Original Article

Clinical comparison of two human papillomavirus detection assays: GenoFlow and reverse line blot

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Abstract

Introduction: Human papillomavirus (HPV) infection is typically critical in the oncogenesis of cervical cancer. However, available HPV detection kits differ in their ability and sensitivity to detect various types of HPV, and this variability has led to inconsistencies in the reporting of the geographic prevalence of HPV types, especially in developing countries. Here, we compared results of the recently developed GenoFlow HPV array test, which detects 33 HPV genotypes, to those of the well-established reverse line blot (RLB) assay, which detects 23 HPV types.
Methodology: In total, 608 cervical specimens with cytology results ranging from normal to cancer were collected using an endocervical brush from women attending outpatient clinics in Riyadh, Saudi Arabia.
Results: Sixty-nine specimens (11%) were positive for HPV. HPV genotype detection using the GenoFlow test had a sensitivity of 62% and a specificity of 100%. Overall agreement between the two HPV genotyping methods was 97%, with a concordance rate of 95%. Among the GenoFlow test results, 2% indicated additional HPV types that were not detected in the RLB assay, whereas the GenoFlow test missed 0.3% of the HPV types that were detected by the RLB; however, both tests were in agreement in detecting all major HPV types.
Conclusion: The GenoFlow test was reliable, with results comparable to the RLB test. However, because the GenoFlow test is less labor-intensive and takes less total time (3 hours), it is a promising, affordable alternative to the RLB for HPV diagnosis and screening programs.

Key words: Detection and genotyping; HPV-DNA; sexual transmitted infections; women's health.

Introduction

Human papillomavirus (HPV) is an established etiological factor for cervical cancer. Among the 200 known high- and low-risk types, approximately 15 anogenital types are associated with cervical cancer [1]. For cervical cancer screening, in 2011, the American Cancer Society, the American Society for Colposcopy and Cervical Pathology, and the American Society for Clinical Pathology recommended cervical cytology plus HPV testing. However, 2018 guidelines indicate that women 30-65 years of age can opt to have only a high-risk HPV test for cervical cancer screening [2]. HPV testing is generally preformed using either DNA- or RNA-based molecular biological tests. DNA-based assays, such as polymerase chain reaction (PCR), in situ hybridization, reverse line blot (RLB), and the Hybrid Capture 2, are best for early stage detection [3].

To date, the late gene (L1) region of HPV DNA has been the most commonly used target for HPV detection assays. Indeed, the sequence of the L1 region from different HPV types is sufficiently conserved to be used for primer design [4]. Therefore, most HPV DNA detection assays use this region for primer design. Those techniques include, but are not limited to, the use of PCR primer sets MY09/MY11 and GP5+/GP6+ for detection only and the use of RLB and GenoFlow assays for both detection and genotyping. The RLB assay makes use of the L1 consensus sequence, with a biotin-labeled PCR product hybridized to an array of immobilized oligonucleotide probes to detect 23 HPV types [5]. The more recently emerged GenoFlow HPV array test from Diagcor Bioscience Incorporation Limited also uses the L1 region as a conserved region but detects 33 types of HPV [6]. The PCR products are

hybridized to probes spotted on a membrane by a rapid flow-through hybridization process. The GenoFlow test also has a unique probe called a "universal probe spot" that is capable of detecting HPV genotypes outside the panel as well as some HPV variants that can be further investigated.

Because accurate detection and typing of HPVs is essential in screening and disease management protocols, the present study aimed to investigate the accordance in HPV detection and genotyping between the RLB and the GenoFlow assays in a cohort of women in Saudi Arabia.

Methodology

Ethical approval and participant informed consent

This study was conducted in compliance with the Declaration of Helsinki. The study protocol was approved by the Research Advisory Council (Ethics Committee) at King Faisal Specialist Hospital and Research Centre (KFSHRC; RAC #2130 033). All participants signed a written informed consent form.

Cervical specimen collection

In total, 608 cervical specimens obtained from women attending outpatient clinics at the King Faisal Specialist Hospital and Research Centre (KFSHRC) in Riyadh, Saudi Arabia, were included in this study. The inclusion criteria for participation in the study were women who were married, divorced, or widowed, and the exclusion criteria were women who were pregnant or virgin. We exclude virgins because of religious and cultural constraints.

