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High Incidence of Human Papillomavirus in 146 Cervical Carcinomas. A Study Using Three Different Pairs of Consensus Primers, and Detecting Viral Genomes with Putative Deletions

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Polymerase chain reaction (PCR) primer sets and probe-cocktails were used for human papillomavirus (HPV) detection and typing of 146 fresh frozen biopsies of cervical carcinoma. We obtained a high detection rate (96%) by using three sets of consensus primer pairs directed at the L1 and E1 regions of HPV and by probing with a cocktail of random-labelled consensus and type-specific PCR products derived from HPV plasmids. In addition, we performed type-specific PCR amplification with E6-E7 primers. The procedure was designed to detect all HPV-positive cases in a rapid, sensitive and specific way. In addition, by using different regions for amplification, we detected cases with putative genomic deletions in HPV. All the negative PCR and DNA isolation controls were negative. The six negative samples were negative with all probe-cocktails and type-specific primers and three of these negative samples were clear cell carcinomas. The detection rate was similar in squamous carcinomas and in adenocarcinomas and type 16 was most common (65%) in both types of carcinoma. There were no double infections of human papillomavirus 16 and 18.

Key words: HPV, papilloma, PCR, probe, cancer, cervical
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INTRODUCTION

A STRONG relationship between human papillomavirus (HPV) and cervical dysplasia and carcinoma has been established by epidemiological studies [1, 2]. Although HPV is neither necessary nor sufficient to cause cervical carcinoma, it is the strongest recognised co-factor of the disease and several molecular pathways for its action have been suggested [3, 4].

The frequency of HPV involvement in cervical intra-epithelial neoplasia (CIN) and carcinoma is still a matter of debate. If HPV is present in nearly all carcinomas and if CIN with HPV present has a higher likelihood for progression to invasive lesions than CIN without HPV, then the detection and typing of HPV should be an integrated part of cytological screening and surveillance, as suggested by Meijer and coworkers [5, 6]. If, on the other hand, there is a substantial proportion of HPV-negative carcinomas, then HPV detection would not carry the same weight.

The aim of the present study was to establish with polymerase chain reaction (PCR) the detectability of HPV in a Norwegian series of 146 carcinomas using three pairs of "consensus" primer sets. The series included 114 squamous carcinomas, 32 adenocarcinomas including five carcinomas of endometroid type, three clear cell carcinomas and one small cell carcinoma. The primers were directed at the L1 (My09/My11 and Gp5/6) and E1 (CpI/IIG) regions of the HPV open reading frames (ORF). The study also includes typing by primer sets specific for HPV type 11, 16, 18, 31 and 33 directed at the E6 region.

MATERIALS AND METHODS

Cervical cancer tissues

During the period 1991–1993, biopsies for this study were obtained from 146 cases of invasive cervical cancer with FIGO stages from IB to IVB. In all cases, separate biopsies were taken for routine histological evaluation. The biopsies for this study were snap frozen in liquid nitrogen and stored at −70°C. At processing, the frozen tissue was divided into two or three pieces according to the size of the tissue sample. One piece was used

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for DNA analysis and one piece was used to make frozen sections for histological confirmation of the content of tumour tissue. The presence of carcinoma was ascertained in all cases.

DNA extraction

A modification of the method by Mies and colleagues [7] was used for DNA extraction. To avoid contamination we used changes of underlay, gloves, pasteur pipettes and scalpels after the handling of every new sample. The tissue sample for DNA isolation was crushed under aluminium foil with a hammer following freezing with liquid nitrogen. The crushed tissue was then incubated in 1 ml autoclaved 1 X TEN buffer with 1% SDS and 500 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) overnight. The clear solution was extracted with an equal volume of phenol-chloroform followed by an equal volume of chloroform. The extracting mixture was rotated carefully for more than 1 h in a rotary mixer and the upper phase was carefully pipetted off with a wide-needle Pasteur pipette. DNA was precipitated with two volumes of 98% ethanol and left at −20°C for more than 20 min. The precipitated DNA was centrifuged for 20 min at 13 000 rpm and washed once with 70% ethanol. The DNA was precipitated and dried at room temperature overnight and dissolved in double-distilled water at room temperature for more than 1 day. As a control of DNA extraction, i.e. to exclude false negatives, we used a set of D3S32 primers which detect areas of human chromosome 3 [8].

