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ABSTRACT

Background: Robust clinical and analytical validation of human papillomavirus (HPV) tests is a pre-requisite for their use in cervical cancer screening given the transience of most high-risk HPV infections.

Objectives: To evaluate the EUROArray HPV test (PCR-based full HPV genotyping test) using the international validation of HPV Genotyping Test (VALGENT) framework, which offers an opportunity to determine analytical and clinical performance according to internationally accepted performance metrics.

Study design: A total of 1,300 consecutive and 300 abnormal cervical samples derived from the Slovenian screening programme were tested with the EUROArray HPV test. Clinical performance for the detection of cervical intraepithelial neoplasia grade 2 and above (CIN2+) was performed and compared to a standard comparator test (Hybrid Capture 2). Intra- and inter-laboratory reproducibility of the assay was performed in a subset of 500 samples.

Results: The relative clinical sensitivity and specificity of EUROArray HPV vs HC2 was 0.93 (95% Confidence Interval (CI), 0.88-0.99; P non-inferiority$_{(ni)}$= 0.1413) and 1.01 (95% CI, 0.99-1.02; P_{ni}= 0.0001), respectively. Application of an *a-posteriori* cut-off for HPV16 led to the relative values of 0.98 (95% CI, 0.92-1.03; P_{ni}= 0.0076) and 1.00 (95% CI, 0.97-1.03; P_{ni}= 0.007), respectively. The assay showed excellent intra- and inter-laboratory reproducibility (concordance $\geq$ 94%, Kappas $\geq$ 0.85).

Conclusion: At the predefined cut-off, EUROArray HPV was less sensitive than HC2 for the detection of CIN2+. However, when the optimised cut-off was applied, EUROArray HPV fulfilled international criteria for its use in cervical cancer screening.

Keywords: Cervical cancer, Human papillomavirus, VALGENT, EUROArray, Extended HPV genotyping test

BACKGROUND

The association of high-risk human papillomavirus (hrHPV) types with cervical high-grade lesions and cancer[1] has increased the use of HPV testing for cervical screening[2]. A number of HPV DNA tests detect viral nucleic acids with a variety of different read-outs[3] including those with genotyping capability. The introduction of genotyping assays in clinical practice may be useful to discriminate between hrHPV positive women at higher risk of cancer and has already been recommended as a triage of primary HPV infection in certain guidelines[4,5].

The clinical performance of any hrHPV test relies on its ability to detect infections associated with cervical intraepithelial neoplasia of grade 2 or worse (CIN2+). Meijer *et al* (2009) established guidelines and minimal requirements of novel HPV tests in terms of sensitivity, specificity and reproducibility relative to the clinical performance of two clinically validated tests, Hybrid Capture 2 (HC2–Qiagen, Gaithersburgh, MD) and GP5+/6+-PCR enzyme immunoassay (GP5+/6+-PCR EIA, Diassay, Rijswijk, The Netherlands)[6]. Although there are a multitude of HPV tests [3], relatively few have been validated according to these criteria. To address this, the VALGENT (VALidation of HPV GENotyping Tests) framework was created to support the clinical performance evaluation of HPV tests, including those with genotyping capabilities[7–13].

As part of the VALGENT-3 project, the performance of a novel HPV molecular test, the EUROArray HPV (EUROIMMUN, Lübeck, Germany), was assessed. The EUROArray HPV is a PCR based test with a probe-based microarray detection platform which is able to simultaneously detect E6 and E7 gene regions of 30 different anogenital HPV types: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68; 26, 53, 66, 70, 73, 82; 6, 11, 40, 42, 43, 44, 54, 61, 72, 81, 89. The first 13 genotypes (hrHPV types) of this list are causally associated with the development of cervical cancer [14], six are "possible" hrHPV types[15,16], whereas the remainder are low-risk HPVs or types of unknown carcinogenicity. Here, we present assessment of the performance for the hrHPV testing of the EUROArray HPV test relative to the

HC2 in terms of sensitivity and specificity for CIN2+ in addition to intra- and inter-laboratory reproducibility.