Cervical specimens were collected using standard techniques with an endocervical brush (i.e., cytobrush) for use in standard Pap testing (PreservCyt, ThinPrep Pap Test; Boxborough, MA, USA) as well as for the HPV detection and genotyping assays. The cervical specimens were examined by a pathologist, consultant, for normal or abnormal cytology, and the stages of abnormal cytology were identified according to the Bethesda Classification [7] as follows: negative for intraepithelial lesion (NIEL), atypical squamous cells of undetermined significance (ASCUS), low-grade squamous intraepithelial lesion (LGSIL), high-grade squamous intraepithelial lesion (HGSIL), and cervical carcinoma. Demographic and clinical data, including age, nationality, marital status, and cervical cytology results, were collected from participating women.

DNA extraction from cervical specimens

Cervical cells were collected from the specimens using centrifugation, and total genomic DNA was extracted using a Gentra Puregene Cell Kit according to the manufacturer's instructions (QIAGEN; Hilden, Germany). The extracted DNA was eluted in 50 µL of RNase/DNase-free water. The quality and the quantity of the extracted DNA were determined using a NanoDrop spectrophotometer (NanoDrop Technologies; Wilmington, DE, USA). The quality of DNA extracted from cervical specimens was determined with β-globin primers. The amplified products were visualized using 1% agarose gels stained with ethidium bromide.

HPV detection

The well-established MY09/MY11 and GP5+/GP6+ primer sets were used to target sequences located within the L1 region. The MY09/MY11 primer set targets a 450–base pair (bp) conserved sequence and was used for the first round of PCR. The GP5+/GP6+ primer set targets a 150-bp sequence within the 450-bp product and was, therefore, used for nested PCR. The internal control was the β-globin gene, the positive controls were HeLa (for HPV 18) and SiHa (for HPV 16) cells, and the negative controls were UltraPure DNase/RNase-free distilled water.

HPV genotyping by RLB hybridization

The HPV genotyping protocol followed was previously published [8]. The oligoprobes (n = 23; aminolink C12) were synthesized as previously described. The high-risk HPV types assessed were 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, and 73, and low-risk HPV types assessed were 6, 11, 40, 42, 44, and 54. Briefly, 23 oligoprobes were spotted on a carboxyl-coated nylon membrane (Biodyne C membrane, 0.45 µm; Pall Corporation, Pensacola, FL, USA). For hybridization, biotinylated PCR products were added to the membrane. Subsequently, the membrane was incubated with an antifluorescein-peroxidase conjugate. HPV genotypes were detected using an enhanced chemiluminescence (ECL) kit. The RLB assay is based on the use of a mini-blotter for spotting in parallel 23 different oligoprobes containing a 5′-amino group on the carboxyl-coated nylon membrane. This is followed by hybridization then incubation of the membrane with an antibiotin conjugate. The positive samples were detected using ECL detection reagents (Amersham; Buckinghamshire, England) and exposure to film (Hyperfilm; Amersham) for 1–10 minutes. The films were developed using standard techniques.

HPV genotyping by GenoFlow HPV array test

The GenoFlow HPV array test (Diagcor Bioscience Incorporation Limited; Hong Kong) is a reverse dot blot assay with the ability to genotype 33 types of HPV. These include 17 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82) and 16 low-risk HPV genotypes (6, 11, 26, 40, 42, 43, 44, 54, 55, 57, 61, 70, 71, 72, 81, and 84). There is also a universal probe for detection of the 33 HPV genotypes as well as out-of-panel HPV genotypes or some HPV variants. The test uses a modified PGMY primer set to amplify the L1 region of HPV. A rapid flow-through process allows for the hybridization of the PCR products to the 33 HPV probe spots and the universal probe spot adhered to the membrane. According to the manufacturer's instructions, for a positive signal or a negative signal to be considered valid, the signal or lack of signal at a probe spot, respectively, also had to be accompanied by a visible signal at the universal probe spot, at the hybridization control spot, and at the amplification control probe spot. The same three spots must also show a visible signal for an HPV of unknown genotype to be considered valid. The GenoFlow assay was optimized in our laboratory in preliminary tests and found to work well in our hands. We used an internal control (the β-globin gene) as well as a negative control (UltraPure DNase/RNase-free distilled water) along with the samples. Positive amplicons were sequenced at the Sequencing Core Facility at KFSHRC using an ABI 3730xL DNA sequencer (Applied Biosystems; Foster City, CA, USA).

