LINEAR ARRAY Human Papillomavirus Genotyping Test Amenable to Automation by Implementation of GT-Blot 48 – A Pilot Study

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Abstract: *Objectives*: Persistent infection with high-risk human papillomavirus is associated with the development of cervical intraepithelial neoplasia and a key factor in progression to invasive cancer. The Linear Array HPV genotyping test (LA) is a valuable diagnostic tool enabling type-specific detection of DNA from 37 anogenital high- and low-risk HPV genotypes. Its value, however, is hampered by the underlying labour-intensive manual line blot technology. This study was designed to improve the suitability of LA in routine use by implementing the GT-Blot 48 instrument as an alternative instead of using the proposed manual detection protocol.

Methods: Automation of LA processing was evaluated by comparing GT-blot 48 processed strips to the manufacturer recommended manual protocol. For type-specificity we compared the performance of GT-blot 48 processed strips with that of PapilloCheck, another PCR-based HPV typing assay. Furthermore, the performance of HPV DNA detection by GT-blot 48 processed strips to that by Hybrid Capture 2 (hr-HC2) assay was compared, using samples from 122 women attending routine cervical examination.

Results: GT-blot 48 processed strips and the manual method demonstrate equal performance. The overall agreement of HPV prevalence between automated LA and PapilloCheck was 96.6% (kappa = 0.92). A 91.2% concordance was observed between automated LA and hr-HC2 (kappa = 0.82).

Conclusions: Automated LA processing is a valuable alternative to the manual method. Results are highly comparable with those obtained by hr-HC2 and PapilloCheck. Implementation of GT-blot 48 is a reliable easy-to-use format, which offers increased time efficiency and the additional benefit of high-throughput with standardized processing.

Keywords: HPV persistence, cervical cancer.

INTRODUCTION

Although the majority of HPV infections are benign, most often transient and self-limited, it is widely accepted that persistent type-specific HPV infection is a necessary event in cervical cancer precursor lesion and invasion [1-5]. More than 100 different HPV genotypes have been identified and classified as high- or low-risk, based on their association with cervical lesions [6, 7]. The most common clinically important types are the high-risk HPV genotypes (i.e., types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) which can be found in virtually all cervical cancer [8-10].

Sensitive and reliable HPV tests have been well-established in cervical cancer screening like HC2 (Digene Corporation, Gaithersburg, MD) [11-13] and AMPLICOR HPV test (Roche Diagnostics, Mannheim, Germany) [14-16] with the limitation that none of these assays provide information on the specific HPV type present in the patient sample. However, type-specific results provide additional information for clinicians regarding both follow up treatment [17, 18] and vaccination.

At present, two widely used HPV genotyping assays are on the market, among them the SPF_{10}-LiPA HPV genotyping test [19-22] and the Roche HPV LINEAR ARRAY (Roche Diagnostics, Mannheim, Germany) [23, 24]. The LA assay amplifies a fragment from the L1 region of HPV by using a broad-spectrum PCR primer set [25]. Despite its widespread use, however, its value is compromised both by the cumbersome DNA extraction protocol and the labour-intensive manual reverse line blot technology.

For LA the MagNA Pure LC protocol was recently adapted for automated HPV DNA isolation instead of the manual AmpliLute kit (Qiagen, Inc., Valencia, CA) [24-27]. On the basis of these promising results, we sought to automate the back-end detection of LA by implementation of the GT-blot 48 instrument (Bee Robotics Ltd., Cibyn Industrial Estate, Caernarfon, N. Wales, UK). This bench-top instrument is designed for use with line blot strip assay eliminating the need for tedious washing and hybridization steps.

The present study was designed (i) to automate the back-end detection of LA, (ii) to analyze its robustness in the setting of a routine diagnostic laboratory, and (iii) to compare the detection of HPV DNA by GT-blot 48 processed strips to that by hr-HC2 and PapilloCheck (Greiner Bio-One, Frickenhausen, Germany).

MATERIALS AND METHODS

Clinical Specimens

Epithelial specimens from 122 women attending routine cervical examination were collected by DNA Pap HC cervi-

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cal sampler (Digene), suspended in 1ml of ViraPap/Viratype transport medium (Digene) and submitted to the laboratory for HPV detection by hr-HC2. The median age was 36 years, and the range 17 to 63 years. According to previous cytologic results, 34% (n = 41) showed cytological abnormalities; 23% (n = 28) with low-grade squamous intraepithelial lesions (LSILs), 6% (n = 7) with atypical squamous cells (ASCs), and 5% (n = 6) with high-grade squamous intraepithelial lesions (HSILs).

