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Evaluation of the Roche MagNA Pure 96 nucleic acid extraction platform for the Seegene Anyplex II HPV28 detection assay

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Abstract

Aim:

Validate the Roche, MagNAPure96 (MP96) nucleic acid extraction platform for Seegene Anyplex II HPV28 (Anyplex28) detection of Human Papillomavirus.

Methods and Results:

Comparisons were made for Anyplex28 genotyping from 115 cervical samples extracted on the Hamilton, STARlet and the MP96. Two DNA concentrations were used for the MP96, one matched for sample input to the STARlet, and another 5x concentration (laboratory standard).

Agreement of HPV detection was 89.8% (κ=0.798; p=0.007), with HPV detected in 10 more samples for the MP96. There was a high concordance of detection for any oncogenic HPV genotype (κ=0.77; p=0.007) and for any low risk HPV genotype (κ=0.85; p=0.008). DNA extracted at laboratory standard had a lower overall agreement 85.2% (κ= 0.708; p <0.001), with 17/115 discordant positive samples that tested negative after STARlet extraction. Of the discordant genotypes 72.7% were detected in the lowest signal range for Anyplex28 ("+").

Conclusions:

MP96 performed with high concordance to STARlet, though produced DNA with a higher analytical sensitivity on the Anyplex28.

Significance and Impact of Study:

This analysis supports the use of samples extracted on the MP96 for HPV genotyping using the Anyplex28. Furthermore, an increase in DNA concentration increased analytical sensitivity of the Anyplex28, particularly appropriate for prevalence studies.

Introduction

Human papillomavirus (HPV) is the underlying cause of the majority of cervical cancers, and a proportion of other types of anogenital cancers in both males and females (de Sanjose et al., 2010). The Australian Government funded school-based HPV vaccination program commenced in 2007 using the quadrivalent vaccine (Merck, Gardasil) targeting four HPV genotypes (6, 11, 16 and 18). In 2018 the program implemented use of the nonavalent vaccine (Merck, Gardasil 9), protecting against nine genotypes of HPV (6, 11, 16, 18, 31, 33, 45, 52 and 58) which are collectively responsible for approximately 91% of cervical cancers (de Sanjose et al., 2010, Garland et al., 2009). Monitoring changes in prevalence of HPV infection in the population over time is a key indicator of vaccine impact and effectiveness. Surveillance programs require highly sensitive and specific HPV detection assays which provide genotype-specific results, monitoring infection is a key marker of vaccine impact and effectives and has been used in many countries (Machalek et al., 2019, Dillner et al., 2008, Garland et al., 2011, Tabrizi et al., 2014, Australia, 2013, Brotherton et al., 2020).

Historically, the PCR based, reverse line blot hybridisation assay, Linear Array (Roche Molecular Diagnostics; Mannheim, Germany) was widely utilised for HPV prevalence research studies, necessitating the validation for different sample types and extraction platforms (Stevens et al., 2007, Phillips et al., 2015). The increased use of HPV partial/full genotyping for primary HPV screening has led to a 31% increase in the number of commercial HPV detection assays, with up to 254 different detection systems available worldwide in 2020 (91 with individual genotyping ability) (Poljak et al., 2020). However, the use of many of these assays as surveillance tools is yet to be assessed with 82% lacking published data (Poljak et al., 2020). With the discontinuation of the Linear Array assay (Roche Molecular Diagnostics; Mannheim, Germany), there is a need for more comprehensive validation of other genotyping assays.

The Seegene Anyplex II HPV28 (Anyplex28) assay (Seegene, Seoul, Korea) detects 28 HPV genotypes, and is utilised as a surveillance tool for the detection of oncogenic HPV genotypes from routinely collected cervical samples (Bule et al., 2020, Latsuzbaia et al., 2019, Jacot-Guillarmod et al., 2017, Kwon et al., 2014, Estrade and Sahli, 2014, Mboumba Bouassa et al., 2019, Shilling et al., 2020). To date the Anyplex28 assay has been validated for nucleic acid extraction on the Seegene NIMBUS and STARlet DNA extraction systems for cervical samples in PreservCyt (Seegene, 2019). The aim of this report was to validate the use of cervical samples extracted on the MagNA Pure 96 (MP96) automated extraction system against the validated MICROLAB STARlet (STARlet) automated extraction system

(Hamilton, Bonaduz, Switzerland) for the detection of HPV genotypes utilising the Anyplex28.