MATERIALS AND METHODS

Study population

The VALGENT-3 sample panel was collated in Slovenia as previously described[12,13,17]. In brief, between December 2009 and August 2010, 1,300 consecutive cervical samples were collected from 20-64 year-old women (screening set) who participated in the national organised cervical cancer screening programme. This collection was enriched with 300 cytologically abnormal samples (enriched set) collected between January 2014 to May 2015. The enriched set samples were also selected on a continuous basis from women referred to a main gynaecological outpatient clinic in Slovenia to obtain 100-150 histologically confirmed CIN2+ cases used to calculate the clinical sensitivity of the test. All the samples were stored in PreservCyt solution (Hologic, Bedford, MA, USA) with aliquots disseminated to participating laboratories for testing with different HPV tests.

In May 2016, the Scottish HPV Reference Laboratory (SHPVRL) in Edinburgh received 1 ml aliquot of these samples which were stored at -80°C until testing[7].

DNA extraction

DNA extraction was performed from 1ml aliquot of sample using the QIAsymphony® DSP Virus/Pathogen Kit on the automated Qiagen QIAsymphony SP using the Complex_800_V6_DSP protocol according to the manufacturer instructions. Final elution volume was 60μl.

EUROArray HPV test workflow and data collection

EUROArray HPV workflow (EUROIMMUN™) consists of PCR amplification of fragments of E6 and E7 viral oncogenes of 30 different HPV types and detection of type-specific identity via hybridisation with immobilised DNA probes that correspond to the targets within a microarray system[18,19].

EUROArray HPV PCR was performed according to the manufacturer's protocol. Briefly, PCR master mix contained 10μl of Mix A, 10μl of Mix B per sample and 5μl template DNA. PCR was carried out using Applied Biosystems 2720 Thermal Cycler (ThermoFisher Scientific, Waltham MA, USA). A region of ubiquitous human Hsp90 gene, served as an endogenous control to verify DNA extraction and amplification adequacy. PCR products were detected with an oligonucleotide microarray system through the TITERPLANE™ technique (EUROIMMUN). Each PCR product was mixed with 65μl of Hybridisation buffer and incubated on a microarray slide for 1 hour at 45°C. After hybridisation, slides were washed and dried prior to analysis using the Microarray Scanner and EUROArrayScan software (EUROIMMUN). Two spots were available for microarray system and endogenous controls and for each HPV type on the microarray grid. Signal strength was expressed in terms of immunofluorescence units with a specific cut-off for each HPV type (defined by the manufacturer).

Reproducibility of detection of hrHPV by EUROArray

The intra-laboratory reproducibility of EUROArray HPV DNA test was performed in Edinburgh, UK. Aliquots of extracted DNA were sent to the Royal Women's Hospital, Melbourne, Australia, for the assessment of inter-laboratory reproducibility. Reproducibility was assessed according to Meijer et al(2009) protocol using 500 valid specimens derived from the screening set. In order to assure 30% of hrHPV positives and avoid selection bias, 150 HC2 positive and 350 HC2 negative samples were selected using a random number generator (Stata version 13, College Station, TX, USA) .

Clinical outcomes and EUROArray HPV data assessment

At time of sample collection, national cervical screening in Slovenia was performed using cytology – standard recall for cytology negative women is cytology at next screening round. For women with abnormal cytology, follow-up was according to the criteria of the Slovenian National Cervical Cancer Screening Program[20]. As the study was retrospective, and the EUROArray HPV under evaluation, no study results influenced management.

Among the 1,600 samples of VALGENT-3 sample panel, 9 samples were excluded from the analysis because there was no amplification of the endogenous control. Of the remaining samples, 1,338 had sufficient outcome data to enable classification as cases (127/1,591) or controls (1,211/1,591).

