Comparison of Visual and Ultraviolet Light Inspection Versus DNA/Protein Biomarkers to Assess Product Adherence With Vaginal Microbicide Applicators

Andrea Ries Thurman, MD, Terry A. Jacot, PhD,* Thomas D. Kimble, MD,* Christine Mauck, MD, MPH,† Ashley Nelson, BS,* Jill L. Schwartz, MD, MPH,† and Gustavo F. Doncel, MD, PhD**

Background: Objective biomarkers of product use and protocol compliance are urgently needed. We compared the sensitivity and specificity of DNA and protein-based biomarkers, obtained from used vaginal gel applicators, to visual inspection of those applicators under ambient light (visual inspection of returned applicator [VIRA]) and ultraviolet light (UVL).

Methods: Forty women inserted hydroxyethylcellulose placebo gel vaginal applicators under direct observation. Applicators were evaluated by VIRA, UVL, and DNA/protein-based methods at 2 time points: within 7 days of the visit and after storing applicators for approximately 30 days. Semen biomarkers were assayed from vaginal swabs and returned applicators.

Results: The overall sensitivity and specificity of DNA and protein-based biomarkers in determining vaginal insertion versus sham handling of returned applicators were 98.3% and 100%, respectively, at both 7- and 30-day evaluations. The overall sensitivity and specificity of VIRA at 7 and 30 days after collection were significantly lower than those of DNA and protein-based biomarkers. Ultraviolet light inspection also had significantly lower overall sensitivity and overall specificity compared with DNA and protein biomarkers. The sensitivity of DNA and protein-based biomarkers for detecting insertion of wiped applicators was 95%, whereas the sensitivity of VIRA (range of 24%–28%) and UVL inspection (range, 38%–84%) was low for this subset. It was feasible to obtain semen biomarkers from vaginal swabs and returned used applicators.

Conclusions: DNA and protein-based biomarkers offer significantly higher sensitivity and specificity compared with VIRA and UVL assessment. The accuracy of these objective biomarkers is maintained despite storage of returned products for approximately 30 days and under conditions potentially modeling field use.

From the *CONRAD, Department of Obstetrics and Gynecology, Eastern Virginia Medical School, Norfolk, VA; and †CONRAD, Eastern Virginia Medical School, Arlington, VA

Acknowledgments: The authors would like to thank Bela Oza, MS; Suzanne Jackson, MS; and Nancy Gonyea, who did visual and ultraviolet light assessments of returned applicators. Dr Leila Mansoor, PhD, provided training before commencing the study. Dr Thomas Moench of Reprotect donated the ultraviolet light viewing box.

The authors report no conflicts of interest.

This work is made possible by the generous support of the American people through the United States Agency for International Development under Cooperative Agreements (GPO-A-00-08-00005-00 and AID-OAA-A-14-00005) to CONRAD, Eastern Virginia Medical School. The work of the authors has also been supported by an intraagency agreement between CONRAD and the National Institutes of Child Health and Human Development (No. Y1-HD-0083). The views expressed by the authors do not necessarily reflect those of the National Institutes of Health, United States Agency for International Development, or the US Government.

Correspondence: Andrea Ries Thurman, MD, CONRAD, Eastern Virginia Medical School, Room 235 Norfolk, 601 Colley Ave, VA 23507. E-mail: thurmaar@evms.edu.

Received for publication June 25, 2014, and accepted September 23, 2014.
DOI: 10.1097/OLQ.0000000000000209

Copyright © 2014 American Sexually Transmitted Diseases Association
All rights reserved.

Millions of women are and continue to be infected by human immunodeficiency virus type 1 (HIV-1), especially in resource-constrained countries.[1] Among novel efforts to prevent new infections is the development of topical anti-HIV microbicides, which are applied vaginally. The Achilles heel of large clinical microbicide trials, however, has been the ability to accurately and objectively evaluate adherence to product use instructions ("product adherence") and adherence to protocol requirements such as condom use ("protocol compliance"). In past and ongoing HIV-1 prevention trials using topical vaginal microbicides, participants are instructed to insert a prefilled applicator of microbicide or placebo gel into the vagina either daily or in a pericoital manner. In addition, participants are advised to use condoms to further reduce their risk of acquiring sexually transmitted infections and HIV-1. Protocol compliance and product adherence are complex processes influenced by sociobehavioral, biological, and product-related factors.[2] Each has a significant impact on drug pharmacokinetics and pharmacodynamics, and, ultimately, on product safety and effectiveness.[3–5] Recent data from large HIV-1 prevention trials[6,7] have highlighted the importance of accurate, objective measures of product adherence after self-reported product use was found to be significantly higher than adherence estimates based on active drug concentrations.[6,7] Adherence, therefore, must be measured objectively, repeatedly, and in real time, and used to manage the trial as well as help with the interpretation of final results.

There have been several past attempts to measure product adherence and protocol compliance with more objective and accurate measures. In the Follow-on African Consortium for Tenofovir Studies (FACTS 001) microbicide gel trial, visual inspection of returned applicators (VIRA) is being used to assess product adherence to counsel participants during the trial.[8] To perform VIRA, clinic staff members inspect returned gel applicators under ambient light and look for dried gel, pubic hair, vaginal secretions or menstrual blood on the external surface. Applicators that have these components are considered to have been inserted into the participant's vagina. The obvious advantages of VIRA are that it is inexpensive to perform and suitable for low resource environments. However, there are anecdotal reports of participants inserting gel applicators vaginally, but expelling the gel outside the vagina, or "incorrect use." In addition, participants in microbicide gel trials typically save used applicators at home, returning them in bulk at monthly or quarterly clinic visits. Anecdotal evidence suggests that a proportion of women, for hygienic reasons, wipe vaginal secretions off the used applicators before storing them for long periods at home under conditions of high heat and humidity. These important "field conditions" have not been previously assessed in studies of VIRA.

