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RESEARCH ARTICLE

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Clinical performance of the HPV DNA Array genotyping assay in detection of CIN2+ lesions with BS GP5+/6+ MPG Luminex tested cervical samples

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Abstract

Human papillomavirus (HPV) detection is used for screening of cervical cancer and genotype-specific persistence has shown to be mandatory for dysplasia development. Aim of this study was to evaluate the clinical performance of HPV DNA Array for cervical intraepithelial neoplasia 2+ (CIN2+) lesion detection. HPV DNA Array is a polymerase chain reaction-based assay that targets E1 sequences of 29 HPV types (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, 85, and 97). The clinical evaluation was performed against the reference assay, BS-GP5+/6+ multiplex genotyping (MPG)-Luminex, with 600 cervical smear samples of a referral population. HPV DNA Array detected CIN2+ lesions with a sensitivity of 90.2%, identical to that of MPG-Luminex. Detection of CIN3+ lesions was with a sensitivity of 90.3%, as compared with 88.7% of MPG-Luminex. It demonstrated very good agreement for HPV detection, irrespective of type, of 91.5% (κ = 0.832). HPV DNA Array is a simple and robust assay, with a short protocol of 4 hours hands-on time and automated readout by ELISpot AiDot software. It permits testing of up to 96 samples in one run and may be considered for use in organized screening programs and low resource settings.

KEYWORDS

cervical cancer, clinical evaluation, clinical validation, human papillomavirus, preneoplastic lesions

1 | INTRODUCTION

The main cause of cervical cancer is persistent human papillomavirus (HPV) infection.[1] In the majority of women, HPV infections will clear within 2 years.[2] Even if a low-grade intraepithelial neoplastic lesion develops, in most women, it will regress within 3 years.[3] However, if genotype-specific HPV infections and lesions persist, women are at higher risk of developing cervical cancer.[4] The most clinically significant types, labeled high-risk (HR) types are HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 73. They have been associated with 94.5% of all cervical cancers.[5]

In 2013, World Health Organization[6] recommended HR-HPV screening in settings where cytology is difficult to implement. A year later, in 2014, Food and Drug Administration has approved the first HPV assay (Cobas, Roche), with partial HPV genotyping of HPV-16

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HR, high risk; LR, low risk; MPG, multiplex genotyping; NPV, negative predictive value; PPV, positive predicative value.

and -18, for primary cervical cancer screening.[7] A shift in the paradigm from cytology to HPV detection in primary cervical cancer screening is evident.[8]

The high number of HPV assays available in the market,[9] challenges the health care professionals to determine which assays are most efficient for the detection of high-grade lesions. To evaluate the performance of any HPV test, a comparison against a well-validated reference HPV assay is warranted. An assay validation guideline has been established by Meijer et al.[10] In this study, the performance of HPV DNA Array was validated against BS GP5+/6+polymerase chain reaction (PCR) followed by Luminex-based hybridization assay termed multiplex genotyping (MPG), an internationally recognized and clinically validated HPV test.[11,12] The study panel comprised of samples from a referral population.

HPV DNA Array (AID Diagnostika GmbH, Strassberg, Germany) is a full genotyping assay based on amplification of E1 sequences of 29 HPV types: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68, 69, 70, 73, 82, 85, and 97. Subsequently, PCR products are detected by reverse dot blot hybridization with type-specific oligonucleotide probes. These probes are spotted at the bottom of one single well in a 96 well microtiter plate. Hybridization colored patterns are evaluated by AiDot software and ELISpot enabled imaging. HPV DNA Array is approved for in vitro diagnosis within the European Union (CE marked).

Here we report on the cross-validation of the HPV DNA Array against MPG and the clinical performance on the consecutively collected sample material.

