Papilloplex HR-HPV test has non-inferior clinical performance for detection of human papillomavirus infection: assessment using the VALGENT framework

Ramya Bhatia 1,2, Elia Alcaniz Boada, Jesper Hansen Bonde, Wim G V Quint, Lan Xu, Ditte Moller Ejegod, Kate Cuschieri, Marc Arbyn

ABSTRACT

Aim The Papilloplex high-risk human papillomavirus (hrHPV) test (Genefirst, Oxford, UK) is a single tube real-time HPV test which provides multiplex detection and separate identification of 14 hrHPV types. Here, we present the clinical validation of the test in SurePath samples in comparison to a clinically validated reference test, the GP5+/6+Enzyme ImmunoAssay (GP5+/6+EIA) using the VALGENT (VALidation of HPV GENotyping Tests) framework.

Methods Clinical performance was assessed using 998 unsel ected, cervical screening samples enriched with 297 cytologically abnormal specimens (100 atypical squamous cells of unspecified significance, 100 low-grade squamous intraepithelial lesions, 97 high-grade squamous intraepithelial lesions). Cases were defined as women diagnosed with histologically confirmed cervical intraepithelial neoplasia two or more (≥CIN2, N=119) and controls defined as women with two subsequent negative cytology results (N=834).

Results The Papilloplex HR-HPV test has non-inferior sensitivity for detection of cervical precancer (p=0.0001 for ≥CIN2 and p=0.0005 for ≥CIN3) and non-inferior specificity, compared with GPS+6+EIA (p=0.0167). The assay also showed excellent or good agreement for overall hrHPV and nearly all individual HPV types as compared with GPS+6+EIA/Luminex.

Conclusion The Papilloplex HR-HPV applied on cervical specimens stored in SurePath medium fulfills the international clinical accuracy criteria for use in cervical cancer screening.

INTRODUCTION

Detection of high-risk human papillomavirus (hrHPV) in cervical specimens is becoming the mainstay test used in cervical screening because it is more consistent and effective than cervical cytology 1,2 and visual inspection. 3 Currently, there are over 200 different assays that detect nucleic acids of HPV, but relatively few are clinically validated. 4-6 Only clinically validated hrHPV, that assure an optimal balance in sensitivity and specificity for detecting cervical precancer should be used in screening. 7-9

The Papilloplex HR-HPV test (Genefirst, Oxford, UK) is a single tube real-time HPV test which provides multiplex detection and separate identification of 14 hrHPV types 10 and is more consistent and effective than cervical cytology. 4 Only clinically validated hrHPV, that assure an optimal balance in sensitivity and specificity for detecting cervical precancer should be used in screening. 7-9

The Papilloplex HR-HPV test has non-inferior clinical performance for detection of human papillomavirus infection: assessment using the VALGENT framework. The assay is based on multiplex probe amplification (MPA) technology and uses differing melting-curve profiles to allow the differentiation of up to six targets per fluorescence channel of a real-time PCR reaction allowing for extended genotyping information.

The assay has previously shown good analytical sensitivity and specificity and reproducibility for the 14 hrHPV types. 10 Preliminary clinical performance studies, using archived cervical Thinprep samples, demonstrated similar performance of Papilloplex for detection of cervical intraepithelial neoplasia grade 2 (≥CIN2) to that of two clinically validated HPV tests: RealTime hrHPV test (RealTime) and Hybrid Capture 2 HPV test (HC2), along with high agreement for HR-HPV and individual genotypes when compared with Linear Array (Roche) and Optiplex HPV test (Diamex). 11 However, while encouraging, the data described above derived using a convenience sample rather than on a well-defined set of specimens that would enable clinical validation according to internationally recognised criteria.

To address this, the clinical performance of the Papilloplex HR-HPV test was assessed according to the international guidelines of Meijer et al 12 using the VALidation of HPV GENotyping Tests (VALGENT) framework. 13 Other among other things, VALGENT provides a ‘fast track’ to validation by the international guidelines by using disease-enriched sample panels, previously characterised for HPV status and cytopathological information. A total of four iterations of VALGENT have been performed to date. Valgent 2 and 3 (V2–V3) supported assay evaluations in ThinPrep media (Hologic, Bedford, Massachusetts, USA) 15 while the first and the fourth iteration supported assay evaluations in SurePath (Becton Dickinson (BD), USA). 24

