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In vitro inhibition of human papillomavirus following use of a carrageenan-containing vaginal gel☆,☆☆,☆☆☆

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HIGHLIGHTS

- We evaluated the ability of a carrageenan containing sexual lubricant to inhibit PsV16.
- Carrageenan levels decreased over time, PsV16 inhibition remained high eight hours after insertion of the carrageenan gel.
- This is the first clinical study directly looking at carrageenan potential for HPV prevention.

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ABSTRACT

Objective. To assess in vitro efficacy of Divine 9, a carrageenan-based vaginal lubricant that is being studied as a microbicide to inhibit HPV16 pseudovirus (PsV) infection.

Methods. Sexually active US women between 19 and 35 years without prior HPV vaccination or cervical intraepithelial neoplasia were instructed to use Divine 9 vaginally with an applicator either before sex only or before and after intercourse. Women who applied a single dose of gel returned for cervicovaginal lavage (CVL) collection 1, 4 or 8–12 h after intercourse versus those who applied gel before and after intercourse returned 1, 4 or 8–12 h after the second gel dose. Carrageenan concentrations were assessed using an ELISA assay and the inhibitory activity was assessed using a PsV-based neutralization assay against HPV16 infection. Carrageenan concentrations and the percentage of PsV16 inhibition were compared using the Wilcoxon rank sum test.

Results. Thirteen women were enrolled and thirty specimens from different time-points were assessed. 87% of CVL samples had detectable carrageenans with levels decreasing over time from intercourse. 93% of CVL samples had detectable PsV16 inhibition with median inhibition of 97.5%. PsV16 inhibition decreased over time, but remained high, with median inhibition of 98.1%, 97.4% and 83.4% at 1, 4 and 8–12 h, respectively. Higher carrageenan concentrations were associated with higher levels of PsV16 inhibition (rho = 0.69).

Conclusions. This is the first report of a human study investigating in vitro HPV inhibition of a carrageenan-based vaginal lubricant with CVL collected after sexual intercourse. We demonstrate excellent efficacy in preventing PsV16 infection.

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1. Introduction

Human papillomavirus (HPV) is the most common sexually transmitted infection in the United States and is necessary for the development of cervical cancer. Although HPV vaccination is highly effective, HPV and cervical disease remain a major public health concern in the US, in part, due to the poor uptake of the HPV vaccine. The Centers for Disease Control and Prevention (CDC), for example, reported an uptake rate of only 40% among US adolescents between 13 and 17 years of age as of 2015 [1]. Vaccination of women older than 26 years of age is not currently recommended, due to diminished efficacy in older age groups. Thus, many young women in the US remain unvaccinated and at least several generations of women will not fully benefit from HPV vaccine. Furthermore, the quadrivalent and bivalent vaccines used through early 2015 in the US targeted only a limited number of HPV types associated with cervical disease, and whether revaccination with nonavalent (9-valent) (or other future vaccines) will be recommended is unknown. While HPV vaccination rates are higher in certain other developed nations, similar limitations regarding the use of quadrivalent or bivalent HPV vaccines and the large number of unvaccinated women 27 years of age or older, still apply.

However, the greatest public health concern regarding HPV infection and cervical disease is in areas or countries of medium and limited resource settings, given limited access to HPV vaccine, compounded by limited access to cervical cancer screening and treatment options. In many settings, but in particular in limited resource settings, condom use is a primary means of preventing HPV and cervical disease by decreasing exposure to infectious particles.

Consistent condom use appears to be moderately successful in preventing HPV transmission, with a 70% decrease in transmission reported in young women whose partners used a condom 100% of the time [2]. Thus, development and testing of condom-compatible microbicides to prevent HPV infection has significant clinical implications.

Carrageenans (CG) are a family of sulfated polysaccharides that are extracted from edible red seaweed. They are commonly used in the food industry as thickening agents as well as formulated as personal lubricants. Carraguard, a CG based vaginal gel, has been shown to be safe and well tolerated [3–5]. Although a large randomized, placebo-controlled trial of Carraguard failed to show Carraguard efficacy in preventing HIV infection in women [6], in vitro and in vivo preclinical evaluation has suggested that CG may have high efficacy in blocking HPV infection [7–13]. Furthermore, in a subgroup analysis of the HIV prevention trial, there was a 60% reduction in the prevalence of high-risk HPV infection in women who consistently used Carraguard [14]. However, no a priori trials have been performed to investigate the efficacy of CG to decrease HPV transmission.

