Oral HPV prevalence assessment by Linear Array vs. SPF10 PCR-DEIA-LiPA25 system in the HPV Infection in Men (HIM) study

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ABSTRACT

Introduction: Oral human papillomavirus (HPV) attributable oropharyngeal cancers are on the rise in many countries. Oral HPV infections among healthy individuals are commonly detected using oral gargle samples. However, the optimal method for HPV genotyping oral gargle specimens in research studies has not been previously evaluated.

Materials and methods: Oral gargle samples from 1455 HPV Infection in Men (HIM) study participants were HPV genotyped using two different methods: Linear Array and the SPF10 PCR-DEIA-LiPA25. The sensitivity of the two tests for detecting individual HPV types and grouped HPV types, high-risk HPV, low-risk HPV, grouped 4-HPV-vaccine types, and grouped 9-HPV-vaccine-types, and the degree of concordance between the two tests was assessed. We also examined whether socio-demographic-behavioral factors were associated with concordance between the two assays.

Results: The sensitivity of SPF10 PCR-DEIA-LiPA25 was higher than Linear Array, with the exception of HPV 70, for the detection of oral HPV. The prevalence ratio of SPF10 PCR-DEIA-LiPA25 to Linear Array varied between 1.0 and 9.0 for individual HPV genotypes, excluding HPV 70, and between 3.8 and 4.4 for grouped 4-valent and 9-valent HPV vaccine types, respectively. There was no association between socio-demographic-behavioral factors and discordance in results between the two tests for oral HPV 16 detection.

Discussion: SPF10 PCR-DEIA-LiPA25 was more sensitive than Linear Array for detecting HPV in oral gargle samples. Given the growing importance of detecting oral HPV infection for research studies of oral HPV natural history and vaccine effectiveness evaluation, we recommend using methods with higher sensitivity such as SPF10 PCR-DEIA-LiPA25 for detecting HPV in oral gargle samples.

1. Introduction

Oral human papillomavirus (HPV) is a cause of a sub-set of oropharyngeal cancers (OPCs). The incidence of OPCs attributable to HPV is on the rise among many countries [1,2]. To inform effective preventive and early screening measures as well as to assess HPV prevention vaccine effectiveness it is essential to study the natural history of oral HPV infections and associated risk factors. Many cross-sectional and prospective studies have been conducted to estimate the prevalence and incidence of oral HPV infection in healthy populations such as the Human Papillomavirus Infection in Men (HIM) study [3], the NHANES study [4] and smaller, localized cohorts such as those at the Ohio State University [5] as well as among high risk populations [6].

Most studies of oral gargle HPV utilized the Linear Array method (Roche Molecular Diagnostics, Alameda, CA) for genotyping specimens [3–6]. This test is based on co-amplification of a 450 bp region of the
specimens were analyzed using the SPF10 PCR-DEIA-LiPA25 system. In 2017 and 2018, these same oral
past 6 months; (i) Willingness to comply with 10 scheduled visits every
history of imprisonment, homelessness, or drug treatment during the
ticipating in a HPV vaccine study; (g) No history of HIV or AIDS; (h) No
infection or treatment of a sexually transmitted infection; (f) Not par-
warts prior to the study; (e) No current reports of a sexually transmitted
anal or penile cancer; (d) Were never diagnosed with genital or anal
HPV DNA genotyping tests utilized in studying oral HPV include the
HPV L1 gene using biotinylated primer sets PGM09/PGMY11 [7]. Other
HPV DNA genotyping tests utilized in studying oral HPV include the
SPF10 PCR-DEIA-LiPA25 system (DDL Diagnostic Laboratory, Rijswijk,
the Netherlands), which utilizes amplification of a 65 bp region of the
HPV L1 gene using biotinylated SPF10 primers followed by hybridization of
the resulting amplicons with 17–28 HPV-specific oligonucleotide
probes immobilized on a nitrocellulose strip [7].

Several studies have compared SPF10 based tests and Linear Array for
HPV genotyping of formalin fixed paraffin embedded (FFPE) tumor
tissue [8–13] and exfoliated cervical cells [14,15]; however, to our
knowledge, no study has compared these two tests for HPV genotyping
of oral gargle samples, a sample that is quite different. Unlike FFPE
tumor and exfoliated cervical specimens where the majority of the
sample is the target tissue that may harbor HPV, oral gargle specimens
are comprised of a large diversity of cells, most of which do not harbor
HPV; hence, HPV viral load is significantly lower. Given the increasing
use of oral gargle samples in oral HPV epidemiological studies and HPV
vaccine efficacy and effectiveness studies [16], it is important to com-
pare and assess the suitability of different HPV genotyping methods to
prevent spurious HPV infection estimates.