Papilloplex is part of the fourth iteration of VALGENT, 14 comprising eleven different HPV genotyping assays from eight different manufacturers, using GP5+/6+PCR enzyme immunoassay (EIA) as comparator 14 and SurePath collected cervical screening samples from an organised screening programme. Assays interrogated include those with extended genotyping capability such as Onclarity HPV assay (Becton Dickinson, New Jersey, USA), 15 limited genotyping such as Cobas 4800 HPV assay (Roche, Basel, USA), 16 Harmonia HPV (Liferiver, Shanghai, China), 17 HPV-risk
assay (Self-Screen BV, Amsterdam, The Netherlands) and those with full genotyping abilities—CLART HPV4 assay (Genomica, Madrid, Spain), HPV MassArray assay (Agena Bioscience, Hamburg, Germany), INNO-LiPA Genotyping Extra II test (FujireBio Europe, Ghent, Belgium), Venus HPV (Liferiver, Shanghai, China) and Papilloplex HPV assay (Genefirst, Oxford, UK). The Papilloplex HPV assay is validated analytically on different extraction systems (including NucliSENS easyMAG and QIAamp DNA Mini Kit) and on a commonly used PCR machine (Applied Biosystems 7500) and provides individual genotyping results with no post-PCR steps.

In the current paper, we further explore clinical performance by determining whether its accuracy to detect cervical precancer is non inferior to a standard, clinically validated comparator HPV test (HPV GP5+/6+ PCR EIA).

MATERIALS AND METHODS

Study population and processing

The VALGENT4 panel consists of 998 unselected, consecutive routine samples collected from women aged 30–59 from the Danish cervical cancer screening programme enriched with 297 cytologically abnormal specimens (100 atypical squamous cells of unspecified significance, 100 low-grade squamous intraepithelial lesions, 97 high-grade squamous intraepithelial lesions). A detailed description of the study population, sample collection and processing can be found in Bonde et al. Samples were collected at the Molecular Pathology Laboratory, Department of Pathology, Copenhagen University Hospital, Hvidovre and collected at the Molecular Pathology Laboratory, Department of Pathology, University of Edinburgh, UK, to perform Papilloplex testing and for IC and positive if the CT value was ≤36 for the HPV channel. Samples were deemed invalid if IC CT value was >38 and no HPV type was detected in the sample. Any samples that were invalid were repeated once. No HPV type was detected in the sample. Any samples that were invalid were repeated once.

Screening and outcome history (average follow-up of 33 months (min 32, max 35 months)) was retrieved from the Danish Pathology Data Bank (PatoBank). All clinical follow-up was managed according to Danish guidelines, and the result of the VALGENT HPV test evaluations did not affect clinical follow-up recommendations. Samples associated with ≥CIN2 or worse were considered ‘cases’ and used for the evaluation of sensitivity.

In total, 122 cases with precancer were detected in VALGENT-4 which included 83 with CIN3 or worse (≥CIN3). Controls were defined based on two consecutive negative cytology results (at enrolment and at 12–24 months before enrolment) and used for specificity assessment (2x negative for intraepithelial lesion or malignancy). The McNemar test was applied to assess differences between matched non-inferiority (p<0.05) test was performed according to the instructions for use (IFU-P0111v3.0, May 2017). In brief, 4 µL of sample DNA was added to a total of 20 µL reaction mix. PCR was performed using the ABI7500 Real Time PCR System and amplification analysis using Applied Biosystems Sequence Detection Software V1.4–7500 Fast System SDS software. Baseline settings for the assay were modified from the recommended IFU settings on discussion with the manufacturer to relative fluorescence values for different channels set as follows—Cy5-10 000, FAM-40 000, Hex-10 000 and ROX-40 000 (IFU recommendation was Cy5-50, 000, FAM-100 000, Hex-25 000 and ROX-100 000). Melting profile analysis and report creation was carried out using GeneFirst MPA Analysis Software, version 0.5. The PCR protocol takes approximately 2.5 hours. Testing was performed in batches along with kit controls. Baseline and threshold for the real time amplification curves were standardised between runs.

FAM- HPV16, 18, 31, 32, 59; HEX-39, 58, 68; ROX-33, 35, 45, 51, 56, 66; Cy5-IC.

