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Cross Comparison of AmpFire HPV Genotyping Assay and Roche Human Papillomavirus (HPV) Linear Array for HPV Genotyping of Anal Swab Samples

Kaleigh A. Connors[a], **Stephen Abbott**[b], **Kamwing Jair**[a], **Jason Daniels**[c], **Madison Lintner**[a], **David Klein**[b], **Avery Wimpleberg**[b], **Jeanne A. Jordan**[a,#]

[a]Department of Epidemiology, School of Public Health, The George Washington University, Washington, DC, USA

[b]Whitman-Walker Health, Washington, DC, USA

[c]Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, USA

Abstract

Although anal cancers represent just 0.5% of all new cancer cases in U.S., rates have increased markedly, with highest rates in HIV-infected MSM. American Cancer Society estimates there will be ~9,090 new cases and ~1,420 deaths in 2021. We compared Roche Linear Array HPV Genotyping (Roche) and AmpFire HPV Genotyping (AmpFire) assays for concordance and agreement to detect 15 HR-HPV types from 151 anal specimens. Within run precision of AmpFire was assessed on 50 anal specimens. Specimens with Roche Combo-positive and HPV33, HPV35 and/or HPV58-positive results were further tested using HPV52-specific TaqMan assay. AmpFire generated valid results on 149/151 (98.7%) specimens; 135/149 (90.6%) and 134/149 (89.9%) had detectable HR-HPV DNA by AmpFire or Roche, respectively. Overall concordance was 89.8% (2007/2235, κ=0.65). HPV16 showed highest overall concordance at 93.3% (139/149, κ=0.84). HPV68 had lowest overall concordance at 77.2% (115/149, κ=0.28). Kappa values were interpreted as being moderate or good for all other HR-HPV types. Within run precision generated

[#]Address correspondence to Jeanne A. Jordan, jajordan@gwu.edu, 800 22nd St., NW, George Washington University, Science and Engineering Hall, 7th floor, Room 7870, Washington, DC 20052.

Credit authorship contribution statement

Kaleigh A. Connors: Investigation, Software, Resources, Writing-Original draft preparation
Stephen Abbott: Resources, Writing – Review & Editing
Kamwing Jair: Investigation, Writing – Review & Editing
Jason Daniels: Investigation, Statistics
Madison Lintner: Investigation
David Klein: Resources, Project administration, Writing – Review & Editing
Avery Wimpleberg: Project administration, Writing – Review & Editing
Jeanne A. Jordan: Conceptualization, Methodology, Supervision, Funding acquisition, Writing – Review & Editing

Declaration of Competing Interest

The authors have nothing to disclose.

Declaration of Interest Statement:

None of the authors have any financial relationships or were given any support from Atila Biosystems, which markets the AmpFire HPV Genotyping assay.

744/750 concordant results; R^2 value=0.97 (p<0.0001) (Mantel Test). In conclusion, AmpFire and Roche demonstrated good inter-assay agreement for detecting most HR-HPV types from anal samples, with AmpFire detecting a broader range of HPV68 subtypes and detecting HPV52 without the need for confirmatory testing.

Keywords

HPV; genotyping; anal specimens

1. Introduction

It is estimated that in the U.S. there will be ~9,090 new cases of anal cancer and ~1,420 deaths in 2021 (1). Although anal cancer cases represent only 0.5% of all new cancer cases, it is currently considered one of the most rapidly rising cancers in U.S. with the incidence of anal cancer among men having sex with other men (MSM) living with HIV being highest of any group (77–137 cases/100,000) (2). Globally, the prevalence of HR-HPV anal infections is high (between 83%−93%) in MSM who are living with HIV (3).

Persistent HPV infections of the anus are strongly associated with subsequent risk of anal cancer. Between 80% to 90% of anal cancers have detectable high-risk (HR) HPV DNA, with HPV16 being the most prevalent HR-HPV genotype, found in ~73% of these cases (4–6). Genotyping HR-HPV DNA from anal samples can be an important tool when conducting research studies and performing surveillance studies, especially in the MSM population living with HIV. In this study, we did a cross comparison of the AmpFire HPV Genotyping Assay (AmpFire) with the well-established Roche Linear Array HPV Genotyping assay (Roche) (7, 8) for detecting 15 HR-HPV genotypes from anal swabs.

