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Using the VALGENT-3 framework to assess the clinical and analytical performance of the RIATOL qPCR HPV genotyping assay

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ABSTRACT

Background and objective: The VALGENT framework is developed to assess the clinical performance of HPV tests that offer genotyping capability. Samples from the VALGENT-3 panel are used to identify an optimal viral concentration threshold for the RIATOL qPCR HPV genotyping assay (RIATOL qPCR) to assure non-inferior accuracy to detect high-grade cervical intraepithelial neoplasia (CIN), compared to Qiagen Hybrid Capture 2 (HC2), a standard comparator test validated for cervical cancer screening.

Study design: The VALGENT-3 panel comprised 1300 samples from women participating in the Slovenian cervical cancer screening programme, enriched with 300 samples from women with abnormal cytology. In follow-up, 126 women were diagnosed with CIN2+ (defined as diseased) and 1167 women had two consecutive negative Pap smears (defined as non-diseased). All 1600 samples were analyzed with the RIATOL qPCR. Viral concentration was expressed as viral log10 of the number of copies/ml. A zone of viral concentration cut-offs was defined by relative ROC analysis where the sensitivity and specificity were not inferior to HC2.

Results: The RIATOL qPCR had a sensitivity and specificity for CIN2+ of 97.6% (CI: 93.2–99.5%) and 85.1% (CI: 82.9–87.1%), respectively, when the analytical cut off was used. At a cut off of 6.5, RIATOL qPCR had a sensitivity of 96.0% (CI: 91.0–98.7%) and a specificity of 89.5% (87.6–91.2%). At optimized cut off, accuracy of the qPCR was non-inferior to the HC2 with a relative sensitivity of 1.00 [CI: 0.95–1.05 (p = 0.006)] and relative specificity of 1.00 [CI: 0.98–1.01 (p = 0.0069)].

Conclusions: The RIATOL qPCR has a high sensitivity and specificity for the detection of CIN2 + . By using a fixed cut-off based on viral concentration, the test is non-inferior to HC2. HPV tests that provide viral concentration measurements or other quantifiable signals allow flexibility to optimize accuracy required for cervical cancer screening.

1. Background and objectives

Human papillomaviruses (HPV), common sexually transmitted viruses with more than 200 genotypes, are the principal cause of cervical cancer. Twelve high-risk HPV (hrHPV) genotypes (HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58 and HPV59) are recognised by the International Agency for Research on Cancer (IARC) as class I or IIa carcinogens [1].

Abbreviations: AGC, atypical glandular cells; ASC-H, atypical squamous cells, cannot exclude high-grade lesion (ASC-H); ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; EIA, GP5+/6+ PCR-based enzyme immunoassay; HC2, qiagen hybrid capture 2; HSIL, high-grade squamous intraepithelial lesion; hrHPV, high-risk human papillomavirus; LSIL, low-grade squamous intraepithelial lesions; NILM, negative for intraepithelial lesion or malignancy; RIATOL, qPCR RIATOL qPCR HPV genotyping assay; VALGENT, VALidation of HPV GENotyping tests

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Current cervical cancer screening recommendations are being revised towards primary HPV testing [2,3]. Many HPV assays, that are currently used in clinical practice identify hrHPV genotypes as a group with limited genotyping capacity (often with separate identification of HPV16 and HPV18 only). HPV full genotyping assays (separate identification of all hrHPV genotypes) can be important in risk-based management of screen-positive women, as marker for the detection of minimal residual disease after treatment for cervical lesions and to monitor vaccination effects.

Several HPV assays generate a signal which increases in strength with the amount of virus present in the sample and use this signal to generate a dichotomous result. However, the quantitative value of the signal can be used to adapt the optimal clinical threshold to assure satisfactory accuracy for detection of high-grade cervical intraepithelial neoplasia (CIN) lesions in a screening population. Exact quantitative values are rarely used for reporting HPV results since their clinical/prognostic value is still a matter of ongoing debate [4–9].

Given the multitude of commercially available HPV tests, validated assays which assure high-quality screening needs to be identified. A collaborative framework VALidation of HPV GENotyping Tests (VALGENT) [10] allows verification of minimal criteria that needs to be fulfilled for the use of any HPV assays in primary cervical cancer screening. In clinical practice, only clinically validated assays should be used and continuous monitoring of test performance is necessary to assure optimal safety of HPV-based screening programs [1,11,12].

The RIATOL qPCR HPV genotyping assay is a laboratory developed test (RIATOL qPCR, Antwerp, Belgium) [13] which has been routinely used in Algemeen Medisch Laboratorium (AML, Sonic healthcare, Antwerp, Belgium) for more than 12 years. It has been accredited (ISO15189) and validated according to the international criteria for HPV DNA tests to be used in primary cervical cancer screening settings [11,14]. Besides a qualitative result for 14 hrHPV genotypes (class I and IIA carcinogenic HPV genotypes plus HPV66 and HPV68), the assay also quantifies viral concentration (VC) of each targeted HPV genotype. In this study, we assessed the clinical performance of RIATOL qPCR through the third instalment of VALGENT project (VALGENT-3) and to identify the optimal clinical VC cut-offs to assure that the test fulfils the required accuracy performance criteria for primary cervical cancer screening.

