An evaluation of a novel single tube method for extended genotyping of Human Papillomavirus

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Title: An evaluation of a novel single tube method for extended genotyping of Human Papillomavirus.

Running Title: Evaluation of novel single tube HPV genotyping assay

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Abstract:

Background: The use of high-risk HPV testing for surveillance and clinical applications is increasing globally and it is important that tests are evaluated to ensure they are fit for purpose. In this study, the performance of a new HPV genotyping test -The Papilloplex® HR-HPV test- was compared to two well established genotyping tests. Preliminary clinical performance was also ascertained for the detection of CIN2+ in a disease-enriched retrospective cohort.

Methods: A panel of 500 cervical LBC samples with known clinical outcomes were tested by the Papilloplex® HR-HPV test. Analytical concordance was compared to two assays: Linear Array HPV Genotyping Test and Optiplex HPV Genotyping Test. Initial clinical performance for the detection for CIN2+ samples was performed and compared to that of two clinically validated HPV tests: the RealTime High Risk HPV test and Hybrid Capture 2 HPV Test.

Results: High agreement for HR-HPV was observed between the Papilloplex and LA and Optiplex HPV tests (97% and 95% respectively); with Kappa for HPV 16 and 18 being 0.90 and 0.81 compared to the LA and 0.70 and 0.82 compared to Optiplex. The sensitivity, specificity, PPV and NPV of Papilloplex for detection of CIN2+was 92%, 54%, 33% and 96% respectively and was very similar to that observed with RealTime and HC2.

Conclusion: Papilloplex HR-HPV test shows similar analytical performance to two HPV genotyping tests at the level of HR-HPV and type specific level. Preliminary data on clinical performance look encouraging although further longitudinal studies within screening populations are required to confirm this.

Introduction

The use of high-risk HPV (HR-HPV) testing for the identification of women at risk of developing cervical cancer and for the management of women who have received treatment is increasing globally (1). Additionally, type specific HPV detection methods are valuable both for epidemiological studies and as a triage for primary HR-HPV infection (2). There are now a wide variety of commercially available HPV tests (3) which vary in terms of detection chemistry, complexity, type range, throughput and required equipment. While a component have been clinically validated for use in primary HPV screening through assessment according to internationally accepted criteria, or used extensively in longitudinal research and surveillance endeavours; peer reviewed evidence on the analytical and/or clinical performance of several tests is lacking.

The Papilloplex® HR-HPV test (Genefirst, UK) is a commercially available HPV genotyping test that performs quantitative multiplex detection of 14 HR-HPV types, together with an endogenous human control target, in a single tube (4). Based on Multiplex Probe Amplification (MPA) technology, the assay utilises differing melting curve profiles to allow the differentiation of up to six targets per fluorescence channel within a real-time assay (4). The test is compatible with real-time polymerase chain reaction (PCR) equipment commonly used in clinical and research laboratories and so does not require a specific locked-down platforms.

Here we present results from an evaluation of the Papilloplex HR-HPV assay where its performance is compared to two qualitative, broad spectrum, extended genotyping assays – the Linear Array (LA) HPV Genotyping Test (Roche Molecular Systems Inc., Alameda, CA, USA) and the Optiplex HPV Genotyping Kit (formerly Multiplex HPV Genotyping Kit, DiaMex, Heidelberg, Germany). Preliminary insight into clinical performance of the assay is also presented through its ability to detect CIN2+ in a disease-enriched sample compared to

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two well established clinically validated HPV assays – Hybrid Capture 2 (HC2) HPV DNA Test (Qiagen Gaithersburg, Inc., MD, US) and the RealTime High Risk HPV test (Abbott Molecular, Des Plaines, IL, USA).

Results

Overall HR-HPV positivity in the cohort

The study cohort consisted of 500 Thinprep® liquid based cytology (LBC) samples with known cytology and histology results (Table 1). The sample cohort of 500 was split into two extraction methods (250 extracted using manual QiaAmp DNA mini kit and 250 using automated Nuclisens EasyMag system). The concordance of Papilloplex at overall HR-HPV level and type specific level with LA and Optiplex showed no significant differences based on extraction chemistry (data not shown). The whole study cohort was therefore used for further analysis. Overall HR-HPV positivity for the genotyping tests and the clinically validated tests was similar: 58.4% for Papilloplex, 57.2% for LA, 56.4% for Optiplex, 56.2% for RealTime and 58.6% for HC2 (Table 2).