2. Materials and Methods

2.1 Study Participants and Specimen Collection

Anal swab-based specimens were collected between June 2017 and July 2019 from 151 eligible study participants recruited at Whitman-Walker Health. Signed informed consent was obtained from all eligible participants. Ethics approval was granted by the George Washington University Committee on Human Research, Institutional Review Board. Inclusion criteria consisted of the following: being born male (irrespective of current gender identity); ≥ 18 years of age; history of sex with a male partner, or someone who self-identifies as gay or bisexual; person living with HIV; abnormal anal Pap test (≥ASCUS) or abnormal digital anal rectal exam.

At their clinic visit, participants were scheduled to undergo high-resolution anoscopy (HRA) and biopsy procedures. Anal specimens collected for HR-HPV genotyping were obtained at that same clinic visit, prior to the HRA procedure. A moistened FloqSwab (Cat. #502CS01, Copan Diagnostics, Murrieta, CA) was used to collect cells from the anal canal. Immediately after sampling, the swab was rinsed in a 20 mL vial of PreservCyt® solution (Hologic, Inc., Marlborough, MA) to release the cells.

Specimen vials were stored overnight at 4°C in the clinic before being transported on cold packs to the laboratory where the specimens were again stored at 4°C until processed. For Roche LA, DNA was extracted from the PreservCyt® solution within 2 weeks of sample collection. These PreservCyt® vials continued to be stored at 4°C for up to 15 months before specimen processing and testing was performed using AmpFire.

2.2 AmpFire HPV Genotyping Assay

AmpFire HPV Genotyping assay (Cat. #GHPVF1618-100, Atila BioSystems, Mountain View, CA) uses isothermal amplification and fluorescent detection in a 4-plex assay to detect the following 15 HR-HPV types; HPV16/18/31/33/35/39/45/51/52/53/56/58/59/66/68. Table 1 illustrates primer and probe combinations used to amplify the 15 HR-HPV types along with an internal control (IC) target from the human beta-globin gene. Amplicon detection utilized specific molecular beacons labeled with either FAM, HEX, ROX or CY5 dye to generate a fluorescent signal in real-time. The HR-HPV-specific gene targets used in the assay are proprietary and as such were not available for description (personal communications). The AmpFire assay is not FDA-cleared.

PreservCyt® solution was processed according to manufacturer's instructions to generate a crude cell lysate. Briefly, 1 mL of PreservCyt fluid was transferred to 1.5 mL microfuge tube and centrifuged for 10 minutes (min) at 10,000 rpm. After discarding the supernatant, 100 μL of 1x lysis buffer was added to each cell pellet, briefly vortexed and incubated at 95°C for 10 min. before being allowed to cool to room temperature. During this time, four separate master mixes were prepared for each specimen being tested (Table 1). Using a 96-well plate format, 23 μL of each of the four master mixes were combined with 2 μL each of concentrated cell lysate, for a total volume of 25 μL. Positive and negative kit controls were included in each run. Isothermal amplification of the targets was performed using a BioRad CFX96 Real-Time PCR Detection System (Cat. # 03378012190, Bio-Rad Laboratories, Hercules, CA) for 60 min at 60°C. Fluorescence was read and the exponential amplification curves analyzed using BioRad CFX Manager Software 2.1 (Cat. #184500, Bio-Rad Laboratories, Hercules, CA). For the results of a specimen to be considered valid, the IC target had to demonstrate both a detectable Ct value and an exponential amplification curve. Specimens with valid IC results lacking a positive signal for any of the 15 HR-HPV types were considered negative for HR-HPV. Results lacking an exponential curve with primer mixture #3 in the HEX channel were interpreted as invalid. To assess reproducibility of AmpFire results, we tested a subset of 50 anal specimens in duplicate.

2.3 Roche LA-HPV Genotyping Assay

Total DNA was extracted from 250 μL of each PreservCyt fluid using Qiagen MinElute Media Kit (Cat. #57414, Qiagen, Germantown, MD) according to manufacturer's instructions. DNA were eluted in 120 μL of elution buffer and stored at −20°C until processing. HPV DNA genotyping was performed using Roche LA-HPV (Cat. #04472209190, Roche Molecular Systems, Inc., Branchburg, NJ), which included PGMY09/11 primers to amplify a 450-bp fragment of L1 gene (8, 9). Amplicons were subjected to reverse-line blot hybridization to detect 37 individual HPV genotypes (Cat. #03378012190, Roche Molecular Systems, Inc., Branchburg, NJ). Bands visible on the

linear array strip were read using the template guide to determine the HPV type. If both high and low beta-globin control bands were visible while other HPV bands were not visible, the sample was considered valid and HPV-negative. Linear array strip results were read independently by two separate individuals and their results compared. Discrepant results between readers were re-reviewed and resolved. The same 15 HR-HPV genotyping results generated by AmpFire were included in this comparison.

