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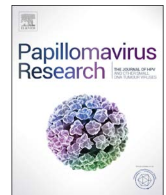
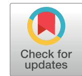


HPV genotype-specific concordance between EuroArray HPV, Anyplex II HPV28 and Linear Array HPV Genotyping test in Australian cervical samples



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ABSTRACT

Purpose: To compare human papillomavirus genotype-specific performance of two genotyping assays, Anyplex II HPV28 (Seegene) and EuroArray HPV (EuroImmun), with Linear Array HPV (Roche).
Methods: DNA extracted from clinican-collected cervical brush specimens in PreservCyt medium (Hologic), from 403 women undergoing management for detected cytological abnormalities, was tested on the three assays. Genotype-specific agreement were assessed by Cohen's kappa statistic and Fisher's z-test of significance between proportions.
Results: Agreement between Linear Array and the other 2 assays was substantial to almost perfect ($\kappa = 0.60 - 1.00$) for most genotypes, and was almost perfect ($\kappa = 0.81 - 0.98$) for almost all high-risk genotypes. Linear Array overall detected most genotypes more frequently, however this was only statistically significant for HPV51 (EuroArray; $\boldsymbol{p = 0.0497}$), HPV52 (Anyplex II; $\boldsymbol{p = 0.039}$) and HPV61 (Anyplex II; $\boldsymbol{p = 0.047}$). EuroArray detected signficantly more HPV26 ($p = 0.002$) and Anyplex II detected more HPV42 ($p = 0.035$) than Linear Array. Each assay performed differently for HPV68 detection: EuroArray and LA were in moderate to substantial agreement with Anyplex II ($\kappa = 0.46$ and 0.62, respectively), but were in poor disagreement with each other ($\kappa = -0.01$).
Conclusions: EuroArray and Anyplex II had similar sensitivity to Linear Array for most high-risk genotypes, with slightly lower sensitivity for HPV 51 or 52.

1. Introduction

Human papillomavirus (HPV) testing and genotyping has multiple applications, in research and clinical practice, and has been identified as a superior test for cervical cancer risk [1]. The genotypes that are of most interest include: 1) those classified as high-risk for cancer development in the mucosal genital region; 2) genotypes included in any vaccine that is available or in development or that may be cross-protected as a result of vaccination; 3) genotypes associated with non-cancer disease endpoints such as genital warts; and 4) rarely-seen genotypes that may have demonstrated carcinogenic potential and/or have been attributed to disease outcomes in immune-compromised patients [2–4]. In view of this, accurate and sensitive genotyping data is essential to produce reliable data to inform disease attribution; vaccine efficacy and surveillance including potential type replacement and cross-protection; and to detect differences in populations, within and between geographical regions [5–8].

Genotyping assays are either “partial” (a small number of genotypes are able to be individually identified, with the remainder as a pooled positive/negative result) or “full” (all genotypes able to be detected by the assay are individually identified). PCR-based assays available for full HPV genotyping can be divided into consensus primer amplification and genotype-specific primer amplification. Most common detection methods include reverse hybridisation (line probe), microarray (chip) and multiplexed real-time PCR [9]. The Roche Linear Array (LA) HPV Genotyping test is one such assay used as a research and

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epidemiological tool, with high analytical sensitivity. LA is a well-established, well-characterised and widely used reverse hybridisation assay based on the L1 gene PGMY09/11 consensus primer set [10]. More recent commercially-available full HPV genotyping tests include the Anyplex II HPV28 Detection and EuroArray HPV assays. Anyplex II HPV28 is a multiplexed real-time type-specific PCR assay, which uses advanced probe-tagging technology to enable discrimination of genotypes based on fluorescent spectra combined with precise melting temperatures [11]. The EuroArray HPV assay is a multiplexed E6/E7 gene target PCR assay combined with microarray capture [12].

The performance of Anyplex II and EuroArray assays for detection of CIN2+ has previously been described [12,13]. This study aimed to determine the relative performance of each of these assays in detection of individual genotypes compared to LA. Each was used to genotype cervical ThinPrep samples collected from unvaccinated Australian women with abnormal cytology results.