2 | MATERIALS AND METHODS

2.1 | Study design

To assess the clinical performance of HPV DNA Array 600 samples were consecutively collected from women undergoing colposcopy at the outpatient referral dysplasia clinic of the Clinic for Gynecology, Charité-Universitätsmedizin Berlin, Germany. Patients consented to use residual material for research (IRB No. EA1/168/13). Cervical scrapings were taken by cytobrush rinsed in ThinPrep (Hologic, Bedford, Massachusetts), and stored at +4°C until analysis. QIAamp DNA Mini Kit (Qiagen, Hilgen, Germany) was used to extract DNA of 2 mL from 20 mL total volume of sample, according to the manufacturer's instructions. Nucleic acid was eluted to a final volume of 160 µL. HPV genotyping was performed with MPG using 5 µL per PCR reaction and with HPV DNA Array 4.8 µL of DNA of each sample. The person performing HPV DNA Array was blinded to the MPG genotyping results and histology status of the samples collected.

2.2 | HPV DNA Array

The assay is capable of genotyping 18 HR (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) and 11 low-risk (LR; 6, 11, 40, 42, 44, 54, 67, 69, 70, 85, 97) HPV types. Initially, the HPV-specific E1-gene sequences are amplified by multiplex PCR with specific biotin-labeled primers. Per PCR reaction 4.8 µL DNA and 20.2 µL of Master Mix, in a total volume of 25 µL was used. The amplified gene fragments were then detected by a hybridization reaction with oligonucleotide probes specific for each HPV type. All probes were spotted as triplets and immobilized on the bottom of each well of a 96 well microtiter plate. Colored spots were evaluated by ELISpot reader and AiDot evaluation software (AID Diagnostika GmbH). The assay incorporates three internal controls: a GAPDH control for verification of adequate DNA content, a conjugate control for correct test execution, and a specificity control to exclude unspecific binding.

2.3 | MPG with Luminex-based hybridization following BS-GP5+/6+ PCR

The MPG is a well-validated genotyping assay proficient for detecting L1 sequences of the HPV: 6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 43, 45, 51, 52, 53, 54, 56, 57, 58, 59, 66, 68, 70, 72, 73, 82, and 90 with high analytical sensitivity.[11] An internal control, the cellular β-globin, is included to follow if sufficient DNA amount of each sample is present. Testing was performed as described.[13,14] However, in our laboratory the final PCR volume was adjusted to 25 µL vs 50 µL used in the publications.

In our laboratory, MPG assay performance was validated by participation in EQUALIS proficiency panel testing.[15]

2.4 | Data analysis

Main outcomes were agreement, sensitivity, and specificity, positive predictive values (PPV) and negative predictive values (NPV). For determining the agreement of HPV detection between the assays only 23 HPV types covered by both assays were included in the analysis (HPV-6, 11, 16, 18, 26, 31, 33, 35, 39, 42, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, 73, and 82). The agreement between assays was evaluated using Cohen's Kappa. The *k* value was interpreted as follows[16]: poor (<0.20), fair (0.21-0.40), moderate (0.41-0.60), good (0.61-0.80), very good (0.81-1.00). The values of the McNemar's test were used to determent the significance of discordant cases between the assays. Statistical analysis was performed with IBM SPSS Statistics for Windows (Version 21.0; IBM Corp, Armonk, NY) and MedCalc 15.8 (MedCalc Software, Ostend, Belgium).

2.5 | Ethics statement

Patients consented to use residual diagnostic material for research (IRB Charite-Universitätsmedizin Berlin, no. EA1/168/13).

3 | RESULTS

3.1 | Characteristics of the study population

Six-hundred consecutive samples were collected from women aged 18 to 94 years, with an average age of 39 years. One hundred fifty-one woman were younger than 30 years, and 449 were 30 years or older. Histology result was available for 348 patients: 195 women

had normal histology, 30 were positive for 1 (CIN1), 61 for CIN2, 47 CIN3, and 15 had cervical cancer.

Routinely MPG was performed: 262 women were HPV negative, 161 women had a single HPV infection, and 177 women had multiple HPV infections. Three hundred twenty-two women had at least one HR-HPV type positive, 193 were HPV 16 positive, and 26 were HPV 18 positive. Two women had a single infection with HPV 90, type not detected by HPV DNA Array, and they were additionally put into the HPV negative group for analytical purposes.

3.2 | HPV DNA Array initial results and sample retesting by both assays

After initial testing, in 135 samples, an HPV DNA Array discordant result to MPG was discovered. To exclude operational mistakes, the discrepant samples were retested two times by both assays. Each retesting was performed with 10 concordant samples as a control.

