

Type specific prevalence of oncogenic HPV types in Telemark, Norway

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Materials and Methods

Material

Cervical cytobrush samples from women aged 25–69 with LSIL, ACUS or unsatisfactory samples (Norwegian Cancer Registry recommendations) including follow-up samples from women testing HPV positive.

Age	Number of patients	Number of samples
25–35	422	527
35–45	406	484
45–55	253	288
55–65	115	134
>65	30	36
N	1 213	1 470

Catchment area: Telemark, Southeastern Norway. Population 150 000.
Study Period: 2005–2007

HPV testing

PapType13 reverse line blot test

- Sample extraction (10 ml): Roche MagNAPure. Elution in 100µl.
- L1 consensus PCR (6348–6557) using biotin-labelled reverse primer.
- Reverse line blot hybridisation to type-specific probes
- Detection: ECL chemiluminescent detection of bound biotin labelled amplicon using peroxidase-streptavidin conjugate

Types detected

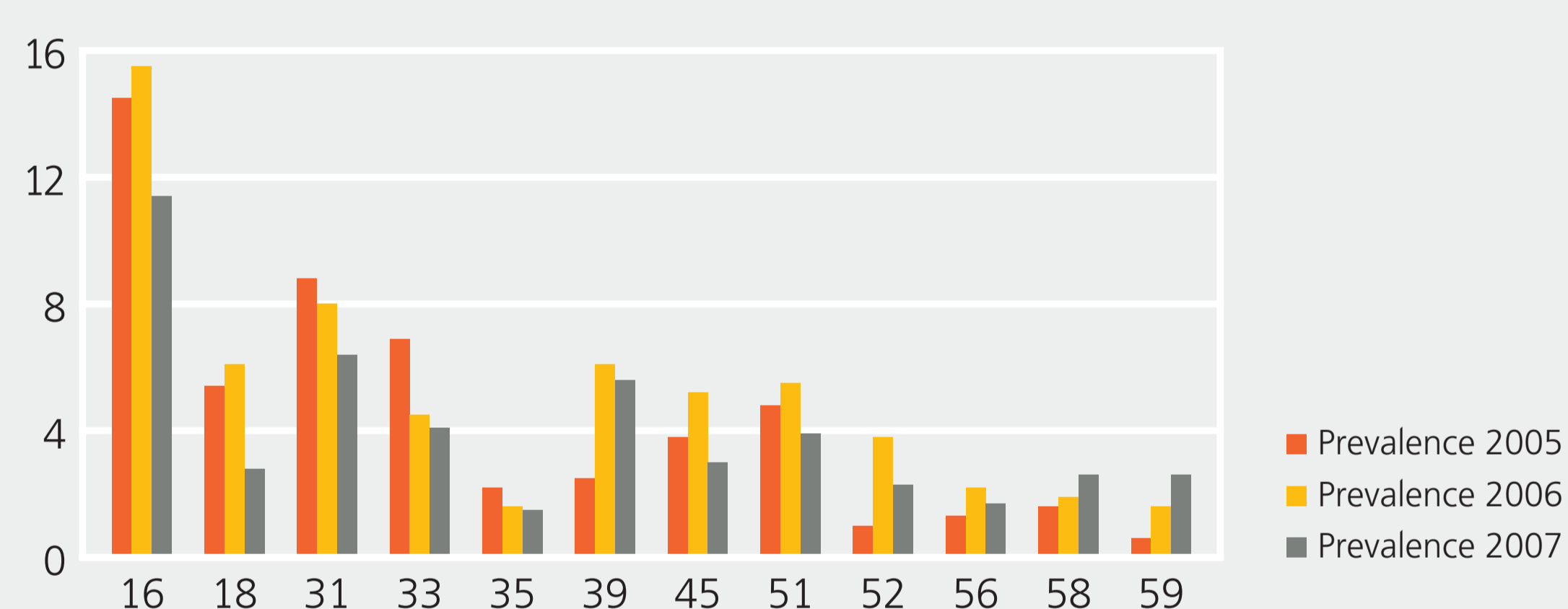
- HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68

Sensitivity

- HPV16, 18, 31, 33, 35, 39, 45, 51, 58, 59: 40 copies
- HPV56 400 copies
- HPV52, 68 4000 copies

Results

HVP type distribution, Telemark 2005-2007



Type distribution

- HPV16 was the most prevalent type (16%)
- HPV31 was the second most prevalent type (8.8%)
- Moderate prevalence (4–6%) of HPV18, 33, 39, 45 and 51
- HPV35, 52, 56, 58 and 59 are scarce types in Telemark (<2.5%)
- Overall prevalence of oncogenic HPV types was 41%

Time dependent prevalence

Year	N	Positive	%
2005	377	125	33
2006	758	308	42
2007	484	174	36

No temporal trend is apparent

Age-dependent prevalence

HPV prevalence declined with age.