Inspection of returned gel applicators under ultraviolet light (UVL) at 365 to 385 nm is another proposed objective method of assessing product adherence. Again, the major advantages of UVL inspection are that it is relatively easy and inexpensive to perform.

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

The UVL inspection unit is small, portable, and suitable for clinic environments.[9] However, microbicide gels, in addition to bodily fluids, including semen, all fluoresce under UVL, limiting the ability to differentiate them with this test. The sensitivity of UVL inspection is approximately 65% when applicators are inserted vaginally without prior vaginal gel exposure (such as might be done with precoital dosing) and improves to 95% when applicators are inserted after gel has previously been expelled into the vagina (such as might be done with a postcoital or daily dosing regimen).[9] Thus, the UVL method may have greater use in detecting compliance with a daily or more frequent dosing protocol rather than an episodic, precoital dosing regimen.

A better marker of product adherence and protocol compliance to a vaginal gel introduced with an applicator would be a triple marker, with an objective assessment of proper vaginal insertion of the applicator, detection of prior semen exposure in the vagina, if any, and measurement of active drug or placebo. Ideally, this triple marker should be obtained noninvasively, such as from the outside shaft of returned applicators.

To detect vaginal insertion of a microbicide gel applicator, we have developed a panel of DNA and protein biomarkers based on vaginal bacterial DNA species present in the vagina and cytokeratin 4 protein (CK4), which is present in vaginal epithelium, but not in the epidermis. Using a previously developed and clinically validated multiplex polymerase chain reaction (PCR) amplifying biomarkers of semen exposure,[10] we incorporated primers amplifying DNA from 7 various species of bacteria reported in the vagina[11] to determine vaginal insertion of gel applicators. Therefore, the resulting multiplex PCR could determine both vaginal insertion and semen exposure simultaneously. For further confirmation of vaginal specificity, CK4 immunocytochemistry was added to the analysis.

The objective of this study was to estimate the sensitivity and specificity of VIRA, UVL inspection, and our vaginal bacterial DNA/CK4 biomarker panel using the gold standard of witnessed use of the applicators in the clinic. Second, we studied how various potential field conditions, such as wiping off an applicator after insertion, alter the sensitivity and specificity of each method. Assessments of applicators that were inserted with and without the presence of previous gel were carried out. The effect of storage time on biomarker sensitivity and specificity was also investigated. Finally, timed sexual intercourse was not required or prohibited in this study, but the presence of semen biomarkers from a vaginal swab and returned used applicators was tested for feasibility based on participants' report of condom use and number of days since last vaginal intercourse.

MATERIALS AND METHODS

This study was approved by the Chesapeake Institutional Review Board (Pro00008154) and was registered with ClinicalTrials.gov (NCT01804023). Healthy women were recruited from the local community (Hampton Roads, VA) through institutional review board–approved print and electronic advertisements. This was a cross-sectional study of 40 healthy, nonpregnant, HIV-1–negative women aged 18 to 50 years. All volunteers had to be at least 3 days from their last menstrual bleeding episode and could not have used vaginal creams, gels, or lubricants in the past 3 days. All volunteers provided written informed consent before any study procedures. Participants were screened with point-of-care tests for pregnancy (hcg Dipstick; Consult Diagnostics, Jacksonville, FL) and HIV-1 (OraQuick; Orasure Technologies, Bethlehem, PA). Women were queried as to whether they currently had a male sexual partner and if this partner had had a vasectomy or had ever been diagnosed as having a low sperm count. We queried participants on the timing of their last sexual intercourse and whether they used a condom.

Once the point-of-care screening tests were confirmed negative, each participant was given 12 prefilled, single-dose HTI polypropylene applicators containing hydroxyethylcellulose (HEC) universal placebo gel. All volunteers were seen in a single visit. The processing of the 12 applicators is detailed in Table 1.

TABLE 1. Applicator Assignments and Assessments

Applicator No.	Condition	Time Until Processing ~7 d	Time Until Processing ~30 d
1	Handled, not inserted vaginally, VIRA and UVL	n = 40	
2	Handled, not inserted vaginally, VIRA and UVL		n = 40
3	Handled, not inserted vaginally, DNA/cytokeratin biomarkers	n = 40	
4	Handled, not inserted vaginally, DNA/cytokeratin biomarkers		n = 40
5	Inserted vaginally by clinician, gel expelled into waste container, VIRA and UVL	n = 40	
6	Inserted vaginally by clinician, gel expelled into waste container, VIRA and UVL		n = 40
7	Inserted vaginally by clinician, gel expelled into waste container, DNA/cytokeratin biomarkers	n = 40	
8	Inserted vaginally by clinician, gel expelled into waste container, DNA/cytokeratin biomarkers		n = 40
9	Inserted vaginally by clinician, gel expelled into vagina, VIRA, UVL, DNA/cytokeratin biomarkers	n = 20	n = 20
10	Inserted vaginally by clinician, gel expelled into vagina, VIRA, UVL, DNA/cytokeratin biomarkers	n = 20	n = 20
11	Inserted vaginally by clinician, gel expelled into waste container, applicator wiped clean, VIRA/UVL, DNA/cytokeratin biomarkers	n = 40	
12	Inserted vaginally by clinician, gel expelled into waste container, applicator wiped clean, VIRA/UVL, DNA/cytokeratin biomarkers		n = 40
Total number of applicators evaluated by VIRA and UVL		n = 160	n = 160
Total number of applicators evaluated by DNA/cytokeratin biomarkers		n = 160	n = 160
Total number of applicators evaluated by either method		n = 240	n = 240

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

Applicators 1 to 4 were handled by the participant but not inserted vaginally, and the gel was then expelled into the trash ("sham"). The investigator then obtained a vaginal swab from participants as a positive control to confirm that the bacterial species present in the vagina were also found in the DNA isolated from used, inserted applicators. Semen markers were also assayed from this vaginal swab. Next, 8 applicators (applicators 5–12, see Table 1) were inserted into the participant's vagina, one at a time, by the investigator. Applicators 5–8 (Table 1) were inserted into the participant's vagina and removed, and the gel was expelled into the trash, to simulate "incorrect use." Applicators 9 and 10 (Table 1) were inserted into the participant's vagina, and the gel was expelled vaginally. Finally, applicators 11 and 12 (Table 1) were inserted vaginally and removed, gel was expelled into the trash, and each applicator was wiped clean with a dry tissue. All participants received a follow-up telephone call from the nurse coordinator 7 to 14 days after the study visit to check for adverse events or any health issues.

