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ORIGINAL PAPER

Fecal Coliform Bacterial Detection to Assess Enema Adherence in HIV Prevention Clinical Studies

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Abstract
Evaluating the efficacy of any HIV prevention strategy is dependent on ensuring and objectively monitoring adherence to the intervention. Medicated rectal enemas are a potential method for providing topical, episodic HIV prophylaxis during receptive anal intercourse. Assessing adherence to recommended enema dosing regimens is essential in evaluating the utility of this strategy. We utilized fecal coliform bacteria on used enema tips as a marker for enema use. Enema tip coliforms were tested by repurposing a microtiter plate-based water quality test designed to detect fecal contamination of water. Coliform detection occurred with 100% sensitivity and specificity when tips were assayed on day of use. The assay performed well post-7 day sample storage at room temperature, yielding a sensitivity of 80% and specificity of 93%. All (n = 64) samples collected in a subset of the DREAM-01 rectal microbicide enema clinical trial tested positive, even when tips were evaluated > 7 days post-reported use. The coliform-based enema tip assay allows monitoring of adherence in interventions involving rectal enemas in a sensitive, specific and inexpensive manner. The test performs well in clinical trial settings.

Keywords PrEP · Adherence · Enema · Topical · Validation

Introduction

HIV remains a major public health concern; current estimates indicate there are nearly 37 million individuals living worldwide with the disease, with more than 2 million new infections occurring annually [1]. Antiretroviral therapy (ART) is the primary modality for sustained viral suppression in infected individuals. Pre-exposure prophylaxis (PrEP) has also shown success for the prevention of HIV in high-risk settings [2–4]. The relative risk reduction of HIV acquisition is greater than 90% in individuals who are highly adherent to PrEP regimens. However, several social and economic factors have impeded the uptake of PrEP in at-risk populations. Issues regarding risk perception, stigma associated with PrEP use, concerns regarding potential drug-related side effects, and cost, are all factors that may impact PrEP adoption in both men and women [5–7]. While systemic prevention using a daily oral dose of antiretrovirals is the only clinically approved strategy, episodic and topical preventive interventions for sexual transmission are of pertinent interest.

One of the biggest challenges in both treatment and prevention is adherence to prescribed regimens. Thus, adherence assessment is essential for evaluation of safety and efficacy in clinical trials. There are a number of behavioral and biomedical measures of adherence. These include behavioral assessments such as self-report or audio computer-assisted self-interview, indirect measures of adherence such as pill counts and refills, and more objective approaches such as drug quantification in a variety of biological matrices [8–12]. Challenges associated with behavioral and indirect assessments of adherence are well described, and such assessments often over-report adherence in clinical trials [8, 9, 13]. While biomedical measures provide the most objective evaluation of adherence, barriers for widespread

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implementation include cost associated with drug testing, inability to assess adherence in a placebo arm, potential white coat effect (defined as improved compliance during short time intervals immediately preceding clinic visits), and interpretation of drug concentrations in the background of both oral and alternative drug delivery systems [14–16].

Topical microbicides, such as those in gel, ring, or enema formulations, have been studied or are currently being evaluated to characterize the safety, tolerability and efficacy of locally administered antiretrovirals for HIV prevention. In addition to adherence assessment methods used in oral dosing trials, vaginal microbicide trials have tested superficial biologic residue on the applicator itself to assess product adherence [17]. Several dye-based approaches have been described to evaluate vaginal applicator use via staining of vaginal cells or mucus present on used microbicidal applicators [18–21]. However, these methods are associated with limitations, and can be confounded by cells or biological secretions sticking to the applicator, an inability to clearly distinguish color changes of the indicator dye, and potential interactions of delivery device components with the assay dye [22]. More recently, in the Microbicide Trials Network ASPIRE trial (MTN-020), a randomized, double-blind, placebo-controlled clinical trial, pharmacological assessments of plasma drug concentrations, as well as remnant drug in returned intravaginal rings, were used to determine adherence to a topical study product [23]. This approach is currently being recapitulated in a number of ongoing clinical trials. However, not all delivery systems are amenable to drug measurements in returned products. Further, vaginal and rectal compartments are physiologically and histologically distinct, and results obtained from vaginal studies/products are not necessarily generalizable to the rectal compartment.