2. Material and methods

Samples were selected from a cohort of 1679 women referred with cytological abnormalities to the Royal Women's Hospital, Parkville, Victoria, Australia between May 2001 and June 2005 [14]. Residual cells collected on cervical brushes were rinsed into ThinPrep vials containing 20 mL of PreservCyt following preparation of Pap smear slides [15]. Overall, samples from 403 women were randomly selected from this cohort; 336 (83.4%) with histologically confirmed high-grade abnormalities ≥CIN2 and 67 (16.6%) with histologically confirmed low-grade abnormalities ≤CIN1 to represent a range of disease grades. Stored frozen PreservCyt samples were thawed, 500 μL was pelleted by centrifugation at 24,000 × g for 20 min and re-suspended in 200 μL phosphate-buffered saline. DNA was extracted on the MagNAPure 96 (Roche Diagnostics GmbH, Penzberg, Germany) using the DNA and Viral Nucleic Acid Small Volume Kit (Pathogen Universal 200 protocol) and eluted in a final volume of 50 μL. Two 5 μL aliquots of DNA were tested using Anyplex II HPV28 (1 aliquot each for mixtures A and B) and a third 5 μL aliquot amplified by PCR and tested on the EuroArray HPV assay (EUROIMMUN, Luebeck, Germany) according to the manufacturers' instructions [12]. A comparison of the relative sample volumes for each assay tested is shown in Table 1. The ≥95% limits of detection for each genotype, where reported for each assay, are listed in Supplementary Table 1. Full genotyping results from these assays were compared with previously-obtained results for these samples from testing on Linear Array HPV Genotyping test (LA; Roche, Branchburg, NJ 08876, USA) [14,16]. The method of DNA extraction used for LA testing was a deviation from the manufacturer's protocol, using 1 mL of cervical sample as opposed to the recommended 250 μL as shown in Table 1, and has been previously validated [17]. LA genotype testing included confirmation of HPV52 in the presence of HPV33/35/58 using a genotype-specific HPV52 qPCR assay as previously described [18]. For the purposes of analysis, results for HPV82 and IS39 (HPV82v) on LA were combined as a single result for HPV82. Statistical analyses were performed in Stata/IC 14.1 for Windows (StatCorp LP, College Station, TX, USA). Genotype-specific agreement between tests were assessed by Cohen's kappa statistic. The primary outcome of this study was whether the test assays (Anyplex II and EuroArray) detected a significantly lower proportion of each genotype than the reference assay (Linear Array). To test this, differences between proportions were assessed by one-tailed Fisher's z-test of significance between proportions. Kappa statistic values were assigned the following strengths of agreement: < 0.00 Poor, 0.00 − 0.20 Slight, 0.21–0.40 Fair, 0.41 – 0.60 Moderate, 0.61 – 0.80 Substantial, 0.81 – 1.00 Almost perfect [19]. Clopper-Pearson confidence intervals (95% CI) for proportions were calculated assuming a normally-distributed sample. The potential influence of sampling variability and/or sample degradation between the original Linear Array test and subsequent re-extraction and comparison genotype testing was evaluated. Forty-three (43) samples were sequentially selected from the original cohort of 403 such that each sample contained one or more of any genotypes that had originally been detected at significantly different frequency on Linear Array than one or both of the comparison assays, to enrich for these genotypes. The most recently extracted DNA samples from these 43 samples, being the same DNA samples that were tested on EuroArray and Anyplex II assays, were tested on Linear Array HPV Genotyping test and compared against the original Linear Array result as well as the EuroArray and Anyplex II results.

3. Results

Prevalence of individual genotypes detected by Linear Array, Anyplex II HPV28 and EuroArray are presented in Table 2. All three tests returned a valid result with a positive internal control, for the 403 samples tested. Overall, 352 (87.3%) were positive on at least one assay for one of the 25 genotypes common to all three tests. Prevalence of any of the 25 common HPV genotypes was highest on LA (86.5%) compared with Anyplex II (85.4%) and EuroArray (83.4%), however the decreases were not significant ($p = 0.326$ and 0.109, respectively). Overall LA detected 18 of the 25 common genotypes more frequently than Anyplex II and/or EuroArray; however prevalence was only significant lower for HPV51 (EuroArray; $\boldsymbol{p = 0.0497}$), HPV52 (Anyplex II; $\boldsymbol{p = 0.039}$) and HPV61 (Anyplex II; $\boldsymbol{p = 0.047}$).

HPV16 was the most common genotype detected by all three assays, with 42.4% (LA), 47.6% (Anyplex; $p=0.553$) and 46.4% (EuroArray;

Table 1
Comparison of input volumes of HPV genotyping assays.