2. Study design

2.1. Sample collection

The collection of specimens used for the present iteration of VALGENT-3 was performed in Slovenia, as previously reported [15–19]. In brief, from December 2009 to August 2010, 1300 consecutive cervical samples were collected from women aged 25–64 years who participated in the Slovenian national cervical cancer screening programme (screening population). Additionally, from January 2014 to May 2015, this collection was enriched with 300 cytological abnormal specimens (enrichment population), which included 100 women with atypical squamous cervical cells of undetermined significance (ASC-US), 100 women with low-grade squamous intraepithelial lesion (LSIL) and 100 women with high-grade squamous intraepithelial lesion (HSIL). Ethical approval was obtained from the Medical Ethics Committee of the Republic of Slovenia (consent numbers: 83/11/09 and 109/08/12).

All samples were stored in ThinPrep PreservCyt solution (Hologic, Marlborough, MA, USA) at −70 °C with aliquots disseminated in the Laboratory for Molecular Microbiology of the Faculty of Medicine, University of Ljubljana, Slovenia to participating laboratories for testing with different HPV assays.

In January 2016, samples of 1 ml of the original ThinPrep aliquot was sent on dry ice to AML, Department of Molecular Diagnostics, Antwerp, Belgium. Samples were handled with care to avoid contamination during storage, aliquoting, transfer to and reception at AML. Upon arrival at AML, samples were stored at −80 °C until further processing.

2.2. Cytology

Conventional cytology was performed in accordance with the standard routine gynaecological practice in Slovenia and in agreement with the European guidelines [20]. Results were classified according to the 2001 Bethesda system [21].

2.3. Hybrid capture 2 testing

Hybrid Capture 2 (HC2) testing was done according to the manufacturer's instructions. HC2 detects 13 hrHPV genotypes in aggregate ((class I and IIA carcinogenic HPV genotypes plus HPV68) and was used as the standard comparator test for the assessment of the clinical performance of the RIATOL qPCR.

2.4. RIATOL qPCR

The RIATOL qPCR is a fully automated, clinically validated laboratory developed HPV test [13,22]. Processing of the samples was performed in batches of 91 samples. After thawing, samples were vortexed rigorously and transferred manually to a 96 deep-well block. DNA was extracted from the cervical samples using the Cervista MTA system (Hologic, Bedford, MA, USA), in combination with the Genfind DNA extraction kit. Subsequently, the DNA was amplified using a series of real-time qPCR reactions on the LightCycler 480 type I (Roche Molecular Systems, Pleasanton, California, USA). The presence of 14 different hrHPV genotypes is determined using TaqMan based real-time PCR reactions targeting type specific) [13] sequences of viral genes (HPV16 E7, HPV18 E7, HPV31 E6, HPV33 E6, HPV35 E6, HPV39 E7, HPV45 E7, HPV51 E6, HPV52 E7, HPV56 E7, HPV58 E6, HPV59 E7, HPV66 E6, HPV68 E7). The PCR reactions are done in ultra-low volume (6 µl) and are performed in 8 multiplex reactions. Cellularity control is performed on every sample by amplification of the beta-globin gene.

Quantification of the amount of HPV in a sample was determined from type specific standard curves constructed from serial dilutions of known quantities of type specific synthetic gene constructs (g-block, Integrated DNA Technologies(IDT), Coralville, Iowa, USA). These results, expressed as the number of copies per µl extracted DNA were used to calculate the VC of type specific HPV copies per ml ThinPrep suspension using the following equation:

$$VC = C_{dna} \times V_{elu} / V_{inp}$$

Where:

VC = viral concentration (copies HPV per ml sample)
C_{dna} = copies HPV per µl DNA (copies/µl)
V_{elu} = elution volume DNA extraction (µl)
V_{inp} = input volume sample for DNA extraction (ml)

The lower level of detection (LOD) for the Riatol qPCR assay is 4 $\log_{10}$ copies/ml. Results were reported as hrHPV negative, hrHPV positive or inconclusive. Based on the beta-globin standard curve, DNA concentration (ng/µl) was determined in every sample. Samples with a DNA concentration below 0.12 ng/µl were considered as invalid and reported as inconclusive. This cut-off was chosen based on extensive analyses demonstrating that, below this cut-off, consistency is not guaranteed. An inconclusive result included no or insufficient material/cells for analysis. A sample was considered analytically HPV negative if none of the 14 hrHPV tests showed a positive signal and the beta-globin DNA concentration was above 0.12 ng/µl. HrHPV positivity was defined using two types of cut-off: 1) an analytical cut-off, which corresponded with the lowest threshold yielding a measurable signal, and 2) an optimised clinical cut-off, yielding the best compromise in clinical

accuracy, as explained below. For all positive samples, VCs were expressed as the $\log_{10}$(copies/ml). In samples with multiple hrHPV infections, only the concentration of the hrHPV genotype with the highest concentration was used for further analysis.