The cytological and histological results were classified, respectively, according to the Bethesda System[21] and CIN nomenclature[22]. The clinical performance of the EUROArray HPV was evaluated in the total population and in women ≥30 years (1,372/1,600). HrHPV positivity for EUROArray HPV was based on presence of minimum one of the 13 hrHPV types targeted by HC2. Samples associated with CIN2+ were considered 'cases' whereas women that had two negative smears (at enrolment and after at a next screening visit 12 to 48 months later) were classified as no-disease controls. The clinical performance of the EUROArray HPV was also assessed with cases at the level of CIN3+.

The chi2 McNemar test was applied to assess differences between matched proportions (P_{McN}) and a $P_{McN} > 0.05$ indicated that sensitivity or specificity of the test was not significantly different from HC2. The test was considered not inferior to HC2 when the p value for non-inferiority (P_{ni}) was <0.05.

In a secondary analysis, we explored the distribution of the quantitative signal of EUROArray HPV for each of the 13 hrHPV types to enable an optimised cut-off for improved clinical accuracy.

RESULTS

The characteristics of the screening and enriched populations in terms of cytological results have been described previously[12,13]. The prevalence of the HPV infection in the Slovenian screening set (n=1,294) as determined by EUROArray HPV, specified by HPV type, is shown in Table S1 (Supplemental material). The composition of the screening and enrichment sets as well as cases and controls groups are described in a flow chart in Figure 1.

Clinical performance of EUROArray HPV

Absolute clinical sensitivity and specificity

EUROArray HPV was positive (for at least one of 13 hrHPV) for 114/127 CIN2+ cases and negative for 1,094/1,211 CIN1 or less, corresponding to an absolute clinical sensitivity for CIN2+ of 89.8% (95% CI, 83.1-94.4) and an absolute clinical specificity for ≤CIN1 of 90.3% (95% CI, 88.5-91.9). With respect to accuracy for CIN3+, EUROArray HPV detected 73/82 cases with an absolute sensitivity for CIN3+ of 89.0% (95% CI, 80.2-94.9) and an absolute specificity for ≤CIN1 of 87.4% (95% CI, 85.5-89.2). Data on the clinical sensitivity and specificity of EUROArray HPV separately for women ≥30 years are available in Table S2 A in Supplemental material.

Relative sensitivity and specificity of EUROArray HPV compared to Hybrid Capture 2

The cross-tabulation of the hrHPV positive and negative results of EUROArray HPV and HC2, according to clinical outcomes, is shown in Table 1. In the whole study population, HC2 was positive in 122/127 CIN2+ whereas the EUROArray HPV was positive for 114/127 cases. Two cases of CIN2+ were negative for both tests. Amongst 11 hrHPV discordant (EUROArray-/HC2+) cases, four samples were EUROArray HPV positive for other types (HPV42, 53, 73 or 82). When these 11 discordant samples (EUROArray-/HC2+) were retested with EUROArray HPV, two of them were HPV16 positive. When CIN3+ was used as the outcome, 71/82 samples were positive for both tests and nine hrHPV discordant (EUROArray-/HC2+) cases were reported.

A total of 1,065/1,211 samples of women without disease (≤CIN1) were hrHPV negative by both tests while 26 and 29 samples were discordant by the EUROArray HPV and HC2, respectively. Table S2 B in Supplemental material provides cross-tabulation data on women ≥30 years.

In the overall population, the test was less sensitive for CIN2+ and CIN3+ with a similar specificity compared to HC2 (Table 2A). The EUROArray HPV was non-inferior to the comparator test for

specificity but inferior for sensitivity for both CIN2+ and CIN3+. Similar results were observed in women ≥30 years (Table S2 C in Supplemental material).

Results are shown for hrHPV positivity defined as presence of at least one of 14 HPV types (HC2 types plus HPV66), to allow comparison with other VALGENT studies (See Tables S3A, B, C of Supplemental material).

Relative accuracy after modification of the EUROArray HPV cut-off for HPV16

Decreasing the EUROArray HPV cut-off for HPV16 was performed ("Optimisation of the EUROArray HPV test cut-off and the influence of HPV16", Fig.S1 of Supplemental material). This resulted in a sensitivity and specificity for CIN2+ of 93.7% (95% CI, 88.0-97.2) and 89.9% (95%CI, 89.0-92.3), respectively, which was statistically non-inferior to HC2 (Pni<0.001) (Table S4 A, Supplemental material).