HPV genotyping assay	Nucleic acid extraction method	Original sample input volume (mL)	Elution volume (mL)	PCR template volume (mL)	Total post-PCR volume (mL)	Total detection volume (mL)	Fraction of original sample detected	Equivalent original sample volume tested (mL)
This study:								
Anyplex II[a]	MagNAPure 96	0.50[a]	0.05	0.005	0.01	0.01	0.1	0.05
Linear Array[a]	MagNAPure 96	1.00[a]	0.10	0.05	0.20	0.075	0.05	0.047[b]
EuroArray[a]	MagNAPure 96	0.50	0.05[a]	0.005	0.09	0.065	0.01	0.007
Manufacturers' recommendations:								
Anyplex II	various	1.00	0.05 – 0.10	0.005	0.01	0.01	0.05 – 0.10	0.05 – 0.10
Linear Array	AmpliLute Liquid Media Extraction	0.25	0.10	0.05	0.20	0.075	0.05	0.012[b]
EuroArray	QIAmp or NucleoSpin	0.50	0.06 – 0.10	0.005	0.09	0.065	0.007 – 0.012	0.004 – 0.006

[a] Volumes apply to this study and are a deviation from the manufacturer's recommendations.
[b] Extraction volumes have been validated to ensure equivalent sensitivity using DNA extracted by MagNAPure 32 automated system compared with the manufacturer-specified AmpliLute manual system.

Table 2
Prevalence of individual HPV genotypes detected in 403 cervical samples, by assay.

