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Use of the Dye Stain Assay and Ultraviolet Light Test for Assessing Vaginal Insertion of Placebo-filled Applicators Before and After Sex

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Abstract

Background—Applicator dye staining and ultraviolet (UV) light have been used in trials to measure adherence, but not in the setting of before and after sex gel dosing (BAT-24). This study was designed to determine if semen or pre-sex gel dosing impacts the sensitivity and specificity of a dye stain assay (DSA) for measuring vaginal insertion of placebo-filled applicators with BAT-24 dosing.

Methods—Healthy monogamous couples received Microlax®-type applicators filled with hydroxyethylcellulose placebo gel. Women were instructed to vaginally insert one dose of gel before and a second dose after sex and to return applicators within 48 hours after sex. Applicators were stained to detect semen followed by UV then DSA and scored by two readers. Positive and negative controls were randomly included in applicator batches.

Results—Fifteen couples completed the study. Each female returned at least six applicators over a 30-day period. The sensitivity for insertion of post-sex applicators was higher for UV (97%) compared to DSA (90%) and the specificity was similar (96%). For pre-sex applicators, the sensitivity and specificity were higher for DSA (100%) compared to UV testing (87% sensitivity, 96% specificity). Among returned post-sex applicators, 95% tested positive by UV compared to 87% by DSA. Agreement between readers was significantly better on the pre-sex applicators for DSA than for UV and for post-sex readings agreement was less than half that for UV, although the results were not statistically significant.

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Conclusions—Applicator tests are feasible for measuring adherence in trials with gel dosing before and after sex.

Introduction

Interpretation of microbicide trial results has been compromised by challenges in accurately assessing product use, coital frequency, and condom use, which are typically measured by self-report, and are unreliable due to recall and social desirability bias. While behavioral measures and biological markers of applicator insertion have been extensively evaluated to assess adherence in microbicide trials, most studies have focused on daily or pre-sex dosing of microbicides [1]. Little is known about the performance of these measures on applicators inserted after sex.

Several studies have used trypan blue [2–4] and FD&C Blue No. 1 [5–12] dye stain assay (DSA) techniques to detect vaginal mucus as a proxy for vaginal insertion of applicators and gel use daily, twice daily, or prior to sex. It has been hypothesized that different surface qualities of various types of plastics as well as frequency of dosing may influence the retention of vaginal mucus in a distinct streaking pattern [8]. The DSA reached high accuracy for the identification of vaginal mucus on polyethylene HTI Plastics applicators [3] as well as on low-density polyethylene (LDPE) Microlax® applicators [2, 5, 8] used once daily. However, the sensitivity and specificity for the DSA was lower on HTI Plastics polypropylene applicators used once or twice daily [7, 10]. A recent test using ultraviolet (UV) light fluorescence to measure daily vaginal insertion of polypropylene applicators demonstrated 84% sensitivity and 83% specificity for daily use [13].

To date, no prior studies have systematically evaluated the DSA or UV light to discern whether gel-filled applicators were vaginally inserted both before and after sex. The so-called “BAT-24” dosing regimen (using one dose of gel within 12 hours before sex and a second gel dose as soon as possible within 12 hours after sex and no more than two gel doses in a 24-hour period) was recently utilized in the CAPRISA 004 trial, which demonstrated a 39% reduction in HIV acquisition among women who used 1% tenofovir gel compared to hydroxyethylcellulose (HEC) placebo gel [14]. The BAT-24 regimen is the dosing strategy for the ongoing FACTS 001 trial designed to confirm whether tenofovir gel used before and after sex is effective in preventing HIV infection. It is thought that the presence of semen and pre-sex gel dosing can alter the vaginal environment such that it may alter the results of the DSA or UV methods.

A study was undertaken at the Albert Einstein College of Medicine and Montefiore Medical Center (Einstein-Montefiore), Bronx, New York (NY), to determine the sensitivity and specificity of the DSA for confirming vaginal insertion of HEC-filled Microlax®-type applicators used after sex. A secondary objective was to compare the DSA and UV light method for assessing applicator insertion both before and after sex. To confirm that women used applicators post-sex, a novel assay was included to test for the presence of male ejaculate on applicators used after sex [15, 16]. The Rapid Identification of Semen (RSID™-Semen) test, developed by Independent Forensics (Lombard, IL), is an immunochromatographic assay that uses two antihuman semenogelin monoclonal antibodies

to detect semenogelin, a unique protein specific to seminal fluid. The test is highly specific to human semen, detecting <2.5 nL of semen, can detect semen from a variety of materials and surfaces, and does not cross-react with saliva, vaginal secretions, menstrual blood or vaginal products [16, 17].

Methods

The study was approved by the Institutional Review Boards of Einstein-Montefiore and the Population Council (NY, NY). Before undergoing any procedures, all participants provided written informed consent in accordance with established guidelines and ethical standards for research with human subjects.