The current study was designed to assess genital tract concentrations of carrageenan as well as inhibition of HPV infection using an in vitro pseudovirion assay after application of a commercially available carrageenan-containing vaginal lubricant prior to penetrative sexual intercourse.

2. Materials and methods

2.1. Eligibility criteria

After obtaining approval from the Albert Einstein College of Medicine Institutional Review Board, women were recruited between February and May 2014 from gynecology clinics at Montefiore Medical Center in Bronx, NY. Eligible subjects were healthy, sexually active women between the ages of 19–35, with an intact cervix, engaging in at least three intercourse events per month, and using an effective contraceptive method for at least 3 months. Exclusions included a history of surgical excision or hysterectomy for cervical intraepithelial neoplasia, a history of HPV vaccination, malignancy, HIV or other immunosuppressive disease, genital warts or ulcers, pelvic inflammatory disease, pregnancy, and breastfeeding. Informed consent was obtained from each participant prior to enrollment. At the screening visit, all subjects underwent laboratory testing including: urine pregnancy test, oral (saliva-based) HIV screening test, liquid-based monolayer Pap testing with HPV typing using a brush, testing for gonorrhea and chlamydia and cervicovaginal lavage (CVL) with 10 cm^3 saline. Screening for Trichomonas and/or a wet mount for bacterial vaginosis and candidiasis was performed for symptomatic patients.

2.2. Intervention gel

Divine 9 containing Carragel (CarraShield Labs, Orlando, FL) is a non-contraceptive water-based lubricant that is clear, odorless and tasteless and made with a mixture of lambda and kappa carrageenans naturally derived from red seaweed. The gel is formulated with 2% carrageenans, which is similar to gels that have previously been investigated in microbicide trials [6,15,16]. It is manufactured in a U.S. FDA GMP-compliant facility and approved as a Class II medical device. All of its ingredients are GRAS (Generally Recognized as Safe) under CFR Title 21 and are food-grade. It has been directly marketed to consumers for over ten years and there have been no reported safety concerns to date.

This interventional gel was supplied to participants in single-use disposable applicators from HTI Plastics (Lincoln, NE) containing 2.5 ml of gel, which delivers on average 2 ml of gel. Divine 1, a Carrageenan-Free Gel (CarraShield Labs, Orlando, FL), a gel with similar rheological properties without carrageenan, was used as a control for the in vitro studies.

2.3. Intervention

Participants were assigned alternately to one of two groups: The *precoital* group was instructed to insert Divine 9 within 12 h prior to vaginal intercourse, have intercourse, and return at an assigned time-point. A second group was instructed to insert one dose of Divine 9 within 12 h before vaginal intercourse, have intercourse, and then insert a second dose of gel as soon as possible, but within 12 h after sex and no more than two doses in a 24-hour period. Hence, the dosing strategy is referred to as *BAT24*. As per participant report, most inserted gel immediately before and in the *BAT24* group, immediately after sex. After an act of sexual intercourse the participants called the research team and those in the *precoital* group were asked to return to the clinic for CVL collection using 10 ml of 0.9% saline solution at 1, 4 or 8–12 h after intercourse, while those in the *BAT24* group returned for CVL collection 1, 4 or 8–12 h after insertion of the second dose of Divine 9. CVLs were stored at −80 °C.

For each coital act, only one time-point was measured as gel is washed out with CVL. However, participants were allowed to return for repeat measurements at different time-points after a coital act as long as there was at least a one-week washout period between study visits. Patients were compensated at IRB-established rates for each return visit.

2.4. In vitro assay for anti-HPV activity

HPV16 pseudovirions (PsV) were prepared based on reagents and methods obtained from the Schiller lab, as previously described [17]. Briefly, 293TT cells were co-transfected with codon-modified HPV16 capsid genes (L1 and L2) together with a reporter plasmid encoding secreted human placental alkaline phosphatase (SEAP). Efficient purification of the HPV16 PsV was achieved by Optiprep™ (Sigma-Aldrich, Allentown, PA) density gradient ultracentrifugation (iodixanol). A titration of the HPV16 PsV stock was routinely tested to determine the minimum amount of HPV16 PsV required giving a robust inhibition signal at the neutralization assay.