Data and statistical analyses

Demographic and clinical characteristics of the participants, including age, religion, marital status, and cervical cytology results were collected. All data collected were stored and analyzed using SAS software, version 9.4. Univariate and descriptive statistics were used to estimate the proportions. Significant associations between HPV status and study variables were assessed using a χ^2 test. Sensitivity and specificity statistical measures (concordant, compatible, and discordant) were used to compare the RLB assay results with those of the GenoFlow assay. The agreement

Table 1. Demographic and clinical characteristics analysis by HPV status.

Characteristic	Patients, % (No.)			Chi square (*P*)
	HPV Positive **11.35% (n = 69)**	**HPV Negative** **88.65% (n = 539)**	**Total** **(n = 608)**	
Age, y				
11–30 (n = 95)	2.30 (14)	13.32 (81)	15.63 (95)	
31–45 (n = 264)	5.43 (33)	37.99 (231)	43.42 (264)	**3.64 (0.30)**
46–60 (n = 209)	2.8 (17)	31.58 (192)	34.38 (209)	
>60 (n = 40)	0.82 (5)	5.76 (35)	6.58 (40)	
Mean (SD)	40.71 (10.62)	43.29 (11.42)	*t* = 1.78 *P* = .075	
Religion				
Muslim (n = 561)	9.57 (58)	83 (503)	88.61 (561)	
Non-Muslim (n = 45)	1.82 (11)	5.61 (34)	11.39 (69)	**8.2 (0.004*)**
Unknown (n = 2)[a]				
Nationality				
Saudi (n = 486)	8.07 (49)	71.99 (437)	80.07 (486)	
Non-Saudi (n = 121)	3.29 (20)	16.64 (101)	19.93 (121)	**3.59 (0.31)**
Unknown (n = 1)[a]				
Marital status				
Married (n =530)	9.74 (59)	77.72 (471)	87.46 (530)	
Divorced (n = 16)	0.50 (3)	2.15 (13)	2.64 (16)	
Widowed (n = 15)	0 (0)	2.48 (15)	2.48 (15)	**15.79 (.0013)***
Single (n = 45)	1.16 (7)	6.27 (38)	7.43 (45)	
Unknown (n = 2)[a]				
Histology grade				
NIEL (n = 552)	10.15 (60)	83.25 (492)	93.40 (552)	
ASCUS (n = 18)	0.51 (3)	2.54 (15)	3.05 (18)	
LGSIL (n = 14)	0.51 (3)	1.86 (11)	2.37 (14)	
HGSIL (n = 4)	0.17 (1)	0.51 (3)	0.68 (4)	**3.1 (0.52)**
Cervical cancer (n = 3)	0	0.51 (3)	0.51 (3)	
Unknown (n = 17)[a]				

ASCUS, atypical squamous cells of undetermined significance; HGSIL, high-grade squamous intraepithelial lesion; HPV, human papillomavirus; LGSIL, low-grade squamous intraepithelial lesion; NIEL, negative for intraepithelial lesion; [a]Unknown data were excluded in the statistical test; *Indicates statistical significance.

between the tests results was assessed by Cohen's kappa statistic. For the statistical analysis comparing the two assays, any negative or undetected (no DNA) results from both assays were excluded from the final analysis. All *P* values reported are 2-sided and were considered to be statistically significant at *P* < 0.05.

