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Clinical performance of the HPV-Risk assay on cervical samples in SurePath medium using the VALGENT-4 panel

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ABSTRACT

Background: The VALidation of HPV GENoyping Tests (VALGENT) framework is designed for comparison and clinical validation of HPV assays.
Objectives: To evaluate the accuracy of the HPV-Risk assay within VALGENT-4, relative to clinically validated comparator HPV tests.
Study design: The VALGENT-4 panel comprises consecutive SurePath cervical samples from routine screening (n = 998), of which 51 had abnormal cytology and 13 women had cervical intraepithelial neoplasia (CIN) grade 2 or worse (CIN2+), enriched with SurePath cervical samples from 297 women with abnormal cytology and 109 CIN2+. HPV-Risk assay was performed on DNA extracted panel samples (n = 1,295), blinded to clinical data, cytology results, and results from other HPV assays evaluated in VALGENT-4. All assay results were reported to the central VALGENT coordination institute for data and statistical analysis. HPV prevalence was analysed and accuracy for detection of CIN grade 3 or worse (CIN3+) and CIN2+ were assessed relative to GP5+/6+-PCR-EIA and GP5+/6+-PCR-EIA-LMNX.
Results: The sensitivity of the HPV-Risk assay for detection of CIN3+ and CIN2+ was similar to that of GP5+/6+-PCR-EIA (relative sensitivity for CIN3+ 1.01; 95%CI: 0.97-1.06; $p_{McN} = 1.000$, and for CIN2+ 1.01; 95%CI: 0.96-1.06; $p_{McN} = 1.000$) at significantly higher specificity (relative specificity 1.04; 95%CI: 1.02-1.06; $p_{McN} < 0.001$). The accuracy of the HPV-Risk assay for CIN3+ and CIN2+ was non-inferior compared to GP5+/6+-PCR- EIA and GP5+/6+-PCR-EIA-LMNX, with all p-values ≤ 0.002. HPV16/18 genotype agreement between HPV-Risk assay and GP5+/6+-PCR-LMNX was high.
Conclusions: The HPV-Risk assay demonstrated non-inferiority to clinically validated comparator assays on cervical samples in SurePath medium using the VALGENT-4 panel, and is therefore suitable for cervical cancer screening.

1. Background

Human papillomavirus (HPV) testing is increasingly incorporated in clinical protocols and cervical screening guidelines given superior clinical performance over cytology [1,2]. However, careful clinical validation of an HPV assay on cervical screening samples is required before use in cervical screening to ensure an optimal distinction between HPV infections associated with cervical intraepithelial neoplasia (CIN) grade 2 and 3 or worse (CIN2+/3+), and clinically irrelevant, transient HPV infections [3–5]. To support clinical validation and comparison of HPV assays, the VALidation of HPV GENotyping Tests (VALGENT) framework was designed. The VALGENT validation panels

Abbreviations: CIN2+, cervical intraepithelial neoplasia grade 2 or worse; CIN3+, cervical intraepithelial neoplasia grade 3 or worse; CI, confidence interval; CT, threshold cycle; EIA, enzyme immunoassay; HPV, human papillomavirus; hr, high-risk; LMNX, luminex; McN, McNemar; ni, non-inferiority; VALGENT, VALidation of HPV GENoyping Tests

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take into account different sample collection media and include samples from women attending routine screening enriched with cytological abnormal samples [6,7]. In order to allow comparison with other HPV tests, each VALGENT panel includes a comparator assay that is clinically validated for cervical screening purpose. The fourth instalment of the VALGENT framework, VALGENT-4, specifically comprises a panel of samples collected in SurePath preservative fluid (BD SurePath™, Becton, Dickinson and Company) as of to date most clinical validation of HPV assays for use in screening had been undertaken on ThinPrep collected samples. The objective of VALGENT-4 is to evaluate HPV assays using two comparator assays based on PCR amplification with GP5+/6+ primers [6], with enzyme immunoassay (EIA) for pooled detection and a luminex-based assay (LMNX) for individual genotyping of the 14 targeted high-risk (hr) HPV types.

Here, we report on the clinical performance of the HPV-Risk assay within the VALGENT-4 panel, relative to hrHPV GP5+/6+-PCR-EIA and GP5+/6+-PCR-LMNX. The HPV-Risk assay is a real-time PCR-based assay that targets the E7 region of 15 HPV types. The assay provides partial genotype information with individual reporting of HPV16 and HPV18 and a pooled detection of 13 other HPV types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 67 and 68) [8]. The assay has previously been shown to be non-inferior on a validation panel of ThinPrep collected cervical samples against clinically validated HC2 and hrHPV GP5+/6+-PCR-EIA [8,9]. Moreover, the HPV-Risk assay meets the cross-sectional accuracy and reproducibility criteria of the international guidelines for HPV test requirements [8]. Previous evaluations of the HPV-Risk assay on SurePath collected samples demonstrated compatibility of the assay with this sample type [8], but formal clinical validation remained to be done.