HC2

The FDA-approved HC2 is a signal amplification assay that uses antibody capture of HPV DNA and RNA probe hybrids and chemiluminescence signal detection. The HC2 includes a mixture of probes for the following high-risk (hr) cancer-associated HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). The low-risk probe set for HPV genotypes (types 6, 11, 42, 43, 44) was not used. All samples with a relative light units/cutoff (RLU/CO) ratio of >1.00 were considered positive. The HC2 was performed according to the manufacturer's instructions.

MagNA Pure

Automated sample preparation was done on the MagNA pure (MP) instrument by using the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche Applied Science, Mannheim, Germany), as described by the manufacturer. In brief, total DNA was isolated from 200 µl of a ViraPap/Viratype aliquot by using the MP. A negative control was included in each DNA extraction run to monitor the DNA isolation procedure. DNA was resuspended in 120 µl of PCR-grade H_2O, and stored at -20°C until further processing by LA and PapilloCheck.

LINEAR ARRAY

The LA HPV genotyping test is based on reverse hybridization of amplicons to immobilized membrane-bound probe. HPV DNA was detected in cervical samples by multiplex PCR targeted to the conserved L1 region of the viral genome. Also, the system includes co-amplification of the human ß-globin gene as an internal control. PCR was performed in a final volume of 100 µl with 50 µl of kit supplied master mix and 50 µl of extracted DNA, following the manufacturer's instructions. After amplification, the biotinylated PCR products were denatured immediately by addition of 100 µl NaOH. Stringent hybridization of the denatured amplicons (75 µl) and genotyping using the LA protocol were performed on the GT-Blot 48 as described below. Only samples positive for ß-globin were considered for analysis. LA genotyping strips were interpreted using the HPV reference guide provided by the manufacturer.

GT-Blot 48

The GT-blot 48 is designed for use of DNA STRIP technology-based reverse hybridization kits including the screening of human polymorphisms (e.g. Factor V Leiden or hereditary haemochromatosis) and identification of microbiological organisms (e.g. methicillin-resistant Staphylococcus aureus). The instrument is equipped with a moving arm to both aspirate and dispense reagents across the tray, 7 peristaltic pumps on board, 2 built in pre-heater for warming up reagents prior to being dispensed into the tray, and also with a waste tube. The detection is performed within a 48 well tray, which is held on a heatable platform to ensure uniform temperature control during the different steps of the assay. The minimum number of samples that can be processed during a run is 2 and the maximum is 48 at a time. The GT-blot 48 can be programmed from a PC using the supplied editor software for individual application requirements and downloaded onboard for future use. Stored programs can be selected for use *via* the GT-blot 48 integral keypad. The temperature setting is ambient to 55°C. At the end of the assay the instrument is cleaned with two pre-programmed settings. In addition, the trays should be cleaned with a washing solution (e.g. incubation of 5% SDS for at least 2 hours and subsequently purification of the wells with a brush, followed by a rinse with de-ionised water). The tubing of the instrument should be flushed with 0.5% chloric solution, followed by de-ionised water.

PapilloCheck DNA Chip

The PapilloCheck test kit is intended to be used for the detection and differentiation of 24 types of HPV in clinical specimens (Greiner Bio-One). The assay was performed according to the manufacturer's instructions. The assay principle is based on the detection of the E1 gene from HPV, amplified and fluorescence-labelled (Cy5) in the presence of specific fluorophore-labelled primers. In the same reaction a fragment of the human ADAT1 gene is amplified to monitor DNA extraction and template quality for the PCR. The amplified products are then hybridized to complementary DNA-probes present in replicates of 5 on each array. For one reaction the following components were mixed in a total volume of 25 µl: 19.8 µl PapilloCheck mastermix, 0.2 µl AmpliTaq Gold polymerase (5U/µl; Applied Biosystems, Branchburg, NJ), 5 µl extracted DNA. The amplification steps were 10 min of initial denaturation at 95°C, followed by 40 cycles with 25 s of annealing at 55°C, 45 s of elongation at 72°C, and 30 s of denaturation at 95°C. An additional 2-step cycling followed by 15 cycles with 45 s of annealing/extension at 72°C, and 30 s of denaturation at 95°C. After amplification, 5 µl of the PCR-products were mixed with 30 µl PapilloCheck Hybridization buffer, and 25 µl of the mix transferred into each compartment of the chip. After hybridization at room temperature in a humid atmosphere, the chips were subsequently washed at room temperature in wash solution I for 10 s, at 50°C in wash solution II for 60 s, followed by the final washing step at room temperature for 10 s. The PapilloCheck DNA-chips are scanned with the CheckScanner at excitation wavelengths of 532 and 635 nm as directed by the manufacturer. To control the hybridization reaction, the Cy3-labelled probe in the hybridization buffer will react with the adequate complementary DNA probes spotted on the chip. Analysis and acceptance of individual results were done automatically with the CheckReport software (version 2.0.3).