Agreement between assays

Agreement of overall HR-HPV positivity between Papilloplex and the two extended genotyping tests is shown in Table 3. High proportional agreement of 97% (95% CI- 95-98) was observed between Papilloplex and LA. Similarly, high proportional agreement of 95% (95% CI- 92-97) was observed between Papilloplex and Optiplex.

Type specific concordance(s) between the Papilloplex and the two genotyping assays for HR-HPV types 16,18,31,33,35,39,45,51,52,56,58,59,66 and 68 are shown in Table 4. Two by two tables for each type detected by Papilloplex (vs comparator test) are also presented in Supplementary Data (Table S1). When comparing the Papilloplex to the Optiplex test there

was at least "substantial" agreement (defined according to a kappa of 0.61 to 0.80) for all types except HPV 68 (0.548). The equivalent comparison of Papilloplex to LA showed at least substantial agreement (defined according to a kappa of 0.61 to 0.80) for all types except HPV 68 (0.573) and HPV 59 which at a Kappa of 0.614 was at the lower end of substantial agreement. Papilloplex detected fewer samples as positive for HPV 16 (N=98) compared to both LA (N=108) and Optiplex (N=146). Similarly for HPV 59, Papilloplex detected fewer samples as positive (N=20) compared to LA (N= 73) and Optiplex (N=28) which is reflected in the aforementioned Kappa value. Conversely, Papilloplex detected a higher number of HPV 31 (N=64) infections compared to LA (N=54) and Optiplex (N=40), and a higher number of HPV 33 (N=44) infections vs Optiplex (N=36). Papilloplex also detected a higher number of HPV 56 (N=32) infections compared to LA (N=22) but this was lower than those detected by Optiplex (N=43) (Table S1).

Clinical performance for detection of Cervical intraepithelial neoplasia 2 or worse (CIN2+)

Of the 500 samples in the panel 87 were associated with CIN2+. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the Papilloplex test for the detection of CIN2+ is summarised in Table 5, with values of 92%, 54%, 33% and 96% respectively. These values were similar to the clinical performance of the HC2 and RealTime assays.

Discussion

Papilloplex HR- HPV test is a single tube test for the quantitative multiplex detection of 14 HR-HPV types, together with an endogenous human control target. This study provides the first analytical assessment of the Papilloplex test compared to two commercially available HPV tests that offer extended genotyping capability: LA and Optiplex. Further, to gain

insight into the potential clinical performance of the assay a preliminary evaluation was undertaken to determine its ability to detect CIN2+ in a disease enriched population.

Papilloplex showed high concordance to Optiplex and LA at the level of overall HR-HPV positivity with a proportional agreement of 95-97% and kappa of 0.90- 0.93. Type specific proportional agreement for all 14 HR-HPV types covered by Papilloplex was generally high although there were some type specific differences. Papilloplex showed moderate concordance to LA and Optiplex for HPV 16 and 59, detecting less infections and clearly, HPV 16 is an important type for both epidemiological and clinical applications. On the other hand, Papilloplex detected more HPV 31 infections compared to both comparator genotyping tests. Type specific differences between genotyping tests have been reported previously (5) and such differences are perhaps inevitable given the range of chemistries available. Nevertheless, these data reinforce the notion that for longitudinal surveillance exercises (in which monitoring prevalence and trends of HPV types is important), consistent use of the same test is important to avoid real changes being confounded by test chemistry. Furthermore, it is notable that the clinical performance of the Papilloplex assay was similar to that of two well established clinically validated tests indicating that type-specific differences (including for HPV 16) may not have significant implications for the detection of disease.

