amplification products were approximately 244-613 bp in size (Fig. 2), and HPV DNA type was confirmed using the above DBH method (Fig. 3).

The detection limit of this method was determined by measuring the specific amplified products of all the positive controls by quantitative gel electrophoresis and ethidium bromide staining ([16]). Briefly, the amplification of each PCR product was estimated by comparing the staining intensity obtained from a known amount of HPV cDNA to that of a lambda phage DNA run as a control. The amplification factor varied depending on the primer sequence. The limits were estimated to be 1-20 and 2-250 viral copies per 10,000 cells for type specific and consensus primers respectively.

Statistics

Fisher's exact test (2 tailed) was used to test for associations between positivity among HPV DNA types. Agreement between the two methods for specific HPV DNA types was calculated using Kappa statistics. Sensitivity and specificity were measured using the methods of Galen and Gambino. McNemars test was used to test for differences in the prevalence of HPV DNA between the two detection methods. Differences in prevalence estimates and 95% confidence intervals were determined.

RESULTS

The study group was comprised of 234 cervical scrape samples. All positive and negative controls for each method reacted appropriately, and all samples demonstrated amplification of the β globin gene. Regardless of method, a single infection was more common than a multiple infection (Table 2). Multiple infections, i.e., the presence of more than one type of HPV (inclusive or exclusive of unidentified types) were 9.5 times more frequent by PCR/DBH. DBH identified only double infections. In contrast, by PCR/DBH there were cases of double, triple, and quadruple infection. A consistent pattern of HPV type associations amongst the multiple infections was not apparent, although there were statistically significant associations between types 6 and 18 ($p=0.002$), and types 33 and 16 ($p=0.042$).

The overall HPV detection rate, regardless of type was 2.9 times greater with PCR/DBH than with DBH, i.e., 70%

Table 1. – *Specifications of Oligonucleotides Used as Primers for HPV Amplification With PCR.*

Type	Prm No.	Nucleotide sequence	(nt)	Region	Localization	Amplimer size (nt)
HPV 6	P1	5'TAGGGGACGGTCCTCTATTC-3'	20	LCR	7195 - 7214	
HPV 6	P2	5'-GCAACAGCCTCTGAGTCACA-3'	20	LCR	7434 - 7443	259
HPV 11	P1	5'-GAATACATGCGCCATGTGGA-3'	20	L1	6842 - 6861	
HPV 11	P2	5'-AGCAGACGTCCGTCCTCGAT-3'	20	L1	7179 - 7198	357
HPV 16	P1	5'-AAGGCCAACTAAATGTVAC-3'	19	URR	7763 - 7781	
HPV 16	P2	5'-GGCTTTTGACAGTTAATACA-3'	20	E6	410 - 429	571
HPV 18	P1	5'-TATGGCGCGTTTGAGGATC-3'	20	E6	104 - 123	
HPV 18	P2	5'-TTGTGTTTCTCTGCGTCGTT-3'	20	E6	556 - 575	471
HPV 31	P1	5'-TGAACCGAAAACGGTTGGTA-3'	20	E6	49 - 68	
HPV 31	P2	5'-CTCATCTGAGCTGTCGGGTA-3'	20	E7	642 - 661	613
HPV 33	P1	5'-AGTAGGGTGTAACCGAAAGC-3'	20	E6	28 - 47	
HPV 33	P2	5'-CTTGAGGACACAAAGGTCTT-3'	20	E6	429 - 448	411
HPV 35	P1	5'-GAATTACAGCGGAGTGAGGT-3'	20	E6	215 - 234	
HPV 35	P2	5'-CACCGTCCACCGATGTTATG-3'	20	E6	485 - 504	290
Consensus	P1	5'-CGTAAACGTTTTCCCTATTTTTTT-3'	24	L1	(5500 - 5790)*	
Consensus	P2	5'-TACCCTAAATACTCTGTATTG-3'	21	L1	(5800 - 6000)*	244 ~ 256
β-globin	P1	5'CTAGCAACCTCAAACAGACA-3'	20		132 - 151	
β-globin	P2	5'-TGCCTATCAGAAACCCAAGA-3'	20		316 - 335	204

LCR = Long Control Region URR = Upstream Regulatory Region (*) Proximate base position