Testing was performed according to the instructions for use (IFU-P0111v3.0, May 2017). In brief, 4 µL of sample DNA was added to a total of 20 µL reaction mix. PCR was performed using the ABI7500 Real Time PCR System and amplification analysis using Applied Biosystems Sequence Detection Software V1.4–7500 Fast System SDS software. Baseline settings for the assay were modified from the recommended IFU settings on discussion with the manufacturer to relative fluorescence values for different channels set as follows—Cy5-10 000, FAM-40 000, Hex-10 000 and ROX-40 000 (IFU recommendation was Cy5-50, 000, FAM-100 000, Hex-25 000 and ROX-100 000). Melting profile analysis and report creation was carried out using GeneFirst MPA Analysis Software, version 0.5. The PCR protocol takes approximately 2.5 hours. Testing was performed in batches along with kit controls. Baseline and threshold for the real time amplification curves were standardised between runs.

Samples were considered valid if a CT value of ≤38 was obtained for IC and positive if the CT value was ≤36 for the HPV channels. Samples were deemed invalid if IC CT value was >38 and no HPV type was detected in the sample. Any samples that were invalid were repeated once.

Papilloplex HR-HPV test
Papilloplex HR-HPV test (Genefirst, Oxford, UK) is a CE marked multiplex real time PCR test targeting the L1 region for the detection of 14 hrHPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) individually. The test includes an internal control (IC) targeting ARHGEF11 gene. The targets covered by different fluorochrome channels are as follows:

For assessment of sensitivity: 119 CIN2+ among which 82 ≥CIN3. For assessment of specificity: 824 women with two subsequent NILM results.

Figure 1 Flow chart representing the study population. A total of 119 ≥CIN2 and 82 ≥CIN3 samples were analysed. ASC-US, atypical squamous cells of unspecified significance; CIN2, cervical intraepithelial neoplasia grade 2; HSIL, high-grade squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; NILM, negative for intraepithelial lesion or malignancy.
at the Unit of Cancer Epidemiology (Sciensano, Belgian Cancer Centre) in Brussels, Belgium.

RESULTS
Hpv prevalence

All samples (n=1295) tested were valid for GP5+/6+EIA but 77 (5.9%) samples were invalid for Papilloplex as defined by a cut-off of ≥38 for IC with no HPV type detected (figure 1). HrHPV prevalence with the Papilloplex test in the screening cohort (n=927) was 15.1% (95% CI: 12.9% to 17.6%), while prevalence of the comparator assay, GP5+/6+EIA was 14.3%. Prevalence of individual HPV types detected by Papilloplex test ranged from 0.2% (HPV35) to 2.59% (HPV45) (table 1).

Overall hrHPV and type specific agreement between Papilloplex HR-HPV and GP5+/6+EIA and GP5+/6+ Luminex, respectively

Table 2 shows the overall concordance for hrHPV (14 hrHPV types) between Papilloplex, HPV-HPV GP5+/6+EIA and the type specific concordance between Papilloplex HPV-HPV GP5+/6+Luminex). Overall concordance of the two assays for 14 hrHPV types was 92.4% (k=0.727), when compared with GP5+/6+EIA and 92.1% (0.823) when compared with GP5+/6+ (Luminex) LMNX, indicating excellent agreement. The level of agreement was also excellent for detection of HPV16 (k=0.867), HPV31 (k=0.903), HPV33 (k=0.809), HPV39 (k=1.000), HPV45 (k=0.802) and HPV66 (k=0.811). For all other HPV types the level of agreement was good except HPV59 which had moderate agreement between the two assays.

Absolute sensitivity and specificity of Papilloplex HR-HPV test
Papilloplex detected 114 of 119≥CIN2 cases (95.8%, 95% CI: 0.91% to 0.99%) and 80 of the 82≥CIN3 cases (97.6%, 95% CI: 0.92% to 1.00%). Absolute specificity for the 2x NIML cohort for ≤CIN1 was 89.1% (95% CI: 0.87% to 0.91%).

Sensitivity and specificity of Papilloplex HR-HPV test when compared with GP5+/6+EIA
Relative sensitivity and specificity can be seen in table 3. Papilloplex HR-HPV test has non-inferior sensitivity and specificity to GP5+/6+EIA for detection of ≥CIN2 and ≥CIN3 (p=0.0001 for ≥CIN2 and p=0.0005 for ≥CIN3). Of the 119≥CIN2 cases, Papilloplex detected 114 and GP5+/6+EIA detected 112 cases. Of the 82≥CIN3 cases, Papilloplex identified 80 cases while GP5+/6+EIA detected 78 cases. The specificity of Papilloplex was also non-inferior to GP5+/6+EIA (relative specificity: 1.003 (95% CI: 0.98 to 1.02, p=0.0167)).