The cross-tabulation of EUROArray HPV vs HC2 with the optimised cut-off for HPV16, in women with CIN2+ and ≤CIN1, is shown in Table S4B (Supplemental material). In the whole study population, relative sensitivity for CIN2+ was 0.98 (95%CI, 0.92-1.03) with a PMcN of 0.3173, Pni of 0.0076 and the relative specificity was 1.00 (0.97-1.03) with a PMcN of 0.7963, Pni of 0.0070 (Table 2B).

Intra- and inter-laboratory reproducibility of the EUROArray HPV test for hrHPV

The intra- and inter-laboratory reproducibility of the EUROArray HPV considering 13 hrHPVs is presented in Table 3 whereas additional data about reproducibility of the test considering 14 hrHPVs (all HC2 types plus HPV66) is presented in Table S5 of the Supplemental material.

A total of 122/500 samples and 370/500 were identified as hrHPV positive and negative, respectively, over the two intra-laboratory runs with eight discordant results. Overall hrHPV agreement was 98.4% (95%CI, 96.9-99.3); Kappa value of 0.96 (95% CI, 0.91-1.00).

Eight invalid samples were excluded from the inter-laboratory reproducibility analysis due to internal amplification control failure. A total of 102/492 positive and 363/492 negative samples were concordant

after inter-laboratory testing; agreement of 94.5% (95%CI, 92.1-96.4); Kappa value of 0.85 (95%CI, 0.80-0.89). The HPV16 optimised cut-off yielded similarly high reproducibility values (Table S6).

DISCUSSION

EUROArray HPV enables the simultaneous detection of 30 different anogenital HPVs in a single reaction through the PCR amplification of fragments of E6 and E7 viral oncogenes. As EUROArray HPV is a relatively novel test, few data have been published regarding its clinical performance[18] and none where performance has been related to the Meijer 2009 criteria.

When compared to HC2 test, EUROArray HPV was non-inferior with respect to specificity for ≤CIN1 but inferior for sensitivity for the detection of CIN2+ and CIN3+; irrespective if the analysis was performed in women older or younger than 30.

It is possible that the initial performance evaluation of EUROArray HPV and HC2 was affected by specimen storage time given that the evaluation was not contemporaneous. However, there is strong evidence suggesting that samples stored in PreservCyt solution at -80°C are suitable for retrospective HPV testing[23]; two other HPV tests, which were performed two and seven years after initial HC2 testing[12,13], showed non-inferior clinical sensitivity compared to HC2.

In a recent study, Cornall *et al*(2016) demonstrated that clinical sensitivity and specificity of EUROArray HPV was similar to a number of HPV tests including HC2[18] and was non-inferior to HC2 in terms of sensitivity. However, that study focussed on a disease-enriched population of 404 women of which 336 had CIN2+. The composition of the VALGENT panels is different to this and designed to determine accuracy (both clinical sensitivity and specificity) in a screening setting which may well explain the variance in these observations[7,24].

According to our results, EUROArray HPV missed 11 CIN2+ cases which were HC2 positive: this could be due to a limited capability of detecting signals for certain hrHPV types. For example, for each HPV

type the instrument is set to detect a minimum fluorescent level (cut-off) generated by the hybridisation of labelled PCR products with the corresponding DNA probe. Optimisation of the EUROArray HPV cut-off for HPV16 yielded a gain in sensitivity without substantial loss in specificity resulting in a non-inferior accuracy for CIN2+ compared to HC2. However, one must recognise that this cut-off optimisation was the result of an *a posteriori* analysis on a given population. More studies in other screening populations are required to confirm whether the clinical performance of EUROArray HPV at this modified cut-off may fulfil the international requirements for screening tests.