2.4 HPV52-specific TaqMan Assay

The Roche LA-HPV genotyping assay was designed to indirectly detect HPV52 DNA using a cross reactive probe that hybridizes to HPV33/35/52/58. Co-infection with HPV52 cannot be ruled out in those specimens testing positive for both the HPV 52/33/35/58 strip (Combo-positive result) and one or more of the individual reaction strips for HPV33, HPV35 and/or HPV58. Therefore, confirmatory testing was run on those specimens having both Combo-positive and HPV33, HPV35 and/or HPV58 positive results using a previously described HPV52-specific TaqMan® assay (10).

Briefly, 10 μL 2x TaqMan® Fast Advanced Master Mix (Cat. #4444556, ThermoFisher Scientific, Waltham, MA), 1.8 μL of each HPV-52 specific primer (10 μM), 0.5 μL dual labeled BHQ HPV-52 specific TaqMan probe (10 μM) and 2.5 ng of DNA extract were combined, and a volume of distilled water added to achieve 20 μL per reaction. Real-Time PCR was performed using QuantStudio® 3 Real-Time PCR System (Cat. #A28136, Applied Biosystems, Foster City, CA) with the following conditions according to manufacture procedures: 50°C, 2 min for 1 cycle; 95°C, 10 min for 1 cycle; 95°C, 15 sec followed by 60°C, 1 min for 40 cycles. Each run included four external controls consisting of two known HPV52 positive specimens, and two known HPV52 negative specimens.

2.5 Statistical Analyses

Statistical analyses were performed in RStudio (RStudio, Inc., Boston, MA). Percent concordance, Cohen's kappa (95% CI) and percent prevalence were calculated when comparing AmpFire and Roche HR-HPV genotyping results. The within-run precision testing of duplicates used the Mantel Test to calculate the R^2 value. Cohen's kappa values between 0.81–1.0 were interpreted as having excellent agreement, values between 0.61–0.80 as good agreement, values between 0.41–0.6 as moderate agreement, values between 0.21–0.4 as fair agreement and those values ≤0.2 are interpreted as having poor agreement beyond chance (11, 12).

3. Results

3.1. HR-HPV Detection.

Of the 151 anal samples tested, 149 (98.7%) had valid AmpFire test results (i.e., generated a positive IC value) and were included in this comparison. Of those 149 specimens, 90.6% and 87.9% tested positive for HR-HPV DNA using AmpFire and Roche, respectively. The most prevalent HR-HPV type present was HPV16 at 32.2% and 30.9% using AmpFire and Roche, respectively. The anal specimens tested by AmpFire had an average or median number of genotypes per specimen of 2.9 and 2.0, respectively, with a range of 0–10 genotypes per

specimen, while those tested by Roche had an average or median number of genotypes per specimen of 2.2 and 2.0, respectively, with a range of 0–8 genotypes.

3.2 Inter-assay agreement of HR-HPV genotypes.

Table 2 illustrates the comparison data categorized as concordant positive, discordant or concordant negative for the 15 individual HR-HPV types assessed by AmpFire and Roche from 149 anal specimens. Overall concordance for these 15 HR-HPV types was 89.8% (2007/2235) with good agreement (κ=0.65). HPV16 demonstrated the highest concordance (93.3%, 139/149) of the 15 HR HPV types, with excellent agreement (κ=0.84), while HPV68 had the lowest overall concordance (77.2%, 115/149) with fair agreement (κ=0.28).

When analyzing HPV52 results generated using the Roche LA-HPV assay, 61/149 (40.9%) specimens were found to be Combo-positive; of which 17/61 (27.9%) were negative for HPV33/35/58. According to Roche LA-HPV guidelines, these specimens are to be interpreted as HPV52 positive. The remaining 44/61 (72.1%) specimens being both Combo-positive and positive for HPV33, HPV35 and/or HPV58 required confirmatory testing based on Roche's guidelines that HPV52 cannot be ruled out in these cases. Confirmatory testing using the previously published HPV52-specific TaqMan assay (10) was conducted and resulted in 23 of the 44 specimens (52.3%) testing positive for HPV52, while the remaining 21 (47.7%) tested negative for HPV52. The data in Table 2 reflects the HPV52 confirmatory results for Roche LA, when concordance and agreement were calculated. The comparison revealed 84.6% concordance with good agreement (κ=0.61).