2. Objectives

To evaluate the accuracy of the HPV-Risk assay within VALGENT-4, relative to clinically validated comparator HPV tests.

3. Study design

3.1. VALGENT-4 panel

The VALGENT-4 protocol has been described in detail before [6]. In short, the panel is standardised, comprising consecutive SurePath cervical samples from 998 women aged 30 – 59 years participating in the organized national cervical cancer screening program of Denmark (screening population) of which 51 samples had abnormal cytology and 13 women diagnosed with CIN2+. In addition, SurePath collected cervical samples from 297 women aged 30 – 59 years with abnormal cytology and 109 CIN2+ were included in the panel (enrichment population). DNA extracted panel samples (n = 1,295) were used in this study and shipped from the parent laboratory in Copenhagen to the testing laboratory in Amsterdam to perform HPV-Risk assay. The clinically validated hrHPV GP5+/6+-PCR with EIA for pooled detection of 14 targeted hrHPV types was used as comparator assay for clinical performance in VALGENT-4. In addition, LMNX readout for individual genotyping of the 14 targeted HPV types [10] was used. GP5+/6+-PCR products were subjected to LMNX irrespective of EIA assay results. For LMNX, an internal probe control for a human DNA was added to verify the quality of a sample. Samples negative for both HPV and the internal control (threshold MFI of 50) were scored as inadequate. GP5+/6+-PCR-EIA and GP5+/6+-PCR-LMNX were performed at DDL Diagnostic Laboratory (Rijswijk, The Netherlands).

3.2. HPV-Risk assay

The HPV-Risk assay (Self-screen BV, Amsterdam, The Netherlands) was performed on the DNA samples essentially as described before using 5µl of input DNA, equalling 1/200 of original SurePath sample [8,9]. Samples were considered HPV positive when threshold cycle (*CT*) values for HPV16, HPV18, and/or other HPV types were $\leq$ 36. If no HPV signals were obtained and the *CT* value for the *β-globin* target was $\leq$ 33, samples were considered HPV negative. Samples were considered invalid when the *CT* value for HPV was > 36 and that for *β-globin* > 33.

All HPV testing was performed blinded to the clinical data, cytology results and results from other HPV assays evaluated in VALGENT-4. Testing results were sent to the Unit of Cancer Epidemiology, Scientific Institute of Public Health (Brussels), where data were compiled and statistical calculations were conducted.

3.3. Data and statistical analysis

Based on the HPV-Risk assay results, the overall prevalence of hrHPV infection and type-specific prevalence of HPV16 and HPV18 infection were assessed. The level of genotype agreement between HPV-Risk assay and GP5+/6+-PCR-LMNX was determined by using kappa statistic. For this purpose, genotype results for GP5+/6+PCR products were categorized as (i) HPV16, (ii) HPV18, or (iii) other HPV types, including HPV31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and/or -68.

For clinical validation, women with histologically confirmed CIN2, CIN3, or cervical cancer were classified as having high-grade disease (disease group). In January 2019, the Danish National Pathology Registry (PatoBank) was re-accessed and reviewed with regards to follow-up data for the VALGENT-4 study population. In total, 122 CIN2+ cases were registered within this period of 33 months (range 32-35 months) after sample collection. Women with previous screening round negative sample (3-5 years prior) and a current negative cytology outcome were classified as having no disease (control group). The accuracy of the HPV-Risk assay for detection of both CIN3+ and CIN2+ was assessed and compared to the hrHPV GP5+/6+-PCR-EIA as the first comparator assay for clinical performance. GP5+/6+-PCR-LMNX was used as second comparator assay. Sensitivity and specificity were calculated from cross-tabulation of test results with exact 95% confidence intervals (CIs). Relative sensitivities and relative specificities with 95% CIs were calculated. The McNemar (McN) test was applied to assess differences between matched proportions. A P_{McN}-value of > 0.05 indicated that the sensitivity or specificity of the HPV-Risk assay was not significantly different from that of the comparator assay. Non-inferiority (ni) of the HPV-Risk assay compared to the comparator assay was assessed according to the international validation criteria, using 0.90 and 0.98 as bench marks for relative sensitivity and specificity, respectively [3,11]. In order to demonstrate non-inferiority, the one-sided p_{ni}-values had to be < 0.05, which means that the 90% lower confidence interval bounds around the relative sensitivity and specificity had to be higher than the benchmarks.