HPV Genotype	Linear Array[a] n [% (95% CI)]	Anyplex II[b] n [% (95% CI)]	p[c]	EuroArray[d] n [% (95% CI)]	p[c]
Any HPV[e]	**348** [86.5 (82.6 – 89.6)]	**344** [85.4 (81.5 – 88.7)]	0.326	**336** [83.4 (79.4 – 86.9)]	0.109
6	**14** [3.5 (1.9 – 5.8)]	**13** [3.2 (1.7 – 5.4)]	0.407	**11** [2.7 (1.4 – 4.8)]	0.256
11	**2** [0.5 (0.06 – 1.8)]	**1** [0.2 (0.006 – 1.4)]	0.235	**1** [0.2 (0.006 – 1.4)]	0.235
16	**191** [47.4 (42.4 – 52.4)]	**192** [47.6 (42.7 – 52.6)]	0.553	**187** [46.4 (41.4 – 51.4)]	0.388
18	**26** [6.5 (4.2 – 9.3)]	**26** [6.5 (4.2 – 9.3)]	0.500	**23** [5.7 (3.7 – 8.4)]	0.318
26	**1** [0.2 (0.006 – 1.4)]	**1** [0.2 (0.006 – 1.4)]	0.500	**11** [2.7 (1.4 – 4.8)]	0.999
31	**45** [11.2 (8.3 – 14.7)]	**46** [11.4 (8.5 – 14.9)]	0.536	**46** [11.4 (8.5 – 14.9)]	0.536
33	**28** [6.9 (4.7 – 9.9)]	**30** [7.4 (5.1 – 10.4)]	0.609	**30** [7.4 (5.1 – 10.4)]	0.609
35	**16** [4.0 (2.3 – 6.4)]	**16** [4.0 (2.3 – 6.4)]	0.500	**13** [3.2 (1.7 – 5.4)]	0.271
39	**27** [6.7 (4.6 – 9.6)]	**21** [5.2 (3.3 – 7.9)]	0.184	**25** [6.2 (4.1 – 9.0)]	0.386
40	**2** [0.5 (0.06 – 1.8)]	**7** [1.7 (0.7 – 3.5)]	0.949	**3** [0.7 (0.2 – 2.2)]	0.643
42	**24** [6.0 (3.9 – 8.7)]	**38** [9.4 (6.8 – 12.7)]	0.965	**29** [7.2 (4.9 – 10.2)]	0.754
43	*N/A*	**6** [1.5 (0.50 – 3.2)]	*N/A*	**17** [4.2 (2.5 – 6.7)]	*N/A*
44	*N/A*	**7** [1.7 (0.7 – 3.5)]	*N/A*	**3** [0.7 (0.2 – 2.2)]	*N/A*
45	**10** [2.5 (1.2 – 4.5)]	**7** [1.7 (0.7 – 3.5)]	0.214	**4** [1.0 (0.3 – 2.5)]	0.052
51	**35** [8.7 (6.1 – 11.9)]	**29** [7.2 (4.9 – 10.2)]	0.216	**23** [5.7 (3.7 – 8.4)]	***0.0497***
52	**40** [9.9 (7.2 – 13.3)]	**26** [6.5 (4.2 – 9.3)]	***0.039***	**31** [7.7 (5.3 – 10.7)]	0.135
53	**22** [5.5 (3.5 – 8.1)]	**16** [4.0 (2.3 – 6.4)]	0.158	**15** [3.7 (2.1 – 6.1)]	0.111
54	**16** [4.0 (2.3 – 6.4)]	**16** [4.0 (2.3 – 6.4)]	0.500	**14** [3.5 (1.9 – 5.8)]	0.354
55	**4** [1.0 (0.3 – 2.5)]	*N/A*	*N/A*	*N/A*	*N/A*
56	**20** [5.0 (3.1 – 7.6)]	**20** [5.0 (3.1 – 7.6)]	0.500	**20** [5.0 (3.1 – 7.6)]	0.500
58	**30** [7.4 (5.1 – 10.4)]	**28** [6.9 (4.7 – 9.9)]	0.392	**26** [6.5 (4.2 – 9.3)]	0.308
59	**16** [4.0 (2.3 – 6.4)]	**16** [4.0 (2.3 – 6.4)]	0.500	**11** [2.7 (1.4 – 4.8)]	0.153
61	**19** [4.7 (2.9 – 7.3)]	**10** [2.5 (1.2 – 4.5)]	***0.047***	**11** [2.7 (1.4 – 4.8)]	0.066
62	**20** [5.0 (3.1 – 7.6)]	*N/A*	*N/A*	*N/A*	*N/A*
64	**0** [0 (0 – 1.0)][f]	*N/A*	*N/A*	*N/A*	*N/A*
66	**20** [5.0 (3.1 – 7.6)]	**18** [4.5 (2.7 – 7.0)]	0.369	**16** [4.0 (2.3 – 6.4)]	0.247
67	**10** [2.5 (1.2 – 4.5)]	*N/A*	*N/A*	*N/A*	*N/A*
68	**6** [1.5 (0.5 – 3.2)]	**10** [2.5 (1.2 – 4.5)]	0.845	**3** [0.7 (0.2 – 2.2)]	0.138
69	**1** [0.2 (0.006 – 1.4)]	**1** [0.2 (0.006 – 1.4)]	0.500	*N/A*	*N/A*
70	**10** [2.5 (1.2 – 4.5)]	**11** [2.7 (1.4 – 4.8)]	0.571	**5** [1.2 (0.4 – 2.9)]	0.085
71	**0** [0 (0 – 1.0)][f]	**0** [0 (0 – 1.0)][f]	0.500	*N/A*	*N/A*
72	**0** [0 (0 – 1.0)][f]	*N/A*	*N/A*	**1** [0.2 (0.006 – 1.4)]	0.816
73	**17** [4.2 (2.5 – 6.7)]	**18** [4.5 (2.7 – 7.0)]	0.583	**14** [3.5 (1.9 – 5.8)]	0.303
81	**9** [2.2 (1.0 – 4.2)]	*N/A*	*N/A*	**6** [1.5 (0.5 – 3.2)]	0.230
82	**4** [1.0 (0.3 – 2.5)]	**4** [1.0 (0.3 – 2.5)]	0.500	**3** [0.7 (0.2 – 2.2)]	0.321
83	**5** [1.2 (0.4 – 2.9)]	*N/A*	*N/A*	*N/A*	*N/A*
84	**15** [3.7 (2.1 – 6.1)]	*N/A*	*N/A*	*N/A*	*N/A*
89	**27** [6.7 (4.6 – 9.6)]	*N/A*	*N/A*	**4** [1.0 (0.3 – 2.5)]	< ***0.0001***

[a] Linear Array HPV (Roche).
[b] Anyplex II HPV28 (Seegene).
[c] Fisher's z-test of significance between proportions; *p* value for the hypothesis that the type prevalence was **lower** than detected on Linear Array. $p < 0.05$ considered significant and indicated in ***bold italics***.
[d] EuroArray HPV (EUROIMMUN).
[e] 25 common genotypes only included.
[f] 97.5% CI.

$p = 0.388$) of samples testing positive. Compared to LA, detection of each individual bivalent (HPV16/18) and quadrivalent vaccine-targeted genotype (HPV6/11/16/18) was not significantly lower by Anyplex II and EuroArray *($p > 0.05$ for each)*. Detection of the remaining individual nonavalent vaccine-targeted genotypes (HPV31/33/45/52/58) was not significantly lower by Anyplex II and EuroArray *($p > 0.05$ for each)*, with the exception of HPV52, which was significantly less often detected by Anyplex II HPV28 assay *($p = 0.039$)*. LA detected significantly more HPV89 than EuroArray; Anyplex II does not detect this genotype. There were 2 genotypes that were detected by one of the other assays significantly more frequently than by LA: HPV26 (detected more frequently by EuroArray, $\boldsymbol{p = 0.002}$) and HPV42 (detected more frequently by Anyplex II, $\boldsymbol{p = 0.035}$) (Table 2).