Age group	N	Prevalence of oncogenic HPV
25–35	527	58%
35–45	484	41%
45–55	288	23%
55–65	134	17%

Limitations

- Repeat samples.
- 257 of 1470 samples (17%) are repeat samples from the same patient.
- Repeat samples are preferentially follow-up samples from HPV-positive patients. The prevalence HPV in such samples is expected to be higher than the baseline prevalence.
- This could lead to an maximum overestimate of baseline prevalence of 12% if all follow-up samples were positive.
- Sensitivity. Test sensitivity for HPV types 52, 56 and 68 is lower. The prevalence of these types may be underestimated.

Conclusions

- HPV prevalence in low-grade cytological samples from Telemark was 41%.
- HPV types 16 and 31 were the dominant types.
- Prevalence declined with age

Detection and typing of 13 oncogenic HPV types by consensus multiplex quantitative real-time PCR

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Summary

We describe a consensus multiplex realtime PCR test that can be completed in 8 hours with 2.5 hours hands-on time, which runs in a closed system and which provides sensitive detection and typing of oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 59 and 66.

Typing

Tests that provide typing have the following advantages.

- Distinguish between sequential transient (type-variable) and persistent (type-constant) infections. Only persistent infections progress to neoplasia.
- Distinguish between types with differing degrees of oncovirulence.
- Provide epidemiological information on type distribution and the population effects of vaccination.

Realtime PCR

Realtime PCR is preferable to conventional PCR with typing by post-PCR hybridisation for the following reasons:

- Closed system. Reduces need for stringent laboratory hygiene and use of aggressive chemicals for decontamination.
- Immediate readout of results.
- Quantitative data obtainable.

Quantitation

Quantitation has the following advantages.

- In clinical use, some studies report an association between high viral load and progression to high-grade dysplasia.
- In epidemiological use, type-specific prevalence studies may be biased by type-specific differences in sensitivity. This bias may be eliminated by using a constant-level cutoff in a quantitative test.

Characteristics of the test

Target

L1 gene segment corresponding to 6321–6579 on HPV16. Primer target region is conserved. Intervening region is variable. Amplicon is 258 bp.

Primers:

One consensus forward primer.

Four group-optimised consensus reverse primers.

Variable positions mixed nucleotides or inosine wildcard.

Probes

Multiplex 5' endonuclease (TaqMan) probes labelled with FAM, VIC, NED and ROX.

Format

Four parallel multiplex realtime PCR reactions:

- **Group 1.** HPV18, HPV52, HPV59. Fluorophors: FAM, ROX, NED.
- **Group 2.** HPV39, HPV51, HPV56, HPV66. Fluorophors: ROX, FAM, VIC, NED.
- **Group 3.** HPV16, HPV45, HPV58. Fluorophors: FAM, VIC, NED.
- **Group 4.** HPV31, HPV33, HPV35, HPV6 internal control. Fluorophors: VIC, ROX, FAM, NED.

Internal Control

4000 copies of HPV6 control are added to Group 4 reactions.

Method

Realtime PCR. 45 cycles. Applied Biosystems AB7000/AB7300

Test Performance

Analytical sensitivity

HPV type	Analytical sensitivity (copies) (500 ng human DNA)	Analytical sensitivity (copies) (50 ng human DNA)
HPV16	40	4
HPV18	40	4
HPV31	4	4
HPV33	400	40
HPV35	400	40
HPV39	40	4
HPV45	400	4
HPV51	4	4
HPV52	400	40
HPV56	40	4
HPV58	400	40
HPV59	40	4
HPV66	400	40

Analytical sensitivity tests were performed on serial dilutions of HPV DNA in human DNA using the multiplex formulations. Analytical sensitivity was 4–400 copies. Lowering the concentration of human DNA increased the sensitivity. 500 ng of human DNA corresponds to 7.5×10^4 cells.

Clinical Samples. Comparison with Hybrid Capture

Comparison of consensus multiplex realtime PCR with Hybrid Capture for 37 LSIL and ASCUS samples.

Hybrid Capture		Consensus realtime	
		+	–
+	+	9	1
	–	4	23

Details for positive samples are given below

Sample	Result HCII	Result Consensus Multiplex
1	+	HPV16
4	+	HPV16, 35, 52
6	+	HPV31, 52
8	+	HPV51, 56
9	+	HPV51
12	–	HPV51, 66
18	+	HPV39
20	–	HPV51
21	–	HPV16, 45
25	+	–
30	±	HPV45
32	+	HPV16
34	–	HPV31
35	+	HPV51, 52

As expected from the analytical sensitivity the consensus multiplex test detects more positive samples than the hybrid capture test. One sample is false negative relative to hybrid capture. Sequencing will be performed in order to determine the type.