This said, we accept that the clinical evaluation performed in this study was preliminary and that the sample used was enriched in nature and did not represent women from a cross section of the screening population. Consequently, the clinical performance observed in this study, will not be representative of performance in a screening population. Nevertheless, determining initial sensitivity (the key measure of performance for screening applications) of a novel HPV test for CIN2+ using a sample with high disease-prevalence has precedent (6, 7) and arguably showing performance relative to that of an assay in which clinical efficacy has been demonstrated also has value, even at an early stage. Furthermore, future clinical

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validation of the test which builds on the present work but involves a longitudinal screening population and assessment according to internationally recognised validation criteria is planned (8, 9).

The variety of HPV tests available with their different scope and capabilities provides users with options to choose the most appropriate test for a particular context and population. Papilloplex HPV is a single-tube assay that identifies 14 HR-HPV types. The ability to perform individual genotyping within a single closed-tube format reduces time and risk of contamination associated with more "open" genotyping systems. The assay is amenable to several DNA extraction chemistries, requires a low amount of input DNA and can be performed with existing real-time 96 well PCR platforms that are available in routine research and clinical laboratories. In terms of analytical performances we have shown that this assay compares favourably to existing more established extended genotyping assays. While initial data on clinical performance is encouraging, further longitudinal assessments will determine its potential use for screening and disease management.

Material and methods

Samples and approvals

A total of 500 liquid based cytology samples (LBCs) were obtained from the Scottish HPV Archive (www.shine.mvm.ed.ac.uk/archive) which is a biobank designed to support HPV Research. The East of Scotland Research Ethics Service has given generic approval to the Scottish HPV Archive as a Research Tissue Bank (REC Ref 11/AL/0174) for HPV related research on archived samples. The Scottish HPV Archive is also registered with National Research Scotland (NRS) Lothian Bioresource. Samples were made available for the present

project through application to the archive steering committee (HPV Archive Application Ref 0016).

The samples used for the study included 473 samples collected from women attending their first routine smear at the age of 20 in Scotland, supplemented by 27 samples from women attending colposcopy clinics due to abnormal cytology (in order to enrich for CIN2+). Routine cytology classification was as per British Society for Clinical Cytology criteria (10). Cytology results were classed as negative (for any abnormality), low grade (borderline squamous changes, koilocytosis, and low grade dyskaryosis) and high grade (which includes moderate and severe dyskaryosis). Subsequent cytology and histology results were obtained through data linkage via Information Services Division, Scotland and samples were classified as 2x cytology negative (with 2 subsequent negative cytology results at least 1 year apart), ≤CIN1 or CIN2+ (Table 1). Samples had originally been collected between 2010 and 2012 and stored in the archive at -80^{o}C.

HPV DNA testing

Samples were retrieved and aliquots prepared for HPV testing with Papilloplex HR-HPV test, HC2, Optiplex HPV genotyping test, LA and RealTime HR-HPV test. Papilloplex test was performed in Genefirst laboratories (Oxford, UK). All other tests were performed at the Scottish HPV Reference Laboratory and HPV Research Group (Edinburgh). All tests were performed according to manufacturer's instructions although a brief description of assay characteristics is provided in Table 2 and a detailed description of the Papilloplex HR-HPV test is provided in the next section. The Optiplex genotyping test has been used for longitudinal immunisation surveillance in Scotland (11–13) and has been adjudicated as proficient for detection of HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 according to the last three consecutive WHO laboratory network (WHO LabNet) HPV DNA

proficiency schemes (when testing was performed in Edinburgh). LA is also associated with good performance on WHO LabNet proficiency panels as outlined in Eklund et al (2014) where it was the most frequently applied assay to the scheme (7).

Papilloplex HPV test

The Papilloplex HR-HPV test was performed on DNA extracted using two different methods. Half the samples were extracted using QiaAmp DNA mini kit (Qiagen,Germany) and half using automated Nuclisens EasyMag system (BioMérieux, France). The method of extraction was randomly allocated to samples.

