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Comparison of Hybribio GenoArray and Roche Human Papillomavirus (HPV) Linear Array for HPV Genotyping in Anal Swab Samples

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Human papillomavirus (HPV) is causally associated with anal cancer, as HPV DNA is detected in up to 90% of anal intraepithelial neoplasias and anal cancers. With the gradual increase of anal cancer rates, there is a growing need to establish reliable and clinically relevant methods to detect anal cancer precursors. In resource-limited settings, HPV DNA detection is a potentially relevant tool for anal cancer screening. Here, we evaluated the performance of the Hybribio GenoArray (GA) for genotyping HPV in anal samples, against the reference standard Roche Linear Array (LA). Anal swab samples were obtained from sexually active men who have sex with men. Following DNA extraction, each sample was genotyped using GA and LA. The overall interassay agreement, type-specific, and single and multiple genotype agreements were evaluated by kappa statistics and McNemar's χ^2 tests. Using GA and LA, 68% and 76% of samples were HPV DNA positive, respectively. There was substantial interassay agreements for the detection of all HPV genotypes ($\kappa = 0.70$, 86% agreement). Although LA was able to detect more genotypes per sample, the interassay agreement was acceptable ($\kappa = 0.53$, 63% agreement). GA had poorer specific detection of HPV genotypes 35, 42, and 51 ($\kappa < 0.60$). In conclusion, GA and LA showed good interassay agreement for the detection of most HPV genotypes in anal samples. However, the detection of HPV DNA in up to 76% of anal samples warrants further evaluation of its clinical significance.

Anal cancers are relatively rare malignancies, accounting for approximately 4% of lower gastrointestinal tract malignancies (1). Human papillomavirus (HPV) infection is causally associated with anal cancer, with HPV DNA being detected in up to 90% of anal intraepithelial neoplasias and anal cancers in both men and women (2, 3). Similar to what is seen with cervical cancers, the high-risk (HR) HPV 16 is the most prevalent HPV genotype found in anal intraepithelial neoplasia and anal cancer samples (4–8). With the gradual increase of anal cancer rates (9–12), there is now a growing need for more routine anal cancer screenings and to establish reliable, reproducible, and clinically relevant methods to detect precursors to anal cancer, especially in high-risk populations like human immunodeficiency virus (HIV)-positive individuals (13). In resource-limited settings, due to the limited availability of anal cytology and high-resolution anoscopy, the detection of HPV DNA is a potentially relevant tool for anal cancer screening (14). Validated methods of HPV DNA detection and genotyping are also important for natural history studies designed to better understand the clinical relevance of these infections in the cervix and in the anal canal (15–18).

In this study, we evaluated the performance of Hybribio Rapid HPV GenoArray test kit (GA) (Hybribio Limited, Hong Kong) in genotyping HPV DNA extracted from anal swab samples against that of the well-established Linear Array (LA) HPV genotyping kit (Roche, USA), a widely accepted reference standard for comparison and assessment of new HPV genotyping assays (19). GA is designed to identify 21 (including 13 HR) HPV genotypes, while LA is able to identify 37 (including 18 HR) HPV genotypes. The levels of interassay agreement between the overall and type-specific HPV infections and between single and multiple HPV infections were evaluated.

MATERIALS AND METHODS

Anal swab samples. Anal swab samples were obtained from 200 male participants from an ongoing HPV study. Participants all were self-reported sexually active men who have sex with men. Anal swabs were collected by physicians using a sterile Dacron swab prewetted in normal saline solution. The swab was gently inserted 2 to 3 cm into the anal canal and removed with 360-degree rotation to collect the squamous epithelium until the anal margin was reached. The swabs were placed in a container with phosphate-buffered saline (PBS) and kept at −20°C until they were transported to the laboratory, where they were stored at −80°C until use. For DNA extraction preparation, samples were digested with 20 μg/ml proteinase K for 1 to 2 h at 37°C, before DNA was extracted from 2,400 μl of sample using the QIAamp DNA blood kit (Qiagen, USA) (20). The extracted DNA was then HPV genotyped using both LA and GA.