In 115 of 135 samples retested with HPV DNA Array, results stayed the same, discordant to MPG. For 20 of 135 samples, the result changed. Eighteen samples now matched MPG (11 samples were originally positive with single infection and then became negative, four samples were originally negative then became positive with a single infection (HPV-16, 18, 51, and 53) and in three samples with multiple infections and the additional type was detected). The rest two samples remained discordant to MPG (the result changed by losing an HPV type that was positive the first time in multiple infection). In 117 samples a discordance was concluded.

These 117 samples were retested with MPG. We found that in 58 samples, the MPG result remained the same, discordant, and in 59 samples, the MPG result changed. In 45 samples it now matched the HPV DNA Array results (33 samples were originally positive with a single infection then turned negative, 12 samples were originally negative then became positive with a single infection (eg, HPV-11, 16, 33, 42, 66). In 11 samples MPG result changed but was still discordant (the result changed by losing an HPV type that was positive the first time in multiple infection).

Finally, after retesting by both assays, in 69 samples a discordance was concluded. We present the analysis on the reevaluated results.

3.3 | CIN2+ and CIN3+ lesion detection

Both MPG and HPV DNA Array demonstrated high sensitivity for detection of CIN2+ lesions of 90.2% (95% CI, 83.58%-94.86%). The specificity was 44% (95% confidence interval [CI], 37.41%-50.75%) for MPG and 47.5% (95% CI, 40.88%-54.30%) for HPV DNA Array. The PPV was 46.8% (95% CI, 95% 40.35%-53.40%) and 48.5% (95% CI, 41.84%-55.15%), and the NPV was 89.2% (95% CI, 81.88%-94.29%) and 89.9% (95% CI, 83.05%-94.68%) for MPG and HPV DNA Array, respectively (Table 1).

Sensitivity remained similar for CIN3+ lesions detection, however, HPV DNA Array detected one case more than MPG, resulting in a sensitivity of 90.3% (95% CI, 80.12%-96.37%) vs 88.7% (95% CI, 78.11%-95.34%) of MPG. Specificity, PPV, NPV for MPG were 38.4% (95% CI, 32.56%-44.45%), 24.8% (95% CI, 19.24%-30.99%), and 93.7% (95% CI, 87.44%-97.43%). HPV DNA Array had a specificity of 39.9% (95% CI, 34.23%-45.84%), PPV 24.4% (95% CI, 19.03%-30.55%), and NPV 95% (95% CI, 89.52%-98.16%).

TABLE 1 Analytical comparison for HPV detection stratified to histology

		HPV DNA Array		MPG	
		n/N[a]	%	n/N[a]	%
	Normal	96/195	49.23	103/195	52.82
	CIN1	22/30	73.33	23/30	76.66
	CIN2	55/61	90.16	56/61	91.80
	CIN3	45/47	95.74	44/47	93.62
	CxCa[b]	11/15	73.33	11/15	73.33
Clinical sensitivity	CIN2+	111/123	90.24	111/123	90.24
	<30	37/38	97.37	37/38	97.37
	≥30	74/85	88.23	74/85	88.23
Clinical sensitivity	CIN3+	56/62	90.32	55/62	88.71
	<30	17/17	100.00	16/17	94.11
	≥30	39/45	86.66	39/45	86.66

Abbreviations: CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; MPG, multiplex genotyping.

[a]n, number of samples found HPV positive by a respective HPV test with the indicated histology; N, total number of woman with the indicated histology.

[b]Of the four cases missed: two were histologically confirmed epithelial carcinoma, one was adenocarcinoma, and one was vaginal carcinoma post radiation.

The CIN2+ and CIN3+ detection were stratified according to age and the results are represented in the Table 1. A difference in younger than 30 years age group for detection of CIN3+ can be observed, where HPV DNA Array had a sensitivity of 100% compared with 94.1% of MPG, due to one case less detected. Surprisingly, four cases with histology-confirmed cervical cancer demonstrated an HPV negative result for both assays.