Before commencing the study, the principal investigator was trained on VIRA and UVL assessment by a member of the Centre for the AIDS Programme of Research in South Africa (CAPRISA) team who had provided training in VIRA and UVL inspection for several HIV-1 prevention trials in Africa. The principal investigator trained the 3 readers at Eastern Virginia Medical School on VIRA and UVL inspection, before commencing the study. All readers and the laboratory staff were blinded to the applicator assignment in the clinic.

Inspection and laboratory analyses of VIRA and UVL of used applicators were to be done within 7 days of the participant's visit for 240 of the applicators and were to be performed on the additional 240 applicators after approximately 30 days of storage at ambient room temperature.

The 3 VIRA and UVL readers analyzed all applicators separately, so that individual responses did not influence other readers. At each VIRA and UVL assessment, VIRA assessments were performed on all applicators before assessing the batch of applicators by UVL. The order of the applicators for the separate VIRA and UVL assessments was changed so that a VIRA result would not influence the subsequent UVL interpretation. Readers evaluated between 1 and 8 participants' applicators at each sitting and graded each applicator as "inserted" or "not inserted" vaginally.

The UVL box was donated by Reprotect LLC and slightly modified to have a 365-nm light as opposed to the 385-nm light used in the previous publication.[9] The 365-nm light (Compact UV Light, Product #36582-045; VWR Corporation, Sacramento, CA) was recommended (T. Moench, personal communication) because it produced less ambient heat than the 385-nm light. Readers viewed applicators through a UVL filter (B&H Photo, New York, NY; Item TIUV2A34) for safety.

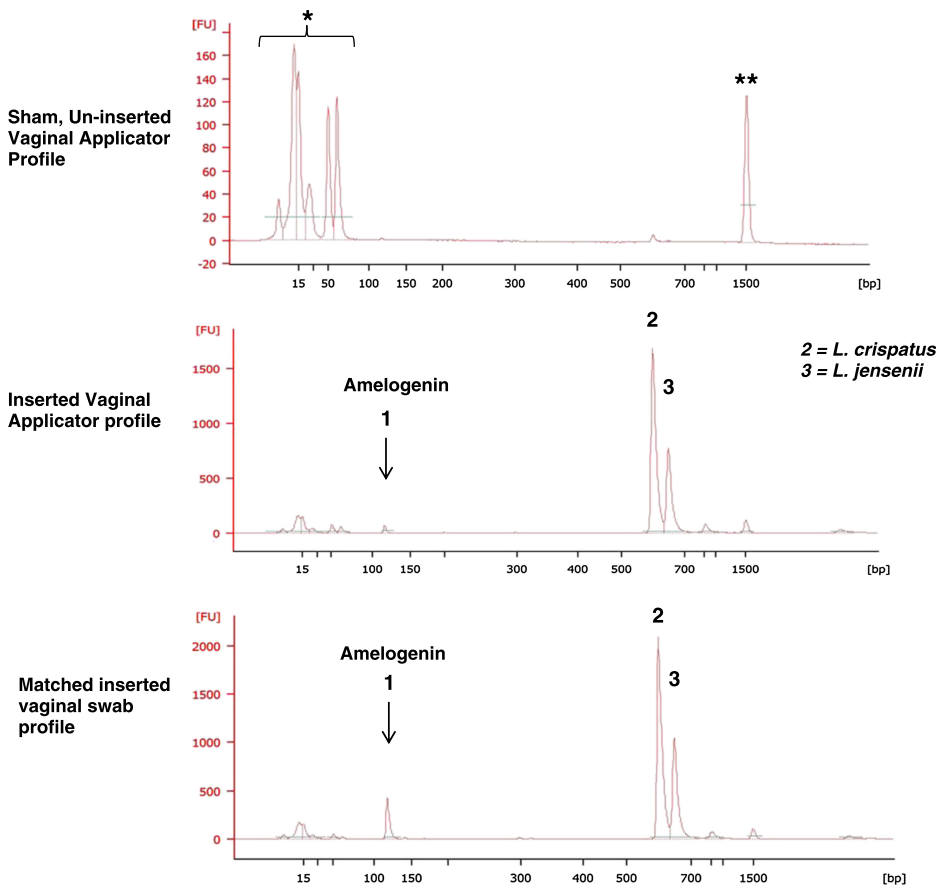

Figure 1. DNA biomarkers distinguish vaginally inserted applicators from sham applicators. DNA was isolated from vaginally inserted and sham applicators and analyzed through multiplex PCR. PCR products were visualized in the electropherograms shown above. The control gene (no. 1), amelogenin, and 2 vaginal bacterial markers (nos. 2 and 3), *L. crispatus and L. jensenii*, are detected in DNA from a vaginally inserted applicator but not from a sham applicator handled by the same participant. The vaginal biomarker profile matches that of the vaginal swab from the same participant. *Nonspecific primer dimers/DNA marker. **DNA marker (for sizing PCR products).