Genotype-specific agreement results are presented in Table 3. Agreement between EuroArray and Anyplex II HPV28 for individual genotype detections were substantial to almost perfect ($\kappa = 0.60 – 1.00$) for most genotypes (Table 3). Comparison of the detection of high-risk HPV genotypes showed almost perfect agreement ($\kappa = 0.81 – 0.98$) for detection of HPV16, 18, 31, 33, 35, 39 (EuroArray only), 45 (Anyplex II only), 51 (Anyplex II only), 52 (EuroArray only), 56, 58, 59 and 66; and substantial agreement for detection of HPV39 (Anyplex II), 51 (EuroArray), 52 (Anyplex II) and 68 (Anyplex II). Agreement between LA and EuroArray for detection of HPV45 was moderate ($\kappa = 0.57$). Each assay performed differently for the detection of HPV68: whilst EuroArray and LA were in moderate to substantial agreement with Anyplex II ($\kappa = 0.46$ and 0.62, respectively), they were in poor disagreement with each other ($\kappa = -0.01$). LA and EuroArray were completely discordant for detection of HPV68 positive samples, detecting 6 and 3 HPV68 positive samples, respectively, with no samples positive on both assays. Anyplex II detected 10 HPV68 positive samples, 3 of which were also detected by EuroArray, and 5 of which were also detected by LA. LA detected 1 HPV68 positive sample that was negative on Anyplex II. With regards to non-high-risk genotypes, only slight to fair agreement was observed for HPV26 ($\kappa = 0.16$, $\boldsymbol{p = 0.002}$), 40 ($\kappa = 0.19$, $p = 0.192$), 43 ($\kappa = 0.24$, $\boldsymbol{p = 0.021}$). EuroArray detected significantly more HPV26 and 43 than Anyplex II.

Genotype-specific agreement between pairs of assays was also calculated restricted to samples that were positive for a single genotype on any of the 3 assays ($n = 117$) or for multiple genotypes (≥ 2) on any of the 3 assays ($n = 226$). The numbers of samples in each of these categories for each genotypes is listed in Supplementary Table 2. Single genotype-specific detections were less frequent than multiple-genotype

Table 3
Genotype-specific agreement between Anyplex II HPV28 and EuroArray, Linear Array HPV in cervical samples (n = 403). κ values ≥ 0.61 have been indicated in bold.