4. Results

4.1. HPV-Risk assay results

A valid HPV-Risk assay result was obtained for 1,286 (99.3%) samples, comprising 990 samples from the screening population and 296 samples from the enrichment population. HPV-Risk assay results overall and stratified for the two populations are shown in Table 1. Of 990 women in the screening population, 114 (11.5%) women tested hrHPV-positive with the HPV-Risk assay. Prevalence of HPV16 and HPV18 was 2.1% (21/990) and 1.1% (11/990), respectively. Of 296 women in the enrichment population, 256 (86.5%) women tested hrHPV-positive. Prevalence of HPV16 and HPV18 was 23.3% (69/296) and 6.4% (19/296), respectively.

For comparison, 143/990 (14.4%) in the screening population and 252/296 (85.1%) in the enrichment population tested hrHPV-positive by GP5+/6+-PCR-EIA. Prevalence of HPV16 and HPV18 as determined by LMNX was 3.1% (31/985) and 2.8% (28/985),

Table 1
HPV-Risk assay and GP5+/6+-PCR-EIA/-LMNX results within the two study populations of the VALGENT-4 panel, i.e., screening population and enrichment population.

	Screening population		Enrichment population		Total	
	n	%	n	%	n	%
HPV-Risk assay						
hrHPV-positive	114	11.5%	256	86.5%	370	28.8%
HPV16-positive	21	2.1%	69	23.3%	90	7.0%
HPV18-positive	11	1.1%	19	6.4%	30	2.3%
hrHPV-negative	876	88.5%	40	13.5%	916	71.2%
GP5+/6+-PCR-EIA						
hrHPV-positive	143	14.4%	252	85.1%	395	30.7%
HPV16-positive[#]	31	3.1%	71	24.0%	102	7.9%
HPV18-positive[#]	28	2.8%	22	7.4%	50	3.9%
hrHPV-negative	847	85.6%	44	14.9%	891	69.3%
Total	990	100.0%	296	100.0%	1,286[*]	100.0%

HPV = Human Papillomavirus; hrHPV: high-risk HPV; n = number of cases.
[*] A total of 9 samples dropped for the analysis (including one CIN2+) given invalid results in HPV-Risk assay.
[#] As determined by LMNX.

respectively, in the screening population, and 24.0% (71/296) and 7.4% (22/296), respectively, in the enrichment population. Genotyping agreement (i.e., HPV16, -18, other) between HPV-Risk assay and GP5+/6+-PCR-LMNX in the screening population was 99.0% (95% CI: 98.1–99.5%) with kappa value of 0.80 (95% CI: 0.74–0.86) for HPV16, 98.3% (95% CI: 97.3–99.0%) with kappa value of 0.56 (95% CI: 0.50–0.61) for HPV18, and 94.8% (95% CI: 93.1–96.0%) with kappa value of 0.72 (95% CI: 0.65–0.78) for other types. In the enrichment population, these figures were 98.7% (95% CI: 96.6–99.6%) with kappa value of 0.96 (95% CI: 0.85–1.08), 99.0% (95% CI: 97.1–99.8%) with kappa value of 0.92 (95% CI: 0.81–1.04), and 90.9% (95% CI: 87.0–93.9%) with kappa value of 0.81 (95% CI: 0.69–0.92), respectively.

4.2. Clinical accuracy of the HPV-Risk assay

Absolute sensitivity and specificity of the HPV-Risk assay for detection of CIN3+ and CIN2+ are shown in Table 2. Sensitivity for detection of CIN3+ was 95.2% (95% CI: 88.1–98.7%) and specificity 89.2% (95% CI: 87.0–91.1%). For detection of CIN2+, sensitivity and specificity were 93.4% (95% CI: 87.4–97.1%) and 92.6% (95% CI: 90.7–94.2%), respectively.

Absolute sensitivity and specificity of GP5+/6+-PCR-EIA and GP5+/6+-PCR-LMNX for detection of CIN3+ and CIN2+ are also shown in Table 2. Cross tabulations of HPV-Risk assay and GP5+/6+-PCR-EIA results or GP5+/6+-PCR-LMNX results stratified by clinical outcome are shown in Table 3. Corresponding relative sensitivities for CIN3+ and CIN2+ as well as relative specificities for ≤CIN1 of the HPV-Risk assay versus GP5+/6+-PCR-EIA or GP5+/6+-PCR-LMNX are shown in Table 4.