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

TABLE 2. Participant Information

Variable	
Total number of women screened for study	40
Total number of women enrolled	40
Analysis population, provided 12 applicators	40
Self-identified ethnicity, n (%)	
Hispanic	4 (10)
Non-Hispanic	36 (90)
Self-identified race, n (%)	
Black	17 (42.5)
White	22 (55)
Mixed	1 (2.5)
Demographic Data	
Body mass index, mean ± SD (range), mg/kg^2	30.0 ± 9.1 (20.3–57.6)
Age, mean ± SD (range), y	32.8 ± 7.7 (21–49)
Years of education, mean ± SD (range)	14.3 ± 1.8 (11–18)
No. participants who reported having a male sexual partner, n (%)	32 (80)
Among sexually active women, average number of days since last vaginal intercourse with male partner, mean ± SD (range)	9.7 ± 13.9 (1–60)
Average number of past pregnancies, mean ± SD (range)	2.6 ± 2 (0–8)
Average number of days since last menstrual bleeding, mean ± SD (range)	13.6 ± 8.2 (4–45)
Reported being diagnosed with a vaginal infection in the past year, n (%)	8 (20)
Contraception used by participants. n (%)	
None	5 (12.5)
Copper intrauterine device	1 (2.5)
Condoms	13 (32.5)
Abstinence	3 (7.5)
Female sterilization	13 (32.5)
Oral contraceptive pills	3 (7.5)
Levonorgestrel intrauterine system	2 (5)

All members of the laboratory staff who performed the DNA and cytokeratin analyses were blinded to the assignments of the applicators in the clinic. To isolate bacterial DNA and vaginal cells, returned applicators were swabbed using a double headed swab. One swab head was used to isolate DNA, whereas the other swab head was used to isolated vaginal cells for CK4 immunocytochemistry. DNA from the swabbed applicators and vaginal swabs was analyzed by multiplex PCR using the Qiagen Multiplex Plus PCR Kit (Qiagen, Valencia, CA), and amplified PCR products were compared through electropherograms generated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) in conjunction with its DNA 1000 kit (Fig. 1). Vaginal insertion versus sham handling was demonstrated when 1 or more vaginal bacterial markers plus the control gene, amelogenin, were amplified. Vaginal insertion could further be confirmed by positive CK4 expression in vaginal cells. The multiplex PCR also amplifies 2 Y chromosomal genes, the sex determining region of the Y chromosome (SRY) and testis-specific Y encoded 4 (TSPY4), from vaginal swab DNA if semen exposure occurred.[10] If the multiplex PCR amplified one or both of these genes, it was concluded that semen exposure occurred within approximately 7 days of applicator insertion.

Statistical analysis was performed using SAS version 9.3 (Carey, NC). Specificity was calculated using the sham applicators (applicators 1–4, Table 1) for each participant, by correctly identifying sham applicators as not inserted. The sensitivity was calculated using applicators 5–12 (Table 1), by the total number of applicators deemed to be inserted based on applicator reading over the total number actually inserted. Comparisons of sensitivity and specificity data were done using χ^2 or Fisher exact test, as appropriate. Comparisons of interreader variability (IRV) were done by comparing the 3 individual readers' sensitivity and specificity results for each subgroup, by χ^2 or Fisher exact test, as appropriate. Descriptive statistics were used to describe the participant's demographic data.

RESULTS

A total of 40 women were screened for this study, and a total of 40 women underwent the study procedures, with each woman providing 1 vaginal swab (total n = 40) and 12 returned applicators for analysis (total n = 480). Table 2 details demographic, social, and medical information of the 40 enrolled participants. There were no adverse events during the study or the 2-week follow-up period. Figures 1 and 2 are representative examples of determining vaginal insertion using our composite panel of DNA and CK4 biomarkers. Figure 1 (middle graph) shows the presence of 2 bacterial DNA species, *Lactobacillus crispatus* and *Lactobacillus jensenii* (peaks 2 and 3,) and the control gene, amelogenin (peak 1) amplified from the DNA isolated from a vaginally inserted applicator. This is identical to the DNA isolated from a vaginal swab obtained from the same participant (bottom graph, Fig. 1). However, no PCR products, including the control gene, are amplified from the DNA isolated from a sham applicator in Figure 1 (top graph). Figure 2 shows further confirmation of vaginal insertion, by CK4 detection, of the inserted applicator shown in the middle panel of Figure 1. Isolated vaginal cells from the same vaginally inserted applicator demonstrated CK4 expression further confirming results of the DNA analysis. Sham applicators do not provide the number of cells seen with vaginally inserted applicators and have no CK4 staining (data not shown). Figure 3 is a representative DNA analysis of a vaginally inserted applicator showing vaginal insertion and semen exposure. The DNA of this participant had both Y-chromosomal genes, SRY and TSPY4, amplified in addition to the vaginal bacterial markers.

Assessments of VIRA and UVL were performed at an average of 1.4 ± 1.6 days after the participant's visit (range, 0–5 days) for the early (<7 day) assessment and were performed at an

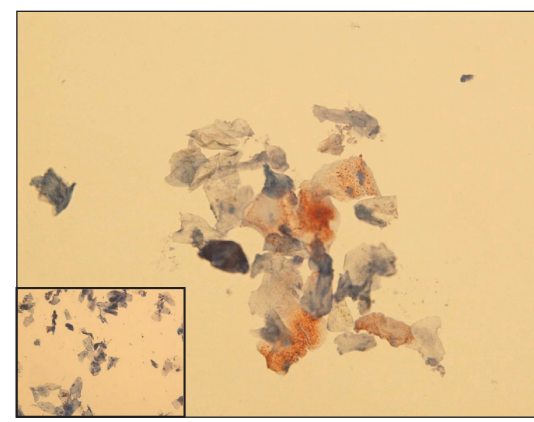

Figure 2. Vaginal insertion confirmed by CK4 immunocytochemistry. Vaginal cells were isolated from vaginally inserted applicators, fixed, and spotted onto slides for CK4 determination. Vaginal cells are positive for CK4 (red color), a marker previously determined to be absent in epidermal cells. Inset picture: nonspecific rabbit IgG-negative control.