DISCUSSION

Papilloplex HR-HPV assay is a PCR based HPV test that detects 14 hrHPVs individually. Based on a simple and rapid (<3 hours) real-time PCR system, the assay is validated on different extraction systems (including NucliSENS easyMAG and QiAamp DNA Mini Kit) and on a commonly used PCR machine (Applied Biosystems 7500) offering flexibility to laboratories using the assay. The ability to provide individual genotyping within a single closed tube assay offers advantages over other genotyping assays in reducing reaction times, number of steps required for assay setup and in reducing contamination related to multiple reactions. The assay was set up manually in this study and analysis was performed using two softwares. However, work around automation of the setup of PCR reactions and streamlining data analysis which will improve the throughput and ease of use of the assay for large scale screening is currently being undertaken by the manufacturer.

In this study, we had an invalidity rate, where samples were IC negative (CT value >38) and negative for HR-HPV types (CT value >38), of 5.8%. This is substantially above what has recently been reported for HPV-based cervical screening. Increasing the IC CT value cut-off to ≥39, ≥40 or ≥41 reduced the invalidity rate to...
In conclusion, the Papilloplex HR-HPV test is a simple, rapid test with high clinical sensitivity and specificity for detection of ≥CIN2 and ≥CIN3. The test fulfills the international clinical accuracy criteria for use in cervical cancer screening on SurePath samples. As a cross-platform PCR based test with full genotyping, Papilloplex HR-HPV has promise for both cervical screening and epidemiological workflows. Further data on the use of the test in clinical settings and in the widely used ThinPrep PreservCyt medium along with intralaboratory and interlaboratory reproducibility of Papilloplex HR-HPV of the assay is warranted.

Handling editor Runjan Chetty.

Competing interests KC, RB and EAB’s institution has received research funding or grants cerebromedician to support research from the following commercial entities in the last 3 years: Cepheid, Euroimmun, GeneFirst, SelfScreen, Hiantis, Seegeen, Roche, Abbott and Hologic. MA and LX are supported by the RISC Network (Grant No. 847845), coordinated by the Free University of Amsterdam (The Netherlands), and the SME Instrument Grant GA 806551 (HPV OncoPredict), funded by the Horizon 2020 Programme of DG Research and Innovation, European Commission (Brussels, Belgium). Sciensano, the institute where MA and LX are employed, received support from Becton Dickinson, Genomics SAU, Agena Biotech GmbH, Zhanghai Biotech (LifeRiver), GeneFirst and FujiRebio for methodological and statistical work as described in the VALGENT protocol (Arbyn et al, J Clin Virol 2016). VALGENT is a researcher induced study protocol where manufacturers can participate under the condition of providing test kits and covering research costs. Researchers do not receive any financial advantage by collaborating in VALGENT.

Patient consent for publication Not applicable.

Ethics approval The study was approved by the Danish Data Inspection Agency J. No. AHV-2017-024, J-Suite: 05356.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available on reasonable request.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/.

ORCID iD Ramya Bhatia http://orcid.org/0000-0001-5294-0668

REFERENCES
1. Ronco G, Diller JM, Elfstrom KM, et al. Efficacy of HPV-based screening for prevention of invasive cervical cancer: follow-up of four European randomised controlled trials. Lancet 2014;383:524–32.
2. Arbyn M, Ronco G, Anttila A, et al. Evidence regarding human papillomavirus testing in secondary prevention of cervical cancer. Vaccine 2012;30 Suppl 5:F88–99.
3. Sankaranarayanan R, Nene BM, Shastri SS, et al. HPV screening for cervical cancer in rural India. N Engl J Med 2009;360:1385–94.
4. Arbyn M, Simon M, Peeters E, et al. 2020 list of human papillomavirus assays suitable for primary cervical cancer screening. Clin Microbiol Infect 2021;27:1083–95.
5. Arbyn M, Snijders PJF, Meijer CJLM, et al. Which high-risk HPV assays fulfill criteria for use in primary cervical cancer screening? Clin Microbiol Infect 2015;21:817–26.
6. Poljak M, Kocjan BJ, Ostrbenk A, et al. Commercially available molecular tests for human papillomaviruses (HPV): 2015 update. J Clin Virol 2016;76 Suppl 1:13–13.
7. download.pdf [Internet]. Available: https://www.fda.gov/media/122799/download [Accessed 09 Mar 2021].
8. Arbyn M, Anttila A, Jordan J, et al. European Guidelines for Quality Assurance in Cervical Cancer Screening. Second edition—summary document. Ann Oncol 2010;21:448–58.
Original research

9  Fu G, Miles A, Alphey L. Multiplex detection and SNP genotyping in a single fluorescence channel. *PloS One* 2012;7:e30340.