The sensitivity of the HPV-Risk assay for both CIN3+ and CIN2+ were similar to that of GP5+/6+-PCR-EIA (both $p_{McN} = 1.000$), at significantly higher specificity ($p_{McN} < 0.001$). The accuracy of the HPV-Risk assay was clinically non-inferior to GP5+/6+-PCR-EIA with respect to sensitivity and specificity for CIN3+ and CIN2+, with all p-values ≤ 0.002.

The sensitivity of the HPV-Risk assay for both CIN3+ and CIN2+ were also similar to that of GP5+/6+-PCR-LMNX (both $p_{McN} = 1.000$), at significantly higher specificity ($p_{McN} < 0.001$). The accuracy of the HPV-Risk assay was clinically non-inferior to GP5+/6+-PCR-LMNX

Table 3
Comparison between HPV-Risk assay and GP5+/6+-PCR-EIA or GP5+/6+-PCR-LMNX for hrHPV detection stratified by clinical outcome.

		GP5+/6+-PCR-EIA			GP5+/6+-PCR-LMNX		
	HPV-Risk assay	Positive	Negative	Total	Positive	Negative	Total
CIN3+							
	Positive	77	2	79	77	2	79
	Negative	1	3	4	1	3	4
	Total	78	5	83	78	5	83
CIN2+							
	Positive	109	4	113	109	4	113
	Negative	3	5	8	3	5	8
	Total	112	9	121	112	9	121
≤CIN1[*]							
	Positive	56	10	66	56	9	65
	Negative	41	783	824	56	764	820
	Total	97	793	890	112	773	885[#]

HPV = Human Papillomavirus; CIN3+(2+) = cervical intraepithelial neoplasia grade 3 or worse (2 or worse).
[*] including two consecutive negative cytology results (control group).
[#] 5 samples tested inadequate with LMNX among samples with valid HPV-Risk assay result.

Table 2
Sensitivity and specificity of HPV-Risk assay, GP5+/6+-PCR-EIA and GP5+/6+-PCR-LMNX for detection of CIN3+ and CIN2+.

	Sensitivity									Specificity								
	n	/	N	%		95% CI				n	/	N	%		95% CI			
HPV-Risk assay																		
CIN3+	79	/	83	95.2%	(	88.1	–	98.7	%)	828	/	928	89.2%	(	87.0	–	91.1	%)
CIN2+	113	/	121	93.4%	(	87.4	–	97.1	%)	824	/	890	92.6%	(	90.7	–	94.2	%)
GP5+/6+-PCR-EIA																		
CIN3+	78	/	83	94.0%	(	86.5	–	98.0	%)	797	/	928	85.9%	(	83.5	–	88.1	%)
CIN2+	112	/	121	92.6%	(	86.3	–	96.5	%)	793	/	890	89.1%	(	86.9	–	91.1	%)
GP5+/6+-PCR-LMNX																		
CIN3+	78	/	83	94.0%	(	86.5	–	98.0	%)	777	/	923[#]	84.2%	(	81.7	–	86.5	%)
CIN2+	112	/	121	92.6%	(	86.3	–	96.5	%)	773	/	885[#]	87.3%	(	85.0	–	89.5	%)

Table 4
Relative sensitivity for CIN3+ and CIN2+ and relative specificity for ≤CIN1 of HPV-Risk assay versus GP5+/6+-PCR-EIA and GP5+/6+-PCR-LMNX in the total study population.

		Relative sensitivity (95% CI)	Relative specificity (95% CI)	McNemar P (P_{McN})	Non-inferiority P (P_{ni})
GP5+/6+-PCR-EIA					
	CIN3+	1.01 (0.97-1.06)		1.000	0.0016
	CIN2+	1.01 (0.96-1.06)		1.000	0.0006
	≤CIN1*		1.04 (1.02-1.06)	< 0.001	< 0.001
GP5+/6+-PCR-LMNX					
	CIN3+	1.01 (0.97-1.06)		1.000	0.0018
	CIN2+	1.00 (0.95-1.05)		1.000	0.0017
	≤CIN1*		1.06 (1.04-1.08)	< 0.001	< 0.001

CIN3+(2+) = cervical intraepithelial neoplasia (CIN) grade 3 or worse (2 or worse); CI = confidence interval.
* Including two consecutive negative cytology results (control group).

with respect to sensitivity and specificity for CIN3+ and CIN2+, with all p-values $\leq$ 0.002.