PCR

The PCR was performed at four different laboratory stations in order to minimise the risk of contamination, and a negative control (water or a breast cancer sample) was included after each seventh sample. The buffer and primers were mixed in a DNA-free room with DNA-free equipment. The DNA, which was isolated in a separate room, was transferred to the PCR buffer with filter pipettes. Three pairs of consensus primers were used. The My primers My09 and My11 in L1 [9] were used with the following final 50 μl PCR solution: 0.5–20.0 μl of the DNA, 50 mM Tris–HCl (pH 8.4), 10 mM KCl, 2.0 mM $MgCl_2$, 1% β-mercaptoethanol, 0.05% bovine serum albumin, 0.2 mM of each dNTP, 20 pmol of each primer and 2.5 units of Taq polymerase (Boehringer Mannheim). The Gp primers Gp5 and Gp6 also in L1 [10] were used under the same conditions as the My primers but with the following exceptions: 1.5 mM $MgCl_2$ and 50 pmol of each primer. The Cp primers in E1 [11] were used under the same conditions as the My primers but with the following exceptions: 3.0 mM $MgCl_2$ (pH 8.8), 17 pmol of CP-I and 26 pmol of Cp-IIG. The My and Gp primers were run with 40 cycles and Cp with 35 cycles. All the PCR products were hybridised as described below.

HPV 11, 16, 18, 31 and 33 type-specific primers from the HPV region E6-E7 were used as described previously [12], i.e. similarly as for My primers but using 1.5 mM $MgCl_2$. The HPV type-specific PCR products were stained with ethidium bromide (Sigma, St Louis, Missouri, U.S.A.) or SYBR Green I (Molecular Probes, Eugene, Oregon, U.S.A.) in polyacrylamide gel (7.5%) and positive cases were registered directly without a following hybridisation. The type-specific PCR was performed in sequences: all cases were tested for type 16 and 18, other types in negative cases only.

Hybridisation of the PCR products

After amplifying plasmid HPV DNA of types 11, 16, 18, 31 and 33 with My, Cp or Gp consensus primers, fluorescein-labelled probes were produced using the random-labelled HPV plasmid PCR product kit (RPN 3031, Amersham, U.K.). The Cp consensus primers did not produce a consensus PCR product, but type-specific products. The fluorescein-labelled probes were mixed into a "cocktail", either of types 16, 18, 31 and 33 or types 11, 16, 18 and 33. Either of these probe mixes was used on patient sample amplificates, produced as described in the preceding section.

Eleven microlitres of the consensus PCR products (50–70 specimens per batch) were run on polyacrylamide gels, transferred to Hybond N^+ membranes (Amersham, U.K.) by Electroblot (Biorad, Hercules, California, U.S.A.) and fixed to the membranes with ultraviolet transilluminator (Alfa-Lab, Vilber Lourmat, France). The blot was then prehybridised for 30 min before 45 μl of the fluorescein-labelled probe was applied. Fluorescein-labelled My, Cp and Gp PCR product mixes from plasmid HPV 16, 18, 31 and 33 were used as probes in the first test run of the DNA samples. In the second run of DNA samples, which were negative in the first round (16 cases), we increased the amount of DNA four times in the PCR and used My and Cp PCR products from plasmid HPV 11, 16, 18, and 33 as probes. After overnight hybridisation, the blots were washed and incubated with anti-fluorescein conjugate labelled with HRP. After washing and signal generation with enhanced chemiluminescence assay, the Hyperfilm-ECL (Amersham, U.K.) was exposed to enhanced light for 2–120 min.

RESULTS

The distribution of histology types in relation to HPV infection and the PCR and blot results for the individual primers and probe mixes are shown in Tables 1 and 2. The age of the patients ranged from 22 to 86 years (median 55). Of 146 invasive cervical cancer cases, 140 (96%) were HPV-positive with one or more of the consensus primers and/or probe mixes. If the three clear cell carcinomas were excluded from the series, 98% of the cases were HPV-positive. The six negative samples were negative with all primer sets and probe mixes and they were negative on retest with newly extracted DNA from the same tumour. Figure 1 shows the different consensus PCR primer products. More than 80% of the samples were identified to be positive with consensus primer PCR products only stained with ethidium bromide, but hybridisation was nevertheless performed in all cases. The different HPV types were identified in 130 of 146 cases (89%) with the type-specific primers (Table 2).