2.5. Clinical outcome and performance measurements

Follow-up and management of the patients with abnormal cytology result was done according to the Slovenian national guidelines [23], and the detailed algorithm has been described in previously published reports [15–19,24].

The histologically confirmed cervical intraepithelial neoplasia grade 2 or worse (CIN2+) were considered as the clinical disease outcome and used for the computation of clinical sensitivity. We considered Women with two consecutive cytological results of negative for intraepithelial lesion or malignancy (NILM) (at enrolment and subsequent screening 12–48 months later) were grouped as non-diseased and used for the computation of clinical specificity for ≤ CIN1.

2.6. Statistical analysis

The clinical sensitivity and specificity of RIATOL qPCR for CIN2+ and CIN3+ were computed and compared to HC2 using the non-inferiority score test [14,25], accepting 0.90 and 0.98 as benchmarks for relative sensitivity and relative specificity, respectively [26]. Statistically significant non-inferiority was accepted when the one-sided p value was < 0.05 [25]. All analyses were performed using STATA version 14 (College Station, TX, USA).

ROC curve analysis was performed to assess the trade-off between sensitivity and specificity as a function of the VC. Subsequently, the range of VC was identified where the clinical sensitivity and specificity was not inferior to that of the HC2. Statistically this translated to find the minimal and maximal VC, where the lower 90% confidence interval (CI) around the relative sensitivity exceeded 0.90 and the lower 90% CI around the relative specificity exceeded 0.98. Ninety percent CIs were used since this correspond approximately with 0.05 confidence level for one-sided non-inferiority testing [11].

3. Results

3.1. RIATOL qPCR HPV analytical genotyping prevalence

The characteristics of the VALGENT-3 population in terms of demographics, cytological and histological results has been reported previously [15–19,24]. Of the 1600 VALGENT-3 samples analysed with the RIATOL qPCR, 56 (3.5%) had a human DNA concentration below the cut-off of 0.12 ng/µl. These samples were considered as invalid and excluded from further analysis, although these group comprises one CIN2+ case. Of the 1544 remaining samples, 217 (17.4%) women in the screening population (N = 1249) and 80 (27.1%) women in the enrichment population (N = 295) tested positive for the presence of hrHPV. The overall and type-specific prevalence of 14 hrHPV genotypes stratified according to the baseline cytology is shown in Table 1. HPV was more prevalent in women with abnormal cytology compared to women with normal cytological results. When samples were tested with RIATOL qPCR considering the 13 hrHPV genotypes targeted by HC2, the prevalence of hrHPV in NILM, ASC-US, LSIL and HSIL were 13.9%, 45.3%, 72.7% and 84.5%, respectively.

3.2. Accuracy of the RIATOL qPCR using the analytical cut-off

The RIATOL qPCR and HC2 results stratified for the outcomes CIN2+, CIN3+ and ≤ CIN1 using the analytical cut-off are presented in Table 2. The clinical sensitivity of the RIATOL qPCR using the analytical cut-off was 97.6% (95%CI, 93.2–99.5%) and 100.0% (95%CI, 95.5–100%) for CIN2+ and CIN3+, respectively. When the analysis is

Table 1
Overall and type-specific prevalence of hrHPV genotypes in the total study population by baseline cytology result, using HC2 and RIATOL qPCR (with analytical cut-off).