Healthy couples were enrolled between May 2012 and January 2013 and asked to apply one applicator of HEC gel vaginally before and one applicator after sex at least twice a week for three to four weeks. Couples were eligible if they were in a mutually monogamous relationship for at least six months, were at low risk for sexually transmitted infections (STI), defined as no reported STI in previous six months, had no history of intravenous drug use, and were using a form of non-barrier contraception. Exclusion criteria included pregnancy, breastfeeding, HIV, and any genital abnormality at screening.

At the screening visit, all participants underwent a genital exam and a rapid oral or serum HIV ELISA test. Women also had a urine pregnancy test. If a woman was diagnosed at screening with candidiasis, bacterial vaginosis, or a urinary tract infection, she was treated and was eligible for enrollment one month after completing treatment. Once enrolled, female participants were issued Microlax®-type LDPE vaginal applicators (Tectubes, Åstorp, Sweden) filled with 7 mL of 2.7% HEC placebo gel [18] (Clean Chemical Sweden AB, Borlänge, Sweden) to vaginally deliver approximately 3.5 mL of gel. After a demonstration, a study staff member witnessed each participant insert her first applicator, which served as the participant's own positive control for DSA and UV validation. Study staff explained that returned applicators would be tested to determine if they had been inserted and results would be compared using two different methods. Participants were instructed to insert one dose of gel vaginally up to 12 hours before sex and a second dose as soon as possible, but within 12 hours, after sex with no more than two doses in 24 hours (BAT-24 dosing). Women were instructed to return each pair of used applicators within 48 hours after each sex act. Participants were issued pre-labeled plastic bags to store each individually used applicator. Each bag had a unique identifying number linking the applicator to the participant and a detachable label for the participant to indicate the date and time of applicator use, whether the applicator was used before or after sex, and the approximate time of sexual intercourse. Participants were also issued tips of cotton swabs to plug used applicators to prevent gel leakage during storage and transport.

At each study visit, applicators were collected and participants were given additional HEC-filled applicators, as needed. Study staff reviewed the label on each bag with the participant, counted used applicators and clarified any discrepancies based on applicator count and a review of the bag labels with the participant. Following review and prior to bags containing the used applicators being brought to the laboratory for testing, the detached labels were

placed in the participant's study chart for data entry. Staff documented any adverse events or changes in the participants' health since their prior visit.

Applicator test procedures

Three laboratory personnel were trained on both the DSA and UV light tests. All returned applicators were initially tested using the RSID™-Semen test (Independent Forensics, Lombard, IL), which can detect semenogelin up to 48 hours after intercourse [16]. The 'smooth' area of the applicator neck was swabbed with a cotton tipped swab pre-moistened with 50 µL of RSID Universal buffer and then placed into 150 µL of the buffer and tested per manufacturer's instructions. The smooth area of the applicator was used to prevent interference with the DSA, which is conducted on the seam located along the applicator neck. The RSID test was considered the "gold standard" measure that an applicator was inserted post-sex. HEC placebo gel was previously shown not to interfere with the RSID™-Semen test (Independent Forensics, personal communication).

Returned used applicators, positive controls (first applicator applied in the clinic) and negative controls (generated in the laboratory by dispensing gel *ex vivo*) were randomly placed into a test rack by one staff member, then evaluated and scored independently by two other trained personnel who were blinded. Applicators were first tested using the UV light procedure, as previously described [13], with the following modification: use of a single spotlight containing 60 x 385 nm UV wide angle LED bulbs without a short pass <400 nm excitation filter. Applicators with a streaked or speckled pattern of blue-green fluorescence were scored as 'positive'. Applicators were then tested with the DSA as previously described and those with the characteristic grainy/streaked pattern were scored as 'positive' [8]. The DSA was considered the "gold standard" measure of adherence of pre-sex applicator use because it had been validated in prior studies [6, 8, 9, 12].

Statistical Analysis

The sensitivity and specificity of the DSA and UV light test were calculated with 95% confidence intervals (CI) using all positive controls (true positive for pre-sex applicators), negative controls (true negative for pre- and post-sex applicators) and post-sex applicators that were RSID-positive (true positive for post-sex applicators). The specificity of the RSID test was calculated using the negative controls (true negative for post-sex applicators). The agreement between two pre- and post-sex applicator readings was compared between DSA and UV tests using generalized estimating equation (GEE) models to take into account the correlation between readings from the same subject.

A subset of applicators (n=18) was initially tested by RSID and UV (time 1) and then stored for repeat RSID and UV testing at 7–16 days (time 2) to determine if results were consistent over time and if applicator testing could be delayed or batched for future clinical trials. Thirty-six post-sex applicators were initially tested by RSID and stored for DSA testing at time 2 (n=18) or at 28–31 days (time 3, n=18). Agreement between time 1 and time 2 for RSID was assessed using the kappa statistic.