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

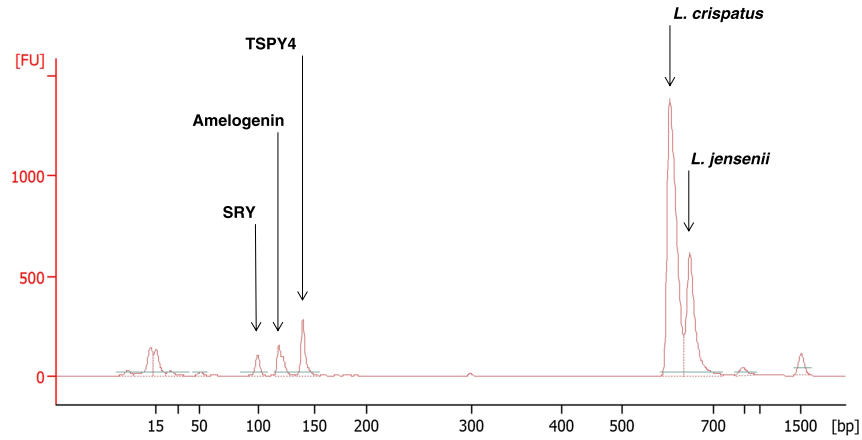

Figure 3. Semen biomarker detection in DNA from vaginally inserted applicators. An electropherogram demonstrating the amplification of Y-chromosomal genes, SRY and TSPY4, in addition to the vaginal bacterial markers and amelogenin.

average of 31.2 ± 1.7 days after the participant's visit (range, 28–34 days) for the 30 day assessment. DNA and protein biomarker assessments were performed at an average of 3.1 ± 2.0 days after the participant's visit (range, 0–7 days) for the 7-day assessment and 35.2 ± 5 days (range, 28–49) for the 30-day assessment.

Table 3 details the overall mean sensitivity and specificity of VIRA and UVL, calculated by pooling the 3 readers' responses, and notes the individual sensitivity and specificity results of each of the 3 readers in parentheses for each subgroup of applicators. Also noted in Table 3 is the *P* value for IRV for each subgroup. There was a statistically significant increase in the specificity of VIRA assessments at 30 days. There was also a statistically significant increase in the overall sensitivity of UVL assessments at 30 days, which was largely due to an increase in the sensitivity of detecting insertion of wiped applicators at 30 days compared with within 7 days. There was no significant IRV for any subgroup measurement for the VIRA assessments within 7 days (all *P* values > 0.15, Table 3). For various subgroups, there were significant interreader differences in sensitivity and specificity results for all other assessments (VIRA at 30 days and UVL assessments at 7 and 30 days, Table 3).

Table 4 illustrates the high sensitivity and specificity of our DNA and protein-based biomarkers of vaginal insertion at the 7- and 30-day evaluation time points. Of note, at least 1 of the 7 vaginal bacterial species tested was present in all 40 vaginal swabs, and some participants had up to 4 of the 7 markers present, supporting the hypothesis that the species selected were representative of an ethnically diverse group of US women.[11]

Tables 5 and 6 compare VIRA and UVL assessment at 7 and 30 days, respectively, with DNA and cytokeratin assessments at the same time points. DNA and protein biomarkers were significantly more sensitive and specific, in all subsets, than VIRA at both 7 and 30 days. In addition, DNA and protein biomarkers had significantly higher overall sensitivity and overall

TABLE 3. VIRA and UVL Assessments at 7 and 30 Days

	VIRA 7 d		VIRA 30 d			UVL 7 d		UVL 30 d		
Variable	**Mean % (individual readers %)**	**IRV *P* value**	**Mean% (Individual Readers %)**	**IRV *P* Value**	***P* Value, VIRA 7 vs. 30 d**	**Mean % (Individual Readers %)**	**IRV *P* Value**	**Mean % (Individual Readers %)**	**IRV *P* Value**	***P* Value, UVL 7 vs. 30 d**
Sensitivity										
All vaginally inserted applicators (360 readings of 120 applicators)	54 (50, 59, 54)	0.36	52 (45, 49, 62)	0.03	0.50	74 (68, 88, 67)	≤0.01	**92 (89, 97, 91)**	0.07	<**0.001**
All vaginally inserted applicators, except wiped applicators (240 readings of 80 applicators)	70 (64, 78, 68)	0.15	64 (59, 59, 74)	0.08	0.18	93 (90, 98, 90)	0.10	96 (95, 99, 95)	0.40	0.07
No gel present (180 readings of 60 applicators)	69 (65, 77, 67)	0.32	62 (55, 57, 75)	0.04	0.15	92 (88, 98, 90)	0.09	95 (93, 93, 97)	0.40	0.28
Gel present (60 readings of 20 applicators)	70 (60, 80, 70)	0.39	68 (70, 65, 70)	0.94	0.84	93 (95, 95, 90)	0.85	100 (100, 100, 100)	1.0	0.06
Wiped (120 readings of 40 applicators)	24 (23, 23, 28)	0.84	28 (18, 30, 38)	0.13	0.46	38 (23, 70, 20)	≤0.01	**84 (78, 93, 83)**	0.17	<**0.001**
Specificity										
All sham-inserted applicators (120 readings of 40 applicators)	49 (55, 45, 48)	0.65	**78 (75, 78, 83)**	0.71	<**0.001**	73 (93, 38, 90)	≤0.01	66 (90, 35, 73)	≤0.01	0.21

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

TABLE 4. DNA/Protein Biomarker Data at 7 and 30 Days

Variable	7 d	30 d	*P*
Sensitivity			
All vaginally inserted applicators (1 reading of 120 applicators; 2 missing applicators from 7-day group, 1 missing applicator from 30-day group)	116/118 (98.3%)	117/119 (98.3%)	1
All vaginally inserted applicators, except wiped (1 reading of 80 applicators; 2 missing applicators from 7-day group)	78/78 (100%)	80/80 (100%)	1
No gel present (1 reading of 60 applicators; 2 missing applicators from 7-day group)	58/58 (100%)	60/60 (100%)	1
With gel present (1 reading of 20 applicators)	20/20 (100%)	20/20 (100%)	1
Wiped (1 missing applicator from 30-day group)	38/40 (95%)	37/39 (95%)	1
Specificity			
All sham-inserted applicators (1 reading of 40 applicators; 1 missing applicator from 7-day group)	39/39 (100%)	40/40 (100%)	1

specificity than UVL assessment at 7 and 30 days. For the subsets of applicators in which sensitivity was assessed DNA and protein biomarkers, there was a nonsignificant trend toward higher sensitivity of DNA and protein biomarkers compared with UVL assessment.