Medicated rectal enemas are a promising delivery system for topical, episodic HIV PrEP during receptive anal intercourse. The Development of Rectal Enema as Microbicide (DREAM-01) phase I clinical trial aims to evaluate the feasibility, safety, and pharmacology of a tenofovir-containing rectal enema for HIV PrEP in men who have receptive anal intercourse [24, 25]. The clinical study plan includes multiple phases of directly observed procedures to determine antiretroviral pharmacokinetics and drug distribution, interspersed with home use phases to determine acceptability. Objective adherence monitoring during home use is a vital part of interpreting feasibility and acceptability data. Collecting and evaluating drug in used, expelled enema fluid as an adherence marker is not feasible in a home use setting. A sensitive, specific, low-complexity assay was therefore required to monitor adherence to enema use regimens. We report the development, validation, and implementation of an enema adherence assay using fecal coliforms (gram-negative bacteria that are present in the stool of warm-blooded animals) present on a used enema tip as a surrogate marker for product use.

Methods

Reagents and Materials

96-well Coliplate™ test plates were purchased from Bluewater Biosciences, Inc. (ON, Canada). Enema bottles were purchased from Health Care Logistics, Inc. (Circleville, OH), sterile normal saline solution from Henry Shein® Medical (Melville, NY), and 4 mL capped vials from Corning, Inc. (Corning NY). Sterile Puritan® polyester swabs, Falcon™ 50 mL conical tubes, and sterile HPLC-grade water were acquired from Fisher Scientific (Fair Lawn, NJ). Plastic enema bottles (125 mL capacity) used for validation and in the clinical study consisted of three parts—a soft, flexible bottle, a screw-top factory lubricated enema tip, and a covering enema tip cap (Cat. No 19389, Health Care Logistics, Ohio or Cat. No. 7299, Total Pharmacy Supply, Texas). Validation enema bottles had no contents and the DREAM-01 home use enema bottles contained 125 mL normal (0.9%) or half–normal (0.45%) saline (Cat. No. 6220, Nurse Assist Inc, Texas).

Sample Collection

All samples were collected following informed consent under Johns Hopkins University School of Medicine IRB-approved protocols. Assay validation required the generation of enema tips that were rectally inserted, as well as stored and transported under controlled conditions; these specimens established performance metrics of the test in the application of identifying enema tip use. Since the marker of enema tip use is the presence of fecal coliforms, enema tips should have confirmed exposure to the anatomical site in a manner consistent with actual use in the field, and validation specimens were delivered to the laboratory in a manner designed to minimize external confounders. Accordingly, enema tips for assay validation were collected under direct observation by clinical study staff. Study volunteers inserted a lubricated enema tip (~ 4.5 cm, attached to an empty enema bottle) into the rectum for ≥ 5 s followed by tip removal. The tip was re-capped and returned to the study team, and each tip was stored capped in a sterile 50 mL conical tube until analysis. During validation studies, testing sets consisted of used enema tips interspersed with unused enema tips as controls, and assays were performed by laboratory staff blinded to sample assignment. Clinical study samples evaluated were from a phase I clinical trial (DREAM-01, NCT02750540) in which research participants were supplied normal or half-normal saline enema fluid and enema bottles for home use

prior to receptive anal intercourse. Study participants were directed to place the used enema tip (capped) in a supplied labeled 50 mL conical tube, record date of use, and mail the sample to the testing laboratory. Samples were assayed within one business day post-receipt.

Coliform Assay

The coliform assay included the Coliplate™ microtiter plate that is utilized for testing fecal coliform contamination of water samples. Manufacturer's specifications stated the lower limit of detection of the test to be 3 coliforms per 100 mL of sample [26].

Used enema tips were visually evaluated prior to assay. Tips with no visible presence of fecal matter were classified as clean (Fig. 1a). Tips with discernible discoloration were classified as used (Fig. 1b). Sample was extracted from the enema tip onto a polyester (Dacron) swab by rubbing the swab at least five times over the tip surface and the junction of the tip and the cap. The swab was transferred to a vial containing 1.6 mL sterile water. The swab shaft was trimmed to allow for vial capping, and the capped vial was vortexed briefly. Samples were incubated at 37 °C for 60 min. After incubation, the swab was removed and extracted material was equally divided into 6 wells of the Coliplate™ (~230 µL/well) microtiter plate. Plates were incubated at 37 °C for 24 h and evaluated visually.