A total of 2µl of DNA was added to the PCR amplification reaction mix (18µl) containing buffer (dNTPs and Mg2+), master mix (Taq polymerase, UNG enzyme and dUTP) and working mix (primers and probes) to obtain a final volume of 20µl per PCR reaction. The PCR was performed on ABI 7500 Fast Real-Time PCR Systems (Applied Biosystems, Warrington, UK). The thermal profile was set to: Amplification stage 1 (50°C for 2 min, followed by 95°C for 3 min), amplification stage 2 (9 cycles of 95°C for 6 sec, followed by 66°C for 45 sec), and amplification stage 3 (42 cycles of 95°C for 3 sec, followed by 60°C for 33 sec, and 63°C for 15 sec). Fluorescence measurements in the ROX, FAM, HEX (JOE), and CY5 channels were recorded during step 2 of amplification stage 3 (60°C for 33 sec). A pre-set dissociation stage (stage 4) was included following the final PCR cycle of amplification (stage 3). The post-amplification melting profile protocol comprised of 95°C for 15 sec, 25°C for 1 min, 75°C for 15 sec, and 60°C for 15 sec. The fluorescence emission data was continually collected during the temperature increase. The negative derivative of the emission reading, with respect to temperature, was plotted against the temperature to form melting curves (per fluorescent channel) generated during the dissociation stage of the reaction (from 25°C to 75°C).

For consistency between experiments, the following threshold values for Ct determination were set (ROX: 100,000; FAM: 100,000; HEX: 25,000 and CY5: 50,000). For each sample, the internal control (CY5 detection channel) and all fourteen HR-HPV types, corresponding to the ROX (HR-HPV types: 33, 35, 45, 51, 56, and 66), FAM (HR-HPV types: 16, 18, 31, 52, and 59) and HEX channel (HR-HPV types: 39, 58, 68) were simultaneously evaluated. Samples were considered positive for HR-HPV DNA types if a Ct value was < 38 for cellular DNA and < 36 in any of the ROX, FAM and HEX fluorescent channels. A sample was considered invalid if the Ct value of cellular DNA was >38. The change in the characteristic melting profile(s) in the sample was compared to the negative control reference melting profile to identify the genotypes present. Samples were tested in batches of 96 samples (including controls) per reaction.

Analysis

HR-HPV concordance of the Papilloplex with comparator tests

Type specific positivity for each HR-HPV type included in Papilloplex was compared to the Optiplex and LA. Concordance, proportional agreement with accompanying 95% confidence intervals (CI) have been presented along with kappa statistics and McNemar's test. The Papilloplex was also compared to the above tests at the level of HR-HPV positivity (for the types covered by Papilloplex only).

Assessment of preliminary clinical performance

Clinical performance of the Papilloplex test was measured as sensitivity, specificity, positive predictive value and negative predictive value for the detection of cervical CIN2+ with 95% CI's around the percentages. The clinical performance of the HC2 and RealTime HPV test was also performed and presented alongside the Papilloplex results. Disease cases were defined as CIN2+ (n=87), whereas no disease was defined as histologically confirmed CIN1 or less or a sample being associated with two consecutive negative cytology results at least 1 year apart (n=349). Pathology data was incomplete to allow this categorisation for 64/500 samples so clinical performance assessment was performed on 436 samples.

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298 **Results Tables**

Underlying Cytology	**N (%)**
Negative	266 (53.2)
Low grade dyskaryosis	156 (31.2)
High grade dyskaryosis	66 (13.2)
Unknown	12 (2.4%)
Total	500
Underlying Histology	
No histology performed (2 x Negative cytology)	263 (52.6)
≤CIN1	86 (17.2)
CIN2+	87 (17.4)
Histology information incomplete	64 (12.8)

299 **Table 1: Cervical pathology associated with study population. Note that clinical**

300 **performance assessment was performed on 436 samples. Samples with incomplete**

301 **histology was not included in this analysis.**

Test	Detection technology	High-risk types identified by the test	Low-risk types identified by the test	High-risk positive (N, %)	High-risk + Low-risk positive (N)
Papilloplex HR-HPV test	Real-time PCR with individual genotyping	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68		292 (58.4%)	
RealTime HR-HPV test	Real-time PCR with partial genotyping	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68		281 (56.2%)	
Hybrid Capture 2 (HC2)	Target amplification followed by Sandwich capture assay	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 [not 66]		293 (58.6%)	
Linear Array HPV Genotyping test	Target amplification followed by hybridisation	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, CP6108	286 (57.2%)	340 (68.0%)
Optiplex HPV genotyping test	Target amplification followed by luminex detection	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68	6, 11, 26, 42, 43, 44, 53, 70, 73, 82	282 (56.4%)	321 (64.2%)

Table 2: Description of assays used in the study with the detection technology, types covered and prevalence of HPV in the study population.