Assay and HPV genotypes	No (%) with virological result, by cytology category:			
	NILM (N = 1189)[a]	ASC-US (N = 128)	LSIL (N = 110)	HSIL+ (N = 113)
HC2				
hrHPV[b]	125 (10.5)	63 (49.2)	85 (77.3)	97 (85.8%)
RIATOL qPCR				
hrHPV (13 genotypes)[c]	165 (13.9)	58 (45.3)	80 (72.7)	95 (84.1)
hrHPV (14 genotypes)[d]	182 (15.33)	64 (50.0)	85 (77.3)	99 (87.6)
HPV 16	25 (2.1)	11 (8.6)	28 (25.5)	56 (49.6)
HPV 18	12 (1.0)	8 (6.3)	9 (8.2)	11 (9.7)
HPV 31	37 (3.1)	18 (14.1)	23 (20.9)	19 (16.8)
HPV 33	11 (0.9)	6 (4.7)	7 (6.4)	7 (6.2)
HPV 35	3 (0.3)	1 (0.8)	1 (0.9)	1 (0.9)
HPV 39	18 (1.5)	3 (2.3)	5 (4.6)	3 (2.7)
HPV 45	5 (0.4)	5 (3.9)	4 (3.6)	6 (5.3)
HPV 51	24 (2.0)	4 (3.1)	9 (8.2)	6 (5.3)
HPV 52	18 (1.5)	9 (7.0)	8 (7.3)	7 (6.2)
HPV 56	11 (0.9)	6 (4.7)	11 (10.0)	6 (5.3)
HPV 58	11 (0.9)	3 (2.3)	11 (10.0)	8 (7.1)
HPV 59	21 (1.8)	3 (2.3)	4 (3.6)	2 (1.8)
HPV 66	21 (1.8)	11 (8.6)	9 (8.2)	6 (5.3)
HPV 68	26 (2.2)	7 (5.5)	9 (8.2)	4 (3.5)
hrHPV (14genotypes)[e]	128 (10.8)	55 (43.0)	81 (73.6)	96 (85.0)

[a] Cytological negative samples (NILM) are only from the screening population.

[b] Positive for at least one of 13 hrHPV genotypes (HPV16, HPV18, HPV31, HPV33, HPv35, HPV39, HPV45, HPV51, HPV52, HPV56, HPV58, HPV59, HPV68).

[c] Positive for at least one of the 13 hrHPV genotypes targeted by HC2 with the RIATOL qPCR.

[d] Positive for at least one of 14 hrHPV genotypes with the RIATOL qPCR (13 genotypes targeted by HC2 + HPV66).

[e] considering the optimised clinical cut-off; NILM, negative for intraepithelial lesion or malignancy; ASC-US, atypical squamous cells of undetermined significance; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion.

Table 2
Relative sensitivity for CIN2+ and CIN3+ and relative specificity for ≤ CIN1 of the RIATOL qPCR assay versus HC2 in the total study population (using the analytical cut-off).

Clinical outcome	HPV test	HC2+	HC2-	Total
CIN2+ (N = 126)	RIATOL qPCR+	120	3	123
	RIATOL qPCR-	1	2	3
	Total	121	5	126
	Relative sensitivity RT/HC2 for CIN2+: 1.02 (0.97–1.06), $p_{n.inf} = 0.0001$			
CIN3+ (N = 81)	RIATOL qPCR +	79	2	81
	RIATOL qPCR -	0	0	0
	Total	79	2	81
	Relative sensitivity RT/HC2 for CIN3+: 1.03 (0.99–1.06), $p_{n.inf} < 0.0001$			
≤CIN1 (N = 1167)	RIATOL qPCR+	100	74	174
	RIATOL qPCR-	20	973	993
	Total	120	1047	1167
	Relative specificity RT/HC2 for ≤ CIN1: 0.95 (0.92–0.98), $p_{n.inf} = 0.9998$			

*$p_{n.inf.} < 0.05$ means that the sensitivity or specificity of the RIATOL qPCR assay are not significantly lower than that of the HC2, accepting the benchmarks of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively.