In this study, we utilized data from the oral sub-cohort in the HIM
study, one of the earliest and largest prospective studies with a study
population of more than 3000 individuals from three different countries
(Mexico, US, Brazil). We compared the sensitivity of the Linear Array
and SPF10 PCR-DEIA-LiPA25 system for HPV genotyping of oral gargle
samples among HIM study participants at the first oral gargle specimen
collection. Oral HPV prevalence is reported using these two methods in
samples derived from the same individuals. The level of concordance in
the results from these two methods for individual and grouped HPV
genotypes was assessed as well as sociodemographic and behavioral
characteristics among the participants may be associated with concordance.

2. Materials and methods

The study population was nested within the oral sub-cohort of the
HIM study which has been previously described [17]. Men were rec-
ruited from São Paulo, Brazil; Caenavaca, Mexico; Tampa, Florida,
and its surrounding areas from March 2005 to December 2009. Baseline
oral specimens were collected between November 27, 2007 and Sep-
tember 29, 2009 in the US, between April 7, 2008 and March 23, 2012
in Mexico, and between December 13, 2007 and November 25, 2009 in
Brazil. Approval of study procedures before the commencement of the
HIM study was obtained from the Centro de Referência e Treinamento
de Doenças Sexualmente Transmissíveis e AIDS, Brazil, National In-
stitute of Public Health of Mexico and the Human Subject Committees
of the University of South Florida. All participants gave written consent.

2.1. Study population

Men included in the HIM study met the following eligibility criteria:
(a) Ages 18–70 years; (b) Residents of one of the three recruitment sites
in Brazil, Mexico or the US; (c) Did not report previous diagnoses of
anal or penile cancer; (d) Were never diagnosed with genital or anal
warts prior to the study; (e) No current reports of a sexually transmitted
infection or treatment of a sexually transmitted infection; (f) Not par-
ticipating in a HPV vaccine study; (g) No history of HIV or AIDS; (h) No
history of imprisonment, homelessness, or drug treatment during the
past 6 months; (i) Willingness to comply with 10 scheduled visits every
6 months for 4 years with no plans to relocate within the next 4 years.
Further details regarding the study population are available elsewhere
[18]. Initially, oral specimens of first 1626 individuals were analyzed
using the Linear Array method. In 2017 and 2018, these same oral
specimens were analyzed using the SPF10 PCR-DEIA-LiPA25 system.
Individuals for whom at least two oral samples were collected and
first oral gargle samples were genotyped for HPV using both Linear
Array and SPF10 PCR-DEIA-LiPA25 system were included in the analyses
(N = 1473).

2.2. Sample collection, DNA extraction, and HPV testing

The oral gargle sample was collected in 15 ml of Scope brand
mouthwash and processed within 24 h of collection. Participants were
asked to perform energetic washing of the oral cavity including the
throat by swishing the mouthwash in their mouth vigorously for ap-
proximately 15 s and were instructed to cover all surfaces of their
mouth. The participant was then instructed to tip their head back and
gargle in the throat for another 15 s and spit the mouthwash back into
the collection tube which was placed at 20 °C until processing. Within
24 h of collection the gargle specimen was centrifuged at 2000 × g for
15 min at 4 °C. The supernatant was decanted and the resulting cell
pellet resuspended in 20 ml cold PBS (4 °C). Centrifugation and pellet
washing was repeated twice with the final cell pellet resuspended in
1.2 ml of PBS and maintained at −80 °C until DNA extraction when
300 µl was used for extraction.

For the Linear Array assay, the DNA concentration was determined
via Nanodrop and concentrations above 1 ng/µl were diluted accord-
ingly. Samples with concentrations below the targeted 1 ng/µl were not
diluted or concentrated prior to use in the assay. Samples were not
initially diluted for the SPF10 LiPA assay as sample concentration and
adequacy were determined during the qPCR portion of the assay. If a
sample was deemed inadequate via qPCR, input DNA concentration was
adjusted to a more acceptable range, typically 10 ng/µl to 100 ng/µl,
and the qPCR portion of the assay was repeated.