Only 4 cases positive with both My and Cp were not positive with one of the type-specific primers. Of the 23 cases positive with only one of the probe mixes, 13 cases were positive with one of the type-specific primer pairs (Table 3). Double-infections with the HPV types 16 and 18 were not observed. Cases that were negative with both My probe mix and Gp probe mix were positive with the Cp probe mix and HPV type-specific primers in 11 and 3 cases, respectively, whereas cases negative with the Cp probe mix were positive with the My probe mix and HPV type-specific primers in 13 and 8 cases, respectively. The Gp probe mix did not probe against 36% of the tested samples and the Gp probe mix did not detect other positive cases than the My or Cp probe mixes. None of the Gp-positive cases were negative with My probes. Figure 2 illustrates Cp PCR probe mix used against PCR products from HPV plasmids type 6, 11, 16, 18, 31 and 33 and Figure 3 shows some of the results of the My probe hybridisation.

All cases with discrepant results are summarised in Table 3. Most squamous cell carcinomas (SCC) were positive on primary

Table 1. PCR blot results in relation to histological and clinical features

No. of cases	SCC 113 (%)	Ac 15 (%)	ASC 12 (%)	CC 6 (%)	Total no. of positive cases (%)	No. tested
My probe mix*	95	73	93	33	90	146
Gp probe mix*	67	50	75	33	64	135
Cp probe mix*	88	93	91	50	87	143

SCC, squamous cell carcinoma; Ac, adenocarcinoma; Asc, adenosquamous carcinoma; CC, one carcinocarcinoma, one signet ring cell carcinoma, one small cell carcinoma and three clear cell carcinomas. * Two probe mixes produced from HPV 16, 18, 31 and 33 or HPV 11, 16, 18 and 33 plasmids.

Table 2. The PCR typing results in relation to histological and clinical features

No. of cases	SCC 113 (%)	Ac 15 (%)	ASC 12 (%)	CC 6 (%)	Total no. of positive cases (%)	No. tested
HPV 11	2	13	0	0	4	16‡
HPV 16	67	33	50	17	65	146
HPV 18	11	14	25	17	13	146
HPV 31	3	0	0	0	2	26†
HPV 33	7	7	0	0	6	75*

See Table 1 for abbreviations.
* Only the HPV 16 and 18 negative samples were investigated with HPV 33 primers. † All the HPV 16, 18 and 33 negative and some of the positive samples were investigated with HPV 31 primers. ‡ All the HPV 16, 18, 33 and 31 negative and some of the positive samples were investigated for HPV 11.

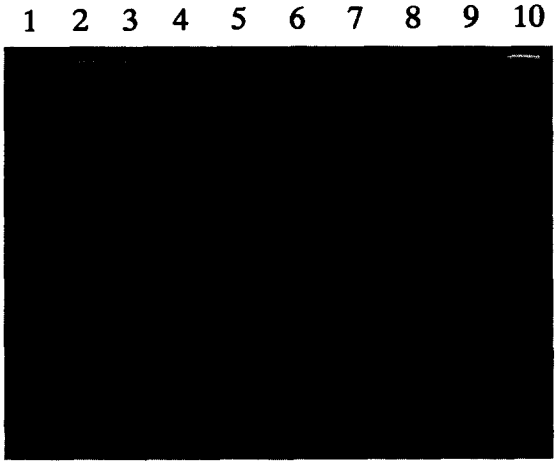

Figure 1. PCR products from My09/11, CpI/IIG and Gp5/6 primers. Numbers 2, 3 and 4 are My PCR products from HPV 33, 18 and 16, respectively. Numbers 5, 6 and 7 are Cp PCR products from HPV 33, 18 and 16, respectively. Numbers 8, 9 and 10 are Gp PCR products from HPV 33, 18 and 16, respectively. Number 1 is a PhiX174 RF DNA-*Hae* III digest (New England Biolabs) and the gel was stained with ethidium bromide (Sigma).

screening by one or more of the consensus primers. Squamous cell carcinomas stage Ib and IIa were all (100%) positive with one of the consensus primers and PCR probe mixes. Four cases positive for HPV type 11 are shown in Table 4. They were all clearly detectable with type-specific PCR (using SYBR-Green as dye), but gave weak or ambiguous results with the consensus primers.

All the negative controls were negative both with PCR and with hybridisation even when many samples (60–70) were run at a time. Thus, the negative controls were not contaminated with HPV DNA and the probe mixes did not crossreact with any other DNA (Figure 4).