Semen biomarkers were obtained from both vaginal swabs and returned, used applicators, with a representative sample from a returned applicator shown in Figure 3.

DISCUSSION

This was the first study to evaluate the sensitivity and specificity of DNA and cytokeratin-based biomarkers, obtained from returned vaginal applicators, used under direct visualization, to objectively determine vaginal insertion. They were tested against VIRA and UVL inspection, modeling standard, and common field conditions, such as wiping and incorrect use of vaginal gel applicators. We found that objective DNA and cytokeratin-based biomarkers were highly sensitive and specific in detecting vaginal insertion versus sham use of HEC placebo applicators. These biomarkers remained robust, with identical sensitivity and specificity, whether applicators were evaluated within 7 days of use or after approximately 30 days of storage at room temperature. We also demonstrated that semen biomarkers could be assayed from returned vaginal swabs and used vaginal product applicators.

Both VIRA and UVL are ultimately subjective assessments of adherence. We found that the sensitivity and specificity of VIRA and UVL readings significantly improved in some subsets with 30-day storage (Table 3). On average, the 7-day VIRA and UVL readings were done the day after the clinic visit. We did not capture whether the gel was still wet or if it had dried at these early readings. However, by 30 days of storage, the gel had dried on used applicators. We hypothesize that the improvements in sensitivity and specificity at 30 days, in some subsets, had little to do with the characteristics of the applicators. In fact, as the gel dries with storage, one would expect for the sensitivity and specificity of adherence assessments to decrease, not increase. We think that the improvements in sensitivity and specificity at 30 days were largely due to a learning curve by the 3 blinded readers. In essence, by the 30-day readings, they had read more applicators. Specifically, the improvement in the overall sensitivity of UVL assessments at 30 days was due to substantial improvements in detecting wiped applicators (an increase in sensitivity from 38% to 84%, Table 3), which subjectively had a streaking pattern that was different from inserted, unwiped applicators. We also noted significant IRV with several of the UVL applicator assessments, supporting our assessment that UVL is a subjective assessment with variability in interreader interpretations.

In the previous study of VIRA and UVL, upon which we based our clinical trial,[9] VIRA and UVL assessments were performed approximately 3 to 6 months after the participant's visit (T. Moench, personal communication). The previous study did not include wiped applicators.[9] Although most participants in the former trial provided applicators under direct observation in the clinic (n = 50), a subset of women (n = 15) inserted the applicators at home.[9] Because of these major differences in study

TABLE 5. VIRA and UVL Assessment at 7 Days Versus DNA/Protein at 7 Days

Variable	VIRA or UVL 7-d Data	DNA/Protein 7-d Data	*P*
Sensitivity			
VIRA sensitivity all applicators	196/360 (54.4%)	116/118 (98.3%)	**<0.001**
VIRA sensitivity all except wiped	167/240 (69.6%)	78/78 (100%)	**<0.001**
VIRA sensitivity no gel present	125/180 (69.4%)	58/58 (100%)	**<0.001**
VIRA sensitivity gel present	42/60 (70.0%)	20/20 (100%)	**0.005**
VIRA sensitivity wiped	29/120 (24.2%)	38/40 (95%)	**<0.001**
Specificity			
VIRA specificity (all sham applicators)	59/120 (49.2%)	39/39 (100%)	**<0.001**
Sensitivity			
UVL sensitivity all applicators	267/360 (74.2%)	116/118 (98.3%)	**<0.001**
UVL sensitivity all except wiped	222/240 (92.5%)	78/78 (100%)	**0.01**
UVL sensitivity no gel present	166/180 (92.2%)	58/58 (100%)	**0.03**
UVL sensitivity gel present	56/60 (93.3%)	20/20 (100%)	0.31
UVL sensitivity wiped	45/120 (37.5%)	38/40 (95%)	**<0.001**
Specificity			
UVL specificity (all sham applicators)	88/120 (73.3%)	39/39 (100%)	**<0.001**

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

TABLE 6. VIRA and UVL Assessment at 30 Days Versus DNA/Protein at 30 Days

Variable	VIRA or UVL 30-d Data	DNA/Protein 30-d Data	*P*
Sensitivity			
VIRA sensitivity all applicators	187/360 (51.9%)	117/119 (98.3%)	**<0.0001**
VIRA sensitivity all except wiped	153/240 (63.8%)	80/80 (100%)	**<0.0001**
VIRA sensitivity no gel present	112/180 (62.2%)	60/60 (100%)	**<0.0001**
VIRA sensitivity gel present	41/60 (68.3%)	20/20 (100%)	**0.004**
VIRA sensitivity wiped	34/120 (28.3%)	37/39 (95%)	**<0.0001**
Specificity			
VIRA specificity (sham)	94/120 (78.3%)	40/40 (100%)	**0.0013**
Sensitivity			
UVL sensitivity all applicators	332/360 (92.2%)	117/119 (98.3%)	**0.02**
UVL sensitivity all except wiped	231/240 (96.3%)	80/80 (100%)	0.08
UVL sensitivity no gel present	171/180 (95%)	60/60 (100%)	0.08
UVL sensitivity gel present	60/60 (100%)	20/20 (100%)	1.0
UVL sensitivity wiped	101/120 (84.2%)	37/39 (95%)	0.09
Specificity			
UVL specificity (sham)	79/120 (65.8%)	40/40 (100%)	**<0.0001**

Bold indicates statistical significance at $P < 0.05$.

design, we did not compare our VIRA and UVL data to this previous study.

In the field, it is likely that a batch of returned applicators will contain unknown proportions of applicators which are sham, wiped, incorrectly inserted and/or used in the presence of vaginal gel, and therefore, the most important targets are the overall sensitivity and overall specificity of various adherence measurements. We found that the overall sensitivity and overall specificity of the DNA and protein-based methods were significantly higher at 7 and 30 days, compared with VIRA and UVL.