The same purification method was utilized in preparation of sam-
ple for the two HPV genotyping assays. DNA was extracted from oral
gargle cell pellets using the automated BioRobot MDx (Qiagen, Inc.)
following the manufacturer's instructions. After specimens thawed, they
were placed on the universal extraction machine and the extraction
protocol was run. A membrane based procedure was executed by the
machine, utilizing optimized buffers to lyse and stabilize DNA, ethanol
and wash buffers to wash away impurities and water to elute DNA for
use in downstream assays. DNA was eluted into a 96 well plate for easy
transfer during PCR procedures. The unused portion of the original
samples and any unused extracted DNA was stored in a −80 °C freezer.

Qualitative identification of HPV DNA was performed to identify
HPV genotype(s) via two methods: amplification of HPV DNA with
the PGMY09/11 L1 consensus primer and HPV genotyping by Linear Array
method (Roche Molecular Diagnostics, Alameda, CA), and a SPF10 PCR-
DEIA-LiPA25 system (DDL Diagnostic Laboratory, Rijswijk, the
Netherlands). Per protocol, for the Linear Array assay, 50 µl (1 ng DNA/
µl) of the processed sample was added to the master PCR mix, and 10 µl
(median DNA concentration: 8 ng/µl) of the processed sample was
utilized for the SPF10 PCR-DEIA-LiPA25 system.

Details regarding the protocol for the Linear Array method has been
made available elsewhere [17]. The SPF10 PCR-DEIA-LiPA25 system is
an in vitro reverse hybridization assay (RHA) [19]. The LiPA25 targets a
65 base pair fragment of the L1 region of the HPV genome. This assay
requires a three step process: (a) qPCR that determines sample ade-
quacy; (b) a DNA enzyme immunosassay (DEIA) or ELISA method that
detects 65 HPV types; and (c) a LiPA25 genotyping multiplex PCR that
selectively identifies the following HPV types by reverse hybridization:
6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56,
58, 59, 66, 68/73, 70, and 74. All samples that were considered as
adequate in step (a) were further analyzed via steps (b) and (c).

2.3. Statistical analyses

We included 1455 individuals in our analyses whose oral gargle
samples were HPV genotyped using both methods, and whose demo-
graphic data were available. Distribution of sociodemographic and
behavioral characteristics among the participants were assessed
(Table 1). We compared the prevalence of individual and grouped 4
Table 1
Sociodemographic characteristics of the HIM study population for whom oral HPV genotyping was conducted through both Linear Array and SPF10 PCR-DEIA-LiPA25 System.

| Characteristic                        | n (%)       |
|---------------------------------------|-------------|
| Country (N = 1455)                    |             |
| Brazil                                | 436 (30.0)  |
| Mexico                                | 513 (35.3)  |
| USA                                   | 506 (34.8)  |
| Age: Mean (SD)                        | 34.0 (11.7) |
| Age: Median (Interquartile Range)     | 32.0 (24.0, 41.5) |
| Age Category (N = 1455)               |             |
| 16-30 years                           | 664 (45.6)  |
| 31-40 years                           | 393 (27.0)  |
| 41-50 years                           | 254 (17.5)  |
| 51-73 years                           | 144 (9.9)   |
| Race (N = 1434)                       |             |
| White                                 | 670 (46.7)  |
| Black                                 | 32 (2.2)    |
| Asian/Pacific Islander                | 201 (14.0)  |
| Mexican                               | 467 (32.6)  |
| Other                                 | 64 (4.4)    |
| Ethnicity (N = 1446)                  |             |
| Hispanic                              | 683 (47.2)  |
| Non-Hispanic                          | 763 (52.8)  |
| Education (N = 1448)                  |             |
| Completed 12 Years or Less            | 640 (44.2)  |
| Completed 13-15 Years                 | 408 (28.2)  |
| Completed at least 16 Years           | 400 (27.6)  |
| Marital Status (N = 1450)             |             |
| Single                                | 617 (42.6)  |
| Married/Cohabiting                    | 123 (8.5)   |
| Divorced/Separated/Widowed            | 710 (49.0)  |
| Smoking Status (N = 1450)             |             |
| Never                                 | 868 (59.9)  |
| Former                                | 310 (21.4)  |
| Current                               | 272 (18.8)  |
| Use of Chewing Tobacco or Snuff (N = 1434) |         |
| Never                                 | 1362 (95.0) |
| Sometimes                             | 20 (1.4)    |
| Daily                                 | 52 (3.6)    |
| Number of Alcoholic Drinks Consumed in the Past Month (N = 1430) |  |
| None                                  | 362 (25.3)  |
| 1-30 drinks                           | 656 (45.9)  |
| More than 30 drinks                   | 412 (28.8)  |
| Sexual Orientation (N = 1376)         |             |
| MSWa                                  | 1246 (90.6) |
| MSMb                                  | 48 (3.5)    |
| MSMWc                                 | 82 (6.0)    |

a MSW: Men who have sex with women only.

b MSM: Men who have sex with men only.

c MSMW: Men who have sex with women and men.