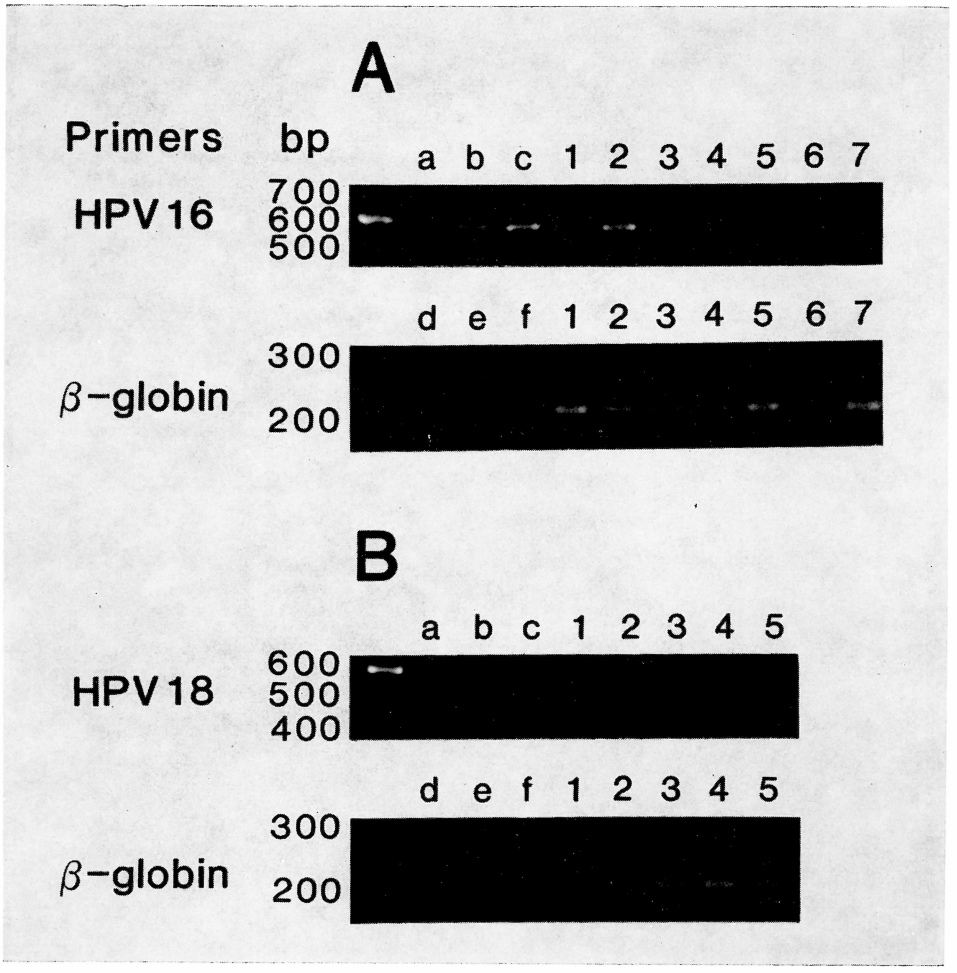

Fig. 2. — Electrophoresis of amplified HPV sequences and the human β-globin gene from patient samples. A) HPV 16 primer and human β-globin primer. Lanes 1-7, patient samples. Lane a, negative control; lanes b, c, positive controls (HPV 16 cDNA). Lane d, negative control (no target); lanes e, f, positive controls (human kidney DNA). All patient samples amplify with the β-globin primer, and patient samples 1-5 with the HPV 16 primer; B) HPV 18 primer and human β-globin primer. Lanes 1-5, patient samples. Lane a, negative control; lanes b, c, positive controls (HPV 18 cDNA). Lane d, negative control (no target); lanes e, f, positive controls (human kidney DNA). All patient samples amplify with the β-globin primer, and patient samples 1-4 with the HPV 18 primer.

(165) and 24% (55) respectively. PCR/DBH detected at least one specific HPV type in 57% (134) of samples and unidentified types in 13% (31). Both methods identified specific HPV types 6, 11, 16, 18, 31, 33, and 35 (Table 3). HPV types 16 and 18 were the most and types 6 and 11 the least frequent, regardless of method. Compared to DBH, the overall prevalence of specific types was 3.5 times greater and the prevalence of individual specific types, except type 11 significantly