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TABLE 1 Detection of HPV genotypes using GA and LA

No. of HPV genotypes detected	No. (%) of samples[a]					
	Total		Containing HR and/or pHR HPV		Containing HPV 16	
	LA[b]	GA[c]	LA	GA	LA	GA
0[d]	39 (24.2)	60 (37.3)				
1	20 (12.4)	43 (26.7)	12/20 (60.0)	39/43 (90.7)	1/20 (5.0)	5/43 (11.6)
2 or 3	46 (28.6)	36 (22.4)	40/46 (87.0)	34/36 (94.4)	6/46 (13.0)	7/36 (19.4)
4 or 5	30 (18.6)	15 (9.3)	30/30 (100)	15/15 (100)	9/30 (30.0)	5/15 (33.3)
>5	26 (16.2)	7 (4.4)	26/26 (100)	7/7 (100)	9/26 (34.6)	3/7 (42.9)

[a] $n = 161$ samples.
[b] GA detects 21 HPV genotypes, including 13 HR HPV, 2 pHR HPV, and 6 LR HPV genotypes.
[c] LA detects 37 HPV genotypes, including 17 HR HPV, 5 pHR HPV, and 15 LR HPV genotypes.
[d] Samples were HPV DNA negative.

All participants in this study had provided informed written consent. No personal identifiers were accessed during testing and data analysis. This study was approved by the Cayetano Heredia Peruvian University institutional review board (IRB; number 57659).

This study was intended as a method comparison study using blinded samples from this ongoing study. Hence, no clinical information on the participants is presented in this paper.

Roche Linear Array HPV genotyping kit. The Roche Linear Array HPV genotyping kit (LA) detects 37 HPV genotypes, including 17 HR types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 67, 68, 73 [MM9], 82 [MM4], and 82var [IS39]) and 15 low-risk (LR) types (types 6, 11, 40, 42, 54, 55, 61, 64, 70, 71, 72, 81, 83 [MM7], 84 [MM8], and 89 [CP6108]), and 5 unknown-risk/probable-high-risk (pHR) types (types 26, 53, 62, 66, and 69) (21, 22). The tests were performed according to the manufacturer's protocol (23), with broad-spectrum amplification using the PGMY09/11 primer pool (24) and reverse line blot hybridization for genotype discrimination (25). Samples negative for human β-globin gene amplification are considered insufficient for HPV DNA testing. After exclusion, only 161 samples were used for subsequent comparison in this study. The detection limit of the kit for 18 of the HPV genotypes, as reported by the manufacturer, is 53 to 8,089 copies per ml of the original sample tested. In samples coinfected with HPV 33, 35, and 58, LA could not specifically identify HPV 52, due to potential cross-hybridization of the probe for HPV 52 detection on the Roche HPV XR strip with the other genotypes (24, 26).

Hybribio Rapid GenoArray test kit. The Hybribio Rapid GenoArray test kit (GA) detects 21 HPV genotypes, including 13 HR types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), 6 LR types (types 6, 11, 42, 43, 44, and CP8304 [81]), and 2 pHR types (types 53 and 66). The tests were performed according to the manufacturer's protocol (27). Briefly, extracted DNA was subjected to PCR amplification using HPV L1 consensus PCR primers (not the same as LA's primers MY09 and MY11). This was followed by flowthrough hybridization on a probed membrane for the detection of HPV genotypes. The results were manually interpreted using the provided guide. The average detection limit of the kit, as reported by the manufacturer, is around 500 copies per μl of target HPV DNA. There is no reported cross-reactivity of the amplification/detection of all the 21 HPV genotypes.

Statistical analysis. McNemar's χ^2 test for matched pairs was used to compare the performance of both HPV genotyping assays. Kappa statistics were calculated to assess agreement between the two methods as well. A kappa value of 0.41 to 0.60 was considered to indicate moderate agreement, while 0.61 to 0.80 was considered to indicate substantial agreement (28). All analyses were performed using STATA 11.2 (StataCorp, College Station, TX).

RESULTS

HPV detection. According to LA, 122 of the 161 samples (75.8%) were HPV DNA positive (Table 1). Among these samples, 102 (83.6%) had multiple HPV genotypes (>1 genotypes) and 109 (89.3%) contained at least 1 HR and/or pHR HPV genotype. Twenty-four of the 25 samples that contained HPV 16 (96%) had multiple HPV genotypes. The highest number of HPV genotypes detected in a single sample was 15. The HPV genotypes most commonly detected were HPV 16 (found in 25 samples), 53 (24 samples), 84 (23 samples), 51, 52, 58, and 59 (21 samples each).