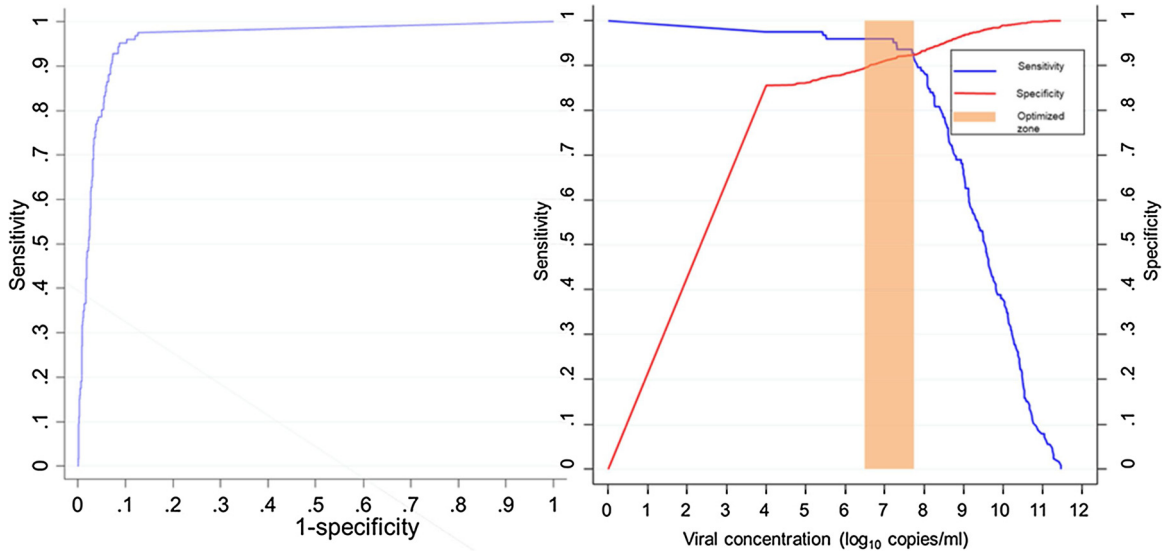

Fig. 1. Part a. ROC curve of the sensitivity as a function of the false-positivity rate (1-specificity) of the RIATOL qPCR to detect CIN2 + . Part b: Variation in sensitivity and specificity for CIN2+ as a function of the viral concentration expressed as $\log_{10}$ (HPV copies/ml of sample). In case of infection with multiple genotypes, the HPV type with the highest concentration was chosen. Within the orange bar (range: 6.493.–7.747), international accuracy requirements for cervical cancer screening tests are fulfilled.

restricted to women > 30 years, absolute sensitivities were similar (Supplementary Table 1). The specificity of the RIATOL qPCR for ≤ CIN1 was 85.1% (95%CI, 82.9–87.1%) in the total study population and slightly higher when analysis was restricted to women > 30 years, 87.5% (95%CI, 85.3–89.5%).

The relative clinical performance of the RIATOL qPCR compared to HC2 is presented in Table 2. When using the analytical cut-off, the RIATOL qPCR had a relative clinical sensitivity of 1.02 (95%CI, 0.97–1.06, $P_{n.inf} = 0.0001$) for CIN2+ and 1.03 (95%CI, 0.99–1.06, $P_{n.inf} < 0.0001$) for CIN3 + . The relative clinical specificity of the RIATOL qPCR assay for ≤ CIN1 was 0.95 (95%CI, 0.92 to 0.98, $P_{n.inf} = 0.9998$).

3.3. Clinical performance of the RIATOL qPCR with the optimized cut-off

Since non-inferiority was not reached for clinical specificity when using the analytical cut-off, an algorithm was developed to calculate a clinically relevant cut-off, which would result in non-inferior clinical accuracy when compared to HC2. A ROC curve of the sensitivity as a function of the false-positivity rate of the RIATOL qPCR to detect CIN2+ was constructed (Fig. 1a). Subsequently, the variation in sensitivity and specificity for CIN2+ was plotted as a function of the VC (Fig. 1b). Next, the range of VC was identified where the clinical sensitivity and specificity where not inferior to HC2 (Figs. 1b and 2).The range where the requirements of non-inferior accuracy of the RIATOL qPCR compared to HC2 are fulfilled was between 6.493 and 7.747. As optimized clinical cut-off, the VC in this range with the highest sensitivity is chosen for further analysis at 6.493.

Using the optimized cut-off, the absolute clinical sensitivity of RIATOL qPCR for CIN2+ and CIN3+ in the total study population was 96.0% (95%CI, 91.0–98.7%) and 98.0% (95%CI, 91.4–99.7%), respectively (Table 3), while the absolute clinical specificity for ≤ CIN1 was 90.0% (95CI, 87.6–91.2%). Comparable results are found when the analysis was limited to women aged 30 years or older (Supplementary Table 2).

The relative clinical sensitivity and specificity of the RIATOL qPCR compared to HC2 is presented in Table 3. When using the optimized clinical cut-off, the RIATOL qPCR had a relative sensitivity of 1.00 for CIN2+ (95%CI, 0.95–1.05) and 1.00 for CIN3+ (95%CI 0.95–1.05) with a $p_{n.inf}$ of 0.0006 (CIN2+) and 0.0045 (CIN3+), and therefore

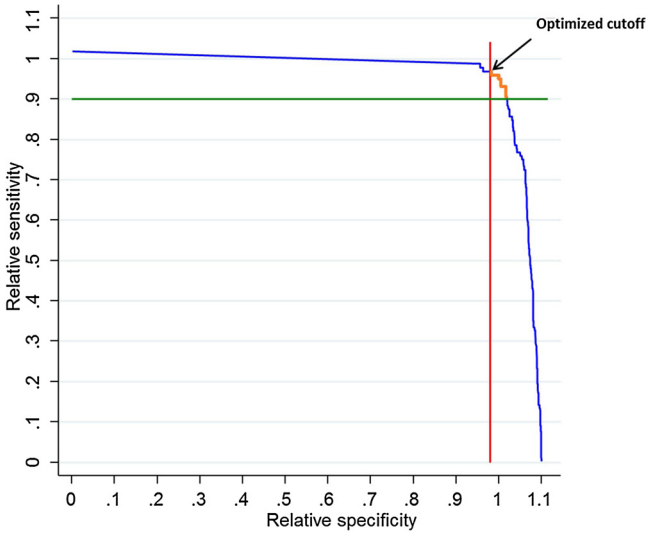

Fig. 2. Plot of the left 90% confidence interval bound around the relative sensitivity for CIN2+ against the relative specificity of the RIATOL qPCR vs HC2 corresponding to viral concentration cut-off points. The orange zone of the line (upper right corner) indicates the range where the requirement of non-inferior accuracy compared to HC2 is fulfilled (viral concentration expressed as $\log_{10}$[copies/ml]) ≥6.493 and < 7.747). Optimized cut-off = 6.493.