In addition, the discordant results between the two tests could be explained by reported cross-reactivity of HC2 to other HPVs beyond the 13 hr types. Cross-reactivity of the HC2 has been shown for 22 different HPV types and most frequently for HPV6, 40, 42, 53, 54 and 66[25]. Notably, 4/11 HC2 positive/hrHPV negative- EUROArray cases showed type specific positivity for HPV42, 53, 73 or 82. It will be interesting to verify the type specific positivity of these discordant cases according to all tests that are under evaluation in the VALGENT-3 project.

Furthermore, it could be argued that HC2 may not be the most optimal comparator test for the validation of genotyping tests. Indeed, Latsuzbaia et al (2016) showed EUROArray HPV and Anyplex II HPV28[19], showed higher agreement between each other, as full genotyping tests, than between each and the comparator assay which only typed HPV16, 18 and 45[26].

Finally, the EUROArray HPV reported excellent intra- and interlaboratory reproducibility which indicated a good reliability in the test and in the laboratories involved.

In conclusion, EUROArray HPV is a novel test that offers full genotyping results for 30 different HPV types. Considering its currently defined cut-off of test positivity, the test has sufficiently high clinical specificity for ≤CIN1 but a clinical sensitivity that is lower than HC2. However, by lowering the current detection cut-off for HPV16, the clinical sensitivity of EUROArray HPV became non-inferior compared to HC2 without compromising the relative specificity and test reproducibility.

FUNDING

CONFLICT OF INTEREST

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TABLES

Table 1. Cross-tabulation of EUROArray HPV and HC2 results for detection of 13 hrHPV types in CIN2+, CIN3+ and ≤CIN1 in women of all ages.

Clinical endpoint	EUROArray HPV result	HC2 result		
		Positive	Negative	Total
CIN2+	Positive	111	3	114
	Negative	11[a]	2	13
	Total	122	5	127
CIN3+	Positive	71	2	73
	Negative	9	0	9
	Total	80	2	82
≤ CIN1	Positive	91	26	117
	Negative	29	1,065	1,094
	Total	120	1,091	1,211

[a] EUROArray HPV detected 4 samples as HPV42, 53, 73 or 82 positive and 2 samples as HPV16 positive after the second analysis with the assay.

Table 2. A) Relative sensitivity for CIN2+, CIN3+ and relative specificity for ≤CIN1 of the EUROArray
HPV test compared to HC2 in women of all ages. **B)** Relative sensitivity for CIN2+ and relative
specificity for ≤CIN1 of the test considering an optimised cut-off for HPV16.

A)

Outcome	Relative sensitivity (95% CI)	Relative specificity (95% CI)	P_{McN} [a]	P_{ni} [b]
CIN2+	0.93 (0.88 - 0.99)		0.0325	0.1413
CIN3+	0.91 (0.84 - 0.99)		0.0348	0.3788
≤ CIN1		1.00 (0.98 - 1.02)	0.6858	0.0008

B)

Outcome	Relative sensitivity (95% CI)	Relative specificity (95% CI)	P_{McN} [a]	P_{ni} [b]
CIN2+	0.98 (0.92 - 1.03)		0.3173	0.0076
≤ CIN1		1.00 (0.97 - 1.03)	0.7963	0.0070

[a] p values for the McNemar test >0.05 determines that the sensitivity or specificity of the evaluated test
is not different from HC2.

[b] Non-inferiority p values <0.05 indicate that the evaluated test has non-inferior accuracy compared to
HC2.

Table 3. Intralaboratory (top) and interlaboratory (bottom) reproducibility of EUROArray hrHPV[*] results for 500 samples evaluated in the laboratories of Edinburgh and Melbourne.

Second analysis in Edinburgh	First analysis in Edinburgh		
	Positive	Negative	Total
Positive	122	2	124
Negative	6	370	376
Total	128	372	500

Second analysis in Melbourne	First analysis in Edinburgh		
	Positive	Negative	Total
Positive	102	3	105
Negative	24	363	387
Total	126	366	492[a]

[*] hrHPV defined as presence of at least one of the following types: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.