Methods

Sample processing (STARlet)

Cervical samples were collected and processed as part of an HPV surveillance study described previously (Shilling et al., 2020). Briefly, de-identified residual cervical specimens resuspended in PreservCyt from women aged 16–24 years submitted for opportunistic *Chlamydia trachomatis* screening were collected from a pathology laboratory in Victoria and stored at room temperature, as per the manufacturer's recommendations. Samples were extracted for DNA on the STARlet using 200 µl of PreservCyt sample, eluted in 100 µl of STARlet elution buffer and tested on the Anyplex28 as per manufacturer validated protocol, utilising the Bio-Rad CFX96/384 real time thermocycler.

Sample processing (MP96)

Samples were also prepared according to a PreservCyt sample extraction protocol that is routinely used in our laboratory (Centre for Women's Infectious Diseases, The Royal Women's Hospital, Melbourne), as previously described (Stevens et al., 2006). Briefly, 1 ml of PreservCyt sample, was centrifuged at 16,200 g for 15 min and resuspended in 200 µl of PBS. DNA was extracted on the MP96 and eluted in 100 µl of MP96 elution buffer (standard extraction). Extracted DNA from the MP96 was tested on the Anyplex28 according to manufacturer instructions (115 samples), and subsequently after a 1/5 dilution with sterile water (matched extraction) (108 samples) to provide an equivalent sample input to the STARlet extraction protocol. Samples were stored between MP96 and STARlet extractions for up to 1 year at room temperature. There was also a 1 year delay in testing diluted DNA samples on the Anyplex28, with all DNA extracts stored at -30°C.

HPV genotyping and analysis

For each extraction method, HPV genotype results were assessed for the frequency/prevalence of oncogenic HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and low-risk HPV genotypes (6, 11, 40, 42, 43, 44, 54, 61, 63, 64, 70, 73 and 82). HPV genotype frequency is defined as the total number of genotypes detected and identifies any genotype bias between extraction method. HPV genotype prevalence is according to total number of samples and indicates any differences in genotype detection

between the extraction methods. The overall agreement of any HPV detection for each sample at both concentrations of the MP96 eluted DNA (laboratory standard and matched) were compared to any HPV detection determined by the STARlet eluted DNA, using McNemar's test.

In addition to overall HPV genotype detection, the Seegene (Seegene Viewer on the Bio-Rad CFX manager) analysis parameters enable crossing points to be grouped based on the following thresholds: ≤ 31 cycles (+++), 31 cycles to 39 cycles (++), and 40 cycles to 50 cycles (+) (hereafter, signal strength)(Seegene, 2019). The trend of association with these 3 groups and within-sample concordance for each genotype detected was evaluated using Fisher's exact test, comparing the STARlet DNA with both MP96 DNA sample input concentrations (laboratory standard and matched). Concordance, agreement, and positive agreement of HPV detection between the MP96 DNA (matched) and the STARlet DNA was assessed according to HPV genotype groupings (as per IARC guidelines) (Bouvard et al., 2009) of any HPV (HPV 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 66, 68, 69, 70, 73, 82), any low risk HPV (HPV 6, 11, 26, 40, 42, 43, 44, 53, 54, 61, 69, 70, 73 and 82), any oncogenic HPV (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68), any non-vaccine targeted oncogenic HPV (HPV 35, 39, 51, 56, 59, 66 and 68) and any vaccine-targeted HPV (HPV 6, 11, 16, 18, 31, 33, 45, 52 and 58), with disagreement assessed using McNemar's test. Due to increased detection of HPV genotypes associated with the laboratory standard DNA, genotype groupings were not assessed. Within-sample concordance was evaluated using Cohen's kappa statistic, with the agreement interpreted as none ($\kappa<0.2$), weak ($\kappa=0.2–0.4$), moderate ($\kappa=0.401–0.6$), strong ($\kappa=0.601–0.8$), near perfect ($\kappa=0.801–0.99$), and perfect ($\kappa=1.0$) (Estrade and Sahli, 2014). All statistical analyses were performed using Stata 16 (StataCorp, Texas).

Results

Study population

The study analysed 115 cervical samples in PreservCyt, with 7 samples unavailable for the MP96 matched extraction due to insufficient DNA. All samples were detected for the Anyplex28 internal control (appendix 1).