considered as non-inferior to HC2. The relative specificity of the RIATOL qPCR assay for ≤ CIN1 was 1.00 (95%CI, 0.98–1.01) and also non-inferior to HC2 ($p_{n.inf} = 0.0069$). Similar results were obtained when the analysis was restricted to women > 30 years (Supplementary Table 2) (Table 4).

4. Discussion

In the present study, the clinical performance of the RIATOL qPCR was compared to HC2 within the VALGENT-3 project. At the analytical cut-off, non-inferiority criteria for screening, as defined by Meijer et al. [26], was not reached for the clinical specificity. Therefore, a clinically relevant viral concentration cut-off were analysed post-hoc, balancing both sensitivity and specificity to meet the defined criteria. A zone of

Table 3
Relative clinical sensitivity for CIN2+ and CIN3+ and relative clinical specificity for ≤ CIN1 of the RIATOL qPCR versus HC2 in the total population (using the optimized clinical cut-off of 6.493 log_{10} copies/ml).

Clinical outcome	HPV test	HC2 +	HC2 -	Total
CIN2+ (N = 126)	RIATOL qPCR+	118	3	121
	RIATOL qPCR -	3	2	5
	Total	121	5	126
	Relative sensitivity RT/HC2 for CIN2+: 1.00 (0.95–1.05), $p_{n.inf}$ = 0.0006			
CIN3+ (N = 81)	RIATOL qPCR+	77	2	79
	RIATOL qPCR -	2	0	2
	Total	79	2	81
	Relative sensitivity RT/HC2 for CIN3+: 1.00 (0.95–1.05), $p_{n.inf}$ = 0.0045			
≤CIN1 (N = 1167)	RIATOL qPCR +	94	28	122
	RIATOL qPCR-	26	1019	1045
	Total	120	1047	1167
	Relative specificity RT/HC2 for ≤ CIN1: 1.00 (0.98–1.01), $p_{n.inf}$ = 0.0069			

*$p_{n.inf.}$ < 0.05 means that the sensitivity or specificity of the RIATOL q-PCR assay are not significantly lower than that of the HC2, accepting the benchmarks of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively.

VCs was identified by relative ROC curve analysis, where the accuracy of the RIATOL qPCR was non-inferior to HC2. Within the defined range, the minimum cut-off value was chosen as threshold. This calculated threshold for screening purposes yields the highest sensitivity with the specificity that still fulfils the proposed criteria.

In cases where multiple HPV infections were present, VC of the hrHPV type with the highest concentration was used for further analyses. When the analysis was performed with the cumulative hrHPV concentration, defined as the logarithm of the sum of the genotype-specific concentrations of all present hrHPV genotypes, comparable results were found (data not shown). Little is known about the influence of an individual HPV genotype in the presence of multiple genotypes on the carcinogenicity [27], therefore further research is necessary to understand the complexity of multiple HPV infections. Since we reported the VC for each HPV genotype separately, for the convenience of the clinician a cut-off per genotype will facilitate the interpretation of the results. For this reason, we choose to calculate the cut-off based on the highest concentration. We are aware of this possible limitation.

Since HPV genotyping information of the biopsies was not available, it was impossible to determine a genotype-specific cut-off. In addition, the cohort used in this study was too small to get reliable and statistically significant results per each targeted hrHPV genotype. However, the authors agree that this would be the ideal situation to have a clinical cut off value per HPV genotype. More methodological research is needed to find feasible clinical and or analytical validation concepts for HPV genotyping tests at the type-specific level.

Riatol qPCR has been clinically validated based on a fixed Ct value (Ct ≤34.00) [22]. This is the first study to calculate an optimized clinical cut-off defined in terms of VC within the VALGENT-3 study. Although there is an international WHO standard available for HPV 16 and 18, comparison of viral load measurements with other quantitative assays is difficult. Consensus quantification strategies for calculation of the amount of HPV particles present in a liquid based cytology sample have not yet been reached and currently different calculation options are used.

The presence of a large sample set and well documented follow-up database, makes VALGENT study well suited for clinical validation of multiple HPV genotyping test at the same time. To insure correct interpretation of CT values, an additional QC standard must be included in the assay.

VALGENT contributes in defining the list of HPV tests, which fulfil the defined minimal requirements of HPV genotyping assays for use in primary cervical cancer screening.