[a] Invalid samples were excluded from the interlaboratory reproducibility analysis.
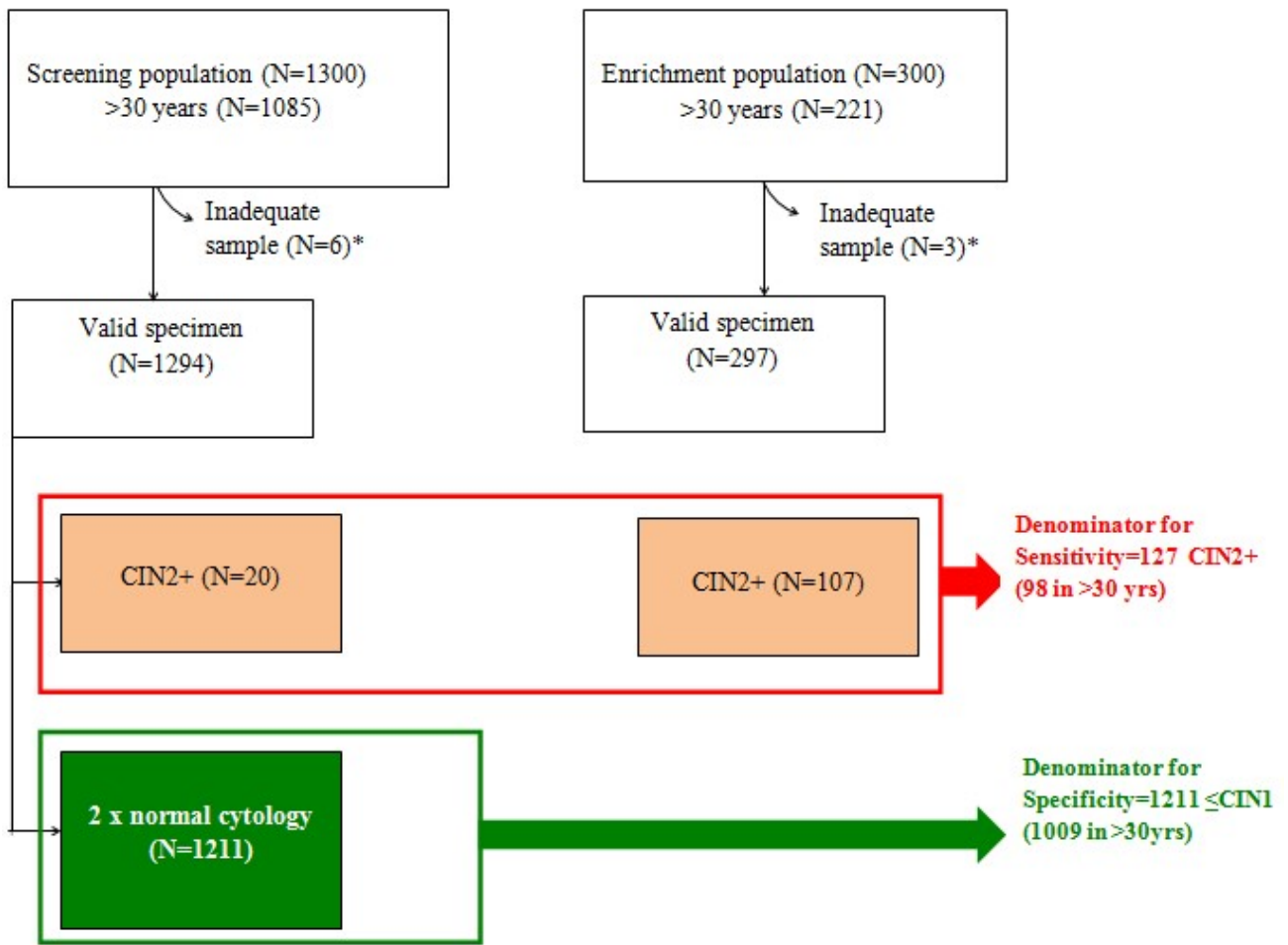
Figure 1. Flow chart showing selection of cases with CIN2+ used as denominator for sensitivity and cases without CIN2+ (women with 2 consecutive negative cytology results) used to compute specificity.