Serial dilutions of Divine 9, Divine 1, and PC-525, another CG-containing gel developed by Population Council, New York, NY, were evaluated. After dilution testing of HPV16 PsV preparations to identify

the amount of material outside the linear range, samples for evaluation were mixed with a 1:1000 dilution of HPV16 PsV and inoculated onto 293TT cells. Infection of 293TT cells was monitored by SEAP activity in the culture supernatant using a FLUOstar OPTIMA Plate Reader luminometer (BGM Labtech, Inc., Cary, NC) and HPV16 PsV inhibition was detected by a reduction in SEAP activity [18]. A 50 μl aliquot of undiluted CVL sample is mixed with 150 μl DMEM solution containing HPV16 PsV, each sample was repeated twice, and the mean value relative light unit of SEAP activity was calculated. The anti-monoclonal antibody 16.V5 (the kind gift of Neal Christensen) that recognizes a neutralization epitope on the HPV16 virus was used as a positive control to demonstrate inhibition. CVLs collected at post-coital visits were assayed for HPV16 PsV inhibition in a similar fashion.

2.5. CG concentration assay

CVLs were assayed for CG concentrations as previously described [12,13]. Briefly, the assay is a sandwich ELISA using a rabbit anti-CG polyclonal IgG antibody (Pacific Immunology, Ramona, CA) coating the well followed by adding samples and standards and finishing with biotinylated rabbit anti-CG polyclonal IgG antibody (Pacific Immunology). HRP-based detection system with TMB substrate followed by stop solution (Zeptometrix) was used to complete the ELISA and absorbance was measured at 405 nm with an Emax Molecular Devices microplate reader (Molecular Devices, Sunnyvale, CA).

For samples with CG levels below the threshold of detection using the ELISA (<400 ng/ml) anti-HPV activity was tested in HeLa cells. This assay was performed to obtain the median effective dilution (IC_{50}) of each CVL sample (dilution that blocks 50% of the infection by HPV16 PsV) and the IC_{50} for a CG control with a known CG concentration. The CG concentration in CVL samples was roughly estimated by dividing the IC_{50} value of the CG control by the IC_{50} value of the CVL sample. The antiviral assay was performed using a previously published assay [12,13] and different from the one described in the previous section. Briefly, CG control gel (PC-525) and CVL samples were diluted in medium to obtain a total of six different dilutions per sample in triplicates and plated on HeLa cell monolayers. Fifty microliters of HPV 16 PsV (5×10^5 copies) was added to all wells with the exception of cell controls and incubated for 72 h at 37 °C, 5% CO_2 and 95% humidity. The final range of CVL dilutions tested in the antiviral assay was 0.25 to 0.0078. Cells were lysed after the 72 hour incubation to detect luciferase activity using the Pierce Firefly Luciferase Glow Assay with Pierce Firefly Signal Enhancer (Thermo Scientific) as described by the manufacturer. Luminescence was read on a Gemini EM microplate reader (emission 542 nm, Molecular Devices) using Softmax Pro 3.2.1 software.

Cytotoxicity of each CVL sample was also measured to ensure the selectivity of the antiviral activity. For this purpose, HeLa cells were exposed to the same CVL dilutions under the same conditions described in the antiviral assay but in the absence of HPV 16 PsV. Cell viability was measured using the XTT colorimetric assay as previously described [12,13].

2.6. Baseline samples and endogenous PsV16 activity assessment

In order to assess if endogenous activity played a role in Psv16 inhibition, we assessed fourteen baseline CVL samples, collected at the screening visit prior to any carrageenan use, including five from women who contributed to the specified time-points. To benchmark the endogenous activity, these were compared to four random samples from those included in the complete analysis demonstrating high levels of carrageenan.

In detail, 100 μl of 293TT cell suspension (300,000/ml) in DMEM media without phenol red (Dulbecco, Invitrogen, Carlsbad, CA) was preplated to a 96-well tissue culture plate two to 5 h before adding the HPV16 PsV. Titrated HPV16 PsV to 1:1000 dilution were combined with 50 μl of diluted controls, CVL sample suspension, or DMEM medium (background control with no HPV16 PsV). The combined HPV16 PsV were incubated on ice for an hour, prior to addition to the plated cells. After 72 h post-infection, the cell supernatant was monitored for HPV16 PsV inhibition by measuring the SEAP activity on FLUOstar OPTIMA Plate Reader luminometer. Hence, inhibition = (1 − mean of activity of HPV16 PsV with test substance / activity of HPV16 PsV at baseline) × 100. Each control/CVL sample suspension was repeated twice, and the mean value (RLU, relative light unit) of SEAP activity was used.

