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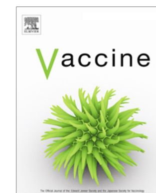

# Evaluation of HPV-16 and HPV-18 specific antibody measurements in saliva collected in oral rinses and merocel® sponges

Katherine H. Parker [a], Troy J. Kemp [a], Yuanji Pan [a], Zhen Yang [a], Anna R. Giuliano [b], Ligia A. Pinto [a,*]

[a] HPV Immunology Laboratory, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute, Frederick, MD, USA
[b] Center for Infection Research in Cancer, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, USA

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ABSTRACT

*Background:* Current Human papillomavirus (HPV) L1 VLP vaccines protect against HPV-16 and HPV-18-associated cancers, in females and males. Although correlates of protection have not been identified, HPV-specific antibodies at sites of infection are thought to be the main mechanism of protection afforded by vaccination. Oral sampling has gained increased attention as a potential alternative to serum in monitoring immunity to vaccination and understanding local immunity in oral cancers.
*Methods:* Serum was collected via venipuncture, and saliva was collected via oral rinses and Merocel® sponges from healthy volunteers: 16 unvaccinated females, 6 females (ages 24–41) and 6 mid-adult aged males (ages 27–45) recipients of three doses of the HPV-16/18/6/11 vaccine (Gardasil®). Mid-adult male vaccine trial participants were compared to female participants. Samples were tested for anti-HPV-16 and anti-HPV-18 immunoglobulin G levels by an L1 virus-like particle-based enzyme-linked immunosorbent assay (ELISA).
*Results:* All vaccinated participants had detectable serum anti-HPV-16 and anti-HPV-18 antibodies. Optimal standard concentration range and sample serial dilutions for oral rinses were determined. The standard curve was not affected by the type of solution examined. Reproducibility of HPV-16 and HPV-18 antibody titers in mouthwash (overall CV < 10%) or in Merocel® extraction buffer was robust (CV < 13%). Excellent assay linearity ($R^2 > 0.9$) was observed for sera spiked controls in both solutions. HPV-16 and HPV-18 specific antibodies were detectable in saliva from vaccine recipients, both in mouthwash and in Merocel® sponges but levels were several logs lower than those in serum.
*Conclusions:* This study confirms the application of HPV-16 and HPV-18 ELISAs currently used in sero-epidemiological studies of immunogenicity of HPV vaccines for use with oral samples. Oral samples may be a useful resource for the detection of HPV-16 and HPV-18-specific antibodies in saliva following vaccination.

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## 1. Introduction

In 2017, it is estimated that 49,670 people will be diagnosed with oral and oropharyngeal cancer in the United States of America (USA), and about 9,700 people will die from this disease [1]. The incidence rates of oral and oropharyngeal cancer are increasing and are more than twice as high in men as women [2]. Persistent infection with oncogenic human papillomavirus (HPV) types, especially HPV-16, is strongly associated with oropharyngeal cancer [3]. Currently in the USA more than 70% of oropharyngeal cancers are attributed to HPV infection, particularly HPV-16 and HPV-18 [4].

Three virus-like particle (VLP) late 1 (L1) based prophylactic vaccines targeting up to nine HPV oncogenic types have been approved by USA Food and Drug Administration (FDA) [5–7]. Gardasil®, first approved in 2006, is comprised of L1 major capsid protein-based VLPs of HPV-6, 11, 16, and 18. The vaccine is highly efficacious at preventing HPV-16 and HPV-18 infections in males and females as well as associated cervical, vaginal, vulvar, and anal lesions [8–11]. Cervarix®, a HPV vaccine comprised of L1 based VLP for HPV-16 and HPV-18 demonstrates robust efficacy against genital HPV-16 and -18 infections in females and in a post-hoc analysis, efficacy at the oral cavity in females [12]. Although the underlying mechanisms of protection have not been fully elucidated, HPV-specific neutralizing antibodies at sites of infection are thought to be the main mechanism of protection against infection [13–15].

* Corresponding author at: Frederick National Laboratory for Cancer Research, Frederick, MD, 21702, USA.
*E-mail address:* pintol@mail.nih.gov (L.A. Pinto).

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Traditionally, serum samples have been the specimen of choice for biomarker detection and immune monitoring of vaccines. However, the site of infection where the cancer originates is of critical importance. Saliva has gained increased attention as an attractive alternative collection site to serum, since oral sampling collection is simple, painless, non-invasive, and presents no risks [16]. Many biomarkers have been measured in saliva, such as hormones, cytokines, and vaccine-induced antibodies, making it a promising matrix for monitoring immune responses both systemically and locally in the oral cavity of vaccinated individuals [17–19]. Previous studies have found oral HPV-specific IgG levels in natural infection; however, the levels are low and only modestly correlate with serum HPV-specific IgG levels [20–24]. Detection of anti-HPV-16 antibodies in saliva from vaccine recipients has been previously reported. However, in that study a luminex bead-based assay was used, and data were reported without the use of a standard curve [25]. Given the interest in measuring HPV-specific antibodies in saliva in clinical/epidemiological studies, we adapted the serum enzyme linked immunoabsorbent assay (ELISA) protocol to assess HPV-specific antibody levels in saliva.