HPV vaccine types (6/11/16/18), grouped 9 HPV vaccine types (6/11/16/18/31/33/45/52/58), any high risk HPV type (16/18/31/33/35/39/45/51/52/56/58/59/68), any low risk type (6/11/42/43/54/55/56/58/66/70) and any HPV type (presence of one of the high or low risk HPV types). Overall, twenty one genotypes that were detected by both Linear Array and the SPF10 PCR-DEIA-LiPA25 system were included in our study.

We used Cohen’s Kappa test to assess the agreement between Linear Array and SPF10 PCR-DEIA-LiPA25 system for each individual and grouped HPV genotype(s) (Table 2). We also visually compared the percentage of samples that tested positive for individual HPV types 6, 11, 16 and 18 and grouped 4 vaccine genotypes (6/11/16/18), grouped 9 vaccine types (6/11/16/18/31/33/45/52/58) and any HPV genotype using grouped bar graphs with standard error bars (Fig. 1).

Going further, we examined the degree of concordance between the results of the two methods for individual and grouped HPV genotype(s) (Table 3) using Cohen's Kappa statistic, prevalence adjusted, bias adjusted Kappa statistic (PABAK) [20] and exact McNemar’s test. The Kappa statistic is affected by both bias between the two tests as well as the overall prevalence [20]. Hence, the PABAK statistic, a version of Kappa that adjusts for bias and takes into account the low prevalence of the outcome measure was included alongside the Kappa statistic. McNemar’s test is used to examine systematic differences between the results of two tests [21]; hence, it was also included in the analyses. Finally, we examined whether self-reported sociodemographic characteristics such as country of residence, age, race, ethnicity, education, marital status, cigarette smoking, alcohol consumption, use of chewing tobacco and/or snuff and sexual orientation were associated with concordance between the two tests in the detection of HPV 16, the most commonly found oral HPV type, using simple and multi-variable logistic regression models (Table 4). We assessed collinearity between covariates, and excluded covariates with high collinearity in the multi-variable logistic regression model. We used naïve Bayesian logistic regression models with weakly informative priors in R using ‘arm’ package [22] when infinitely wide confidence intervals were encountered in the logistic regression models.

3. Results

Overall, 1455 participants of the total 3098 participants in the oral sub-cohort of the HIM study were included in the analyses. Approximately 30% of participants were from Brazil and around 35% of the participants were from Mexico and the US each. The mean and median age of the participants was 34 and 32 years respectively. Almost half (45.6%) of the participants were aged between 18 and 30 years while less than 10% of the participants were older than 50 years. Most participants reported either White (46.7%) or Mexican (32.6%) races. There was an almost equal distribution between Hispanic (47.2%) and non-Hispanic (52.8%) ethnicities. The majority of participants completed 12 years or less of education (44.2%), were single (42.6%), had
Table 3

Degree of oral HPV detection concordance between linear array and SPF10 PCR-DEIA-LiPA25 system within the oral HIM sub-cohort that underwent both tests.