DISCUSSION

The aim of our study was to determine the prevalence of HPV in a large series of invasive carcinomas. By using two primer sets for PCR from the highly conserved L1 region and one primer set from the E1 region, six different probe mixes from the same regions of the HPV genome with strong non-radioactive signal generation and fresh frozen tissue for the DNA extraction, we feel confident to have identified all carcinomas which contained (and retained) these parts of the HPV genome. 7 of the cases with no My hybridisation signals but with strong Cp hybridisation signals may have been deleted in L1, and 9 cases with absent Cp hybridisation but with positive type-specific and My

Table 3. The discrepant hybridisation results

No.	My 09-11 PCR	My 09-11 HYB	Gp 5-6 PCR	Gp 5-6 HYB	Cp I-IIG PCR	Cp I-IIG HYB	HPV 16	HPV 18	HPV 33	HPV other types	FIGO stages
1	3+	6+	3+	6+	4+	–	–	4+	–		SCCIb
2	–	4+	–	–	–	–	4+	–	–		SCCIb
3	1+	2+	–	–	–	–	–	–	–	HP35?	SCCIb
4	3+	1+	1+	–	4+	5+	–	–	2+		SCCIb
5	–	1+?	–		3+	6+	–	–	–		SCCIb
6	–	1+?	–		3+	7+	–	–	–	?	SCCIIa
7	1+	2+	–	–	–	–	1+	–	–	?	SCCIIb
9	2+	3+	–	–	–	–	4+	–	–		SCCIIb
10	–	–	–	–	–	1+	–	–	–	?	SCCIIb
11	3+	8+	4+	5+	2+	–	–	–	2+		SCCIIb
12	4+	–	3+?	–	4+		–	1+			SCCIIb
13	–	3+	–	–	–	–	4+	–	–		SCCIIb
14	4+	8+	2+	4+	–	–	4+	–	–		SCCIIIb
15	–	3+	–	–	–	–	3+	–	–		SCCIIIb
16	3+	7+	–	–	–	–	4+	–	–		SCCIIIb
17	–	–	–	–	–	1+	1+?	–	–		SCCIIIb
18	2+	1+?	3+?		4+	1+	3+	–	–		SCCIIIb
19	–	–	–	–	–	1+	–	–	–	?	SCCIIIb
20	4+	1+	4+		4+	4+	–	–	1+		SCCIVb
21	–	1+	–	–	–	–	–	–	–	?	ACEIIIb
22	–	–	–	–	–	3+	–	–	–	?	ACEIIb
23	–	–	–	–	–	1+	–	–	–	?	ACEIIIb
24	–	–	–	–	–	3+	–	–	–	?	ACEIIIb

SCC, squamous cell carcinoma; ACE, adenocarcinoma endometroid; PCR, polymerase chain reaction results; HYB, hybridisation results with the PCR probe mixes. Carcinoma nos 1 to 20 are squamous cell carcinomas. Carcinoma nos 21 to 24 are endometroid adenocarcinomas. The PCR and hybridisations results from 1+ to 8+: the estimated visual UV light or film blackening density, with an arbitrary scale from 1 to 8. ?, The HPV types have not been determined.

hybridisation signals may have been deleted in E1. By this consensus primer pair combination we only risk missing tumours where the HPV genome is completely integrated and at the same time deleted in the E1 and L1 regions. On the other hand there were no such cases, at least not type 16 and 18, for they would have been detected by the type-specific primer pairs in the E6 region. Deletions are indeed described in studies on established cell lines [13, 14]. In the relatively few clinical cases where deletions have been mapped, the deletion pattern appears, however, to be complex [15–17]. In many cases the integration also appears not to be complete, but allows for both integrated and episomal viral DNA [18, 19]. In view of these findings, and the high incidence of HPV in CIN III and carcinoma found by Meier and coworkers in Holland [5, 6], our finding of a 96% HPV positivity in carcinoma is perhaps not surprising. There is a 20% increase, however, in comparison with earlier studies by ourselves and others, using paraffin-embedded carcinoma tissue for DNA extraction and PCR-based HPV detection [12, 20]. This discrepancy cannot be explained solely on the basis of artifacts due to formalin fixation [21]. Such material precludes, however, the use of long amplificates and Manos My 09-11 primers may give false negative results because of modifications of the DNA by the fixatives. Other causes which lower the optimal HPV detection in previous studies are: (i) use of only one type of "consensus primers"; (ii) lower than optimal DNA concentration and purity (abs 260/abs 280 < 1.7); (iii) competetive/inhibiting structures in the PCR solution [6]; (iv) use of only one consensus probe or probe mix. Each of these factors has to be taken into account when results from different laboratories are compared.