Our study demonstrates that HPV tests, providing viral concentrations (or other quantifiable signals) allow flexibility to optimize the clinical accuracy required for primary cervical cancer screening. This technique is already applied in the paper of Viti et al. [18] where a modification of the EUROArray HPV cut-off for HPV 16 makes the test non inferior to HC2.

In the future, HPV assays that generate quantified outputs might have an advantage compared to assays only producing a qualitative output because of their adaptability for particular specimens (vaginal self-samples, urine samples, other non-cervical specimens) or specified clinical settings (vaccinated women, follow-up after treatment) or for certain surveillance or research purposes (HPV vaccination trials, epidemiological studies, post vaccination surveillance of HPV infections). Our team strongly supports the application of different cut-off values, predefined according to specific situations/needs, i.e. primary screening, follow-up, sample type, etc. Insights are based on historical routine data, suggesting to be superior versus exploiting a fixed cut-off (unpublished data). Future research is needed to confirm this.

In conclusion, by using the optimised cut-off based on viral concentration, the RIATOL qPCR test shows non-inferior sensitivity and specificity for CIN2+ compared to the HC2 and fulfils the international accuracy criteria for primary cervical cancer screening.

Declaration of Competing Interest

IB and JB are clinical pathologist working in the private laboratory AML. DVB is a molecular biologist working in the private laboratory AML. AML has received research support in the form of free kits, reduced prices or funding from Abbott, Hologic, Cepheid, Roche, Becton Dickinson, Seegene, Biomérieux, Rover Medical devices, Aprovix and My Sample. IB and JB have also received travel grants to attend symposia, conferences and meetings from Hologic and Abbott.

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CRediT authorship contribution statement

I. Benoy: Investigation, Writing - original draft. **L. Xu:** Data curation, Formal analysis, Writing - original draft. **D. Vanden Broeck:** Investigation, Writing - review & editing. **M. Poljak:** Supervision, Validation, Writing - review & editing. **A. Oštrbenk Valenčak:**

Table 4
Absolute sensitivity and specificity of hrHPV testing of the RIATOL qPCR with different cut-offs to detect CIN2+ and the relative accuracy compared to HC2.

Cut-off (log_{10} copies/ml)	Sensitivity RIATOL qPCR, %	Relative sensitivity of RIATOL qPCR/ HC2 (90% CI)	$p_{n.inf}$	Specificity RIATOL qPCR, %	Relative specificity of RIATOL qPCR/ HC2 (90% CI)	$p_{n.inf}$
Analytical	97.6	1.02 (0.97–1.06)	0.0001	85.1	0.95 (0.92–0.98),	0.9998
6.493	96.0	1.00 (0.95–1.05)	0.0006	89.5	1.00 (0.98–1.01)	0.0069
7.747	91.3	0.95 (0.90–1.00)	0.0399	92.5	1.03 (1.01–1.06)	< 0.001

* $p_{n.inf.}$ < 0.05 means that the sensitivity or specificity of the RIATOL q-PCR assay are not significantly lower than that of the HC2, accepting the benchmarks of 0.90 and 0.98 for relative sensitivity and relative specificity, respectively.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.09.008.