According to GA, 101 of the 161 samples (67.7%) were HPV DNA positive (Table 1). Among these samples, 58 (57.4%) had multiple HPV genotypes (>1 genotype), and 95 (94.1%) contained at least 1 HR and/or pHR HPV genotype. Fifteen of the 20 samples that contained HPV 16 (75%) contained multiple HPV genotypes. The highest number of HPV genotypes detected in a single sample was 8. The HPV genotypes most commonly detected were HPV 52 (found in 24 samples), 53 (22 samples), 58 (21 samples), 16 (20 samples), and 18 (19 samples).

Interassay agreement on HPV genotypes detectable by both assays. A total of 19 HPV genotypes are detectable by both assays. These include HR HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68, LR HPV 6, 11, 42, and 81, and pHR HPV 53 and 66.

For the detection of all 19 assay-common HPV genotypes, there was good overall interassay agreement between GA and LA, with a kappa value of 0.72 and 87.0% agreement. No significant difference was observed between the assays (McNemar's P value = 0.28). Overall, both assays were in agreement in showing that 93 samples (57.8%) were HPV DNA positive while 47 samples (29.2%) were HPV DNA negative. Among the samples with discordant results, 8 (5.0%) were positive by GA only, while 13 (8.7%) were positive by LA only.

For the detection of the 15 HR HPV genotypes detectable by both assays, there was good overall interassay agreement between GA and LA, with a kappa value of 0.70 and 85.7% agreement. No significant difference was observed between assays (McNemar's P value = 0.30). Overall, both assays were in agreement in showing that 86 samples (53.4%) were HR HPV DNA positive, while 52 samples (32.3%) were HR HPV DNA negative. Among the samples with discordant results, 9 (5.6%) were positive by GA only, while 14 (8.7%) were positive by LA only.

To observe the efficiency of the assays in detecting individual HPV genotypes present in samples with multiple HPV infections, the total number of HPV genotypes detectable by both assays in each sample sample was analyzed (Table 2). It was found that LA was able to detect more HPV genotypes in each sample, while GA had more samples with only 1 HPV genotype. There was moderate

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TABLE 2 Comparison of the number of HPV genotypes detected in each sample by GA and LA[a]

No. of genotypes detected by LA	No. (%) of samples with no. of genotypes detected by GA						
	0	1	2	3	4	>5	Total
0	**47 (29.2)**	8 (5.0)	0	0	0	0	55 (34.2)
1	6 (3.7)	**20 (12.4)**	3 (1.9)	0	0	0	29 (18.0)
2	7 (4.4)	9 (5.6)	**15 (9.3)**	3 (1.9)	0	0	34 (21.2)
3	0	4 (2.5)	4 (2.5)	**7 (4.4)**	0	0	15 (9.3)
4	0	2 (1.2)	1 (0.6)	2 (1.2)	**3 (1.9)**	3 (1.9)	11 (6.8)
>5	0	0	0	3 (1.9)	4 (2.5)	**10 (6.2)**	17 (10.6)
Total (%)	60 (37.3)	43 (26.7)	23 (14.3)	15 (9.3)	7 (4.4)	13 (8.1)	161 (100)

[a] Comparison was restricted to the 19 HPV genotypes detectable by both assays. κ = 0.53, agreement = 63.35%. Boldface indicates agreement for the total number of HPV genotypes detected, irrespective of the type of HPV genotypes detected.

overall interassay agreement between assays, with a kappa value of 0.53 and 63.4% agreement. The highest total numbers of HPV genotypes detectable by both assays found in a single sample were 8 and 9 for GA and LA, respectively.

When a comparison was made on a genotype-specific level, the most frequently detected assay-common HPV genotypes were HPV 52, 53, 58, 16, and 18 for GA and HPV 16, 53, 51, 52, 58, and 59 for LA (Table 3). There was good interassay agreement (kappa value > 0.60, agreement > 94.0%) on all but 2 HR genotypes (HPV 35 and 51) and 1 LR genotype (HPV 42). There were also significant interassay differences (McNemar's *P* value < 0.05) for the detection of HPV 51 and 59. In general, LA detected more incidences of HPV genotypes than GA.

