Evaluation of Two Types of Sponges Used To Collect Cervical Secretions and Assessment of Antibody Extraction Protocols for Recovery of Neutralizing Anti-Human Papillomavirus Type 16 Antibodies


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Immunogenicity evaluations in human papillomavirus (HPV) vaccine trials have relied on serological samples, yet cervical antibodies are likely to be most relevant for protection against infection. In order to assess functional antibody levels at the cervix, the secreted-alkaline-phosphatase neutralization assay (SEAPNA) was used to measure HPV-neutralizing activity. We assessed the variability of the SEAPNA with serum samples after vaccination with an HPV type 16 (HPV16) L1 virus-like particle vaccine and whether the SEAPNA can be used to monitor neutralizing activity at the cervix. The SEAPNA has an overall coefficient of variation of 29.3%. Recovery from ophthalmic sponges was assessed by spiking V5 (mouse anti-HPV16) antibody onto and extracting it from sterile Merocel and Ultracell sponges and sponges used to collect specimens from participants. V5 recovery from sterile Merocel sponges was complete, yet that from Ultracell sponges was null. The mean V5 recoveries from participant Ultracell and Merocel sponges were 61.2% and 93.5%, respectively, suggesting that Merocel sponges are more appropriate for specimen collection. The SEAPNA can be applied to determine the surrogates of protection and to examine the durability of protection at the cervix.

Prophylactic human papillomavirus (HPV) L1-based virus-like particle (VLP) vaccines have been shown to be safe, immunogenic, and protective against cervical infection and the associated lesions by homologous HPV types (7–9, 11, 17, 18). In addition, HPV VLP vaccinations induce neutralizing antibodies not only in the periphery but also at the cervix (6, 12). Several assays for monitoring HPV type-specific antibody responses have been developed; however, validation efforts for these assays have focused on serum rather than the cervix, where protection is executed.

HPV VLP enzyme-linked immunosorbent assays (ELISAs) have been used to determine serum antibody titers in epidemiological studies of HPV infection and in HPV vaccine trials (8, 9, 15, 16, 19). Enzyme-linked immunosorbent-based assays have the advantage of being applicable for large-scale studies. However, these assays lack the ability to discern between neutralizing and nonneutralizing antibodies and may have the disadvantage of detecting antibodies to yeast and baculovirus-derived proteins, compromising their specificity. It is therefore still unclear whether ELISAs are optimal for the evaluation of the levels of protective antibodies at the cervix.

Recently, a pseudovirion (PsV) neutralization assay (a secreted-alkaline-phosphatase neutralization assay [SEAPNA]) was developed by Pastrana et al. to evaluate the neutralizing potential against HPV (13). This assay has the advantage of specifically measuring the biological activity believed to be relevant for protection (i.e., neutralization).

Here, we explored whether the SEAPNA could be used to monitor neutralizing antibody levels at the cervix by the use of two different ophthalmic sponges (Merocel and Ultracell) commonly used in large-scale studies for cervical mucus sampling. We evaluated the effect of a standard extraction buffer (EB) previously used for cervical secretion extraction and examined the recovery levels of V5 (mouse anti-HPV type 16 [HPV16] monoclonal type-specific neutralizing antibody) (3) from sterile, unused Merocel and Ultracell sponges and from Merocel and Ultracell sponges used to collect specimens from participants via the SEAPNA. Finally, we evaluated the use of a mouse monoclonal immunoglobulin G (IgG) for spiking and recovery from study participant specimens as a means of controlling for recovery efficiency in future efforts and attempted to improve recovery from collection devices.

MATERIALS AND METHODS

SEAPNA with serum. (i) Participant specimens. Sera collected 1 month after the first vaccination or 1 month after the second vaccination from 12 participants enrolled in a phase I trial of a VLP HPV16 vaccine were used (9). This study was conducted according to the guidelines established by the Joint Committee for Clinical Investigation of the Johns Hopkins University School of Medicine and its institutional review board for human experimentation.