Logistics

Using the MagNAPure automatic DNA extractor and ABI Prism 7300 realtime PCR instrument the test can be completed from sample preparation to result analysis in 8 hours of which 2.5 hours is hands-on time. The most time-consuming step is sample preparations (4.5 hours). 20 samples can be analysed in 96 well plates. PCR throughput could be increased by use of 384-well PCR plates.

Quantitation

This test can be run quantitatively by running parallel dilutions of the target in order to give standard curves.

For quantitation it would be advantageous to type first, then quantify to avoid running excessive numbers of standards.

The difference in analytical sensitivity when the amount of human DNA is changed from 50 to 500ng implies that levels of human DNA should be standardised to achieve accurate quantitation.

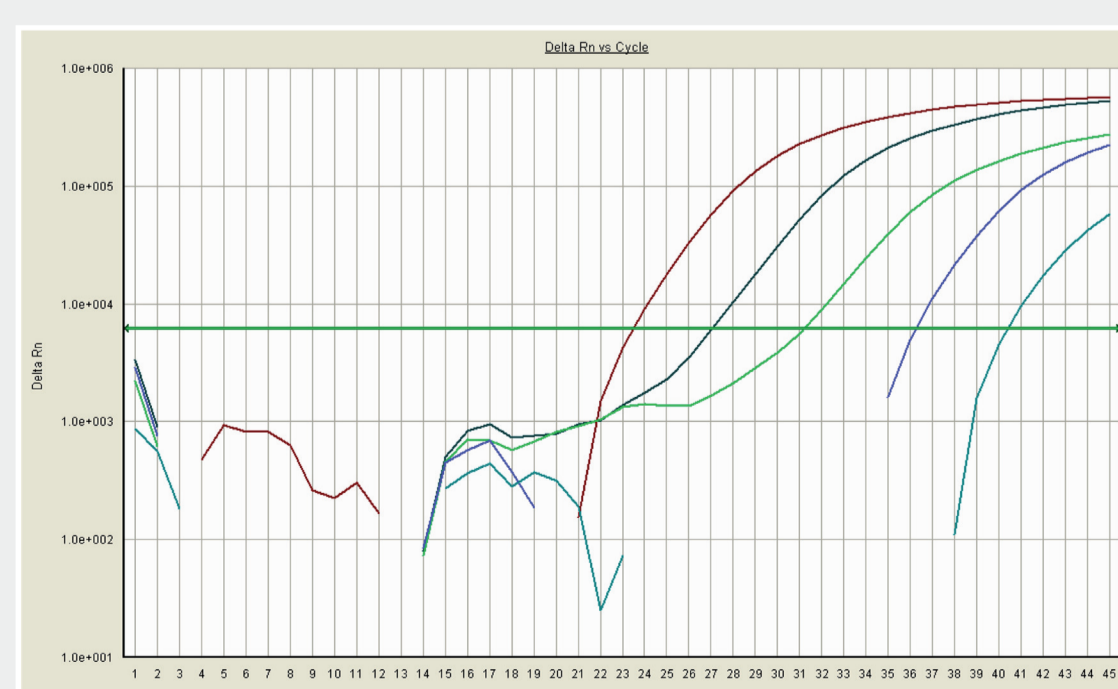
Conclusions

- Consensus multiplex realtime PCR for HPV typing of all oncogenic types has been achieved.
- The test is sensitive and all stages can be completed in a working day
- The test is logistically suitable to medium throughput laboratories. Higher throughput would require high throughput DNA extraction and 384 well PCR instruments.
- Sensitivity is affected by the presence of human DNA. For quantitative experiments the amount of human DNA will need to be standardised.