Apart from these technical considerations, there may of course be geographical differences in HPV involvement, as shown for instance in comparisons between Denmark and Greenland [22] or Spain and Colombia [1, 2]. It would be surprising, however, if this was the cause of the difference between the present series of Norwegian patients and our previously published series of Swedish patients [12]. Kristiansen and coworkers [23] with similar material as ours obtained only about an 80% presence of HPV but nearly the same number of HPV 16-positive cases. Our present high detection rate is probably due to technical factors, such as (i) the use of fresh frozen tissue samples for DNA extraction; (ii) multiple primers, both consensus and type-specific ones; (iii) a sensitive and specific procedure for hybridisation; and (iv) the use of four different consensus probe mixes and two different HPV type-specific probe mixes. Our strategy was to amplify, in a first step, all possible cases with three pairs of consensus primers. The second step was to detect all consensus PCR products with both a mix of consensus probes derived from the My 09-11 and Gp HPV plasmid amplificates and a mix of type-specific probes derived from the Cp HPV plasmid amplificates. Together with a highly sensitive non-radioactive labelling, this strategy gave us a quick (2–4 days) and safe method with a high detection rate. These procedures are similar to those used by Meijer, van Den Brule and coworkers [5, 6], with the difference that they omitted the DNA extraction and we used a probe mix similar to that used by Tieben and

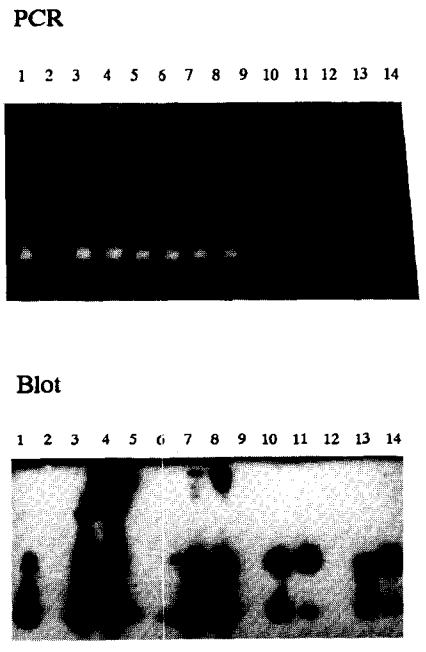

Figure 2. The Cp probe mix (HPV 16, 18, 33, 31) is not probing HPV 6 and 11. PCR products with Cp consensus primers CpI/CpII-G and HPV16, 18, 31, 33 Cp probe mix hybridisation. No. 11 is HPV 16, 18 and 33 PCR-negative samples with type-specific primers, numbers 10 and 13 are HPV 16 PCR-positive samples and no. 14 is a HPV 33 PCR-positive sample. No. 12 is My and Gp PCR-probe positive but clearly not Cp PCR-probe positive and not HPV 16, 18, 31 and 33 positive. No. 1 is Cp PCR-positive control (a mixture of different HPV Cp PCR-positive samples). Numbers 2 and 9 are PCR-negative controls (with double-distilled water). Numbers 3–8 are PCR probe analysis of HPV 31, 33, 6, 11, 18 and 16 plasmids, respectively. Note that HPV 6 and 11 are completely negative.

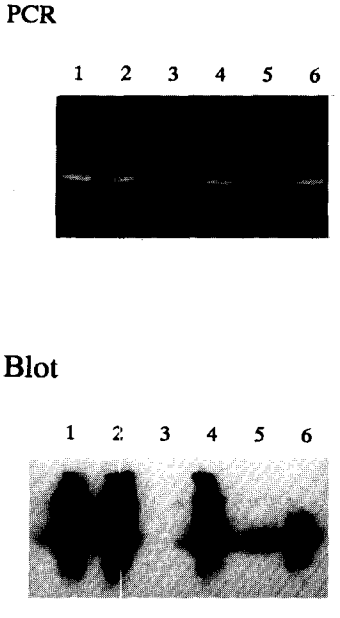

Figure 3. My positive samples. PCR products with My consensus primers My09/My11 and HPV16, 18, 31, 33 My probe mix hybridisation. Numbers 1, 2 and 5 are HPV 16-positive samples and number 4 is a HPV 18-positive sample. Number 3 is a PCR-negative control, and number 6 is a My PCR-positive control.