| HPV Genotype | SPF10+/LA- | SPF10+ /LA+ | SPF10+/LA- | SPF10+ /LA- | Kappa Statistic<sup>b</sup> | PABAK<sup>d</sup> | McNemar test<sup>e</sup> p-value |
|--------------|------------|-------------|------------|-------------|--------------------------|----------------|-----------------------------|
| 6            | 1440       | 1           | 2          | 12          | 0.12                     | 0.98           | 0.01                        |
| 11           | 1452       | 2           | 0          | 1           | 0.80                     | 1.00           | 1.00                        |
| 16           | 1426       | 4           | 3          | 22          | 0.24                     | 0.97           | < 0.01                      |
| 18           | 1451       | 0           | 0          | 4           | 0                        | 0.99           | 0.12                        |
| 31           | 1451       | 0           | 0          | 4           | 0                        | 0.99           | 0.12                        |
| 33           | 1454       | 0           | 0          | 1           | 0                        | 1.00           | 1.00                        |
| 35           | 1450       | 0           | 1          | 4           | 0.001                    | 0.99           | 0.37                        |
| 39           | 1447       | 0           | 1          | 7           | 0.001                    | 0.99           | 0.07                        |
| 40           | 1455       | 0           | 0          | 0           | N/A<sup>c</sup>          | 1.00           | 1.00                        |
| 42           | 1455       | 0           | 0          | 0           | N/A<sup>c</sup>          | 1.00           | 1.00                        |
| 45           | 1453       | 0           | 0          | 2           | 0                        | 1.00           | 0.50                        |
| 51           | 1440       | 1           | 2          | 12          | 0.12                     | 0.98           | 0.01                        |
| 52           | 1447       | 0           | 1          | 7           | 0.001                    | 0.99           | 0.07                        |
| 53           | 1445       | 0           | 1          | 9           | 0.001                    | 0.99           | 0.02                        |
| 54           | 1453       | 0           | 0          | 2           | 0                        | 0.99           | 0.01                        |
| 56           | 1447       | 0           | 0          | 8           | 0                        | 0.99           | 0.01                        |
| 58           | 1452       | 1           | 0          | 2           | 0.50                     | 1.00           | 0.50                        |
| 59           | 1452       | 1           | 1          | 1           | 0.50                     | 1.00           | 1.00                        |
| 66           | 1441       | 2           | 2          | 10          | 0.25                     | 0.98           | 0.04                        |
| 68           | 1454       | 0           | 0          | 1           | 0                        | 1.00           | 1.00                        |
| 70           | 1453       | 1           | 1          | 0           | 0.67                     | 1.00           | 1.00                        |
| Any HPV      | 1316       | 15          | 13         | 111         | 0.17                     | 0.83           | < 0.01                      |
| Any high risk HPV type | 1372       | 9           | 7          | 67          | 0.18                     | 0.90           | < 0.01                      |
| Any low risk HPV type | 1396       | 6           | 6          | 47          | 0.17                     | 0.93           | < 0.01                      |
| HPV 4 vaccine types | 1404       | 7           | 5          | 39          | 0.23                     | 0.94           | < 0.01                      |
| HPV 9 vaccine types | 1388       | 8           | 6          | 53          | 0.2                      | 0.92           | < 0.01                      |

<sup>a</sup> SPF10: SPF10 PCR-DEIA-LiPA25 system; LA: Linear Array method.

<sup>b</sup> Was obtained using Cohen's Kappa test, a test of agreement, between Linear Array and SPF10 PCR-DEIA-LiPA25 System.

<sup>c</sup> Kappa statistic was not available when both tests had zero detection for an oral HPV genotype.

<sup>d</sup> PABAK: Prevalence adjusted, bias adjusted Kappa estimate.

<sup>e</sup> Exact McNemar's test was used.
Among the 21 individual HPV genotypes, the sensitivity of the SPF10 PCR-DEIA-LiPA25 system was substantially more sensitive than the Linear Array method for detecting HPV in oral gargle samples; detection of HPV 16, 4 HPV vaccine types, 9 HPV vaccine types, and any HPV genotype was 3.9 times, 4.4 times and 2.0 times higher, often substantially, for the SPF10 PCR-DEIA-LiPA25 system compared to the Linear Array method. This difference is much higher than previous, FFPE tumor tissue based studies [11], where the increase in detection was not more than 2 fold. Any discordance found between the results from the two methods were not systematic i.e. they were not associated with the sociodemographic or behavioral risk factors for oral HPV infection.

The limit of detection for HPV 16, HPV 18, HPV 31, and HPV 45 has been reported to be 6, 41, 96, and 49 HPV DNA copies/PCR reaction respectively for the Linear Array assay [23] and 2, 1, 3, and 9 HPV DNA copies/PCR reaction respectively for the SPF10 PCR-DEIA-LiPA25 system (personal communication with Dr. Maurit de Koning of DDL Diagnostic Laboratory). The large discrepancy of level of detection between the Linear Array and SPF10 PCR-DEIA-LiPA25 systems can likely be

### Table 4

Univariate and multivariate adjusted association between sociodemographic characteristics and concordance in oral HPV type 16 detection by linear assay and SPF10 PCR-DEIA-LiPA25 system.