DISCUSSION

Detection of anal HPV is underevaluated compared to that of HPV in the cervix. In view of the increasing incidence of anal cancer, it is therefore important to establish a reliable method for clinical use and for large cohort studies. This study evaluated the performance of GA against the well-established LA in the anal HPV genotyping and found that there was overall good interassay agreement for the detection of all HPV and HR HPV genotypes. However, LA showed greater efficiency in the detection of more individual genotypes in samples with multiple genotype infections.

As HPV is now widely associated with cervical carcinogenesis, HPV genotyping has been recommended as an alternative screening method to be used in combination with traditional cytology-based cervical screenings or visual inspection with acetic acid (29–31). In recent years, similar guidelines for anal screening and management have also been developed (32, 33). Many HPV genotyping assays are currently available. The World Health Organization (WHO) global HPV laboratory network (LabNet) evaluated some of these commercially available assays (34–36), including LA (37), Digene Hybrid Capture (38), restriction fragment length polymorphism (RFLP) (39), and GA (27). Among all these assays,

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TABLE 3 Interassay agreement between GA and LA for the detection of specific HPV genotypes[a]

HPV		No. of samples positive by:					
Risk group	Genotype	GA	LA	Both	% agreement	Kappa	McNemar's *P* value
HR	16	20	25	18	94.4	0.77	0.10
	18	19	15	13	95.0	0.74	0.16
	31	12	13	11	98.1	0.87	0.56
	33	8	8	6	97.5	0.74	1.00
	35	1	4	1	98.1	0.39	0.08
	39	14	17	11	94.4	0.68	0.13
	45	9	12	8	96.9	0.75	0.18
	51	5	21	4	88.8	0.27	0.002
	52	24	21[b]	16	96.3	0.85	0.41
	56	7	11	7	97.5	0.85	0.05
	58	21	21	18	96.3	0.84	1.00
	59	14	21	14	95.7	0.78	0.01
	68	6	10	6	97.5	0.74	0.05
LR	6	18	19	16	96.9	0.85	0.65
	11	10	11	10	99.4	0.95	0.32
	42	3	8	2	95.7	0.35	0.06
	81	14	13	11	96.9	0.80	0.65
pHR	53	22	24	20	96.3	0.85	0.41
	66	11	12	10	98.1	0.86	0.56

[a] Comparison was restricted to the 19 HPV genotypes detectable by both assays.
[b] LA cannot specifically distinguish HPV 52 in samples coinfected with HPV 33, 35, or 58.

LA has consistently been shown to be most proficient in terms of efficiency of detection and in terms of having the fewest false-positive results (34, 35), and thus, it has been widely used as a standard reference assay for the assessment of new assays.

GA is increasingly being used for studies conducted in resource-limited laboratories, especially in Asia (40–43). The cost of GA is almost a quarter of the cost of LA, and its hybridization time is approximately half of LA's (27). In addition, GA has the ability to distinguish and identify HPV 52, one of the most common HR HPV genotypes in women in eastern and southeastern Asian (44–47). Although GA has been shown to have high agreement with several other HPV genotyping assays, including LA (27), the Amplicor HPV test (48), and PCR-RFLP (43), to the best of our knowledge, GA has not yet been evaluated for HPV genotyping in anal swab samples. Screening of anal and cervical swab specimens may be different, as anal swabs may contain smaller starting amounts of DNA than cervical swabs due to their different anatomical structures and the different brushes used for sample collection, as well as possible interference in the assay by stool present in the anal samples (49).

When the performance of GA in HPV detection and genotyping was evaluated in this study, emphasis was placed on the detection of HR HPV and the ability to identify multiple HPV genotypes present in a single sample. Although the importance of infections with LR HPV should not be disregarded, in resource-limited settings, it is more clinically relevant for an assay to be able to identify HR HPV genotypes previously associated with anal cancers (50–52).

In a previous study where GA was similarly compared against LA for HPV genotyping of cervical swab samples (27), the overall interassay agreement for all HPV and HR HPV genotypes were reported to be 97.5% ($\kappa = 0.83$) and 97.8% ($\kappa = 0.87$), respectively, higher than the agreement found in this study. However, when only types detectable by both assays are considered, both studies showed good agreement for the total number of HPV genotypes detected per sample, irrespective of the type of HPV genotypes detected, and for the detection of individual HPV genotypes, with the notable exception of the agreement for HPV 35. Interestingly, the kappa values for HPV 42 and 51 were low in both studies ($\kappa = 0.54$ and 0.52 in reference 27; $\kappa = 0.35$ and 0.27 in this study), with LA detecting more of these HPV types than GA. This suggests that GA may have lower overall efficiency or specificity in the detection of HPV 35, 42, and 51 than LA. Some potential contributors to this lower efficiency include less efficient PCR primer designs, out-competition of PCR in samples with mixed HPV genotypes, and less efficient probing of the membrane by the assay, which subsequently reduces the detection of those genotypes.