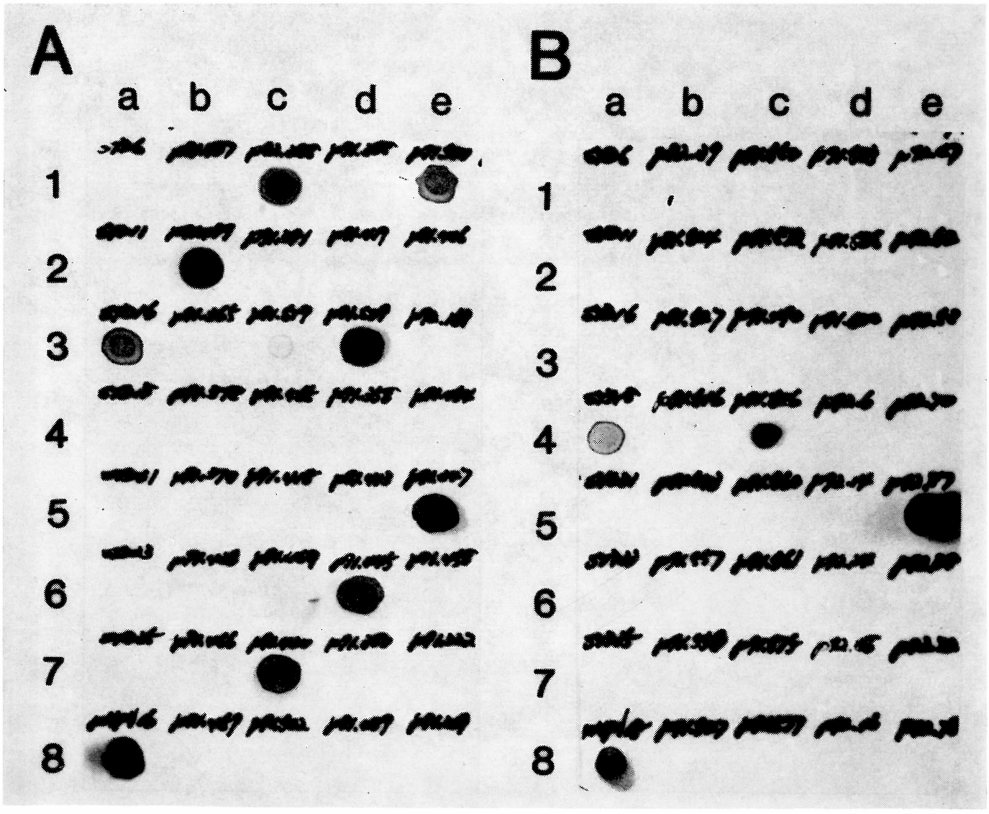

Fig. 3. — Dot-blot hybridization using biotinylated HPV DNA probe. A) With HPV 16 specific probe. Column a-1, 100 pg of HPV 6 cloned DNA standard, -2, HPV 11, -3, HPV 16, -4, HPV 18, -5, HPV 31, -6, HPV 33 and -7, HPV 35 (without amplification); a-8, 250 fg HPV 16 cDNA (amplified); Column b to e, the amplified products of clinical specimens. 2 μl (4%) of the products were applied to the membrane. Amplified patient products b2, c1, c7, d3, d6, e1, and e5 are positive; B) With HPV 18 specific probe. Column a-1, 100 pg of HPV 6 cloned DNA standard, -2, HPV 11, -3, HPV 16, -4, HPV 18, -5, HPV 31, -6, HPV 33 and -7, HPV 35 (without amplification); a-8, 250 fg HPV 18 cDNA (amplified); Column b to e, the amplified products of clinical specimens. 2 μl (4%) of the proucts were applied to the membrane. Amplified patient products c4 and e5 are positive.

greater with PCR/DBH. There was very poor agreement between the two methods for concordance of overall HPV positivity and specific HPV typing with Kappa statistics ranging from 0.13 for HPV 6 to 0.31 for HPV 18 (data not shown).

Cases were 2.6 times as likely to be grouped in the high risk category of HPV 16/18 by PCR/DBH versus DBH (41% and 16% respectively), and twice as likely to be in the intermediate risk category of HPV 31/33/35 (14% and 7% respectively). While the prevalence of low risk HPV types 6/11 was extremely low with both methods, still cases were twice as likely to be grouped in this category by PCR/DBH versus DBH (2% and 1% respectively).

Relative to DBH, PCR had a sensitivity of 85%, a specificity of 35%, a false negative rate of 15%, and a false positive rate of 65% (Table 4). The eight false

Table 2. – *Multiple HPV infections.*

Number of infections	DBH		PCR[1]		PCR[2]	
	N	%	N	%	N	%
0	179	76	100	43	69	30
1	49	21	77	33	108	46
2	6	3	39	17	39	17
3			12	5	12	5
4			6	3	6	3

1 Exclusive of unidentified types
2 Inclusive of unidentified types

negative cases comprised five cases of HPV 16, two cases of HPV 31 and one case of mixed HPV 16 and 35 infection. The 118 false positive cases comprised a wide variety of HPV types, and no consistent pattern could be demonstrated.

DISCUSSION

Using a paired comparison format, confounders of HPV detection other than the detection system itself were controlled in this study. As HPV DNA was identified 2.9 times more frequently with PCR/DBH, the detection system was confirmed as the major determinant of the HPV status of cervical smears interpreted as CIN I. This increased positivity rate was partially due to the detection of unidentified HPV types in 13% of specimens, but mostly because PCR/DBH, due to its superior sensitivity level was 3.5 times more effective than DBH in identifying specific HPV types. The increased detection was not type specific, since all HPV types assayed were identified, nor was it skewed towards a specific risk category of HPV types, since PCR/DBH was at least twice as sensitive in detecting low, intermediate, and high risk HPV types. However, this increased detection capacity accounted for the poor concordance between the two methods for overall HPV positivity and specific HPV typing.