Reference Samples

A panel of samples from an external quality assurance program, including HPV16, -18, and -6 and a HPV negative control probe (MRC-5 cells) were used as reference (INSTAND, Düsseldorf, Germany).

Assessment of Results

For direct comparison between hr-HC2 and LA only the assay common high-risk HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) have been considered for assessment of the results. Likewise, to compare between LA and PapilloCheck, only the assay common genotypes (types 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 55, 56, 58, 59, 66, 68, 70, 73 and 82) are considered. Assay unique genotypes are presented in Table **1**.

Table 1. Overview of Assay Unique HPV Genotypes

HPV Type	HC2	LA	PapilloCheck
6	X	X	X
11	X	X	X
16	X	X	X
18	X	X	X
26		X	
31	X	X	X
33	X	X	X
35	X	X	X
39	X	X	X
40		X	X
42	X	X	X
43	X		X
44	X		X
45	X	X	X
51	X	X	X
52	X	X	X
53		X	X
54		X	
55		X	X
56	X	X	X
58	X	X	X
59	X	X	X
61		X	
62		X	
64		X	
66		X	X
67		X	
68	X	X	X
69		X	
70		X	X
71		X	
72		X	
73		X	X
81		X	
82		X	X
83		X	
84		X	
IS39		X	
CP6108		X	

Note: X denotes HPV assay unique genotypes.
Boldface denotes high-risk HPV genotypes.

The genotyping results were termed as follows: concordant (both assays showed identical assay common genotypes), compatible (both assays showed at least one or more assay common genotype(s)), and discordant (no assay common genotype similarities).

Statistical Analysis

We compared the agreement between the used methods by calculating kappa statistics and 95% confidence intervals (CIs).

RESULTS

Precision of automated LA processing was determined by performing replicate testing. Within-run and between-run variations were determined using a panel of reference probes containing one HPV-negative sample (MRC-5-cells) and three HPV-positive samples (HPV6, -16, and -18). Within-run and between-run variations were performed in duplicate. The negative control was negative and the positive probes gave the expected band pattern.

Comparison of manual to automated method was done by retesting 20 cervical specimens in a blinded manner. The testing comprised 10 HPV-negative and 10 HPV-positive samples containing 8 single infections with HPV types 16, 18, 31, 39, 42, 53, 56 and 84 and 2 multiple infections with HPV6 plus HPV16 and HPV31 plus HPV35 plus HPV73. We found completely identical results by comparing the automated GT-blot 48 processed strips to the manufacturer recommended manual method.

Next, we compared the type-specific performance of GT-blot 48 processed strips with the PapilloCheck DNA chip, a different HPV genotyping method (Table **2**). To increase the number of HPV types evaluated, PapilloCheck was applied to 18 specimens that tested positive for HPV by hr-HC2 and LA (single and multiple-type) and to 12 randomly selected hr-HC2-negative samples. One sample was excluded from the analysis due to both a negative internal amplification control and negative HPV result by PapilloCheck. The overall agreement of HPV prevalence for the remaining 29 valid samples between the two methods was 96.6% (28/29) of the samples (kappa = 0.92). Of the 18 LA-positive samples with assay common genotypes, 12/18 (67%) were concordant, 5/18 (28%) compatible, and only 1/18 (5%) showed discordant results (Table **3**). The proportion of types detected by LA and PapilloCheck differ, 25 genotypes compared to 22 genotypes, respectively. Importantly, all 5 compatible results were multiple-type infections. In one case two HPV low-risk types were missed by PapilloCheck (HPV53 plus HPV73), and in two cases one high-risk type (HPV18 and HPV51). Conversely, LA failed to detect high-risk type HPV68 in two cases. One sample gave a discrepant result between the two assays. The PapilloCheck did not detect a single-type infection of HPV high-risk (HPV58), which was found by LA. Unfortunately, due to a limitation in sample availability, this result could not be resolved by retesting the sample.

Finally, we evaluated the concordance of HC2 and automated LA in detecting high-risk HPV among 122 unselected specimens representing various degrees of dysplasia (Table **4**). All specimens tested positive for human ß-globin internal control and were considered valid. Of the 122 specimens, 9

Table 2. Analysis of Concordance Between LA and PapilloCheck[a]

LA Result	No. of Samples Tested by PapilloCheck		
	Negative	Positive	Total
Negative	11	0	11
Positive	1	17	18
Total	12	17	29

Note: [a] HPV6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 55, 56, 58, 59, 66, 68, 70, 73, and 82 (common genotypes).