3.3. Within run precision.

To assess within run precision of AmpFire, 50 of the 149 anal specimens were tested in duplicate for the 15 HR-HPV types. Of the 750 genotyping results generated, 744 (99.2%) results were concordant between the duplicates. Using the Mantel Test, the R^2 value was found to be 0.97 ($p<0.0001$).

4. Discussion

The results from this cross-comparison study between Roche and AmpFire HPV genotyping assays on 149 anal specimens showed an overall concordance of 89.8% with good agreement (κ=0.65) for the 15 HR-HPV types included in the AmpFire HPV genotyping assay.

The Roche assay uses an indirect approach to detect HPV52, so we incorporated an HPV52-specific confirmatory test to retest the 44 Combo-positive, HPV33, HPV35, and/or HPV58 positive specimens. AmpFire detects HPV52 DNA directly using a specific fluorescent probe following target amplification, eliminating the need to perform confirmatory testing. This is an important point as HPV52 contributes to a high burden in both cervical and anal cancers (13, 14), and is one of the seven HR-HPV types in the 9-valent HPV vaccine (15).

The lowest level of concordance and agreement between these two assays was seen with HPV68. This finding is consistent with the report from Eklund et al. and the World Health Organization (WHO) Human Papillomavirus Laboratory Network that state that the primers

used in the Roche LA-HPV assay were designed to detect HPV68 subtype b, but due to several nucleotide mismatches present cannot detect the HPV68 subtype a (16). Primers used in the AmpFire assay are designed to detect both HPV68 subtypes (personal communication).

Discordant results seen can in part be explained by the significant differences in the methods used for sample preparation, target amplification and detection platforms. AmpFire uses a 4-fold larger volume of PreservCyt fluid than the Roche assay, but utilizes a crude cell lysate concentrate for target amplification, while Roche uses DNA extraction prior to PCR. DNA concentrations of the prepared specimens were not measured, nor compared, before the extracts were used for testing, and could have played a role in the differences seen here.

Amplification technologies used by AmpFire and Roche LA-HPV are quite different. The former uses a highly efficient isothermal target amplification, while the latter uses PCR thermocycling. The detection strategies vary as well; AmpFire uses fluorescence, while Roche LA-HPV uses a colorimetric linear array. Lastly, the two assays were designed to have different limits of detection. Roche assay was designed using a clinical cutoff and has a higher limit of detection (LOD) compared to AmpFire assay with LOD between 2–20 copies/μL (9). This helps explain why AmpFire assay detected an overall greater number of the 15 HR-HPV types than did Roche LA-HPV (455 vs. 349).

Although both assays use a multiplex approach to amplify HPV DNA, the number of oligonucleotide pools per reaction are different. Roche LA-HPV incorporates a set of 5 upstream oligos (PGMY11) and a set of 13 downstream oligos (PGMY09) for HPV along with 1 primer pair (PC04 and GH20) for the internal beta globin control; all this is carried out in a single reaction (8). AmpFire incorporates 4 primer pairs per reaction, requiring 4 separate reactions be set up and run for each sample in order to screen all 15 HR-HPV types and the IC. Roche assay can experience greater primer competition for template binding along with using a less efficient target amplification method compared with AmpFire assay.

One limitation to our study included the fact that the two genotyping assays could not be run within the same time period from sample collection; PreservCyt fluids were stored at 4°C for up to 15 months before specimens were tested by the AmpFire assay. Another limitation included the fact that sample processing was not the same for the two assays; AmpFire protocol generates crude cell lysates that are used to detect HR-HPV DNA while Roche protocol requires DNA extraction be performed on specimens before screening for HR-HPV DNA.