(ii) Cell culture. 293TT cells were expanded and cultured as previously described (13).
(iii) SEAPNA. The SEAPNA was performed as previously described with a few modifications (13). Serum samples were serially diluted in fourfold increments with neutralization buffer (NB). Controls, set up in triplicate, included (i) NB alone, (ii) NB plus HPV16 Piv, and (iii) V5 (mouse anti-HPV16) (3) plus HPV16 Piv, and (iv) SBs (mouse anti-bovine papillomavirus 1) (14) plus HPV16 Piv. Diluted serum was incubated with HPV16 Piv at 4°C for 1 h in duplicate wells at a 1:5 ratio. Then, the samples were transferred to the 293 TT cells and incubated for 72 h. Following incubation, supernatants were transferred to 96-well V-bottom plates, clarified by centrifugation, and frozen at ~80°C before SEAP analysis. SEAP was detected using the Great EscAPe SEAP assay kit, according to the manufacturer’s protocol (BD Biosciences-Clontech Laboratories, Inc., Mountain View, CA). Samples were read on a chemiluminescence plate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA). Serum neutralization titers were defined as the reciprocal of the calculated dilution that caused a 50% reduction in SEAP activity based on linear interpolation.

(iv) Testing format. To evaluate within-plate, between-day, and between-technician variability for the SEAPNA, specimens from the 12 participants described above were each tested twice by two technicians, each on two different days.

(v) Statistical analysis. For each technician, a nested, within-person analysis of variance was used to test for assay reproducibility across plates. Data were analyzed on the natural logarithmic scale.

RESULTS

(iii) Collection sponges. The cellulose-based Ultracell ophthalmic sponge (Ultracell Medical Technologies, Inc., North Stonington, CT) and the polyvinyl acetate-based Merocel ophthalmic sponge (Medtronic Ophthalmics, Jacksonville, FL) were used in this study.

(ii) Participant specimens. Cervical secretion specimens were taken from participants in a natural history study of HPV and cervical neoplasia in Costa Rica (1) using either Ultracell (n = 35) or Merocel (n = 20) sponges. The Ultracel or Merocel sponge was gently placed in the cervical os for 30 seconds to allow for passive absorption of cervical secretions (2, 10) prior to Pap smear sampling. Sponges were then stored in cryovials, frozen, and shipped to the United States in liquid nitrogen. Upon arrival in the United States, the samples were stored at ~80°C until the extraction procedure. Specimens obtained from participants in the natural history study were used for spike-recovery experiments, because they were presumed to have low/undetectable levels of neutralizing antibodies. Prior to the extraction process, the sponges were thawed on ice and participant sponge weights were recorded. All participants provided informed consent, and the study was approved by the National Cancer Institute (NCI) and the local institutional review board in Costa Rica.

(iii) Effect of cervical sponge extraction buffer on SEAPNA. Fourfold serial dilutions (1:10 to 1:40,960) of the EB (containing phosphate-buffered saline [Invitrogen, Grand Island, NY], 256 mM NaCl [Sigma-Aldrich, St. Louis, MO], and 100 µg/ml aprotinin [Sigma-Aldrich, St. Louis, MO]) were tested in triplicate. Results were expressed as follows: % of the control [Invitrogen, Grand Island, NY], 256 mM NaCl [Sigma-Aldrich, St. Louis, MO], or 100 µg/ml aprotinin (Sigma-Aldrich, St. Louis, MO)] were spiked with mouse IgG1-TNP to calculate the recovery. The extraction process was performed as described above, and the mouse IgG1-TNP eluted was evaluated by testing each sample in duplicate wells with a mouse IgG1 ELISA detection kit (Bethyl Laboratories, Inc., Montgomery, TX). The recovery was calculated as follows: % recovery = (quantitative value of sample X/quantitative value of control) × 100.

(ii) Effect of BSA on recovery of mouse IgG1-TNP from Ultracell sponges. BSA was tested in three formats as follows. (i) Two sponges were incubated with BSA (300 µg each, 0.5% to 0.00005%) diluted in EB for 1 h at 4°C, and centrifuged in a Spin-X tube for 15 min at 13,000 × g at 4°C. Then, 30 µl of mouse IgG1-TNP (1,000 ng/ml) was spiked onto each sponge and eluted in EB or ELISA buffer as controls. The extraction process was performed as described above, and the amount of mouse IgG1-TNP eluted was evaluated by testing each sample in duplicate wells with a mouse IgG1 ELISA detection kit (Bethyl Laboratories, Inc., Montgomery, TX). The recovery was calculated as follows: % recovery = (quantitative value of sample X/quantitative value of control) × 100.

RESULTS

(iii) Serum SEAPNA. To assess the SEAPNA variability, 12 serum samples from vaccine recipients were tested in duplicate per plate with two technicians setting up two plates for each sample, on two different days. The tier range for the samples from the 12 vaccinees was 1/98 to 1/16,731. The overall coefficient of variation (CV) of the assay was 29.3% for the SEAPNA, with most of the variability occurring within the plate (26.3% CV). The between-plate variability was low (7.4% CV), and the intraclass correlation coefficient was high (91.1%), demonstrating that biological variability among women is much larger than the assay variability.