Previously, our lab monitored the immunogenicity of HPV vaccines by measuring serum anti-HPV-16 and anti-HPV-18 IgG antibody levels in sero-epidemiological studies using a standardized VLP-based direct ELISA [26,27]. Here, we evaluated whether the standardized L1 VLP-based direct ELISA could be used to detect HPV type-specific antibodies in oral samples collected using two types of collection methods, mouthwash and Merocel® sponges following vaccination. Our hypothesis was that the HPV vaccine would induce antibodies against HPV at mucosal sites, in this case saliva, that could be detected by an L1 VLP ELISA. However, because levels at mucosal sites were expected to be much lower than in serum, the assay had to be optimized and qualified for the new matrix: saliva. This study serves as a methods validation paper, accompanying our previous findings of HPV antibodies detected in the oral cavity of vaccinated males [28]. A mouthwash sample is comprised of both oral cells and saliva, and is the standard method for collecting oral specimens for HPV analysis [29]. Mouthwash specimens are commonly archived in many HPV studies; therefore, we chose to assess if this specimen was adequate for oral antibody testing. Merocel® sponges served as a device that collects saliva. The data described here indicate that the standardized L1 VLP-based ELISA can reliably detect HPV-16 and HPV-18 antibodies in both mouthwash and Merocel® sponges. Having an assay that allows for accurate detection of HPV-specific antibodies in saliva provides an alternative means of determining efficacy of HPV vaccines for the prevention of oral cancer.

## 2. Materials and methods

### 2.1. Samples

Saliva from healthy research donor volunteers (Occupational Health Services, FNLCR, Frederick, MD), 16 unvaccinated (ages 32–63) and 6 female (ages 24–41) recipients of three doses of the HPV-16/18/6/11 vaccine (Gardasil®) were collected in mouthwash (Target) and in Merocel® (Beaver-Visitec International, Inc.) sponges. HPV vaccinated donors were chosen based solely on vaccination status, and unvaccinated donors were chosen based on prescreening and testing as HPV seronegative. Six serum samples from male recipients of Gardasil® were used from *The Mid-Adult Male Vaccine Study – The MAM STUDY* (www.clinicaltrials.gov, NCT01432574). This is a single-arm intervention trial that enrolled and vaccinated men ages 27–45, and assessed the antibody responses to Gardasil [30]. Subjects were vaccinated intramuscularly with Gardasil at day 1 of the study and at months 2 and 6. The six male subjects were selected out of 150 men from Tampa, Florida, and Cuernavaca, Mexico, who met eligibility criteria (male sex, age 27–45 years, and completion of 4 years of follow-up in the HPV Infection in Men study) and received at least one dose of vaccine. The six samples chosen were from month 30 of the study (24 months after vaccination).

### 2.2. Mouthwash samples

50 mL tubes (Corning Cat# 352098) were filled with 15 mL of mouthwash solution and donors were asked to swish the mouthwash vigorously for 30–45 s and to expel the mouthwash into an empty collection tube [31]. Samples were centrifuged, aliquoted and stored at −80 °C.

### 2.3. Merocel® sponges

Subjects placed a Merocel® sponge against the central part of the inner cheek for a total of 30 s, 15 s for each side. The sponge was then placed into a sterile 15 mL cryovial. Vials were stored at −80 °C until extraction. The sponges were extracted using a buffer containing PBS (Gibco, Cat# 14190-136), 256 mM NaCl (Fisher Scientific, Cat# S271-10) and 100 μg/mL aprotinin (Sigma, Cat# A-4529-25MG). Extracts were aliquoted and stored at −80 °C. A dilution factor, based on the weight of the collected material, was calculated for each sample, as previously reported [32].

### 2.4. Serum

10 mL of blood was collected in a red top tube (BD, Cat# 366430). Following centrifugation, sera was aliquoted into cryovials and stored at −80 °C until testing.

### 2.5. ELISA

HPV-16 and HPV-18-specific IgG antibody titers were determined by the L1 VLP ELISA. Microtiter plates (Maxisorp, NUNC, Cat# 439454) were coated with HPV VLPs produced in our laboratory, as previously described [33,34]. Starting at 1:2, Saliva was serially diluted 2-fold to 1:256, in the blocking buffer (PBS (Gibco, Cat# 14190-136), 4% Milk (BD, Cat# 232100), 0.2% Tween 20 (VWR, Cat# EM-PX1296-1) was plated and assayed. Positive controls for HPV ELISAs were generated by spiking HPV-antibody positive serum obtained from an HPV vaccine recipient, and diluted into mouthwash or sponge extraction buffer, at ratios of 1:16,666, 1:50,000, and 1:150,000. Negative controls were obtained from Occupational Health Services donors whose serum tested below detection cut offs for HPV-16 and -18 antibodies (negative for HPV-16 and -18 antibodies). Serial dilutions of samples, standards and quality controls were included in each plate and absorbance was measured. The mean optical density (OD) of saliva samples, from HPV-16 and HPV-18 seronegative individuals, plus 3 standard deviations were used to define the cutoff. Antibody levels, expressed as ELISA units (EU)/mL, were calculated by interpolation of OD values from the standard curve by averaging the calculated concentrations from all dilutions that fall within the working range of the standard curve. Assay reproducibility and linearity were determined using mouthwash or sponge extraction buffer spiked with three different known levels of HPV-16 and HPV-18 antibodies (Low: 0.027 EU/mL; Medium: 0.08 EU/mL; High: 0.267 EU/mL). Limit of quantitation (LOQ) was determined by testing 15 seronegative samples, diluted 1:2, and run on three separate days. The mean of the 15 samples plus three times the standard deviation, represents LOQ for the assay. For limit of detection (LOD), absolute mouthwash was diluted 1:2, and ran on 6 separate plates, in three different days. The LOD represents the mean plus two times the standard deviation for the assay (data not shown).

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