2.7. Data analysis

Carrageenan concentrations and the percentage of PsV16 inhibition were compared using the Wilcoxon rank sum test for non-parametric data. As the percentage of PsV16 inhibition had a logarithmic

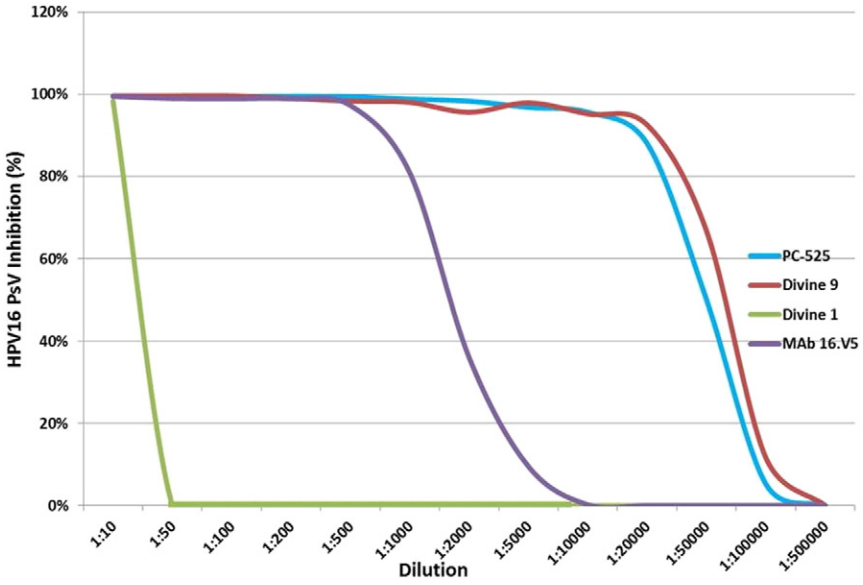

Fig. 1. HPV16 pseudovirus (PsV) inhibition assay. Inhibition of 1:1000 dilution of PsV stock by PC-525, Divine 9 and Divine 1. HPV16 V5, a monoclonal antibody that recognizes a neutralization epitope on the HPV16 virus, was used as a positive control. The IC_{50} for Divine 9 is at a dilution close to 1:100,000.

distribution, correlation between PsV16 inhibition and carrageenan concentration was calculated using the Spearman's rank correlation coefficient. Logarithmic conversion of PsV16 inhibition was also performed to standardize the correlation.

All statistical analyses were performed using Stata v9.0 (StataCorp LP, College Station, TX) with a two-sided p-value of <0.05 considered statistically significant.

3. Results

3.1. In vitro efficacy

Serial dilutions of Divine 1 without CG, Divine 9 and PC-525 gels were performed to determine PsV inhibition (Fig. 1). Similar PsV16 inhibition was seen with both Divine 9 and PC-525 gels. The IC_{50} for Divine 9 was noted at a dilution close to 1:100,000. Divine 1 demonstrated minimal activity with no inhibition noted at dilutions ≥ 1:100.

3.2. Pilot study

We enrolled thirteen women who provided samples for a total of thirty time-points. Women were allowed to participate at more than one timepoint in a separate month. Each of the thirty timepoints was evaluated as a separate event. Baseline demographic data are shown in Table 1. Nearly half the women studied are Hispanic. Three timepoints were measured in the two groups: 1 h, 4 h and 8–12 h after intercourse in the *precoital* use group and 1 h, 4 h and 8–12 h after the second insertion of Divine 9 gel in the *BAT24* group. In total, thirty timepoints were measured, twelve in the *precoital* use group and eighteen in the *BAT24* group. Eleven women (85%) had measurements at more than one timepoint with separate intercourse events (median of 2 timepoints), with 2 (15%), 5 (38%) and 6 (46%) participants at 1, 2 and 3 separate timepoints. Nine women (69%) provided samples using the test agent both as part of the *precoital* group and the *BAT24* group. Cumulatively, a total of 11 samples were available for analysis at the 1 h, 10 samples at the 4 h and 9 samples at the 8–12 hour time-points.