Coliform presence was detected via the production of insoluble blue compounds via bacterial beta-galactosidase enzyme activity on the galactopyranoside X-gal substrate contained within the Coliplate™ microtiter plate. Outcomes were binary; any of the 6 wells that showed enzymatic activity through product formation was considered positive for enema tip insertion into the rectum (Fig. 2). Criteria for evaluation of a positive result were consistent with the package insert, in which a single positive well across a tested volume indicated the presence of coliform bacteria within an aggregate sample. Each assay was run with three controls—unused enema tip, a Dacron swab swiped across the testing bench, and an unused Dacron swab. Alternate rows of the plate were left blank to minimize aerosol cross-contamination.

Assay Validation

The application of the coliform assay for the detection of fecal-based bacteria on enema tips was validated through the assessment of analytical sensitivity and specificity using rectally inserted enema tips. Assay validation was performed with enema tips collected under direct observation, subject to varying storage conditions, and interspersed with negative control samples (unused enema tips in a ratio of one control for every two samples). Storage conditions tested were Day 0-RT (10 samples, 5 negative controls), 7 day-RT (30 samples, 15 negative controls) and 7 day-RT + 30-day 4 °C (10 samples, 5 negative controls, samples stored on bench for 7 days and then at 4 °C for a month). The last cohort was included to evaluate the potential for batch testing in support of clinical research trials.

Environmental contamination was also tested by swabbing hands of selected volunteers before and after enema tip use, and testing the swabs using the aforementioned assay. Interference from enema solution was assessed by collecting tips used during administration of a normal or half-normal saline enema.

Fig. 1 Clean (**a**) and (**b**) used enema tips. The used enema tip shown above yielded six of six evaluated wells positive in the Coliplate™ assay

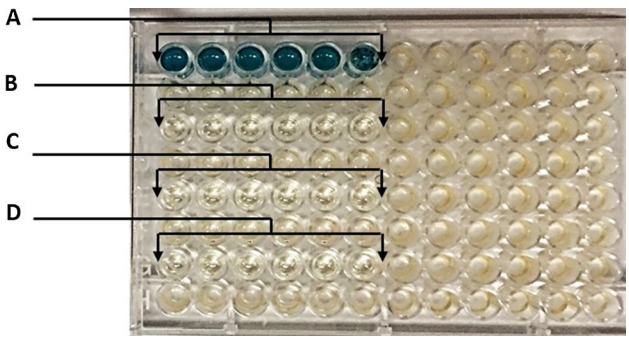

Fig. 2 Representative assay post-24 h incubation at 37 °C. Blue wells indicate beta-galactosidase activity and positive results; transparent wells indicate negative results. **a** Used enema tip sample wells; Negative control wells prepared from **b** an unused enema tip, **c** a bench-top swab, and **d** an unused swab

Analysis

Using known positive samples and controls analyzed by staff blinded to sample origin, we calculated sample stability, assay sensitivity $\left(\%, \frac{true\ positives}{true\ positives+false\ negatives} \times 100\right)$, specificity $\left(\%, \frac{true\ negatives}{true\ negatives+false\ positives} \times 100\right)$, positive predictive value $\left(\text{PPV}\%, \frac{true\ positives}{true\ positives+false\ positives} \times 100\right)$ and negative predictive value $\left(\text{NPV}\%, \frac{true\ negatives}{true\ negatives+false\ negatives} \times 100\right)$. Clinical trial samples were evaluated qualitatively to gauge performance of the assay in field conditions.

Results

Assay Optimization and Stability Assessment

Enema tips were tested on the day of collection (Day 0-RT, 10 samples), incubated for 7 days at room temperature (7 day-RT, 5 samples) or 14 days at room temperature (14 day-RT, 5 samples) prior to testing. All tips assayed on the day of collection (Day 0-RT) and stored on the lab bench for one week (Day 7-RT) tested positive (Table 1). However, only 3 of 5 samples collected under direct observation and stored for 14 days (14 day-RT, Table 1) tested positive. Five additional samples were obtained from tips after the use of saline enemas in order to characterize the effect of enema solution on the assay, and all samples tested positive (data not shown). To evaluate possible sources of contamination during applicator collection, volunteers' hands were swabbed before and after enema tip insertion (n = 5). All hand swabs remained negative, while swabs from applicators yielded positive results. Additionally, all internal negative controls—swabs of unused enema tips, swabs from the work bench, and unused swabs—tested negative in all assays.