3.4 | HPV detection

The HPV positivity rate, irrespective of genotype, of HPV DNA Array was 54.3% (326/600), compared with 55.6% (334/600) of MPG. Agreement between assays was 91.5% with κ 0.832 (95% CI, 78.7%-87.6%) showing very good agreement (Table 2). HPV DNA Array demonstrated a sensitivity for HPV detection of 88.7% (95% CI, 84.8%-91.8%), a specificity of 92.1% (95% CI, 88.2%-95.1%), with PPV of 93.7% (95% CI, 90.3%-96%) and NPV of 86.3% (95% CI, 81.7%-90.1%) in comparison with MPG.

Stratifying according to age, sensitivity for HPV detection within less than 30 years of age group was 93.9% ($\kappa = 0.805$; 95% CI, 69.5%-91.5%), and within ≥30 years of age group 90% ($\kappa = 0.831$; 95% CI, 77.9%-88.2%) demonstrating very good agreement between the assays. Further, when focusing on agreement among CIN2+ and CIN3+ lesions, a sensitivity of 96.4% ($\kappa = 0.631$; 95% CI, 39.5%-86.6%) for CIN2+ and 98.2% ($\kappa = 0.742$;

TABLE 2 Agreement for HPV detection between HPV DNA Array and MPG stratified by age and histology

		MPG					
	HPV DNA Array	Positive	Negative	Sensitivity	Specificity	κ (95% CI)	McNemar's *P*
Overall population (600)	Positive	305	21	91.3%	92.1%	0.832 (78.7% to 87.6%)	0.322
	Negative	29	245				
<30 (151)	Positive	108	4	93.9%	88.9%	0.805 (69.5% to 91.5%)	0.549
	Negative	7	32				
≥30 (449)	Positive	198	16	90.0%	93.0%	0.831 (77.9% to 88.2%)	0.418
	Negative	22	213				
CIN2+ (123)	Positive	107	4	96.4%	66.7%	0.631 (39.5% to 86.6%)	1.000
	Negative	4	8				
CIN3+ (62)	Positive	54	2	98.2%	71.4%	0.742 (46.5% to 100%)	1.000
	Negative	1	5				

Abbreviations: CI, confidence interval; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; MPG, multiplex genotyping.

95% CI, 46.5%-100%) for CIN3+ was observed, demonstrating good agreement. McNemar's *P* values showed that the differences between assays were not statistically significant.

4 | DISCUSSION

Objective of this study was to report the HPV DNA Array's potential to detect high-grade lesions. Testing 600 samples gave us extensive insight into the assay's protocol and performance. HPV DNA Array showed to be an easy and robust assay, particular for its short 4 hour protocol, with hands-on time of 2 hours. It permits high throughput by testing up to 96 samples in one run, with automated readout (3 minutes for a full plate). HPV DNA Array is a full genotyping assay, which may be of advantage to clinicians, as many emerging studies show that an important determining risk factor for dysplasia development is type-specific persistence.[17]

In their publication, Poljak et al,[9] highlighted that there are more than 200 available HPV assays in the market and only 10% to 15% have documented clinical performance. Hence, it is important to perform clinical validation studies in order for health care providers to choose the appropriate assays. In this study we validated the performance of the newly developed HPV DNA Array genotyping test against MPG, an assay with high analytical sensitivity and well-established clinical validation.[11] In our laboratory, MPG assay performance was validated by participation in EQUALIS proficiency panel testing.[15]

Within this study group of a referral population, HPV DNA Array demonstrated good clinical sensitivity by detecting more than 90% of CIN2 and higher lesions, identical to clinical sensitivity of MPG, and slightly higher sensitivity for detection of CIN3+ lesions (90.3% vs 88.7%) than MPG, due to a one more case detected. Surprisingly both assays failed to detect four cervical cancer cases, for which further investigation and search for pathology results post-treatment was conducted. It was found that one patient had an adenocarcinoma, which is less likely to be HPV positive than squamous cervical carcinomas.[18] One patient had recurrent vaginal cancer, treated with radiation therapy. The potential causal connection between HPV and vaginal cancer has been investigated in the literature, however, not all vaginal cancers are HPV+. Studies found that in approximately 60% to 80% of vaginal cancers, an HPV can be found.[19] Also, it has been discovered that viral load decreases significantly post-treatment, which could explain the HPV negative result by both assays if it was an HPV+ cancer.[20] The other two missed cervical cancers were squamous cell carcinomas with no prior therapy. It is possible that they were missed due to deletion of L1 and E1 genes, although such deletions are very seldom[21,22] or these were cancers with etiology other than HPV infection.