Table 3. HPV Type-Specific Results with LA and PapilloCheck

Case No.	HPV Genotype		Classification
	Linear Array	PapilloCheck	
1	16, 68	16, 68	Concordant
2	18, 51	18	**Compatible**
3	16	16	Concordant
4	16	16	Concordant
5	56	56	Concordant
6	58	-	Discordant
7	31	31	Concordant
8	39	39	Concordant
9	56	56	Concordant
10	16	16, 68	**Compatible**
11	16, 18	16	**Compatible**
12	16	16	Concordant
13	31	31, 68	**Compatible**
14	40, 52, 53, 56, 73	40, 52, 56	**Compatible**
15	70	70	Concordant
16	35	35	Concordant
17	70	70	Concordant
18	16	16	Concordant

Note: Boldface denotes compatible results with assay common genotypes.

were excluded since hr-HC2 was negative and LA detected an assay unique genotype (n = 113). The agreement between the two assays for assay common genotypes was 91.2%, and the kappa value was 0.82 (95% CI, 0.72 to 0.93). Among the 59 hr-HC2 positive samples, 51 were positive by LA, whereas two samples were LA positive but hr-HC2 negative. Seven of eight hr-HC2-only-positive samples were positive for the assay unique low-risk genotypes HPV53, HPV70 and IS39. The most frequent high-risk HPV types detected were HPV16 (n = 15) 12%, HPV51 (n = 9) 7%, and HPV31 (n = 8) 6%.

DISCUSSION AND CONCLUSIONS

Laboratory automation has simplified clinical diagnostics while heightening their degree of standardization, efficiency, and capacity. Our goal was to automate the back-end detection of LA by implementation of GT-blot 48. We have successfully automated LA without any modifications like dispense volumes, incubation time and wash parameters. The hands on time was reduced from 120 to 30 minutes between the manual and automated detection. Based on the testing of 8 patients, the total time to complete LA was 7 h, thereby allowing laboratory to provide the result to the clinician in a timely fashion.

Table 4. Analysis of Concordance Between hr-HC2 and LA[a]

LA Result	No. of Samples Tested by hr-HC2		
	Negative	Positive	Total
Negative	52	8	60
Positive	2	51	53
Total	54	59	113

Note: [a] HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (common genotypes).

We found that GT-blot 48 processed strips were specific and showed 100% concordance with results from the recommended manual protocol. Type-specific performance was evaluated by comparing results of GT-blot 48 processed strips with results from PapilloCheck. Currently, there is no gold standard test available for identification of HPV. To increase the discriminatory power, we choose the PapilloCheck instead of the widely used SPF_{10}-HPV-LiPA system as a reference assay, since the PCR primer of the SPF_{10} testing system and LA bind within the conserved L1 open reading frame. The overall agreement of HPV prevalence between LA and PapilloCheck was 96.6%. Similarly, we found an almost perfect agreement, when type-specific results were classified according to cancer-associated HPV types, with at least one positive carcinogenic HPV type. Both HPV PCR-based typing systems showed different profiles for the detection of HPV genotypes within compatible results. The difference in analytical sensitivity may be due to competition for PCR primers in the presence of multiple HPV types, which is consistent with the findings of other studies [20, 22]. Indeed, all of the compatible results are multiple-type infections. One sample was LA positive but negative by hr-HC2 and PapilloCheck. Interpretation of this result is complicated by several factors. As indicated by the manufacturer, the limit of detection of HPV58 is comparable. On the other hand, contamination is a less likely explanation, given that the negative controls included in each run were found consistently negative and no other HPV58 isolate (a possible source of contamination) was detected during our study. Together with cytological findings, this sample was assumed to be truly positive.

The strength of agreement between hr-HC2 and LA is considered almost perfect (91.2.% agreement; kappa = 0.82). A recent study reported a similar agreement of results (91%; kappa = 0.81) [27]. Possible explanations for the discordant results by hr-HC2 and LA could be related to: (1) stochastic sampling error (i.e. where samples with low HPV DNA concentration will not always allow positive aliquots to be made), and (2) cross-reactivity of HC2 with other HPV types not included in the hr-HC2 assay probe set. It is important to note that 6 of 8 hr-HC2 positive samples were positive for the assay unique low-risk type HPV70 and HPV53. The HC2 cross-reactivity with noncarcinogenic HPV observed here is consistent with previous reports [18, 28].

There are some limitations inherent to our study. First, the analysis consisted of women from the general population

representing a low HPV prevalence. The impact, if any, of a high prevalence population on type-specificity should be further studied. Nevertheless, the obtained HPV genotypes provided some insight into the excellent concordance between the two genotyping methods. Second, cytological data available to us are incomplete and precluded a detailed analysis. A recent study, however, using the LA assay, demonstrated a strong association of increasing severity of cytologic interpretation with more-carcinogenic HPV risk categories [23].

Back-end automation of LA is an effective alternative to the manual detection protocol. Our results indicate the validity of the automated platform. The chief features of the assay are its reduced hands-on technician time and its potential for high-throughput. At present, there is a need for type-specific information to manage viral persistence before precancerous lesions develop into progressive disease [29, 30] and to monitor the risk of replacement with non-vaccine types [31].

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