Linear Array (LA) HPV Genotyping test						
		NEG	POS	Proportional agreement	Kappa	McNemars test: p-value
Papilloplex HR-HPV test	NEG	203	5	97% (95, 98)	0.934	0.210
	POS	11	281			
Optiplex HPV genotyping test						
		NEG	POS	Proportional agreement	Kappa	McNemars test: p-value
Papilloplex HR-HPV test	NEG	200	8	95% (92, 97)	0.894	0.076
	POS	18	274			

311 **Table 3: Overall agreement between Papilloplex HR-HPV test and comparator tests.**

312 **Concordance between the samples are indicated and proportional agreement with 95%**

313 **CI (in brackets), kappa and McNemar's test p-value are listed.**

314

	HPV type	Optiplex HPV test	Linear array HPV test	HPV type	Optiplex HPV test	Linear array HPV test
Proportional agreement	**16**	89% (86, 91)	97% (95, 98)	**51**	98% (96, 99)	98% (97,99)
Kappa		0.7	0.902		0.879	0.914
McNemars test: p-value		<0.001	0.021		1	0.727
Proportional agreement	**18**	97% (95, 98)	97% (95, 98)	**52**	96% (94, 97)	*
Kappa		0.822	0.809		0.811	*
McNemars test: p-value		0.286	0.077		0.664	*
Proportional agreement	**31**	95% (93, 97)	97% (95, 98)	**56**	97% (95, 98)	98% (96, 99)
Kappa		0.744	0.846		0.784	0.805
McNemars test: p-value		<0.001	0.021		0.007	0.002
Proportional agreement	**33**	98% (97, 99)	99% (97, 99)	**58**	98% (96, 99)	98% (97, 99)
Kappa		0.966	0.91		0.811	0.886
McNemars test: p-value		0.008	0.453		0.146	0.727
Proportional agreement	**35**	99% (98, 100)	100% (99, 100)	**59**	98% (96, 99)	95% (93, 97)
Kappa		0.774	0.907		0.738	0.614
McNemars test: p-value		0.125	1		0.039	<0.001
Proportional agreement	**39**	97% (96, 99)	98% (96, 99)	**66**	99% (97, 100)	99% (97, 99)
Kappa		0.851	0.937		0.915	0.908
McNemars test: p-value		0.774	0.388		1	0.016
Proportional agreement	**45**	99% (96, 99)	99% (98, 100)	**68**	98% (97, 99)	98% (96, 99)
Kappa		0.867	0.924		0.548	0.573
McNemars test: p-value		1	1		0.07	1

Table 4: Type specific agreement of Papilloplex with Optiplex and Linear array (LA) HPV tests. Proportional agreement with 95% CI (in brackets), kappa and McNemar's test p-value are indicated. *- Linear Array (LA) is unable to identify HPV-52 status in samples also positive for HPV33, HPV35, and/or HPV58. Results for HPV-52 is therefore not presented.

	Papilloplex HR-HPV test	Hybrid Capture 2 (HC2)	RealTime HR-HPV test
Sensitivity	92% (84, 97)	91% (83, 96)	91% (83, 96)
Specificity	54% (48, 59)	54% (48, 59)	56% (50, 61)
PPV	33% (27, 39)	33% (27, 39)	34% (28, 40)
NPV	96% (93, 99)	96% (92, 98)	96% (92, 98)

320 **Table 5: Clinical performance of HPV tests for detection of CIN2+. Sensitivity,**

321 **Specificity, Positive predictive value (PPV) and Negative Predictive value (NPV) along**

322 **with 95% CI (in brackets) are indicated.**