Evaluation of SEAPNA for measurement of antibodies collected using ophthalmic sponges. The EB had little to no effect on the SEAPNA (95% confidence interval [CI] of 98.3% to 111% of the control in two separate experiments). The total mean recovery of V5 from sterile unused Ultracell sponges (n = 16) was only 2.5% (95% CI, −0.6% to 5.6%) (Table 1).
In contrast, Merocel sponges (n = 32) had a mean recovery of 102% (95% CI, 98.1% to 106%) (Table 1).

Subsequently, we examined the recovery of V5 spiked prior to extraction onto the polyvinyl acetate-based Merocel sponges used in the collection of cervical secretions from 20 participants enrolled in an HPV natural history cohort study in Guanacaste, Costa Rica. The combined results from two separate experiments using spikes with two different dilutions of V5 demonstrate that 18 of 20 (90%) participant Merocel sponges had recoveries of >80%, with a mean recovery of 93.5% (95% CI, 86.4% to 101%) (Table 1). The recovery of V5 from the participant Ultracell sponges was more variable than that observed for Merocel sponges, with 18 of 35 (51.4%) having recoveries of >80%. In four experiments with spikes at two different dilutions of V5, the mean recovery of V5 neutralization was 61.2% (95% CI, 44.3% to 78.2%) from participant Ultracell sponges.

It is interesting to note that the participant Ultracell sponges that gave the poorest recoveries also had the lowest weights, on average. The sponges with greater than 50% recovery were heavier (sponge weights were 0.1280 g), while those with less than 50% recovery had a mean weight of 0.0896 g (P < 0.0001, Mann-Whitney test). The menopausal statuses of participants who provided the Ultracell specimens were also indicative of poor recovery status, a phenomenon likely explained by the lower sponge weights obtained from menopausal participants, who are known to have decreased levels of secretions in their genital tracts.

**Strategies to monitor and improve antibody recovery from cervical secretions collected using cellulose-based Ultracell sponges.** The results from Ultracell sponges, either sterile or from participants, were null or very erratic, respectively. Due to the use of the Ultracell sponges in some natural history studies, we focused on improving the recovery of neutralizing antibodies and implementing controls to monitor extraction efficiency. Detergents and BSA were used to improve recovery, while the spiking of mouse IgG1-TNP onto sponges after collection and prior to extraction was tested as an agent to monitor extraction efficiency.

Mouse IgG1-TNP had no adverse effects on PsV infectivity and V5 neutralization (data not shown).

**Strategies to improve recovery from Ultracell sponges included treating Ultracell sponges with three different detergents and blocking nonspecific binding to the cellulose-based Ultracell sponge.** We discovered the maximum concentrations of Empigen BB (0.0005%), Triton X-100 (0.00005%), and Tween 20 (0.0005%) that could be cultured with the 293TT cells without having an impact on cell viability. Similarly, the maximum concentrations of Empigen BB, Triton X-100, and Tween 20 that could be used in the SEAPNA were 0.00005% (Fig. 1A). Next, we tested whether Empigen BB could improve the recovery of antibodies from unused sterile Ultracell sponges. Even at Empigen BB concentrations as high as 0.5%, only 17.1% of the mouse IgG1-TNP could be recovered. Thus, the three detergents tested in this study do not appear to improve the extraction of neutralizing antibodies from Ultracell sponges.

BSA was used in an attempt to block nonspecific binding of the antibodies to the cellulose-based Ulractell sponges. We compared the recoveries of mouse IgG1-TNP antibodies from the following: (i) Ultracell sponges soaked in BSA prior to spiking with mouse IgG1-TNP, (ii) Ultracell sponges spiked with mouse IgG1-TNP diluted in BSA, and (iii) Ultracell sponges spiked with mouse IgG1-TNP and extracted in BSA. The results indicate that BSA at concentrations of >0.05% added to the sponge (Fig. 1B) or mouse IgG1-TNP prior to spiking (data not shown) was sufficient to effectively elute the mouse IgG1-TNP. In contrast, adding BSA to the EB could not sufficiently extract the purified mouse IgG1-TNP previously spiked onto Ultracell sponges (Fig. 1C).