3.3. Carrageenan detection

Twenty-six (87%) CVL samples had detectable carrageenan levels. There was no statistically significant difference in CG level between samples in the *precoital* and *BAT24* groups (median: 2650 ng/ml [interquartile range (IQR): 300–16,550 ng/ml] vs 12,150 ng/ml [IQR: 1834–46,600 ng/ml, p = 0.21]). Combining all samples, carrageenan levels detectable in CVL decreased over time since insertion (Fig. 2) (p for trend: 0.021). However, carrageenan concentrations did not significantly decrease from one to 4 h after insertion (median: 16.9 μg/ml vs 15.5 μg/ml, p = 0.75). Notably, there was a significant decrease in carrageenan concentration by 8 h after insertion (median: 16.9 μg/ml vs 0.34 μg/ml, p = 0.02).

Table 1
Participant demographics.

Age (years)		28.2 ± 4.7
Race	White	5 (38.5)
	Black	5 (38.5)
	Other	3 (23.1)
Ethnicity	Hispanic	6 (46.2)
	Non-Hispanic	7 (53.8)
History of abnormal Pap	Yes	8 (61.5)
	No	5 (38.5)
Number of lifetime sexual partners		5.8 (2.7)
Number of sexual partners in prior six months		1.2 (0.4)
History of STD	Yes	7 (53.9)
	No	6 (46.1)
Smoking status	Ever smoked	3 (23.1)
	Current smoker	1 (7.7)
	Never smoked	10 (76.9)

Data is presented as n (%) for categorical variables or mean ± standard deviation for continuous variables.

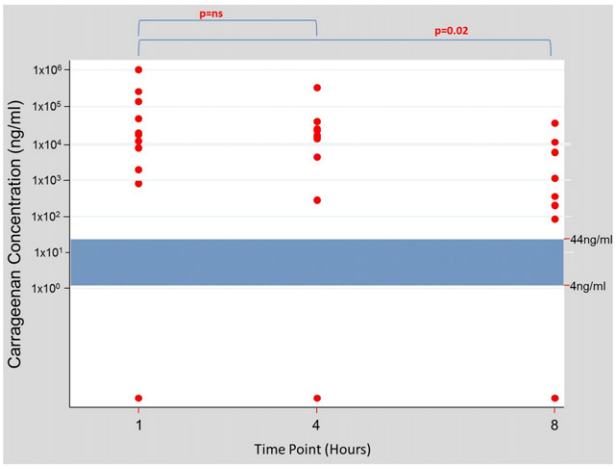

Fig. 2. Carrageenan concentrations in cervicovaginal lavages (CVLs) obtained 1, 4 or 8–12 h after intercourse with a single dose of Divine 9 gel before sex or in CVLs collected 1, 4 or 8–12 h after a second dose in women who applied gel before and after sex. The hatched area represents the range of IC_{50} levels needed that have previously been described for carrageenans [7]. There is a decline in carrageenan concentration by 8 h after insertion but levels remain detectable and above the previously described IC_{50}.

Eighty-seven percent of the CVL samples exceeded the previously described IC_{50} of various carrageenans (>44 ng/ml) [7]. At the 1 h, 4 h and 8–12 hour time-points 91%, 90% and 78% of CVLs, respectively, had carrageenan levels > 44 ng/ml.

3.4. PsV16 inhibition

The CVL samples from the participating women were combined with HPV16 pseudovirions and 293TT cells to assess for inhibition of pseudovirion infection. Twenty-eight (93%) of the CVL samples had detectable PsV16 inhibition with median PsV16 inhibition of 97.5% (IQR: 83.4%–98.5%). There was no significant difference in the percentage of PsV16 inhibition between the *precoital* and the *BAT24* (median: 97.3% vs 97.8%, p = 0.90). PsV16 inhibition decreased over the study time-points with median percent PsV16 inhibition of 98.1%, 97.4% and