References

[1] IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Biological agents: a review of human carcinogens, IARC Monogr. Eval. Carcinog. Risks Hum. 100B (2012) 1–475.
[2] M. Arbyn, G. Ronco, A. Anttila, et al., Evidence regarding HPV testing in secondary prevention of cervical cancer, Vaccine 30 (Suppl 5) (2012) F88–F99.
[3] G. Ronco, P. Giorgi-Rossi, F. Carozzi, et al., Efficacy of human papillomavirus testing for the detection of invasive cervical cancers and cervical intraepithelial neoplasia: a randomised controlled trial, Lancet Oncol. 11 (2010) 249–257.
[4] P.E. Castle, M.A. Schiffman, C.M. Wheeler, Hybrid capture 2 viral load and the 2-year cumulative risk of cervical intraepithelial neoplasia grade 3 or cancer, Am. J. Obstet. Gynecol. 191 (November) (2004) 1590–1597.
[5] E.L. Franco, F. Coutlee, Prognostic value of measuring load of human papillomavirus DNA in cervical samples: an elusive target, J. Natl. Cancer Inst. 101 (February (4)) (2009) 131–133.
[6] C. Constandinou-Williams, S.I. Collins, S. Roberts, L.S. Young, C.B. Woodman, P.G. Murray, Is human papillomavirus viral load a clinically useful predictive marker? A longitudinal study, Cancer Epidemiol. Biomarkers Prev. 19 (Mar) (2010) 832–837.
[7] L.F. Xi, J.P. Hughes, P.E. Castle, et al., Viral load in the natural history of human papillomavirus type 16 infection: a nested case-control study, J. Infect. Dis. 203 (May (15)) (2011) 1425–1433.
[8] X.L. Fu, M. Schiffman, Y. Ke, et al., Type-dependent association between risk of cervical intraepithelial neoplasia and viral load of oncogenic human papillomavirus types other than types 16 and 18, Int. J. Cancer 140 (April (15)) (2017) 1747–1756.
[9] C.E. Depuydt, A.M. Criel, I.H. Benoy, M. Arbyn, A.J. Vereecken, J.J. Bogers, Changes in type-specific human papillomavirus load predict progression to cervical cancer, J. Cell. Mol. Med. 16 (September (14)) (2012) 3096–3104.
[10] M. Arbyn, C. Depuydt, I. Benoy, et al., VALGENT: a protocol for clinical validation of human papillomavirus assays, J. Clin. Virol. 76 (Suppl 1) (2016) S14–S21.
[11] M. Arbyn, P.J. Snijders, C.J.L.M. Meijer, et al., Which high-risk HPV assays fulfil criteria for use in primary cervical cancer screening? Clin. Microbiol. Infect. 21 (April (30)) (2015) 817–826.
[12] L. von Karsa, M. Arbyn, H. De Vuyst, et al., European guidelines for quality assurance in cervical cancer screening. Summary of the supplements on HPV screening and vaccination, Papillomavirus Res. 1 (2015) 22–31.
[13] I.M. Micalessi, G.A. Boulet, J.J. Bogers, I.H. Benoy, C.E. Depuydt, High-throughput detection, genotyping and quantification of the human papillomavirus using real-time PCR, Clin. Chem. Lab. Med. 50 (2012) 655–661.
[14] C.J.L.M. Meijer, P.E. Castle, A.T. Hesselink, et al., Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older, Int. J. Cancer 124 (2009) 516–520.
[15] N.J. Polman, A. Ostrbenk, L. Xu, et al., Evaluation of the clinical performance of the HPV-Risk assay using the VALGENT-3 panel, J. Clin. Microbiol. 55 (2017) 3544–3551.
[16] L. Xu, A. Ostrbenk, M. Poljak, M. Arbyn, Assessment of the Roche linear Array HPV genotyping test within the VALGENT framework, J. Clin. Virol. 98 (2018) 37–42.
[17] L. Xu, E. Padalko, A. Ostrbenk, M. Poljak, M. Arbyn, Clinical evaluation of INNO-LiPA HPV genotyping EXTRA II assay using the VALGENT framework, Int. J. Mol. Sci. 19 (September (11)) (2018).
[18] J. Viti, M. Poljak, A. Ostrbenk, et al., Validation of EUROArray HPV test using the VALGENT framework, J. Clin. Virol. 108 (September (10)) (2018) 38–42.
[19] A. Ostrbenk, L. Xu, M. Arbyn, M. Poljak, Clinical and analytical evaluation of the Anyplex II HPV HR Detection assay within the VALGENT-3 framework, J. Clin. Microbiol. 56 (September (12)) (2018) e01176–18.
[20] M. Arbyn, A. Herbert, U. Schenck, et al., European guidelines for quality assurance in cervical cancer screening: recommendations for collecting samples for conventional and liquid-based cytology, Cytopathology 18 (2007) 133–139.
[21] D. Solomon, D. Davey, R. Kurman, et al., The 2001 Bethesda System: terminology for reporting results of cervical cytology, JAMA 287 (2002) 2114–2119.
[22] C.E. Depuydt, I.H. Benoy, J.F. Beert, A.M. Criel, J.J. Bogers, M. Arbyn, Clinical validation of a type-specific real time quantitative human papillomavirus PCR to the performance of Hybrid Capture 2 for the purpose of cervical cancer screening, J. Clin. Microbiol. 10 (50) (2012 Oct) 4073–4077.
[23] M.Ursic Vrscaj (Ed.). Society for gynecological oncology,colposcopy and cervical pathology,Slovenian guidelines for management of pathological cercical smears. http:, zora.onko-i.si/fileadmin/user upload/dokumenti/strokovna priporocila/2011 Smernice web.pdf, editors. 2011.
[24] M. Poljak, A. Ostrbenk, K. Seme, et al., Comparison of clinical and analytical performance of the abbott realtime high risk HPV test to the performance of hybrid capture 2 in population-based cervical cancer screening, J. Clin. Microbiol. 49 (May) (2011) 1721–1729.
[25] N.S. Tang, M.L. Tang, I.S. Chan, On tests of equivalence via non-unity relative risk for matched-pair design, Stat. Med. 22 (Apr (30)) (2003) 1217–1233.
[26] C.J. Meijer, J. Berkhof, P.E. Castle, et al., Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older, Int. J. Cancer 124 (February (1)) (2009) 516–520.
[27] R. Ghittoni, R. Accardi, S. Chiocca, M. Tommasino, Role of human papillomaviruses in carcinogenesis, Ecancermedicalscience 9 (2015) 526.