Results

Patient demographic and clinical data

In total, 608 cervical specimens were collected from women visiting the outpatient clinics at KFSHRC. The women had a mean (SD) age of 43 (11) years, ranging from 22–80 years. Other demographic characteristics of the cohort and the results of the cytological tests of their cervical specimens by HPV status are given in Table 1. HPV was detected first by PCR targeting the L1 region. Although an association with HPV status was detected for two variables, religion and marital status, no such association was detected for cervical cytology. The distribution of cytology grade by HPV status for the cervical specimens included is shown in Figure 1.
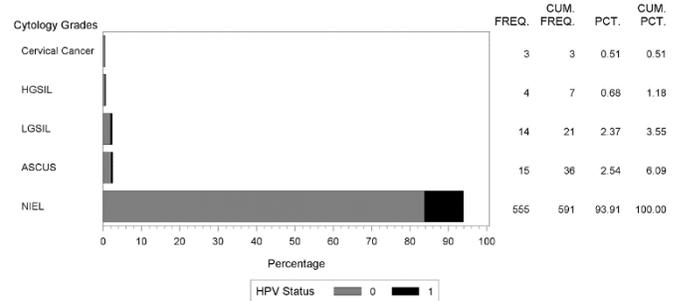
Prevalence and distribution of HPV type in cervical specimens

To compare the results of the RLB and the GenoFlow assays, we ran independent tests on 608 cervical specimens. Figure 2 shows the percentages and frequencies of HPV types detected using the RLB assay by cytology grade. The most frequently detected type was HPV 18 (54%), followed by HPV 16 (18%) and then multiple types of HPV infection (12%). Among specimens positive for HPV 18, approximately 52% had a NIEL (normal) cytology grade, whereas only 2% had an HGSIL grade. Among specimens positive for HPV 16, 16% had a NIEL cytology grade, and 2% had an LGSIL grade. For cervical specimens positive for multiple types of HPV infections, 4% had an ASCUS grade, 4% had a NIEL grade, and 2% had an LGSIL grade. Figure 3 shows the percentages and frequencies of HPV types detected using the GenoFlow test by cytology grade. The most frequently detected type of HPV infection in this assay was from multiple HPV types. Among these, 30% had a NIEL grade, 8% had an ASCUS grade, and 4% had HGSIL and LGSIL grades. Among specimens positive for HPV 18, (21%) had a NIEL grade, whereas the specimens positive for other HPV types mostly had a NIEL grade. One specimen was positive for HPV 56, and this specimen had an ASCUS cytology grade.
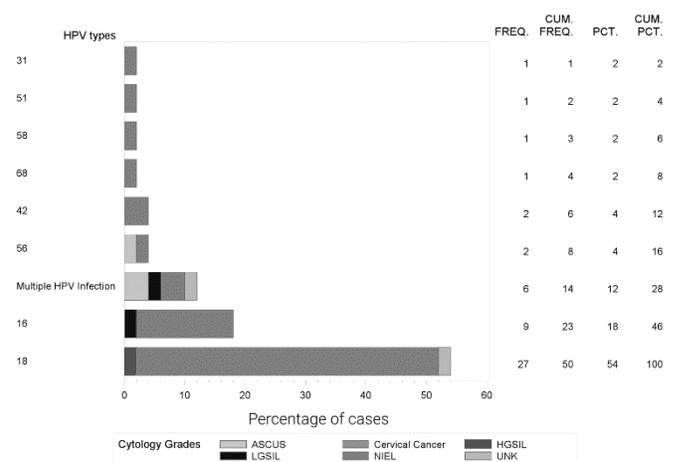
HPV positivity agreement

As seen in Figures 2 and 3, the frequencies of HPV infections detected in the RLB and GenoFlow assays were slightly different. In the RLB assay, most of the specimens were positive for HPV 18, HPV 16, and multiple HPV genotypes, whereas for the GenoFlow assay, most of the specimens were positive for multiple HPV infections, followed by HPV 18 and HPV 42. For the HPV 18 genotype, the RLB test detected 31 positive

Figure 1. Distribution of cytology grade by human papillomavirus (HPV) status.

Of 591 specimens, 93.9% were considered normal, that is, negative for intraepithelial lesion (NIEL), and 10% were positive for HPV. Of the low-grade squamous intraepithelial lesion (LGSIL) and atypical squamous cells of undetermined significance (ASCUS) specimens, six were positive for HPV. For high-grade squamous intraepithelial lesion (HGSIL), only one specimen was positive for HPV. Cervical cancer was detected in no specimen. CUM. FREQ., indicates cumulative frequency; CUM. PCT., cumulative percentage.