Table 4. The HPV 11-positive samples

Sample number	My PCR	HYB	Cp PCR	HYB	HPV11 PCR
693	–	–	–	4+	2+
800	–	1+	–	1+	2+
812	2+?	–	4+?	1+	4+
2749	–	2+	–	3+	3+

My: the My09/11 PCR and hybridisation results. Cp: the CpI/IIG PCR and hybridisation results. HPV 11: positive with the type-specific primers. HYB: Hybridisation results with the PCR probe mixes. The PCR and hybridisations results from 1+ to 4+: the estimated visual UV light or film blackening density, with an arbitrary scale from 1 to 4. Sample number 2749 was negative with the first PCR run, but with ten-fold increase of the DNA amount, the sample had positive results with hybridisation.

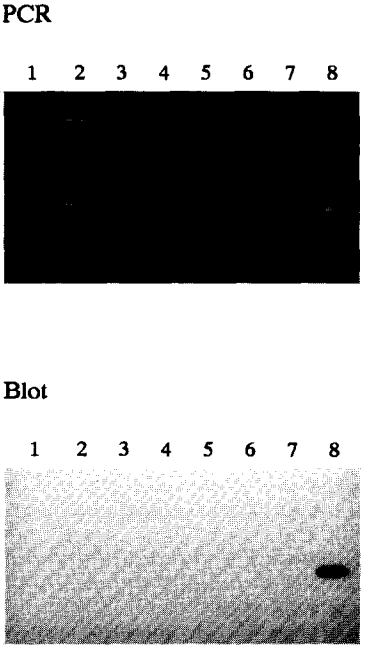

Figure 4. Hybridisation of the negative and positive controls. PCR products with Cp consensus primers CpI/CpII-G and HPV16, 18, 31, 33 Cp probe mix hybridisation. The film was exposed for only 2 min, but the results were the same after 2 h. Number 8 is a Cp PCR-positive control (a mixture of different HPV Cp PCR-positive samples). Numbers 1, 5 and 7 are negative controls with only water. No. 6 is a DNA-negative control. Numbers 2–4 are breast cancer samples as negative controls.

coworkers [11]. The probe mix from Meijer and coworkers (from Gp5/6 primers) gave, however, in our hands, a lower detection rate than the probe mixes from the other two primer pairs. Compared to the method used by Forslund and colleagues [24], our method gives a more rapid detection of HPV, but necessitates individual typing by separate E6-E7 primers. This proved an advantage in some cases with weak or ambiguous PCR products from consensus primers, illustrated particularly by the type 11 cases (Table 4). Whether the poor consensus primer results are due to deletion-integration [15, 17], remains to be determined.

Since our combination of Cp and My primers and probe mixes were effective in HPV detection, it may be useful to combine other Cp probe mixes to separately detect high- and low-risk

HPV types. In this respect, the Cp PCR products from E1 are particularly interesting, since they were efficient in detecting HPV and, in addition, permit individual typing. They are also complementary to the My09/11 primers because they would detect cases deleted in L1. Studies using only L1 primers may miss such cases [2].

The dominant HPV type, as in most other series, was type 16 (65%), followed by type 18 (13%), type 33 (6%), type 11 (4%) and type 31 (2%). Further subtyping will be performed later, including the use of sequencing. Only one of the 140 HPV positive cases (<1%), lacking Cp probe hybridisation and without HPV type-specific PCR amplification, may be of another type than HPV 11, 16, 18, 31 and 33, since the Cp probe mix is type-specific. However, since the Cp primers amplify all HPV types, the Cp probe mix may also hybridise to some of the amplified products from other HPV types. In four of the samples where we got relatively strong hybridisation signals from the Cp probe mix, the HPV typing with the type-specific primers were negative even though the Cp probe mix included the same HPV types.

Our findings show that the method of using the combination of consensus primer and probe mixes together with type-specific probe mix with a rapid and sensitive non-radioactive detection system can find most cases of HPV. Our findings also show that HPV is present in most cervical carcinomas. Further studies on the importance of the virus load and the deletion/integration patterns are, however, urgently needed to elucidate the pathogenetic mechanisms involved. Such studies are in progress on this series of invasive carcinoma in Norway.

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