| Characteristic                | OR* (95% CI) | aOR* (95% CI) |
|------------------------------|--------------|---------------|
| Country                      |              |               |
| Brazil                       | 1.96 (0.72, 5.79) | 2.05 (0.68, 6.23) |
| Mexico                       | 1.49 (0.53, 4.47) | 1.58 (0.48, 5.21) |
| USA                          | Ref | Ref |
| Age Category                 |              |               |
| 18–30 years                  | Ref | Ref |
| 31–40 years                  | 0.77 (0.24, 2.12) | 0.59 (0.20, 1.79) |
| 41–50 years                  | 1.44 (0.49, 3.82) | 0.99 (0.33, 2.97) |
| 51–73 years                  | 1.26 (0.28, 4.11) | 0.66 (0.15, 2.92) |
| Race                         |              |               |
| White                        | Ref | – |
| Black                        | 0.50 (0.13, 1.90) | – |
| Asian/Pacific Islander       | 1.34 (0.21, 8.50) | – |
| Mexican                      | 0.70 (0.29, 1.67) | – |
| Other                        | 0.21 (0.01, 3.62) | – |
| Ethnicity                    |              |               |
| Hispanic                     | 1.35 (0.61, 3.12) | – |
| Non-Hispanic                 | Ref | – |
| Education                    |              |               |
| Completed 12 Years or Less   | Ref | Ref |
| Completed at least 16 Years  | 0.93 (0.34, 2.34) | 0.98 (0.38, 2.56) |
| Marital Status               |              |               |
| Single                       | Ref | Ref |
| Married/Cohabiting           | 1.31 (0.54, 3.36) | 1.38 (0.48, 4.01) |
| Divorced/Separated/Widowed   | 3.23 (0.96, 9.84) | 3.39 (0.93, 12.30) |
| Smoking Status               |              |               |
| Never                        | Ref | Ref |
| Former                       | 0.56 (0.13, 1.68) | 0.63 (0.19, 2.01) |
| Current                      | 0.82 (0.27, 2.10) | 0.76 (0.24, 2.14) |
| Use of Chewing Tobacco or Snuff |              |               |
| Never                        | Ref | Ref |
| Sometimes                    | 0.28 (0.01, 5.10) | 0.3 (0.02, 5.93) |
| Daily                        | 2.29 (0.32, 16.44) | 1.93 (0.26, 14.30) |
| Number of Alcoholic Drinks Consumed in the Past Month | | |
| None                         | Ref | Ref |
| 1–30 drinks                  | 1.11 (0.39, 3.57) | 1.27 (0.43, 3.74) |
| More than 30 drinks          | 1.78 (0.63, 5.75) | 2.08 (0.68, 6.36) |
| Sexual Orientation           |              |               |
| MSW#                         | Ref | Ref |
| MSM#                         | 1.24 (0.07, 6.13) | 1.17 (0.18, 7.46) |
| MSMWH#                      | 0.72 (0.04, 3.51) | 0.72 (0.13, 4.10) |

None of the variables were significantly associated with HPV 16 discordance at α = 0.05 level.

* Unadjusted odds ratio calculated using simple logistic regression.

* 95% confidence interval.

* Adjusted odds ratio; was calculated using multivariable logistic regression with weakly informative default prior distribution for the regression coefficients. Model was adjusted for country of residence, age, education, marital status, cigarette smoking status, using chewing tobacco or snuff, alcohol consumption and sexual orientation.

* 95% Bayesian credible interval; an interval within which an observed parameter value falls with 95% probability.

* Weakly informative prior distribution was applied via naïve Bayesian logistic regression model since simple logistic regression yielded very wide confidence intervals.

* MSW: Men Having Sex with Women.

* MSM: Men Having Sex with Men.

* MSMW: Men having Sex with Men and Women.

never smoked cigarettes during their life (59.9%), never chewed tobacco or used snuff (95.0%), had consumed between 1 and 30 drinks during past month (45.9%) and were heterosexual (90.6%) (Table 1).

Among the 21 individual HPV genotypes, the sensitivity of the SPF10 PCR-DEIA-LiPA25 method was substantially more sensitive than the Linear Array method except for HPV 70. Linear Array did not detect HPV types 18, 31, 33, 54, 56 and 68 whereas the SPF10 PCR-DEIA-LiPA25 system was able to detect 4, 5, 6, 11, 16, and 18, and 49 HPV DNA copies/PCR reaction respectively for the SPF10 PCR-DEIA-LiPA25 system to Linear Array in our study population varied between 1.0 and 9.0, including 3.8 for HPV 16. Among the grouped HPV categories, the prevalence ratio varied between 3.8 and 4.8 (Table 2). Overall, the prevalence of oral HPV for types 6, 11, 16, and 18, grouped 4 and 9 HPV vaccine genotypes, and any HPV genotype was higher, often substantially, for the SPF10 PCR-DEIA-LiPA25 system compared to the Linear Array method (Fig. 1). For grouped 4 HPV vaccine types, 9 HPV vaccine types, and all HPV genotypes, the prevalence was 3.2%, 4.2% and 8.7% respectively by the SPF10 PCR-DEIA-LiPA25 system compared to Linear Array’s 0.8%, 1.0%, and 4.3% respectively. Oral HPV prevalence was 3.9 times, 4.4 times and 2.0 times higher for grouped 4 HPV vaccine types, 9 HPV vaccine types, and all HPV genotypes respectively when the SPF10 PCR-DEIA-LiPA25 system was used compared to the Linear Array method.