HPV Genotype	Anyplex II agreement with Linear Array[c] κ (95% CI)	EuroArray agreement with Linear Array κ (95% CI)	Anyplex II[a] agreement with EuroArray[b] κ (95% CI)
6	**0.89 (0.76 – 1.00)**	**0.79 (0.62 – 0.97)**	**0.91 (0.80 – 1.00)**
11	**0.67 (0.05 – 1.00)**	**0.67 (0.05 – 1.00)**	**1.00 (1.00 – 1.00)**
16	**0.98 (0.95 – 1.00)**	**0.93 (0.89 – 0.97)**	**0.96 (0.93 – 0.98)**
18	**0.96 (0.90 – 1.00)**	**0.89 (0.80 – 0.99)**	**0.85 (0.74 – 0.96)**
26	**1.00 (1.00 – 1.00)**	0.16 (−0.12 – 0.44)	0.16 (−0.12 – 0.44)
31	**0.91 (0.85 – 0.98)**	**0.96 (0.92 – 1.00)**	**0.95 (0.90 – 1.00)**
33	**0.93 (0.85 – 1.00)**	**0.96 (0.92 – 1.00)**	**0.96 (0.91 – 1.00)**
35	**0.87 (0.74 – 1.00)**	**0.89 (0.77 – 1.00)**	**0.89 (0.77 – 1.00)**
39	**0.78 (0.65 – 0.91)**	**0.84 (0.72 – 0.95)**	**0.82 (0.69 – 0.94)**
40	0.44 (0.04 – 0.84)	0.40 (−0.15 – 0.94)	0.19 (−0.14 – 0.53)
42	**0.72 (0.59 – 0.85)**	**0.78 (0.65 – 0.91)**	**0.85 (0.76 – 0.95)**
43	*N/A*	*N/A*	0.24 (0.01 – 0.48)
44	*N/A*	*N/A*	0.60 (0.24 – 0.96)
45	**0.82 (0.62 – 1.00)**	0.57 (0.25 – 0.88)	**0.72 (0.43 – 1.00)**
51	**0.90 (0.82 – 0.98)**	**0.78 (0.66 – 0.90)**	**0.84 (0.72 – 0.95)**
52	**0.77 (0.66 – 0.89)**	**0.86 (0.77 – 0.95)**	**0.91 (0.82 – 0.99)**
53	**0.61 (0.43 – 0.80)**	**0.63 (0.45 – 0.82)**	**0.77 (0.60 – 0.93)**
54	**0.87 (0.74 – 1.00)**	**0.86 (0.73 – 1.00)**	**0.93 (0.84 – 1.00)**
55	*N/A*	*N/A*	*N/A*
56	**0.95 (0.88 – 1.00)**	**0.95 (0.88 – 1.00)**	**0.95 (0.88 – 1.00)**
58	**0.93 (0.85 – 1.00)**	**0.85 (0.74 – 0.95)**	**0.92 (0.84 – 1.00)**
59	**0.87 (0.74 – 1.00)**	**0.81 (0.65 – 0.97)**	**0.81 (0.65 – 0.97)**
61	**0.61 (0.40 – 0.82)**	0.59 (0.37 – 0.80)	**0.85 (0.69 – 1.00)**
62	*N/A*	*N/A*	*N/A*
64	*N/A*	*N/A*	*N/A*
66	**0.89 (0.78 – 1.00)**	**0.88 (0.71 – 1.00)**	**0.88 (0.76 – 1.00)**
67	*N/A*	*N/A*	*N/A*
68	**0.62 (0.34 – 0.90)**	−0.01 (−0.02 – 0.00)	0.46 (0.12 – 0.79)
69	**1.00 (1.00 – 1.00)**	*N/A*	*N/A*
70	**0.95 (0.86 – 1.00)**	**0.66 (0.38 – 0.94)**	**0.62 (0.34 – 0.90)**
71	*N/A*	*N/A*	*N/A*
72	*N/A*	0.00 (. – 1.00)	*N/A*
73	**0.91 (0.81 – 1.00)**	**0.83 (0.69 – 0.98)**	**0.87 (0.74 – 1.00)**
81	*N/A*	0.53 (0.22 – 0.83)	*N/A*
82	**0.75 (0.41 – 1.00)**	**0.86 (0.58 – 1.00)**	**0.86 (0.58 – 1.00)**
83	*N/A*	*N/A*	*N/A*
84	*N/A*	*N/A*	*N/A*
89	*N/A*	0.25 (0.05 – 0.44)	*N/A*

[a] Anyplex II HPV28 (Seegene).
[b] EuroArray HPV (EUROIMMUN).
[c] Linear Array HPV (Roche).

detections for all genotypes, and agreement for samples with single genotype detection was > 96% for all genotypes, with κ > 0.700 for all genotypes except cases where a single case was positive for that genotype on a single assay. The number of genotypes with 100% agreement across assays was high for single detections. Genotype-specific agreement for samples with multiple genotype detection was similar to the whole cohort (Supplementary Table 3).

The most recently-extracted DNA samples from 43 specimens were re-tested on Linear Array and the results compared with the original Linear Array results and the EuroArray and Anyplex II results to examine the potential effect of sampling variability and/or sample degradation over time. The overall genotype-specific agreement between the first and second Linear Array results for these 43 samples was 96.4%. Overall the sensitivity of the second Linear Array for genotype-specific detections was 13% lower than the first within this small subset of samples. The relative prevalences of 6 genotypes (HPV26, 42, 51, 52, 61 and 89) were compared (Supplementary Table 4). In the original analysis the detection of these 6 genotypes was significantly different between Linear Array and either Anyplex II or EuroArray. Detection of HPV51 and 89 were 100% concordant between the first and second Linear Array runs. Detection of HPV26 and 61 remained substantially lower and higher, respectively, than for Anyplex II and/or EuroArray, the same pattern as seen in the original results. In the case of HPV42 and HPV52, the second Linear Array results became more similar to the other assays (increasing and decreasing in prevalence, respectively, compared with the first Linear Array results).