In comparison to DBH, multiple infections were 9.5 times more common with PCR/DBH. Both methods identified double infections, but triple and quadruple infections were only seen by PCR/DBH. Multiple infections can result from cross reacting HPV types, selective increased detection of a specific HPV type, or a generalised increased detection of all types. Some HPV types, e.g., 6 with 11, and 16 with 31 demonstrate a certain degree of genomic homology which can result in a "pseudo" double infection ([17, 18]). This phenomenon however, does not explain the multiplicity of HPV infections by PCR/DBH observed in this study, particularly since statistically significant associations were between the relatively non-homologous types 6 with 18, and 16 with 33. Moreover, the small size and uniqueness of each primer's sequence

Table 3. – *Frequency of specific HPV types and agreement between types.*

HPV	DBH		PCR		95% CI	P value
	N	%	N	%		
6	2	0.9	26	11.1	6.0, 14.6	0.000
11	2	0.9	3	1.3	–2.3, 1.4	1.000
16	26	11.1	65	27.8	9.5, 23.8	0.000
18	12	5.1	48	20.5	9.3, 21.4	0.000
31	9	3.8	28	12.0	3.2, 13.0	0.002
33	6	2.6	21	9.0	2.2, 10.6	0.006
35	4	1.7	24	10.3	4.2, 12.8	0.000
Total	61	–	215	–		

Table 4. – *HPV positivity PCR/DBH versus DBH.*

DBH	PCR/DBH		
	Positive	Negative	Total
Positive	47	8	55
Negative	118	61	179
Total	165	69	234

Abbreviations:
DBH = dot blot hybridization
PCR/DBH = polymerase chain reaction with dot blot hybridization confirmation.

would almost eliminate the potential for cross reactivity between the much larger HPV DNA probe used in the confirmatory DBH step and the amplified PCR product. As the increased detection capacity of PCR/DBH was not confined to a specific HPV type, it is more likely that the increased number of multiple infections with PCR/DBH was due to the greater sensitivity of the technique in detecting all HPV types.

Assuming DBH to be the gold standard, PCR/DBH had a high sensitivity rate of 85%, and a low false negative rate of 15%. The specificity of 35%, however, was extremely low. It is somewhat spurious to label these PCR/DBH results as false positives given the specificity of the annealing process. However, the possibility that all or some of the 118 "false" positive results were actual false positives must be considered. Sample contamination by extraneous virus either at the time of collection or at any stage of specimen preparation within the HPV testing laboratory may cause a false positive result. If the amount of contaminant can only be detected by PCR, then this is of major relevance to studies on HPV prevalence. However, in paired comparison studies such as this one, where the sensitivity of one method is being measured against another, contamination is not of major concern. Consequently, it is reasonable to conclude that the false positive cases arose because of the greater detection capacity of PCR/DBH relative to DBH.

Possible reasons for the eight false negative results include a false positive DBH result or an unsuccessful PCR amplification. A false positive DBH reaction may result from specimen contamination during the actual dot blot hybridization reaction. However, this was an unlikely event, as the negative controls reacted appropriately. Unsuccessful PCR reactions can result from poorly preserved cellular DNA, an inhibitor of the PCR reaction, or a mutated (subtype) HPV virus. In this study, all specimens demonstrated amplification of the β globin gene thus eliminating concerns regarding the appropriateness of the cellular DNA ([10]).

Substances capable of inhibiting Taq DNA polymerase have been identified by a number of investigators, and are extremely difficult to recognise and monitor ([19]). However amplification of all the specimens with either a β globin or HPV primer downplayed the role of an inhibitor as a cause of the false negative reactions. Mutation(s) within the HPV genome would not prevent annealing with the relatively large subgenomic probe used in the DBH reactions, but could interfere with the complimentary binding of the much smaller oligonucletide primer used in PCR amplifications. This possibility is likely responsible for the false negative reactions by PCR/DBH, and could also explain the absence of HPV DNA in 30% of specimens. Of course, failure to identify HPV DNA in these particular specimens may be due to the presence of a type not identified by the probes and primers used in this study, a volume of virus below the detection capacity of PCR/DBH, or alternatively, the total absence of the virus.

In summary, relative to DBH, the study demonstrated a 2.9 times greater ca-

pacity for overall HPV DNA detection in cervical smears interpreted as CIN 1 by PCR/DBH. PCR/DBH identified all specific HPV types with greater frequency than DBH, and detected unidentified types in 13% of cases. HPV types 16/18 were the predominant types regardless of method and multiple infections were 9.5 times more frequent with PCR/DBH. Since each patient acted as her own control in this comparison study, the significant effect of the HPV DNA detection method on the frequency of HPV in cervical smears interpreted as CIN 1 was underscored.

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