5. Discussion

In this study, we evaluated the clinical performance of the HPV-Risk assay for detection of high-grade CIN (CIN3+/CIN2+) and compared its accuracy to GP5+/6+-PCR-based clinically validated comparator HPV tests using the VALGENT-4 panel comprising cervical samples collected in SurePath medium. This study is part of the VALGENT-4 reporting and the first large assessment of HPV-Risk assay performance on SurePath collected cervical screening samples. The results show that the sensitivity of the HPV-Risk assay for detection of CIN3+ and CIN2+ was comparable to that of both comparator assays based on GP5+/6+ primers, at significantly higher specificity on SurePath screening samples. The current study adds important data on the clinical value of the HPV-Risk assay on SurePath cervical screening samples in addition to previous reports on Thinprep cervical screening samples, self-collected (cervico-) vaginal specimens, urine and formalin-fixed paraffin-embedded tumour tissue specimens [8,9,12], Mes et al. submitted]. The study findings highlight assay performance consistency in different study cohorts and in different collection media used in cervical screening.

The strength of this study is the evaluation of the HPV-Risk assay in a completely blinded, formalized and uniform manner in a large sample series from the VALGENT-4 framework. This framework allows for subsequent test comparisons and data pooling in multiple testing meta-analysis [13]. Here, two comparator assays based on GP5+/6+ primers were used, but the HPV-Risk assay has previously also been shown to be clinically non-inferior to the other standard comparator test, i.e., HC2 [9]. Reproducibility of the HPV-Risk assay on cervical screening samples according to the guidelines for HPV DNA test requirements for primary cervical cancer screening has been demonstrated before [8].

In addition to the established EIA read out for the pooled detection of 14 targeted hrHPV types, the LMNX readout was used in this study. A substantial to near perfect genotype agreement between HPV-Risk assay and GP5+/6+-PCR-LMNX has been observed herein. LMNX had been compared earlier to EIA with high agreement for hrHPV detection (kappa = 0.969) and clinical sensitivities and specificities for CIN2+/3+ being non-inferior to that of EIA (all P < 0.001) [10]. Results of this study show that the sensitivity of the HPV-Risk assay for detection of CIN3+ and CIN2+ was also comparable to that of hrHPV GP5+/6+-PCR-LMNX at significantly higher specificity, on SurePath screening samples.

The relatively short follow-up period in VALGENT-4 of at maximum 3 years may be considered as a limitation to this study. However, since hrHPV GP5+/6+-PCR-EIA was validated through randomised trials with follow-up over 14 years, the cross-sectional accuracy of this comparator test is well acknowledged for validation studies. Future linkage of the VALGENT-4 allows the retrieval of longer follow up information from the Danish PatoBank, which may provide information on long term safety at a later point in time.

In conclusion, the HPV-Risk assay has a high sensitivity and specificity for detection of CIN3+ and CIN2+ in SurePath screening samples, and has demonstrated non-inferiority compared to the clinically validated hrHPV GP5+/6+-PCR-EIA as well as hrHPV GP5+/6+-PCR-LMNX in this sample type. Results from this study verify that the HPV-Risk assay can be applied in primary cervical cancer screening using SurePath collection medium.

Author contribution

Design of Protocol: JB, DE, LX, MA.
Panel testing and reporting: DH, WQ, ATH, SD, HP.
Statistical analysis: LX, MA.
Writing of manuscript: DH.
Editing of manuscript: All.
Decision to submit: All.

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Ethical and data inspection agency approvals

The study was approved by the Danish Data Inspection Agency No. AHH-2017-024, I-Suite: 05356. EU-GDPR compliant data handler agreement was established between the principal site Hvidovre Hospital and the Statistical analysis unit at Sciensano, Brussels. All collected samples were verified for non-compliance in the Danish human biological material in health research projects register (Vævsanvendelsesregisteret).

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manufacturers of HPV tests included in VALGENT. The Scientific Institute of Public Health did not receive any funding for the current evaluation of the HPV-Risk assay. DH is minority stakeholder of Self-screen B.V., a spin-off company of VU University Medical Center. Self-screen B.V. holds patents related to the work and has developed and manufactured the HPV-Risk assay, which is licensed to QIAGEN (QIAscreen HPV PCR Test®). DH serves occasionally on the scientific advisory board of Pfizer and Bristol-Meyer Squibb, and has been on the speakers' bureau of Qiagen. AH and SD are employed by Self-screen B.V. JB is the PI of studies funded in part by BD Diagnostics, Agena Bioscience, Genomica SAU, LifeRiver Biotech and QIAGEN. He has received honoraria for lectures from BD Diagnostics, Roche Molecular Systems, QIAGEN and Genomica SAU. JB is an appointed member of the National Danish Cervical Screening Committee by the Danish Health Authority, and member of the Regional cervical screening steering committee of the Capital Region of Denmark. DE and HP attended meetings with various HPV device and assay manufactures. WQ is shareholder of LBP.

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