Figure 2. Distribution of human papillomavirus (HPV) types detected with reverse line blotting (RLB) assay by cytology grades.

ASCUS indicates atypical squamous cells of undetermined significance; CUM. FREQ., cumulative frequency; CUM. PCT., cumulative percentage; HGSIL, high-grade squamous intraepithelial lesion; LGSIL, low-grade squamous intraepithelial lesion; and NIEL, negative for intraepithelial lesion; UNK, unknown.
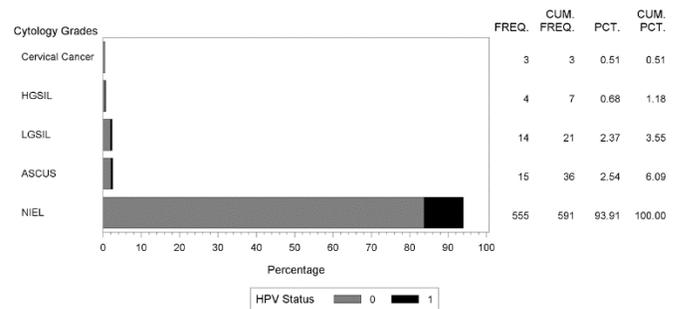
specimens, whereas the GenoFlow test detected 15 positive specimens. Of these 15, the two tests agreed on 13 positive specimens. For the HPV 16 genotype, the RLB test detected 12 positive specimens, whereas, the GenoFlow test detected 4 positive specimens, of which the two tests agreed on 2 specimens. The RLB test detected multiple HPV infections, mostly with HPV 16 coinfection, whereas for the GenoFlow test, most of the detected multiple infections were HPV 18 coinfection with low-risk HPV types. For the high-risk genotypes, the two tests agreed 79% of the time, while they agreed 100% of the time for low-risk HPV genotype detection. We conducted a concordance analysis to compare the detection of the HPV genotypes between the two methods for the cervical specimens tested. The results are shown in Table 2.

The detection of HPV genotypes using the GenoFlow test showed a sensitivity of 62.79% and a specificity of 100%. The false-positive rate was 0%, while the false-negative rate was 37%. There was 97.26% overall agreement between the two methods of genotyping HPV, with a concordance rate of 95%. For the GenoFlow test, 1.72% of the results showed additional HPV types, but the major HPV types agreed with the RLB test, while 0.34% of the results missed detecting HPV types (Table 3). Overall, the agreement rate of these two methods was relatively high with good specificity. However, the sensitivity of the GenoFlow HPV test was not as good as that of the RLB test. The agreement of the tests in the detection of HPV as assessed by Cohen's kappa statistic was 0.63 ($P < 0.0001$), indicating good agreement between the results detected by the GenoFlow and RLB tests.

Discussion

HPV infection is the most common sexually transmitted infection and is particularly common

Figure 3. Distribution of HPV Types detected with the Geneflow assay by cytology grade.

ASCUS indicates atypical squamous cells of undetermined significance; CUM. FREQ., cumulative frequency; CUM. PCT., cumulative percentage; HGSIL, high-grade squamous intraepithelial lesion; LGSIL, low-grade squamous intraepithelial lesion; and NIEL, negative for intraepithelial lesion.

among sexually active young women. To date, there is no universal HPV detection assay. Every laboratory has its own techniques, thus affecting the accuracy of the reported results. Indeed, it has been observed that there is a discrepancy of results from Saudi Arabia, where some research groups have reported high rates of HPV infection [8-10], whereas other groups have reported lower rates [11]. On close inspection, it appears that the groups using amplification-based techniques reported higher rates than those using hybridization-based assays [12]. Therefore, we aimed in the present study to evaluate the accuracy and reliability of two HPV DNA amplification–based techniques, namely, the RLB and GenoFlow assays. Although both techniques use the L1 consensus sequence for HPV detection and genotyping assays, the GenoFlow test detects 33 types of HPV [4,6] and has a universal probe spot that is capable of detecting HPV genotypes outside the panel as well as some HPV variants, whereas the RLB uses a biotin-

Table 2. Concordance between the DiagCor GenoFlow test and reverse line blot test results in 583 samples.