10  Sakellariou GK, Bilski M, Moreau M, et al. Principles and analytical performance of Papillomplex® HR-HPV, a new commercial CE-IVD molecular diagnostic test for the detection of high-risk HPV genotypes. *Diagn Microbiol Infect Dis* 2019;95:46–54.

11  Bhatia R, Serrano I, Wennington H, et al. Evaluation of a novel single-tube method for extended genotyping of human papillomavirus. *J Clin Microbiol* 2018;56:e01687–17.

12  Meijer CJLM, Berkhof J, Castle PE, et al. Guidelines for human papillomavirus DNA test requirements for primary cervical cancer screening in women 30 years and older. *Int J Cancer* 2009;124:516–20.

13  Arbyn M, Depuydt C, Benoy I, et al. VALGENT: a protocol for clinical validation of human papillomavirus assays. *J Clin Virol* 2016;76 Suppl 1:S14–21.

14  Bonde J, Ejegod DM, Cuschieri K, et al. The Valgent4 protocol: robust analytical and clinical validation of 11 HPV assays with genotyping on cervical samples collected in SurePath medium. *J Clin Virol* 2018;108:64–71.

15  Cuschieri K, Geraets DT, Moore C, et al. Clinical and analytical performance of the Onclarity HPV assay using the VALGENT framework. *J Clin Microbiol* 2015;53:3272–9.

16  Heideman DAM, Xu L, Hesselink AT, et al. Clinical performance of the HPV-Risk assay on cervical samples in SurePath medium using the VALGENT-4 panel. *J Clin Virol* 2019;121:104201.

17  Xu L, Oštrbenk A, Poljak M, et al. Assessment of the Roche linear array HPV genotyping test within the VALGENT framework. *J Clin Virol* 2018;98:37–42.

18  Xu L, Benoy I, Cuschieri K, et al. Accuracy of genotyping for HPV16 and 18 to triage women with low-grade squamous intraepithelial lesions: a pooled analysis of VALGENT studies. *Expert Rev Mol Diagn* 2019;19:543–51.

19  Xu L, Oštrbenk A, Poljak M, et al. Clinical and analytical evaluation of the Anyplex II HPV HR detection assay within the VALGENT-3 framework. *J Clin Microbiol* In Press 2018;56:e01176–18.

20  Oštrbenk A, Xu L, Arbyn M, et al. Clinical and analytical evaluation of the Anyplex II HPV HR detection assay within the VALGENT-3 framework. *J Clin Microbiol*. In Press 2018;56:e01176–18.

21  Xu L, Padalko E, Oštrbenk A, et al. Clinical Evaluation of INNO-LIPA HPV Genotyping EXTRA II Assay Using the VALGENT Framework. *Int J Mol Sci* 2018;19:2704.

22  Xu L, Oštrbenk A, Poljak M, et al. Assessment of the Roche linear array HPV genotyping test within the VALGENT framework. *J Clin Virol* 2018;98:37–42.

23  Xu L, Benoy I, Cuschieri K, et al. Accuracy of genotyping for HPV16 and 18 to triage women with low-grade squamous intraepithelial lesions: a pooled analysis of VALGENT studies. *Expert Rev Mol Diagn* 2019;19:543–51.

24  Bhatia R, Boada EA, Bonde J, et al. Evaluation of HarmoniaHPV test for detection of clinically significant human papillomavirus infection using the VALGENT framework. *J Virol Methods* 2021;294:114161.

25  Heideman DAM, Hesselink AT, Berkhof J, et al. Clinical validation of full genotyping CLART® HPV45 assay on SurePath and ThinPrep collected screening samples according to the International guidelines for human papillomavirus test requirements for cervical screening. *BMC Cancer* 2020;20:396.

26  Bhatia R, Boada EA, Bonde J, et al. Evaluation of HarmoniaHPV test for detection of clinically significant human papillomavirus infection using the VALGENT framework. *J Virol Methods* 2021;294:114161.

27  Heideman DAM, Hesselink AT, Berkhof J, et al. Clinical validation of the COBAS 4800 HPV test for cervical screening purposes. *J Clin Microbiol* 2011;49:3983–5.

28  Tang N-S, Tang M-L, Chan ISF. On tests of equivalence via non-unity relative risk for matched-pair design. *Stat Med* 2003;22:1217–33.

29  Cuschieri K, Wilkon A, Palmer T, et al. The challenges of defining sample adequacy in an era of HPV based cervical screening. *J Clin Virol* 2021;137:104756.