HPV genotype frequency for each DNA extraction method

Overall, the HPV genotype frequency (according to the total number of genotypes detected) was not greatly affected by DNA extraction methods, with a similar overall frequency determined for each HPV genotype detected (Figure 1). The greatest discrepancies within the oncogenic genotypes were HPV39 with a 2.9% difference in overall frequency between Laboratory standard (4.7%) and STARlet (7.6%). The Low risk genotypes also had discrepant frequencies in HPV53 (3% discrepant) and HPV54 (3.2% discrepant) (Figure 1). Further interpretation of these anomalies was not performed due to low sample numbers.

The most prevalent HPV genotypes detected per individual using the DNA extracted from the STARlet extraction platform were HPV42, 54 and 51 (10%, 9% and 8%, respectively). The most prevalent genotypes detected from the DNA extracted from MP96 extraction platform were HPV42 and 51 (13% and 10% for the matched and 18% and 13% for laboratory standard extraction of HPV genotypes detected respectively) (Sup Figure 1). HPV genotypes 6, 11, 16 and 18 were not detected from DNA extracted by either method.

Matched extraction methods are concordant for HPV genotype detection

The overall agreement for any HPV detection was 89.8% from matched extraction as compared to the STARlet (κ=0.798; p=0.007) (Table 1). Matched DNA was identified to have an additional 10 discordant HPV positive samples, while the DNA extracted on the STARlet was identified to have only one discordant positive sample (Table 1).

Furthermore, the genotype specific signal strength agreement between matched extraction and STARlet was 72.4% (p=0.081) (Table 2). Of the discordant samples, 72.7% (24/33) (not-detected from STARlet DNA and detected from matched extraction) were detected at the lowest signal strength for Anyplex28 ("+"), with 21% (7/33) detected at midrange signal strength for Anyplex28 ("++") and 6% (2/33) detected at the highest signal range for Anyplex28 ("+++") (Table 2).

Comparison of each extraction method to detect different groupings of HPV genotypes

Concordance of results in samples extracted between each system was highest for any nonavalent vaccine-targeted genotypes at 96.3% agreement (κ=0.861; p=0.317), with no statistical difference from either extraction method for detection of vaccine targeted HPV genotypes (Table 3). The agreement for the detection of any oncogenic genotypes, and any non-vaccine targeted oncogenic genotypes, was strong (κ=0.774 and 89.8% agreement, κ=0.772 and 90.74% agreement, respectively), with detection of HPV genotypes in the

matched extracted samples significantly higher than STARlet (p=0.0067 and p=0.0016, respectively). Detection of any HPV genotype and any low risk HPV types also showed strong agreement (κ=0.798, 89.8% agreement and p=0.01, κ=0.852, 93.52% agreement and p=0.008, respectively).

Laboratory standard extraction methods increase HPV genotype detections compared to STARlet

The overall agreement for any HPV detection was 85.2% from the laboratory standard extraction (i.e. five times concentrated DNA) as compared to the STARlet (κ=0.708; p<0.001) (Table 1). Laboratory standard DNA was identified to have an additional 17 discordant HPV positive samples, with no discordant samples positive from the STARlet only.

Furthermore, the genotype specific single strength agreement between laboratory standard extraction and STARlet was 62.1% with more discordant HPV detections compared to the matched extracted samples (Table 2). Of these, 68.8% (44/64) were positive at the lowest signal strength for Anyplex28 ("+"), with 28% (18/64) at mid-range for Anyplex28 ("++") and 3% (2/64) at the highest signal range for Anyplex28 ("+++") (Table 2).

Discussion

In a direct comparison of extraction methodologies for the Seegene Anyplex II HPV28 assay, the MP96 extraction platform performed comparably for the detection of HPV genotypes from cervical specimens in PreservCyt compared to the manufacturer-validated extraction method (STARlet). There was a difference in hands on and platform processing time between the MP96 and STARlet. The MP96 has an onboard processing time of one hour with a further hour for hands on processing time (two hours total for 94 clinical samples). In contrast the STARlet has a total of 45 min hands on and platform processing time (for 88 clinical). The increased processing time from the MP96 is predominantly due to the MP96 method requiring centrifugation of each sample prior to DNA extraction.