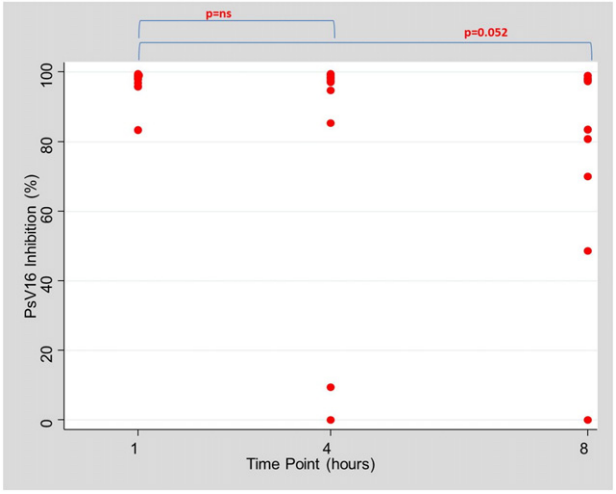

Fig. 3. Percentage of PsV16 inhibition as measured in CVLs obtained 1, 4 or 8–12 h after intercourse with application of Divine 9 gel either before sex or before and after sex. No statistically significant difference was seen between PsV16 inhibition at 1 and 4 h. There is a decline in PsV16 inhibition when comparing one to 8 h (median: 98.1% vs 83.4%, p = 0.052), but inhibition remains high.

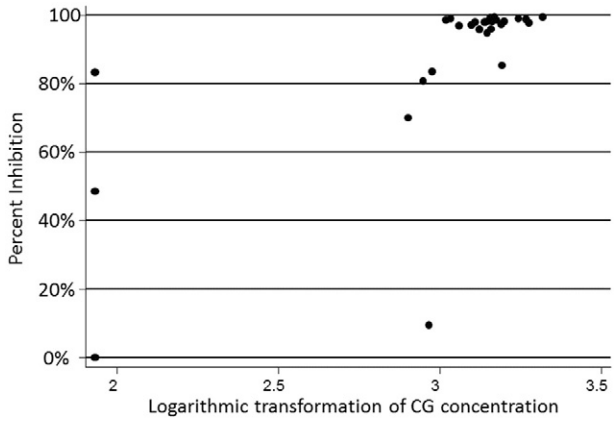

Fig. 4. Correlation of logarithmic conversion of CG concentration and percent PsV16 inhibition. Increasing CG concentrations were associated with higher levels of PsV16 inhibition (rho = 0.74).

83.4% at 1, 4 and 8–12 h, respectively, though the trend did not reach statistical significance (p for trend = 0.06) (Fig. 3).

3.5. Relationship between carrageenan levels and PsV16 inhibition

Higher carrageenan concentrations were associated with higher PsV16 inhibition (rho = 0.74), though there appeared to be a threshold effect rather than a simple linear relationship (Fig. 4). With one exception, all samples having a carrageenan concentration > 400 ng/ml demonstrated ≥95% PsV16 inhibition (median: 97%).

3.6. Endogenous PsV16 inhibition

Fourteen baseline samples, including five from women who contributed to the specified time- points, were collected prior to any carrageenan use. Despite none of the samples having detectable carrageenan levels, 12 (86%) demonstrated PsV16 inhibition (median 85.7%, IQR: 66.0–93.0%). To determine if the PsV16 inhibition found in CVLs of women who received carrageenan gel was solely due to endogenous activity of cervicovaginal secretions, serial dilutions of baseline samples and selected carrageenan treated CVLs was performed (Fig. 5).

While median values for PsV16 inhibition in undiluted baseline samples were high (median 92%), this rapidly decreased with subsequent dilutions. By a 1:5 dilution, median PsV16 inhibition in the baseline CVL samples was 0%. In comparison, median values for PsV16 inhibition in the undiluted and 1:5 dilution of the CVLs of women who received carrageenan gel were 99% and 97.5%, respectively. At every dilution point, the carrageenan containing CVLs had significantly higher PsV16 inhibition as compared to the baseline samples.