Results

Participant characteristics

Nineteen couples were screened, 16 were enrolled and 15 completed the study. One couple elected not to enroll, and two were not eligible (one required a medical procedure and one was not using effective contraception). One couple was lost to follow-up after the enrollment visit. The average age of the men and women was 30.7, 76% self identified as Black/African or Hispanic, 66% attended some or completed college, 47% were married or living with a partner, and 20% reported a prior STI (Table 1).

Performance of the applicator tests

Fifteen positive controls (inserted at the first clinic visit) for DSA and UV, 14 negative control applicators (emptied *ex vivo*) for RSID, and 11 negative controls for DSA and UV were assayed in a blinded fashion. The specificity of the RSID test was 93% (0.642, 0.996). The sensitivity of RSID could not be calculated given the lack of a true positive control.

Each female participant returned between 6–22 applicators. The median number of returned applicators was 12. A total of 190 applicators were returned, including 95 that participants reported had been inserted pre-sex and 95 inserted post-sex. Thirteen women returned 100% of their used applicators, one woman returned all except one pre-sex applicator which was not used and one returned all except one post-sex applicator which was lost.

The primary study objective was to determine the sensitivity and specificity of the DSA for confirming vaginal insertion of HEC-filled Microlax®-type applicators used after sex. The sensitivity and specificity of the DSA on post-sex applicators was estimated to be 90% and 100%, respectively. The sensitivity of the UV light test (97%) was higher than that of the DSA (90%), however the difference was not statistically significant likely due to the small sample size. The specificity of the UV test was 96%, which was similar to that of the DSA. For pre-sex applicators, the sensitivity and specificity were higher for DSA (100%) compared to UV light (87% sensitivity, 96% specificity) (Table 2).

A total of 77 of 94 (82%) post-sex applicators tested positive by RSID. One post-sex applicator was not tested by RSID. As shown in Table 3 and excluding applicators that were stored for later testing, 95% of post-sex applicators confirmed positive by RSID tested positive by UV compared to 87% by DSA. For the pre-sex applicators, 97% tested positive by DSA compared to 80% for UV. The percentage of disagreement between readers for both DSA and UV was similar to that observed with the positive and negative controls (data not shown).

Concordance between readers for DSA vs. UV test

Agreement between readers for results of pre-sex and post-sex readings was compared between DSA and UV tests. The agreement between readers was significantly better on the pre-sex applicators for DSA than for the UV test: OR=13.6, 95% CI = 1.69, 109.15. However, for post-sex applicators, the agreement between DSA readings was less than half

that for UV readings, although the results were not statistically significant (OR=0.48, 95% CI = 0.11, 2.06).

Agreement of results for applicators tested initially and following storage

Fifteen post-sex applicators were used to compare RSID results and nine post-sex applicators were used to compare UV results at time 1 to results obtained at time 2 (stored 7–16 days). The kappa coefficient for agreement of the RSID results is 0.4. Twelve applicators were RSID-positive, one was RSID-negative at both time points and two results were discrepant. For UV, all nine post-sex applicators tested positive at both time points. For post-sex applicators that were RSID-positive and stored for later testing by DSA and UV, seven of seven were positive at the second time point and eight of nine were positive at the third time point for both testing methods.

Discussion

This study demonstrated that the DSA and UV light test accurately detected vaginal insertion of HEC-filled Microlax®-type applicators after sex. In contrast to other biomarkers [1, 17], both tests are non-invasive, require little time and are inexpensive to perform. The sensitivity and specificity for both methods was high. Interestingly, the sensitivity of the DSA for post-sex applicators was lower compared to UV, and agreement between readers on the pre-sex applicators was higher for the DSA and lower for post-sex applicators compared to UV. Further, the RSID test was a useful tool to accurately evaluate whether returned applicators were used post-sex. Only eighty-two percent of returned applicators that were reportedly used after sex tested RSID-positive, which may be explained by several factors. Applicators may not have been inserted post-sex or they may have been inserted but sex did not occur. The applicators may have been rinsed before they were returned, condoms may have been used or male participants may not have ejaculated intravaginally. Given the small sample size, the results should not be over-interpreted.

Although the number of applicators stored for repeat testing by RSID and delayed testing by DSA and UV was small, our findings indicate that it may be feasible to incorporate these methods into future trials of candidate microbicides used before and/or after sex. The kappa coefficient for agreement between time points for RSID testing was moderate, suggesting that the RSID test could possibly be performed on stored applicators approximately once weekly. In addition, RSID testing may be a useful adherence measure in future microbicide trials that include BAT-24 product dosing. UV testing requires less time to perform than the DSA, as a washing step and drying period are not required. Therefore, UV testing could potentially be performed while participants are still at the clinic and results indicating non-use could be discussed at the time, potentially bolstering adherence rates in future trials. However, disclosing results in real time could potentially lead participants to use other strategies to make them appear adherent, such as vaginally inserting applicators without dispensing gel into the vagina.