In Table 3, we assessed the number of positive cases detected by SPF10 PCR-DEIA-LiPA25 system but left out by the Linear Array method and vice versa for individual and grouped HPV genotypes. Overall, detection by the SPF10 PCR-DEIA-LiPA25 system and missed by the Linear Array method occurred much more frequently than vice versa, with the exception of HPV 70. For example, for HPV 16, 22 cases were picked up by the SPF10 PCR-DEIA-LiPA25 system but not Linear Array, whereas 3 cases were picked up by Linear Array but not the SPF10 PCR-DEIA-LiPA25 system. Similarly, for grouped any high risk HPV, the SPF10 PCR-DEIA-LiPA25 system detected 67 cases that Linear Array did not, whereas the Linear Array detected 7 cases that the SPF10 PCR-DEIA-LiPA25 system did not. While the traditional Cohen’s Kappa method found moderate to substantial agreement for only HPV types 11, 58, 59 and 70, the PABAK method found substantial agreement (more than 80%) for all individual and grouped HPV types. The results from the two tests were significantly different for HPV genotypes 6, 16, 51, 53, 56, 66 and all categories of grouped HPV genotypes; for all of these individual and grouped categories, oral HPV prevalence by the SPF10 PCR-DEIA-LiPA25 system was substantially higher than that of the Linear Array method.

We examined factors that may be associated with concordance of oral HPV 16 detection between the two HPV genotyping assays (Table 4). We did not include race and ethnicity in our adjusted model because of the high degree of collinearity between country of residence and the covariates race and ethnicity: more than 91% and 99% of the Mexican participants reported race of ‘Mexican’ and ethnicity of ‘Hispanic’ respectively. None of the unadjusted or adjusted associations achieved statistical significance.

4. Discussion

The SPF10 PCR-DEIA-LiPA25 system was substantially more sensitive than the Linear Array method for detecting HPV in oral gargle samples; detection of HPV 16, 4 HPV vaccine types, 9 HPV vaccine types, and any HPV genotype was 4 times higher with the SPF10 PCR-DEIA-LiPA25 system compared to the Linear Array method. This difference is much higher than previous, FFPE tumor tissue based studies [11], where the increase in detection was not more than 2 fold. Any discordance found between the results from the two methods were not systematic i.e. they were not associated with the sociodemographic or behavioral risk factors for oral HPV infection.

The limit of detection for HPV 16, HPV 18, HPV 31, and HPV 45 has been reported to be 6, 41, 96, and 49 HPV DNA copies/PCR reaction respectively for the Linear Array assay [23] and 2, 1, 3, and 9 HPV DNA copies/PCR reaction respectively for the SPF10 PCR-DEIA-LiPA25 system (personal communication with Dr. Maurit de Koning of DDL Diagnostic Laboratory). The large discrepancy of level of detection between the Linear Array and SPF10 PCR-DEIA-LiPA25 systems can likely be
explained by the fragment of DNA being amplified buy each assay (~450 bp vs 65 bp, respectively), therefore making the SPF10 assay more robust in processes that degrade DNA. In our study, although the SPF10 PCR-DEIA-LiPA25 system was in general more sensitive than the Linear Array method, the Linear Array method was able to detect a few cases which were not picked up by the SPF10 PCR-DEIA-LiPA25 system. One potential explanation may be the extremely low HPV DNA load in oral gargle samples leading to variation in HPV DNA composition when different oral gargle aliquots are used, though they have been aliquoted from the same original sample. A similar phenomenon has been observed in a study comparing carcinogenic HPV detection by the SPF10 PCR-DEIA-LiPA25 system and Hybrid Capture 2 assay in cervical specimens from 5683 women enrolled in the Costa Rican HPV 16/18 Vaccine Trial [24]. In that study, HC2 positive but SPF10 PCR-DEIA-LiPA25 system negative samples had lower HP viral load, and the authors hypothesized that sampling error due to low HP viral load was a potential reason.