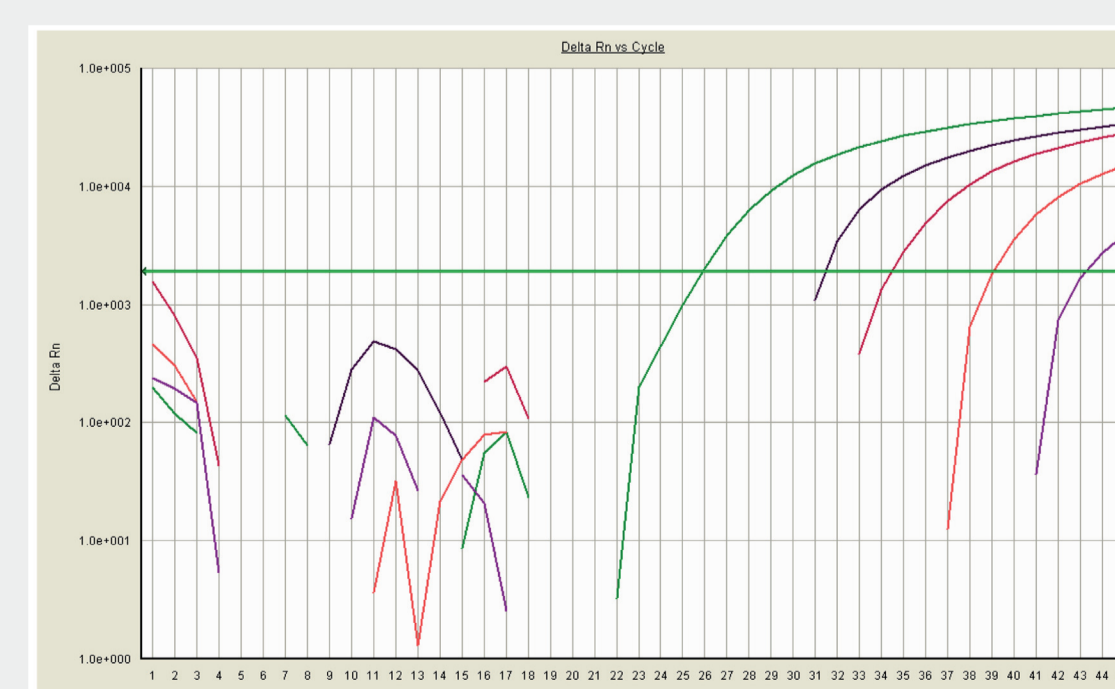
Future Work

Future work will concentrate on:

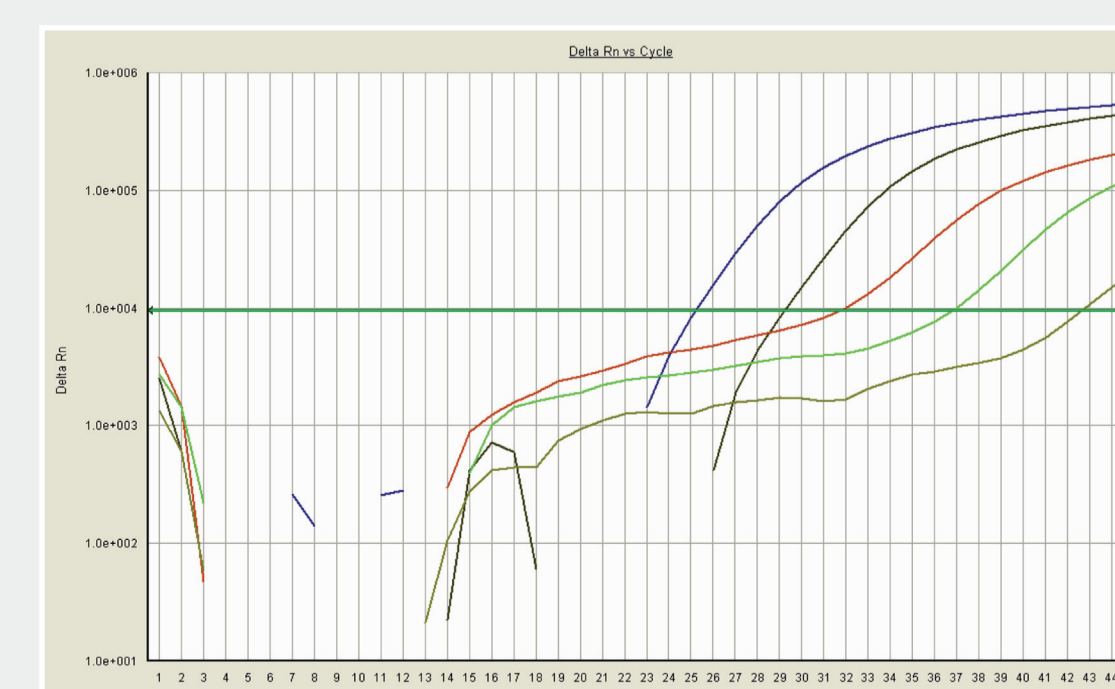
- Improving the robustness of the test and eliminating residual cross-reactions by improving some of the probes.
- Testing the reaction on alternative PCR platforms
- Evaluating the test on clinical material.



Amplification of HPV31 in multiplex PCR. Dilution series from 4×10^5 to 40 copies.



Amplification of HPV33 in multiplex PCR. Dilution series from 4×10^5 to 40 copies.



Amplification of HPV35 in multiplex PCR. Dilution series from 4×10^5 to 40 copies.