4. Discussion

The analytical performance for individual HPV genotypes of two relatively new assays, EuroArray HPV and Anyplex II HPV28, were compared against the well-established Linear Array HPV Genotyping Test, using previously-characterised cervical cytology samples [20]. The performance of these assays for detection of CIN2+, based on aggregated HR-HPV detection, has previously been described in the same sample cohort [12,13]. Linear Array was used as the reference genotyping test as it is widely used, has high sensitivity and has well-characterised performance characteristics [21]. EuroArray detected fewer HPV51 (borderline significant) and HPV89, and significantly more HPV26 than Linear Array. Anyplex II HPV28 detected significantly fewer HPV52 and 61, and more HPV42. These differences can largely be explained by differences in the limits of detection (where reported by each manufacturer), shown in Supplementary Table 1. The increased detection of HPV26 by EuroArray is unexpected and may be an artefact of the assay. Other than these differences, EuroArray and Anyplex II were not significantly different to the performance of Linear Array for individual detection of common genotypes and overall performed comparably.

Agreement for genotype-specific detection between EuroArray and Anyplex II HPV28 was overall very good. EuroArray HPV and Anyplex II HPV28 have recently been compared to a partial genotyping test (Aptima, Hologic) and to each other on a cohort of 150 cervical samples collected from European women [22]. While agreement between EuroArray and Anyplex II were generally similar between the previous and present study, agreement was overall slightly higher in the present study and there were particular genotype-specific differences observed which may be due to population differences in prevalence or genomic sequences. These include HPV 6, 70 and 73 (reported agreement was substantially higher in the present Australian cohort), and HPV18, 45 and 68 (reported agreement higher in the European cohort).

Other than the quality of clinician-collected cervical sample preserved in ThinPrep PreservCyt media, the accuracy of detection and identification of individual genotypes by full genotyping assays is affected by a number of other independent and interdependent factors. The limits of detection inherent to an assay will determine how sensitively each genotype is detected (Supplementary Table 1). Linear Array, when performed according to manufacturer's instructions, has lower limits of detection per PCR reaction than one or both of the other assays, with the exception of genotypes 26, 33, 39, 40, 42, 56 and 82, which explains much of the variation in performance between assays. Primer and/or probe fidelity with the target region of the genome may alter the sensitivity of detection, leading to genotype bias or potentially misidentification by cross-reactivity [23,24]. The relative sensitivities of an assay for individual genotypes can also be a factor in masking of one genotype by one or more other genotypes, in samples with two or more genotypes present, due to competition for assay reagents [5,25]. This is dependent on the chemistry of the assay itself, and is particularly the case for single-reaction PCR-based assays using consensus primer sets. In this study, there was no evidence of systematic masking of any particular genotype(s) in the presence of other genotypes. While agreement between assays was stronger for some genotypes in single-genotype detections than in multiple-genotype detections (most notably HPV42, 51, 52, and 53), this can be explained by the relative limits of detection of each assay. The prevalent genotypes as well as strain differences in a population may have an impact on the detection of some genotypes with some assays [24]. In addition to regional differences in relative genotype and strain prevalence, rates of vaccination and therefore vaccine-targeted genotypes consequently can have an impact

on potential masking of detection of genotypes due to assay competition.

In this study differences in detection of HPV68 were noted across the three assays. This is likely due to variable efficiency of detection of two subtypes of HPV68, a and b, across the three assays utilized. Variations in the primer binding sequences for both Linear Array and EuroArray result in Linear Array only being able to amplify subtype b efficiently [26], and EuroArray only being able to amplify subtype a efficiently (personal communication, Markus Cavalar, August 12, 2016). This was demonstrated by complete discordance between Linear Array and EuroArray for detection of HPV68. The PGMY primer set, on which Linear Array was based, has recently been modified to improve detection of HPV68a, however these changes have not been incorporated into the Linear Array primer mix [11,27]. Anyplex II amplifies both HPV68 subtypes efficiently, detecting all 3 of the samples that were positive for HPV68 on EuroArray, and 5 of the 6 samples that were positive on Linear Array. In this cohort, more HPV68b (Linear Array, 1.5%) was detected than 68a (EuroArray, 0.7%). The prevalence of HPV68 according to Anyplex II was 2.5%. As the prevalence and clinical relevance of HPV68 are generally low, the implications of this limitation are usually not significant. HPV68 is associated with a very small proportion (< 1%) of cervical cancers [28].