Pair-wise analyses of detection for any HPV genotype identified an 89.8% agreement between samples extracted on the STARlet and the matched extraction (i.e. equivalent sample input on the MP96) . The agreement was lower when the laboratory standard extracted samples were used (five times sample input on MP96), presumably due to the increased detection of low abundance genotypes. This validation suggests that the laboratory standard

extraction protocol allows for detection of significantly more HPV DNA compared to the manufacturers validated methodology (STARlet), with 85% of samples showing complete agreement for detection of any HPV genotype between the two extraction systems.

The concordance between extraction methodologies was reflected in the reported signal strength. The majority (25/35) of discordant genotypes on the STARlet were detected at low signal strength ('+'). This suggests that with increased input lower abundance genotypes are more likely to be detected using the Anyplex28 assay, as the highest level of discordance was seen for samples detected late in the PCR cycle. Future studies are warranted to assess the limit of detection for each extraction platform.

The Anyplex28 assay and validated DNA extraction protocol (STARlet) is designed to detect HPV DNA at a sensitivity and specificity threshold to predict grade II cervical intraepithelial neoplasia or greater from female cervical samples. However, HPV DNA detection and genotyping is also vital to monitor the effectiveness of vaccination programs in the community, requiring assays that detect all HPV DNA from a sample (Cornall et al., 2017). The findings presented suggest that the laboratory standard extraction protocol (5 fold increase in sample input) utilising the Anyplex28 genotyping kit could be considered for research where higher analytical sensitivity is desired, such as in studies of HPV vaccine impact and effectiveness. Analysis according to specific genotype groupings show that extraction methods are highly comparable at matched volumes of DNA (equivalent to 200 µl of sample input), specifically in a proportion of the nonavalent HPV vaccine targeted genotypes, which is highly relevant for prevalence studies focusing on these genotypes.

This study has a number of limitations. The extractions and HPV testing were performed at different time points for each methodology: consequently, sample DNA may have degraded between extractions and testing as there was approximately one year between the MP96 and STARlet extractions and another year between the STARlet and matched extraction Anyplex28 PCR detection. Although this delay in DNA extractions may partially explain the difference in the analytical sensitivities, previous studies from stored PreservCyt samples and HPV detection suggest limited DNA degradation would occur over such a timeframe and most likely had limited effect on the findings (Phillips et al., 2016, Agreda et al., 2013). Another limitation was the lack of validated confirmatory assays for discordant genotypes between extraction systems. Signal strength analysis was used to investigate discordant results, which is semiquantitative and should not be used as an indicator of viral load.

Nevertheless, we observed patterns of increasing concordance with increasing signal which support our interpretations. The population cohort utilised (*Chlamydia* screening samples, indicating sexual activity) is potentially a higher risk group than the general population and could result in a higher HPV prevalence. There was an absence in the detection of HPV 6, 11, 16 or 18 DNA in this cohort, presumably due to sustained, high vaccination coverage achieved among young women within Australia or possibly. The total sample size may also have contributed to the lack of HPV 6, 11,16 or 18 DNA detection.

These analyses validate the use of DNA extracted on the automated extraction platform MP96 for genotyping on the Anyplex28 assay, with comparable HPV detection to currently validated extraction methods. Furthermore, DNA extraction from 1ml of PreservCyt sample on the MP96 is a valid method for research-based surveillance studies, with high concordance specifically for vaccine targeted genotypes and higher detection for non-vaccine targeted HPV genotypes. Either machine (STARlet or MP96) could be utilised for vaccine surveillance research. The STARlet extraction platform has been clinically validated for sensitivity and specificity for histologically confirmed Cervical Intraepithelial Neoplasia Grade 2+ (CIN2+). For the MP96 extraction process to be utilised for routine cervical screening a clinical validation would be required.

Declaration

Ethics.

The study was approved by the Melbourne Health Human Research Ethics Committee (HREC number 2017.361).

Consent for publication

Not applicable

Availability of data and materials

All data is presented in the document or appendix material.