4. Discussion

In this study we present the results of in vitro characterization and the correlative results of carrageenan concentrations and in vitro anti-HPV activity of Divine 9, a carrageenan-based sexual lubricant, when inserted by women either before sex or before and after sex. This is the first clinical study directly looking at carrageenan potential for HPV prevention. Inhibition of PsV16 was demonstrated at dilutions of <1:10,000 and was similar to that seen with Carraguard, a different carrageenan-based vaginal gel that is being studied as a potential microbicide. While carrageenan concentrations decreased with increasing time from gel insertion, PsV16 inhibition remained high even at the 8 hour time-point with 6 of 9 samples showing >80% inhibition. These data demonstrate that there was retention of the carrageenan containing sexual lubricant after penetrative sexual intercourse and that despite declining concentrations of carrageenans over time, Divine 9 with Carragel provides high levels of in vitro inhibition against HPV after intercourse.

Two additional sources of HPV inhibition need to be considered, endogenous gel activity in the absence of carrageenan as well as intrinsic immunity from vaginal secretions. Our results show both are not the essential sources of PsV16 inhibition in participants who used Divine 9. The Divine 1 gel, identical to the Divine 9 gel but without carrageenans, demonstrated a thousand fold lower inhibition than the carrageenan-containing gel; this makes it unlikely that the gel matrix itself contributes to PsV16 inhibition. Similarly, serial dilutions of CVL from participants who used Divine 9 gel demonstrated a linear decrease in inhibition, not present in control samples, suggesting that the PsV16

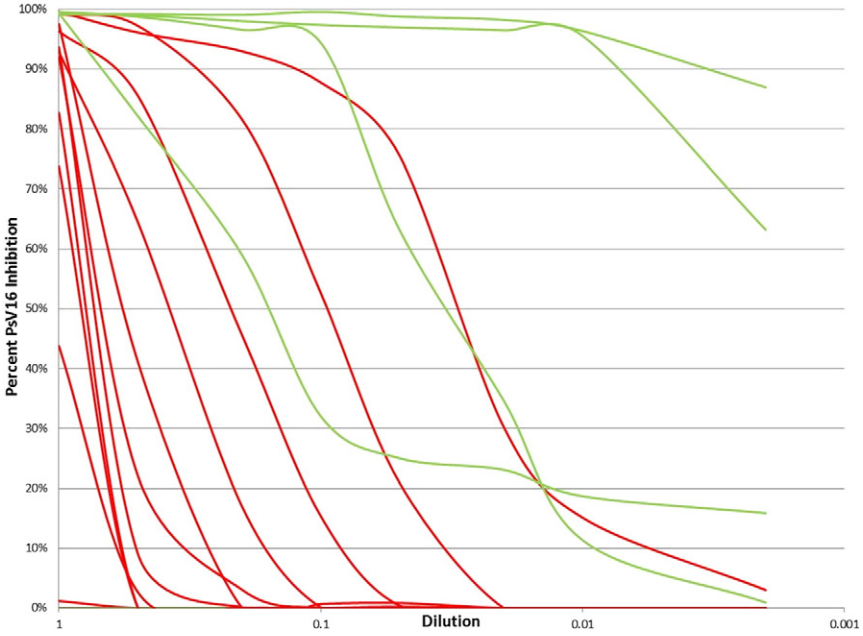

Fig. 5. Serial dilutions of CVLs. Green lines represent carrageenan containing CVLs while red lines represent baseline CVL samples. At all dilutions the median PsV16 inhibition was significantly higher for the carrageenan CVLs as compared to the baseline samples.

inhibition results primarily from the carrageenan compound and not innate immunity.

This study has several limitations, including a small sample size. Additionally, we did not have formal measures of adherence with gel use or with sexual intercourse. Women were compliant by self-report. However, despite reported adherence with gel use, four CVLs were found to have undetectable CG levels. Carrageenan levels were not measured in the absence of coitus, which limits our ability to determine how much gel leakage there is in the absence of coitus and whether the gel is diluted by seminal fluid. The small sample size precludes detection of small differences between dosing regimens and time-points. Lastly, baseline CVL samples in the absence of Divine gel use were not collected for all enrolled subjects. Thus, while these data demonstrate strong HPV16 PsV inhibition, it is a conservative estimate of the ex-vivo inhibitory effect of this gel.

The results of this study suggest that Divine 9 demonstrates high levels of anti-HPV activity in vitro. A randomized phase II trial to determine the clinical efficacy of Divine 9 in preventing incident HPV infections is ongoing. We anticipate that this trial will provide data on the in vivo efficacy of carrageenans to prevent HPV infection, which may lead to further development of an additional strategy to prevent cervical cancer.

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