One of the limitations of the Linear Array is the cross-reactivity of the HPV52 probe with HPV33, 35 and 58, making the use of an extra confirmatory test necessary to confirm the status of HPV52 in such cases [18]. Due to the use of a potentially more-sensitive qPCR assay it is difficult to determine the relative sensitivity of the Linear Array for HPV52 in the presence of HPV33, 35 and/or 58. To partially address this, a sub-analysis was performed on only samples negative for HPV33, 35 and 58 (n = 331; data not shown). The proportion of samples in this subset positive for HPV52 by Linear Array was 9.4%, compared with 7.9% (EuroArray) and 6.7% (Anyplex II), very similar to the proportions detected in the whole cohort of 403 (9.9%, 7.7% and 6.5%, respectively). Within this subset, agreement for HPV52 was slightly higher than within the whole cohort, with κ values of 0.904 (Linear Array and EuroArray) and 0.816 (Linear Array and Anyplex II). This suggests that the confirmatory qPCR assay may be slightly more sensitive for HPV52 than the Linear Array detection strips alone, however the relative performance of EuroArray and Anyplex II compared to Linear Array were largely independent of the confirmatory HPV52 assay.

At present, the main indication for full genotyping assays is for population-level research purposes, where high analytical sensitivity, genotype-specific specificity and the ability to compare results between timepoints (for example pre- and post-vaccination) and populations (for example, different geographical locations, to inform vaccination policy) are important. Access to particular assays differs in different parts of the world; some are more cost-effective than others and therefore more accessible where resources are limited; some require smaller sample volumes than others, and some may fit in better with existing processes and equipment within an individual laboratory. Newer assays are being developed and released regularly and hence it is important to determine relative performance and levels of agreement between different assays.

The clinical value of extended genotyping remains unclear. It is anticipated that genotype-specific identification might be useful for test of cure, further stratification of cancer risk and to differentiate persistent from transient infection [29]. Likely anticipating a future when the clinical value of genotyping has been proven, a significant proportion of the more than 200 commercially available HPV detection assays offer full genotyping, and many are positioned as clinical tests. Many of these assays, however, have not been validated [9]. The VALGENT protocol for validation of genotyping tests for clinical cervical screening has recently been developed [30,31] to complement the earlier Meijer criteria for use of HR-HPV DNA tests in population-based screening [32], and to determine whether extended genotyping offers any additional clinical value. While Linear Array, EuroArray and Anyplex II HPV genotyping assays are yet to be assessed using the VALGENT protocol, Anyplex II HPV28 has been assessed for, and meets, the Meijer criteria for HR-HPV screening [33,34].

The major limitations to this study are the use of samples stored for an extended period between Linear Array testing and the comparison testing with Anyplex II and EuroArray, and not using the exact same DNA sample extracted for Linear Array testing on the other 2 assays. Variability in a comparison can be introduced by preparing separate nucleic acid extracts, as sampling artefacts can be introduced. An additional issue is that it is unclear how stable samples in PreservCyt medium are when stored for extended periods at −80 °C. There is evidence of genomic and HPV nucleic acid stability over time (up to 2.5 years) when samples refrigerated [35] or a year at −80 °C [36], yet evidence of the stability of frozen PreservCyt samples or over longer time periods are lacking. Studies of this nature have been called for to enable evaluation of the feasibility of establishing sample banks [37]. To examine the potential impact of sampling variability and/or possible degradation of sample over time, we re-tested 10% of the samples on Linear Array using the most recently extracted DNA samples. We observed only a small decrease in overall sensitivity within this subset, which may be due in part to age of the original sample and/or freeze-thaw of the DNA sample. We also observed other variations (genotype-specific changes including increased detection) which are likely attributable to the fact that the DNA was re-extracted from a non-identical aliquot of original sample. When we examined specifically those genotypes that were significantly differently detected in the original analysis, overall there was little change in the observed patterns of 4 of these genotypes. The observed variability in the other 2, HPV42 and 52 (increasing and decreasing, respectively), upon re-testing is characteristic of sampling variability. Finally, this study only evaluated the performance of clinician-collected cervical samples in PreservCyt, and extension to other sample types such as self-collected genital swab and urine would be informative, particularly as self-collected samples for HPV testing become more commonly used.

5. Conclusion

EuroArray HPV and Anyplex II HPV28 had similar sensitivity to LA with slightly lower analytical sensitivity for a small number of high-risk HPV genotypes. The data suggests they are well suited for population research, however this study should be repeated in a lower-risk screening population as assay performance may vary with lower prevalence. Further assessment is required to confirm what further clinical benefit can be gained from the full genotyping offered by these assays.

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Conflict of interest

All authors declare that they have no conflict of interest.

Ethical approval

All aspects of this study were endorsed by the Royal Women's Hospital Human Research and Ethics Committees (RWHHREC) 00/30. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

For this retrospective study formal consent was not required from participants. This article does not contain any studies with animals performed by any of the authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.pvr.2017.10.002.

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