HPV DNA Array demonstrated a higher clinical specificity, 47.5% vs 44% of MPG. This is not surprising, as MPG is a very sensitive assay,[15,16] which is of benefit for epidemiology and vaccine effectiveness follow up. HPV DNA Array showed a very good agreement to MPG for HPV detection (>90%; $\kappa = 0.832$). The agreement remained high when focusing on the ≥30-year-of-age group (>90%; $\kappa = 0.825$).

It has to be mentioned, that these were historic sample collections, and therefore clinical data on the underlying disease was mostly available (348/600). The study population consisted of samples collected from women attending the referral clinic and is not a representative of a screening population. A higher number of HPV positive and lesion positive samples was present, in contrast to what is expected in a screening population.[23]

Due to the lack of samples from women attending the regular screening, the guidelines set by Meijer et al[10] could not be fully complied with. Meijer et al[10] recommend that validation studies should be conducted in comparison with internationally recognized and well-established assays; they should demonstrate a CIN2+ sensitivity and specificity of 90% and 98%, respectively; as well as have a high inter- and intra-laboratory reproducibility.

Setting aside the background of samples used, the sensitivity of HPV DNA Array for detection of CIN2+ in women 30 years of age and older was more than 90% of the reference assay, as required by the Meijer guidelines. Additionally, in our referral population, we observed specificity of >98% of the reference assay. Future studies

that will investigate sensitivity, specificity, and the intra- and inter-reproducibility on samples from a screening population, to fulfill these criteria, are warranted.

The main limitation of this study was the necessity of retesting of discordant samples. We observed that in a certain number of initially discordant samples the HPV results changed after retesting with HPV DNA Array (15%, 20/135 discordant) and with MPG (50%, 59/117 discordant). It might seem alarming that 15% and 50% of retested samples demonstrated different outcome after additional testing, however, a number of 135 and 117 samples of 600 in total were repeated. Additionally, these were samples initially discordant, hinting to the problematic of the samples themselves. There was no result change in samples chosen as control, although they were retested several times by both assays.

In most cases, we observed that when results changed it was either a single infection that was lost, or a negative sample becoming a single infection, or losing an HPV type in a sample with multiple infections. We could theorize that the HPV types initially missed were present in low copy numbers, hence missed during pipetting for the first time, but not the second time or vice versa. Or the sequences of the missed HPV types could be more difficult to amplify within PCR due to competition with other HPV types. Or there was an initial operational mistake while pipetting.

It is noteworthy to mention that the MPG assay seems to be more sensitive and therefore, prone to detecting low copy numbers that may fluctuate around the detection limit.

This retesting was performed and included in the analysis with the aim to have the most accurate HPV results, especially for validation purposes. However, we are aware such retesting would not be feasible as part of a real-life screening program. This might be of importance for epidemiological studies, but not relevant for clinical routine as histologically important lesions were detected.

It seems that HPV genotyping assays demonstrate a lower agreement for HPV type-specific detection,[24] they, however, show a very good agreement for detection of CIN. This could be explained by the higher number of viral copies in such lesions.[10]

5 | CONCLUSION

HPV DNA Array demonstrated a very good clinical performance for CIN2+/CIN3+ lesion detection and a very good agreement to the MPG test. HPV DNA Array is a full genotyping assay and may be competitive to other full genotyping assays due to high throughput and ease of handling, what may allow its use in organized screening programs and low resource settings as well.

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CONFLICT OF INTERESTS

A. P. received travel grants from AID/GeinID. M. H. and R. P. are employed at AID/GenID. AID/GenID provided the necessary kits free of charge. They had no role in study design, data collection, and analysis.

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