The major advantages of VIRA and UVL are that both assessments can be performed on site, are inexpensive, and require little ancillary equipment. However, both are ultimately subjective assessments, prone to IRV, which we demonstrated in our study. DNA and protein-based biomarkers obtained from returned used product applicators are not invasive compared with other objective measures of adherence, such as drug analyses of vaginal fluids and/or blood. In addition, by obtaining objective adherence measures from the used returned product applicator, the DNA and protein biomarkers give information about real-time use (and pattern of use) in between clinic visits. In contrast, depending on the half-life of the active drug, drug level analyses provide adherence data over a limited range of time.

We found that DNA and protein methods remained robust for approximately 30 days of storage. Therefore, it may be feasible that these markers can be used in large HIV-1 prevention and women's health trials whereby participants are seen monthly, quarterly, or even less frequently. We are currently testing the sensitivity and specificity of these methods after prolonged (>30 days) storage.

The human vagina has a well-described microflora, which has been shown to differ based on ethnicity and the menstrual cycle.[11] We expanded our previously established semen biomarkers multiplex PCR[10] to include amplification of 7 vaginal bacterial species. Our study participants were primarily non-Hispanic black and non-Hispanic white women. All participants had at least 1 of the 7 bacterial species present on vaginally inserted applicators, and some women had up to 4 markers present. Data support that these species will be present in US women of other races and ethnicities.[11] We plan to determine that these species are present in women from high HIV-1 prevalence regions where topical microbicide trials are currently being conducted, such as South Africa.

Some applicators (numbers 9, 10, 11, and 12; Table 1) were first evaluated by VIRA and UVL and were then swabbed and evaluated using DNA and protein-based methods. Before the study, we confirmed that applicators exposed to UVL at wavelengths as low as 254 nm at a distance comparable with that of the UVL box for 1 minute did not damage the DNA allowing accurate PCR analysis. The current study design, in which some applicators were evaluated by all 3 methods (VIRA, UVL, DNA/protein), was implemented to limit the number of vaginal applicators that needed to be inserted into a participant's vagina at one visit.

We used HEC placebo gel, dispensed in prefilled, single-dose HTI polypropylene applicators, in this study, because it was used in the previous validation study of VIRA and UVL[9] and is currently being used in ongoing HIV prevention trials such as the FACTS 001 study. It has been previously shown that the sensitivity of other objective measures of product use, such as the dye stain assay (DSA) with FD&C Blue Dye No. 1 or Trypan Blue 0.4% of HTI polypropylene applicators, varies from approximately 47% to 100%, whereas the sensitivity of the DSA is more consistent (approximately 91%–98%) with Microlax low-density polyethylene applicators.[12,13] These differences in the performance of the DSA test are thought to be based on surface characteristics of the applicators that affect the streaking patterns of the dye, which, in turn, affects the sensitivity of the DSA test.[12,13] We also have preliminary data showing that the DNA and protein-based biomarkers can be recovered from single-use paper applicators, which are being proposed as an alternative to the plastic applicators. Finally, we have demonstrated that tenofovir 1% gel, currently undergoing phase III clinical testing, does not interfere with the DNA and protein-based biomarkers.

We compared our DNA and protein-based biomarkers with VIRA because this method of assessing product adherence is currently being used in the FACTS 001 trial to enhance adherence counseling.[8] Visual inspection of returned applicator is inexpensive and easy to implement. However, we demonstrated that the overall sensitivity of this method is relatively low (52%–54%), compared with previous estimates of VIRA sensitivity, primarily due to the very low sensitivity (24%–28%) of detecting wiped applicators, which was not modeled in the study by Moench and colleagues.[9]

Ultraviolet light inspection can be used to enhance detection of vaginal secretions on used product applicators, with a reported sensitivity of 84% (range, 79%–87%).[9] We found that the overall sensitivity of UVL assessment was 74% for applicators assessed within 7 days of use and 92% when applicators were stored for approximately 30 days. The specificity of UVL assessment was 73% at the 7-day reading and 66% for applicators stored

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.

for more than 30 days. Our data are in accordance with those of Moench and colleagues,[9] demonstrating that UVL has a higher overall sensitivity compared with VIRA. Our blinded readers underwent a learning curve and demonstrated significantly more accurate UVL assessments at 30 days compared with 7 days, for some applicator subsets. Ultraviolet light assessment of applicators, due to its low cost and improved sensitivity and specificity, likely has a role in adherence monitoring in microbicide gel trials. However, we also found significant IRV with UVL at both the 7- and 30-day readings. Investigators will need to weigh the distinct benefits and disadvantages of UVL assessment when considering how to assess adherence in future clinical trials.

The previous study of UVL inspection noted that the sensitivity of UVL inspection is highest (95%; range, 89%–98%) when applicators were inserted with gel already present in the vagina, as in the postcoital dose of the BAT24 regimen or what might be encountered with a daily or frequent use regimen.[9] We also found that the sensitivity of both VIRA and UVL inspection was higher with applicators inserted in the presence of vaginal gel (applicator 10, Table 1) as opposed to those inserted without vaginal gel being present (applicators 5, 6, 9; Table 1). The DNA and protein-based biomarkers, on the other hand, had 100% sensitivity whether or not gel was present vaginally and remained at 100% despite 30 days of storage.

Before this investigation, UVL inspection had not been tested in the presence of semen,[9,12] which fluoresces under UVL and/or stains with dyes. Although participants enrolled in the study could not have had menses or used vaginal lubricants within 3 days of enrollment, there were no restrictions on sexual activity.