There were several limitations to the study. A placebo gel was used among mutually monogamous, low-risk couples. Therefore, findings may not be generalizable to other populations, such as high-risk individuals using a drug-containing vaginal gel. The LDPE

MicroLax®-type applicator used in the current study differs from the HTI-type applicator used in the CAPRISA 004 trial and in the ongoing FACTS 001 confirmatory trial. Studies of the performance of the DSA and UV test should be considered on HTI-type applicators with BAT-24 dosing. Moreover, although the DSA and UV light test are simple to perform, neither can indicate whether gel was expelled into the vagina nor the precise timing of product application relative to sex or if sex took place at all.

To address the latter limitation, we also included RSID testing and found that applicators could be used to detect the presence of semenogelin as a biomarker of unprotected sexual exposure. However, there were no true positive controls to determine the sensitivity of RSID in this study. In addition, the assay is not quantitative and we did not evaluate whether the time of application of gel following coitus impacts assay performance. Thus it may not allow one to determine if gel was applied shortly after a single sex act, after multiple acts or at a later time more distant from last sexual exposure. There are other biomarkers that may provide a measurement of sexual exposure over a longer time period such as DNA polymerase chain reaction (PCR) testing to detect Y chromosome. In one study in which self-administered vaginal swabs were used, Y chromosome DNA was detectable for up to 15 post-coital days, with a half-life clearance of four days [19]. Whether this assay could be used with applicators is not known. Another innovative biomarker is a DNA multiplex PCR test that can detect 2 Y-chromosome linked-genes, SRY and TSPY4, as well as amelogenin, a gene found on both the X and Y chromosomes. This test can distinguish between female-only DNA (a marker for vaginal applicator insertion) and the additional presence of male DNA presumably as a result of semen exposure, which could indicate unprotected intercourse before applicator insertion in pericoital regimens [20]. However, the value of this test requires further study as it is possible that male DNA may be detected with protected intercourse from skin contact and if DNA persists for prolonged time periods within the female genital tract, the test may not provide an accurate assessment of recent sexual exposure. Cost may also limit the use of PCR testing strategies. Currently an RSID™-Semen kit includes 25 tests/kit and costs approximately \$200 or \$8/test.

The results of this small study indicate that both DSA and UV test may provide a reliable biomarker of vaginal applicator insertion with BAT-24 dosing and that the RSID test may also help identify applicators that were inserted following vaginal sex without barrier protection. The assays are easy to perform and could be incorporated into future clinical trials both as measures of adherence and to provide feedback in time to participants to address some of the behavioral challenges in clinical trials that have thwarted the prevention field. Larger studies as well as testing in high-risk cohorts are needed.

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Table 1

Characteristics of the 15 couples who completed the study

	N=30	%
Mean age (median; range)	30.7 years (29; 22–45)	
Women	30 years (29; 22–45)	
Men	31.5 years (29; 22–44)	
Race/ethnicity (all)		
Black/African	10	33
Hispanic	13	43
White (non-Hispanic)	3	10
Other	4	13
Attended some or completed college	20	66
Married or living with partner	14	47
Current Contraception		
Oral contraceptive pill (OCP)	5	33
Intrauterine device (IUD)	7	47
Tubal ligation	1	7
Hysterectomy	2	13
History of STI (5 females, 1 male)	6	20
Currently smoke cigarettes	3	10
Ever pregnant	14	93

Table 2

Sensitivity, specificity and 95% confidence intervals (CI) of the DSA and UV light test on pre-sex and post-sex applicators

	UV light test		DSA test	
	Pre-sex	Post-sex	Pre-sex	Post-sex
Sensitivity 95% CI	0.867 (0.684, 0.956)	0.968 (0.924, 0.998)	1 (0.859, 1)	0.899 (0.838, 0.939)
Specificity 95% CI	0.955 (0.751, 0.998)	0.955 (0.751, 0.998)	1 (0.815, 1)	1 (0.815, 1)

Table 3

Performance of the DSA and UV light test on all returned applicators

	UV light test			DSA test		
	Positive N (%)	Negative N (%)	Disagreement	Positive N (%)	Negative N (%)	Disagreement
Pre-sex	69 (80.23)	5 (5.81)	12 (13.95)	75 (97.40)	1 (1.30)	1 (1.30)
Post-sex	82 (95.35)	1 (1.16)	3 (3.49)	67 (87.01)	4 (5.19)	6 (7.79)