**DISCUSSION**

The SEAPNA is a novel assay designed to directly measure HPV type-specific neutralizing activity. SEAPNA variability has not been extensively characterized, and the assay has not been formally validated for use with genital tract specimens. In the present study, we defined the components of variability of the SEAPNA using serum and evaluated the ability of the assay to measure neutralizing activity in cervical secretion specimens.

While the SEAPNA is more variable than the ELISA (5) and the competitive Luminex assay (4), the level of variability seen among women was far greater than that observed for the SEAPNA, as indicated by the high intraclass correlation coefficient observed with serum samples (91.1%). Important conclusions from our methodological work to evaluate the ability of the SEAPNA to measure neutralizing activity at the cervix are as follows. First, we have shown that neutralizing antibodies can be completely recovered from Merocel sponges, while recoveries from Ultracell sponges are low and more variable. This is likely explained by the chemical compositions of Merocel (polyvinyl acetate) and Ultracell (cellulose) sponges. Our poor recovery results from Ultracell sponges appear to contrast with a previous study that analyzed the recovery of cytokines and antibodies from different sponge materials (2). However, the Ultracell sponges evaluated in the study by Castle et al. (2) were made of polyvinyl alcohol, in contrast to the cellulose-based Ultracell sponges used in this study. It is intriguing to note that in the study by Castle et al., the performance of the cellulose-based Weck-Cel sponges was not dramatically different from that of the polyvinyl alcohol-based Ultracell or polyvinyl acetate-based Merocel sponges tested here. Cytokines and antibodies in that study may have been spiked onto the sponges in the presence of a carrier protein, which would

<table>
<thead>
<tr>
<th>Sponge type</th>
<th>No. of sponges</th>
<th>Mean % V5 recovery</th>
<th>SD</th>
<th>95% CI</th>
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<tr>
<td>Merocel</td>
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<tr>
<td>Sterile</td>
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<td>15.2</td>
<td>86.4–101</td>
</tr>
<tr>
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<tr>
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<td>5.8</td>
<td>−0.64–5.6</td>
</tr>
<tr>
<td>Participant</td>
<td>35</td>
<td>61.2</td>
<td>49.3</td>
<td>44.3–78.2</td>
</tr>
</tbody>
</table>

*Mean, SD, and 95% CI values were calculated by combining the results obtained from sponges spiked with V5 at dilutions of 1/1,000 and 1/2,000.
have decreased the effect of nonspecific binding with the sponges, as noticed in our study. Second, we have shown that the presence of cervical mucus has little impact on the ability of the SEAPNA to measure neutralizing activity in material extracted from Merocel sponges (90% of the specimens evaluated had recoveries of >80%). This contrasts with the Ultracell results, in which only 51.4% of the sponges tested had recoveries of >80%. It is important to note that Ultracell sponges with the lowest recoveries also had the lowest cervical secretion weights, suggesting that the degree of nonspecific trapping of antibodies onto cellulose sponges was probably dependent on the amount of protein preadsorbed onto the sponge. This was confirmed in additional experiments that demonstrated an increase in antibody recovery from Ultracell sponges that had been presoaked in BSA prior to spiking with V5 compared to those spiked with V5 before the addition of BSA.

Third, we have demonstrated that the spiking of Ultracell or Merocel sponges with mouse IgG1-TNP can be used to assess recovery efficiency during extraction without a negative impact on the SEAPNA. This finding is of particular importance for studies that have used cellulose-based Ultracell sponges to collect cervical secretions, given the variable and incomplete recovery seen with this collection device.

Finally, we explored alternative extraction protocols to increase the recovery of antibodies from Ultracell sponges and demonstrated that the use of detergents and the use of BSA in the EB were not viable options. The levels of detergent required for optimal extraction were cytotoxic in the SEAPNA, and BSA would need to be added prior to specimen collection.
to block nonspecific binding to the sponge. Additional studies will be required to define a protocol capable of efficiently eluting antibodies from non-BSA-treated Ultracell sponges without interfering with the SEAPNA.

In summary, this study demonstrates that SEAPNA variability is modest relative to the variability observed among vaccinated women and that the SEAPNA can be used to measure neutralizing titers in cervical secretions. Collection devices made of polyvinyl acetate were shown to be superior to cellulose-based sponges. For studies that have used cellulose-based collection devices, a method is described to enable direct assessment of recovery efficiency from individual specimens. Additional evaluation of cervical secretion collection, processing methods, and direct comparisons between alternative testing procedures (e.g., the SEAPNA versus the standard ELISA) are needed to define the best approaches to measure immunogenicity at the cervix in clinical trials.

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REFERENCES