DiagCor GenoFlow Test	Reverse Line Blot Test		Total
	Positive	**Negative**	**Total**
Positive	27	0	27
Negative	16	540	556
Total	43	540	583

Table 3. Sensitivity and specificity of the DiagCor GenoFlow test compared with those for the reverse line blot assay.

Sensitivity/specificity	Assay result	No. (%)	No. (%)
Concordant	GenoFlow and reverse line blot the same	12 + 540 (94.68%)	552 (94.68%)
Compatible	Showed additional types but major types were concordant	8 (1.37%)	10 (1.72%)
	Missed types but major types were concordant	20 (0.34%)	
Discordant	Missed or different type	21 (3.60%)	21 (3.60%)
Total		583 (96.40%)	583 (96.40%)

labeled PCR product hybridized to an array of immobilized oligonucleotide probes to detect 23 HPV types [5].

Our results showed that 11% of screened cervical specimens were positive for HPV, consistent with previous studies that have indicated similar prevalence rates in their cohort studies. For example, an observational, epidemiological cross-sectional study conducted between April 2010 and December 2011 at three hospitals in Saudi Arabia included 417 women and found that HPV DNA was detected in 9.8% of these women [9]. In the present study, our demographic data indicated an association between religion and marital status with HPV positivity. Regarding marital status, it has been reported previously that multiple partners are a significant risk factor for HPV infection in Saudi populations [10]. Worldwide, multiple sex partners are a potential independent risk factor for contracting HPV infection [13]. However, the association detected in the present study between religion and HPV positivity may be attributable to a skewed sample collection: our cohort consisted mainly of Muslims (89%). To our knowledge, no published research has indicated an association between religion and HPV positivity. Most studies that have included religion as a potential factor are focused on HPV awareness, vaccine acceptance, and education [14-17].

Although it is rare for a cervical cancer or cervical dysplasia (LGSIL and HGSIL) case to be negative for HPV, it does occur. Indeed, several studies have reported patients with cervical cancer who tested negative for the presence of an HPV infection. For example, in the Belgium annual reports for 2017, almost 15% of their screened cervical cancer cases were negative for HPV. Several explanations might account for cervical cancer tumors that are not induced by the presence of a chronic HPV infection, including the misclassification of endometrial cancers or metastasis of other cancers to the cervix, the loss of HPV expression, or the existence of cervical cancers that are not induced by HPV [18]. Both of the detection tests used in our study have internal and universal detection controls, making it unlikely that both tests would miss detecting the presence of an HPV type that the tests were designed to measure.

In the present study, although the results of the RLB and GenoFlow assays might have initially appeared to differ substantially, the overall concordance between the two assays was 95%, with Cohen's kappa statistic equal to 0.63, indicating that the assays were in close agreement. In compatible cases, the genotypes detected in both the GenoFlow and RLB tests were closely matched, with only minor differences. However, the RLB results suggested that HPV 18 was the most frequently detected type in the study specimens, whereas the GenoFlow assay results indicated that multiple HPV infection was most common. This discrepancy may be attributed to the GenoFlow test detecting more HPV types than the RLB test (33 vs. 23 types). Thus, while both assays detected the same HPV types in the same specimens, the GenoFlow assay detected additional types that were not included in the RLB probe design.

Conclusion

On the basis of our findings, we can reasonably conclude that the genotyping of clinical specimens can be accurately conducted with the GenoFlow test and that the performance of this test is comparable to that of the standard RLB test. Given the simplicity of the GenoFlow test and the relatively short time needed to conduct the assay and return the results, this is a promising affordable addition or even alternative to other tests used in HPV diagnosis and screening programs.

Authors' contributions

FA is the principal investigator of this study. HK and TB ran the molecular assays. DO analyzed the data and provided the statistical analysis. AT provided the cervical specimens and the demographic and clinical data. MF helped in funding the project and reviewing the manuscript for important intellectual content. MA analyzed and interpreted patient data regarding HPV infection and disease progression. All authors helped in writing and reviewing the manuscript. In addition, all authors read and approved the final manuscript.

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