We were able to detect semen biomarkers on vaginal swabs and applicators from a subset of participants who reported unprotected intercourse during the 7 days before the visit. For the semen biomarkers, the comparator in this study was participant self-report of last intercourse, which is not as relevant as a clinical trial involving timed intercourse or vaginal inoculation with the partner's semen in the clinic. We recently conducted such a trial (ClinicalTrials.gov No. NCT00984555), and the data are in the final analysis. Importantly, we demonstrated that SRY and TSPY4 could be recovered by swabbing used returned product applicators, using the same methodology we have used on vaginal swabs. Detecting seminal proteins (semenogelin) from used vaginal applicators has previously been reported.[14] However, seminal proteins such as semenogelin and prostate-specific antigen degrade in the vagina approximately 24 to 72 hours after exposure.[15–17] Our preliminary data support that DNA-based semen biomarkers, including SRY and TSPY, are present in the vagina for up to 7 to 15 days after exposure.

DNA and protein biomarkers are more expensive and labor intensive than VIRA and UVL assessment, but have significantly higher overall sensitivity and specificity. For all the assessment methods (VIRA, UVL, and DNA/protein biomarkers), if applicators are not stored in separate wrappers, biologic material from one applicator will contaminate other applicators. In this study, we took care to use clean technique and hold applicators at the plunger end for VIRA and UVL inspections, whereas DNA and protein samples were obtained at approximately 2 to 3 cm from the tip of the applicator. In the current FACTS 001 trial, used applicators are stored in a stripform portfolio, with separate sleeves for each returned applicator. Clinic staff removed applicators from the sleeves to perform VIRA assessments. It is not known how much cross-contamination of applicators or loss of vaginal sample occurs during this handling and how this might affect the specificity of DNA and protein methods. We are currently embarking on a study to use DNA and protein biomarkers to assess a subset of returned gel applicators from the FACTS 001 trial.

In this study, we demonstrated that DNA and protein-based biomarkers obtained from empty, returned applicators offer the potential for a highly sensitive and specific measure of product adherence with topical microbicides. We have shown that semen biomarkers can be assayed from returned vaginal applicators stored for up to 30 days at room temperature. Because condom use is advocated in topical microbicide trials, a semen biomarker is also a marker of protocol compliance. We also have preliminary data showing the feasibility of the third prong of our adherence panel, assaying active drug or placebo from the shaft of returned applicators. This panel, encompassing vaginal insertion, semen exposure, and drug or placebo expulsion, will provide a highly sensitive and specific objective biomarker panel to noninvasively analyze product adherence and protocol compliance.

REFERENCES

1. UNAIDS. Report on the Global AIDS Epidemic. 2012. Available at: http://www.unaids.org/en/media/unaids/contentassets/documents/epidemiology/2012/gr2012/20121120_UNAIDS_Global_Report_2012_en.pdf.
2. van der Straten A, Montgomery ET, Hartmann M, et al. Methodological lessons from clinical trials and the future of microbicide research. Curr HIV/AIDS Rep. 2013; 10:89–102.
3. Hendrix CW, Cao YJ, Fuchs EJ. Topical microbicides to prevent HIV: Clinical drug development challenges. Annu Rev Pharmacol Toxicol. 2009; 49:349–375.
4. Karim QA, Karim SSA, Frohlich JA, et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Sci Express. 2010; 10.1126:1.
5. Dai JY, Gilbert PB, Hughes JP, et al. Estimating the efficacy of preexposure prophylaxis for HIV prevention among participants with a threshold level of drug concentration. Am J Epidemiol 2013; 177: 256–263.
6. Van Damme L, Corneli A, Ahmed K, et al. Preexposure prophylaxis for HIV infection among African women. N Engl J Med 2012; 367: 411–422.
7. Marrazzo J, Ramjee G, Nair G, et al. Pre-exposure prophylaxis for HIV in women: daily oral tenofovir, oral tenofovir/emtricitabine, or vaginal tenofovir gel in the VOICE Study (MTN 003). CROI 2013; 2013; Atlanta, GA.
8. Rees H. An Update on FACTS 001. 2013. Available at: http://www.mtnstopshiv.org/sites/default/files/attachments/REES-FACTS%20001%20update%20MTN%20Oct%202012.pdf.
9. Moench TR, O'Hanlon DE, Cone RA. Evaluation of microbicide gel adherence monitoring methods. Sex Transm Dis. 2012; 39:335–340.
10. Jacot TA, Zalenskaya I, Mauck C, et al. TSPY4 is a novel sperm-specific biomarker of semen exposure in human cervicovaginal fluids; potential use in HIV prevention and contraceptive studies. Contraception. 2013; 88:387–395.
11. Ravel J, Gajer P, Abdo Z, et al. Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A 2012; 108Suppl 1:4680–4687.
12. Katzen LL, Fernandez-Romero JA, Sarna A, et al. Validation of a dye stain assay for vaginally inserted hydroxyethylcellulose-filled microbicide applicators. Sex Transm Dis. 2011; 38:1050–1055.
13. Austin MN, Rabe LK, Hillier SL. Limitations of the dye-based method for determining vaginal applicator use in microbicide trials. Sex Transm Dis. 2009; 36:368–371.
14. Keller MJ, Buckley N, Katzen LL, et al. Use of the dye stain assay and ultraviolet light test for assessing vaginal insertion of placebo-filled applicators before and after sex. Sex Transm Dis. 2013; 40:939–943.
15. Old J, Schweers BA, Boonlayangoor PW, et al. Developmental validation of RSID-Semen: A lateral flow immunochromatographic strip test for the forensic detection of human semen. J Forensic Sci. 2012; 57:489–499.
16. Bahamondes L, Diaz J, Marchi NM, et al. Prostate-specific antigen in vaginal fluid after exposure to known amounts of semen and after condom use: comparison of self-collected and nurse-collected samples. Hum Reprod. 2008; 23:2444–2451.
17. Jamshidi R, Penman-Aguilar A, Wiener J, et al. Detection of two biological markers of intercourse: prostate-specific antigen and Y-chromosomal DNA. Contraception. 2013; 88:749–757.

Copyright © 2014 by the American Sexually Transmitted Diseases Association. Unauthorized reproduction of this article is prohibited.