Other HPV genotyping assays have been described that also use anal swabs. One study by Low et al., compares Hybribio GenoArray and Roche LA for HPV genotyping of anal samples and found the overall inter-assay agreement to 86% (κ=0.70); findings which are similar to what we found here (89.8% agreement, κ=0.65) (17). Two other HPV genotyping assays have been described that are used to screen anal samples. Bouassa et al. (2018) describes using the Anyplex™ II HPV28 test (Seegene, South Korea) for genotyping HPV DNA from anal swabs in a high-risk group of MSM living with HIV for a molecular epidemiology study in the Central African Republic. Their study found, on average, 2.7

genotypes per anal sample in their HIV-infected MSM group; a value similar to what we found in this study (18). Parisi et al. (2019) describes using the Inno-LiPA HPV Genotyping Extra II test (Fujirebio Europe N.V, Gent, Belgium) to genotype HPV DNA in anal samples collected from a population of HIV-infected MSM where the prevalence was found to be ~89% (19).

In conclusion, to our knowledge, this is the first manuscript describing the performance of AmpFire HPV genotyping test using anal specimens, which revealed high levels of concordance and good agreement with the Roche genotyping test. AmpFire assay combines a simple processing protocol, requires less equipment to run, and has a quick turn-around time (90 minutes) compared to the Roche Linear Array genotyping test (270 minutes) to complete screening. Lastly, the primer and probe combinations used in the AmpFire assay detected a broad range of HPV68 subtypes and could detect HPV52 without the need for confirmatory testing.

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Highlights:

- Overall agreement between AmpFire and Roche-LA was 89.9% for anal specimens.
- Highest concordance of 93.3% was seen for HPV16.
- AmpFire (90 minutes) has a faster turnaround time than Roche-LA (270 minutes).
- High rate of anal infection with HR-HPV (90.6%; AmpFire or 87.9%; Roche-LA) in a population of HIV-infected MSM.
- Multiple HR-HPV genotypes (75.2%; AmpFire or 63.8%; Roche-LA) were found in a population of HIV-infected MSM.

Table 1.

Combinations of primers and specific fluorophore-labeled molecular beacon probes used into the AmpFire HR-HPV Genotyping Assay to amplify and identify 15 different HR-HPV types along with a human beta-globin target (Internal Control).

	Fluorescent	Dye		
Primer Mix	**FAM**	**HEX**	**ROX**	**CY5**
1	HPV-31	HPV-51	HPV-39	HPV-16
2	HPV-35	HPV-68	HPV-18	HPV-59
3	HPV-33	Internal Control	HPV-66	HPV-45
4	HPV-58	HPV-56	HPV-53	HPV-52

Table 2.

Comparing results of AmpFire (Test) and Roche LA-HPV (Reference) for 15 HR-HPV types from 149 specimens (n=2235).

HPV type	P/P	P/N	N/P	N/N	Concordance (%)	Kappa (95% CI)	Prevalence Test	Prevalence Reference
HPV16	42	6	4	97	139/149 (93.3)	0.84 (0.68–1.00)	32.2	30.9
HPV18	17	6	5	121	138/149 (92.6)	0.71 (0.55–0.87)	15.4	14.8
HPV31	9	20	1	119	128/149 (85.9)	0.40 (0.27–0.54)	19.5	6.7
HPV33	19	13	2	115	134/149 (89.9)	0.66 (0.50–0.81)	21.5	14.1
HPV35	23	11	3	112	135/149 (90.6)	0.71 (0.55–0.87)	22.8	17.4
HPV39	10	9	6	124	134/149 (89.9)	0.51 (0.35–0.67)	12.8	10.7
HPV45	12	7	1	129	141/149 (94.6)	0.72 (0.56–0.88)	12.8	8.7
HPV51	20	12	3	114	134/149 (89.9)	0.67 (0.51–0.82)	21.5	15.4
HPV52	29	12	11	97	126/149 (84.6)	0.61 (0.44–0.77)	27.5	26.8
HPV53	29	11	2	107	136/149 (91.3)	0.76 (0.60–0.92)	26.9	21.5
HPV56	13	11	1	124	137/149 (91.9)	0.64 (0.48–0.79)	15.4	9.4
HPV58	23	13	6	107	130/149 (87.2)	0.62 (0.46–0.79)	24.2	19.5
HPV59	16	5	2	126	142/149 (95.3)	0.79 (0.63–0.95)	14.1	12.1
HPV66	14	6	5	124	138/149 (92.6)	0.67 (0.51–0.84)	13.4	12.8
HPV68	12	25	9	103	115/149 (77.2)	0.28 (0.13–0.43)	24.8	14.1
15 HR-HPVs	288	167	61	1719	2007/2235 (89.8)	0.65 (0.61–0.69)		

P; positive, N; negative, Data represented as the ratio of AmpFire/Roche LA-HPV results, Prevalence is shown as percentage.