The Cohen’s Kappa test showed moderate to substantial agreement for only a few HPV genotypes. The finding is different in this study of oral HPV from a previous study [14] where the kappa value was 0.82 when these two tests were compared for any HPV genotyping of cervical specimens. This may be due to difference in HPV prevalence between the two studies. The low Kappa statistic estimates in our study may be due to low HPV viral load in oral gargle specimens compared to cervical specimens. Given that more than 90% (and often more than 99%, depending on HPV type) of the oral specimens are negative for oral HPV, Cohen’s Kappa may not be an ideal method for measuring inter-test agreement [25]. Hence, though we presented Kappa test results in this study to increase comparability of results to previous studies, we believe that these statistics should not be considered as an absolute indicator of inter-test agreement between the SPF10 PCR-DEIA-LiPA25 System and the Linear Array method in oral gargle samples, and other methods such as prevalence adjusted, bias adjusted Kappa estimate (PABAK) may provide better estimates of agreement between the two methods. In our study, good concordance was shown with PABAK despite the poor concordance shown with Kappa test.

None of the unadjusted and adjusted associations between sociodemographic - behavioral risk factors and discordance in HPV 16 results reached statistical significance at α = 0.05. These tests were conducted to examine whether discordance in oral HPV genotyping was random or systematic in nature. The discordance in oral HPV genotyping appears to be unbiased by sociodemographic and behavioral risk factors in our data.

Overall, the SPF10 PCR-DEIA-LiPA25 system had substantially higher sensitivity than the Linear Array method for genotyping HPV in oral gargle samples. Like all studies, this study has a few limitations that should be noted. One of the limitations is the temporal distance between the two methods for testing the oral gargle samples. Since the SPF10 PCR-DEIA-LiPA25 system was used almost a decade after the Linear Array method, there is a possibility of degradation of DNA within the oral samples with storage. However, it has to be noted that degradation would have led to a lower detection, not higher oral HPV detection as observed in this study. The strengths of this study include use of a large number of oral gargle samples originating from a pool of diverse participants residing in three different countries and belonging to a wide range of age groups. It should also be noted that the assays utilized in this study are assays suitable for research, such as studies of oral HPV natural history, vaccine trials with oral HPV as the endpoint, and vaccine effectiveness, but may not be suitable in clinical practice. At the time of this writing, there is no HPV DNA test that we are aware of that has been validated for clinical evaluation of oral HPV.

In summary, these data indicate the need to utilize highly sensitive HPV genotyping assays with smaller primer sizes for oral gargle specimens to assess oral HPV prevalence. Given the difficulty of localizing asymptomatic oral HPV infections, sampling methods such as oral gargle are used to estimate oral HPV infection but tend to be diluted in comparison with methods such as direct sampling of genital and anal epithelia. Therefore, there is a higher chance of underestimation of oral HPV incidence and persistence compared to that of anogenital and cervical mucosa when methods such as Linear Array, or assays with similar sensitivity, are used. In the light of increasing importance of detection of oral HPV, we recommend using methods with higher sensitivity and smaller primer sizes such as the SPF10 PCR-DEIA-LiPA25 system, a test that has already been widely used in HPV vaccine trials.

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CRediT authorship contribution statement

Deepti Bettampadi: Conceptualization, Methodology, Formal analysis, Writing - original draft. Bradley A. Sirak: Methodology, Investigation, Writing - review & editing. William J. Fulp: Methodology, Writing - review & editing. Martha Abrahamsen: Investigation, Writing - review & editing, Project administration. Luisa L. Villa: Investigation, Writing - review & editing. Edoardo Lazzcano-Ponce: Investigation, Writing - review & editing. Jorge Salmeron: Investigation, Writing - review & editing. Kimberly A. Isaacs-Soriano: Investigation, Writing - review & editing. Maria L. Baggio: Investigation, Writing - review & editing. Manuel Quiterio Trenado: Investigation, Writing - review & editing. Anna R. Giuliano: Conceptualization, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Anna R Giuliano is a member of the Merck Advisory Board and her institution has received funding for research through a Merck Investigator-initiated studies program.

Luisa L Villa is a consultant to Merck for the HPV prophylactic vaccine and provides occasional consultancy to BD, Roche and Qiagen concerning HPV tests.

Appendix A. Supplementary data

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