Conflict of interest statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: DAM reports travel grants from Seqirus, travel funding and honoraria to her institute from Merck Sharp and Dohme (MSD), outside the submitted work. DH and MS are investigators on the Compass trial for which VCS

Foundation has received kits and partial funding from Roche. VCS Pathology has also received free HPV test kits from Abbott, AusDiagnostics, Atila Biosystems, BD, Cepheid, Hologic, Roche, and Seegene. VCS Foundation has received travel funding for DH to attend conferences and meetings from Roche, Abbott and Seegene but has had no personal gain from any diagnostics manufacturer. SMG and SP were investigators on a cervical cancer typing study with laboratory testing funded by Seqirus more than three years ago. SMG was an investigator on a recurrent respiratory papillomatosis surveillance study partially funded by an investigator-initiated grant from MSD more than three years ago. SMG has received grants to her institution from Merck for an investigator initiated grant studying HPV and young women. She has received speaking fees from MSD for work performed in her personal time and is a member of the Merck Global Advisory board HPV. DAM is an investigator on a genital wart's surveillance grant funded by Seqiris. All other authors declare no conflicts of interest.

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Author contribution statement

The study was conceived by DAM. SA and HS designed the study and performed the data analysis. SA HS and SP drafted the initial manuscript. SP, GM, MM, JD and DH contributed to the study design, analysis and writing of the manuscript. SA, PB, DH performed/supervised DNA extractions and HPV testing. All authors reviewed and contributed to the manuscript.

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Figure 1: Genotype specific frequency (according to the total number of genotypes detected), as detected in DNA extracted by STARlet (manufacturer recommended 200 µL input, 100 µL elution volume), laboratory standard (1 mL input, eluted in 100 µL), and

matched (1/5 dilution of MP96 extracted DNA). Note that HPV genotypes 6, 11, 16, 18, 26, and 69 were not detected in any sample for any extraction method (Black bars represent the Laboratory standard results, dark grey bars represent the Matched sample results, and the light grey bars represent the STARlet sample results).

Table 1: Comparison of detection for any HPV genotype on the HPV28 assay, with DNA extracted by MP96 at neat (n=115) and 1/5 dilution (n=108), and STARlet

		STARlet				
		Negative	**Positive**	***Total***	**Agreement**	κ **(p-value*)**
Matched#	**Negative**	50	1	*51*	89.80%	0.798 (0.007)
(equivalent to	**Positive**	10	47	*57*		

STARlet)						
	Total	*60*	*48*	*108*		
Laboratory standard (5x concentration)	**Negative**	43	0	*43*	85.20%	0.708 (<0.001)
	Positive	17	55	*72*		
	Total	*60*	*55*	*115*		

*McNemar's test

Seven samples had insufficient DNA remaining to be diluted

Table 2: HPV signal strength comparison for all detected HPV genotypes between samples extracted on the MP96 neat and 1/5 diluted and the STARlet

	STARlet -	**STARlet +**	**STARlet ++**	**STARlet +++**	***Total***	**Total agreement**
Matched -	-	1	1	0	*2*	72.40%
Matched +	24	14	0	0	*38*	
Matched ++	7	20	39	0	*66*	
Matched +++	2	0	6	13	*21*	
Total	*33*	*35*	*46*	*13*	*127*	
Laboratory standard -	-	0	0	0	*0*	62.10%
Laboratory standard +	44	3	0	0	*47*	
Laboratory standard ++	18	35	31	0	*84*	
Laboratory standard +++	2	0	22	14	*38*	
Total	*64*	*38*	*53*	*14*	*169*	

Table 3: Comparison between Seegene Anyplex II HPV28 results from the matched extractions and STARlet (n=115)

	HPV grouping				
	Any	**Low risk**	**Oncogenic**	**non-vaccine targeted oncogenic**	**Nonavalent vaccine-targeted‡**
Matched+/STARlet+	47	31	31	25	15
Matched +/STARlet-	10	7	10	10	3
Matched -/STARlet+	1	0	1	0	1
Matched -/STARlet-	50	70	66	73	89
Agreement %	89.80	93.52	89.81	90.74	96.3
κ	0.80	0.85	0.77	0.77	0.86
Interpretation*	s	np	s	s	np
p-value (McNemar)	0.010	0.008	0.007	0.002	0.317

*n= none (κ<0.2), w=weak (κ=0.2–0.4), m=moderate (κ=0.401–0.6), s=strong (κ=0.601–0.8), np=near perfect (κ=0.801–0.99), and p=perfect (κ=1.0) (Estrade & Sahli, 2014)

‡Includes nonavalent vaccine-targeted types HPV 31, 33, 45, 52 and 58 (no HPV 6, 11, 16 or 18 were detected)

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