Previous studies in populations of men who have sex with men have shown that anal HPV DNA prevalence is 42 to 66% in HIV-negative populations and around 89% in HIV-positive populations (53–58). As our study population includes both HIV-negative and -positive men (up to 49.5% were HIV positive), it is not surprising that up to 76% of our population were positive for anal HPV DNA, and most of them had multiple HPV genotype infections (up to 83%) and at least one HR or pHR HPV (up to 94%). Thus, the clinical utility of HPV testing as a means of early detection of anal intraepithelial neoplasia and anal cancer remains unclear, given the very high HPV prevalence in the anal cavity of sexually active men who have sex with men.

It is important to note that as with cervical HPV testing (59), there needs to be a balance between an assay's analytical sensitivity for anal HPV detection and its clinical utility and relevance. An increase in an assay's analytical sensitivity may result in an increase in false-positive results that may not be associated with clinical specificity or a true increase in risk of disease progression. The clinical relevance of LA in cervical screening has been reported (60); however, there are currently no known published studies on the clinical relevance of GA in cervical screening or on the clinical relevance of both kits in anal screening. Hence, future studies could focus on generating a similar guideline for anal HPV testing, screening, and management, as well as performing larger studies to determine the clinical relevance of both kits in anal screening.

In our population, we found a high prevalence of HPV 16, which is the most common HPV genotype found in anal intraepithelial neoplasia and anal cancers (4–8). Similar to previous findings, HPV 16 infections were most likely to be present in coinfections with other HPV genotypes, suggesting an association of HPV 16 with survival advantage or increased risk for subsequent HPV infections (61, 62). With the demonstrated clinical efficacy of the quadrivalent HPV prophylactic vaccination against HPV 6, 11, 16, and 18 (Gardasil; Merck & Co.) in the prevention of HPV 6, 11, 16, and 18 incidences and persistence of infection and HPV-associated anal intraepithelial neoplasia (63), the vaccine has now been licensed by the U.S. Food and Drug Administration (FDA) for the prevention of anal cancers (64). However, in view of the high prevalence of other HR HPV genotypes, like HPV 51, 52, 53, 58, 59, and 84, new vaccinations should look to cover a wider range of HPV genotypes for broader protection against HPV-associated anal cancers.

This study has some limitations, as the two assays were not concurrently performed in the same laboratory. This comparison was performed on readily available anal DNA specimens. After DNA extraction and LA, a small aliquot of DNA was sent to a separate laboratory for GA analysis approximately 6 months later. Therefore, some degradation of DNA due to the longer storage duration, repeated freeze-thawing, and transportation conditions may have resulted in fewer HPV-positive results for GA. However, DNA degradation would be expected to affect all genotypes. Given the reasonably good type-specific agreement between the assays with only a few exceptions, loss of specimen integrity does not appear to have affected the results substantially. On top of that, some laboratory-to-laboratory variation may be present in these results. Interlaboratory comparisons on the same assay could not be performed, as each laboratory had the facilities to perform only one of the assays. Lastly, we did not verify the actual HPV genotypes present, especially in samples with discordant assay results. This could be done either by using another WHO HPV LabNet-evaluated diagnostic kit or by using more powerful deep next-generation sequencing (65, 66).

In conclusion, HPV genotyping of anal swab samples using GA was reasonably comparable to that achieved with the LA reference standard, with good interassay agreement for the detection of most HPV genotypes. Thus, GA represents a reasonable HPV genotyping assay for both anal and cervical swab samples. Although the clinical utility of HPV testing in anal cancer screening and management has not been thoroughly evaluated, based on the cervical cancer screening guidelines, it is likely that in combination with other screening methods, like anal cytology and anos-

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copy, anal HPV genotyping results could also be a good clinical predictive tool of the risk of development of anal neoplasia and could be useful for the recommendation of screening intervals for both men and women.

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