Table 1 Coliplate™ assay qualification

	Day-0 RT	7-day RT	14-day RT
True positive	10	5	5
Tested positive	10	5	3
Tested negative	0	0	2

Day-0 RT—Store at room temperature, assay on day of collection

7-day RT—Store at room temperature, assay 7 days after collection

14-day RT—Store at room temperature, assay 14 days after collection

Assay Validation

All rectally inserted enema tips collected under directly observed observation (n = 10) tested on the day of collection (Day 0-RT) were positive, and all 5 negative controls remained negative, yielding assay performance metrics of 100% (Table 2). For 7 day-RT samples, 6 of 30 true positive samples (directly observed administration) yielded a false negative result, demonstrating 80% analytical sensitivity. One of the 15 true negative controls tested positive on the plate, yielding a 93% analytical specificity (Table 2). The positive (PPV) and negative (NPV) predictive values for enema tips analyzed following 7 days at RT were 96% and 70%, respectively. The extended storage group demonstrated 100% specificity and 60% sensitivity. The assay PPV was 100%, albeit with a small sample set.

Clinical Trial Samples

Within the DREAM-01 clinical study, a total of 70 tips were received via mail from 18 male trial participants. The median participant age was 45 years old (range 25–63); 72% of the participants were Caucasian and 28% were African

Table 2 Enema adherence assay validation

	Day-0 RT		7-day RT		7-day RT + 30-day 4 °C	
	Used	Not used	Used	Not used	Used	Not used
True	10	5	30	15	10	5
Tested positive	10	0	24	1[a]	6	0
Tested negative	0	5	6	14	4	5
Sensitivity (%)	100 (66–100)[b]		80 (61–92)		60 (27–86)	
Specificity (%)	100 (46–100)		93 (66-100)		100 (46–100)	
PPV (%)	100 (66–100)		96 (78–100)		100 (52–100)	
NPV (%)	100 (46–100)		71 (46–87)		56 (23–85)	

Day-0 RT—Store at room temperature, assay on day of collection

7-day RT—Store at room temperature, assay 7 days after collection

7-dayRT + 30-day 4 °C—Store 7 days at room temperature, then 30 days at 4 °C before assay

[a]Likely due to aerosol cross-contamination

[b]Denotes 95% confidence interval

American. The tips were visually evaluated as clean or used, and tested for coliforms in the laboratory. Using the coliform assay, samples were assayed within one business day of receipt in the laboratory. All wells from all clinical trial samples tested positive in the coliform assay, suggestive of beta-galactosidase activity and the presence of coliform bacteria extracted from the enema tip. From the DREAM-01 clinical trial, 64 tips had an identifiable use date and were included in performance analysis. The time interval between use and assay performance was 9 ± 5 days (mean $\pm$ SD), with a range of 2–41 days. Twenty-one tips were tested in ≤ 7 days post-use. Eighteen of 21 tips were visibly used while 3 appeared clean (Fig. 1). All 21 tips tested positive in the assay. Forty-three used tips took > 7 days to arrive to the laboratory; 17 tips (40%) appeared clean on visual examination. All 43 tips tested outside the established 7 day assay validation period, including tips that appeared clean, tested positive in the assay. One returned tip that appeared clean tested positive after a 41 day use-to-assay interval.

Discussion

A fecal coliform assay has been repurposed to effectively evaluate enema adherence in a clinical trial setting. Our initial qualification revealed that used enema tips could be stored at room temperature for up to 7 days and yield positive results in the plate-based assay, with 100% sensitivity and specificity. Presence of saline solution on the tip pursuant to enema use did not affect the assay performance, indicating that enema solution does not interfere with the assay. Efficacy of the assay on used enema tips even after a period of room temperature storage suggests that the assay may be implemented in field conditions where used samples are shipped to a central testing laboratory for analysis. Storage beyond 7 days led to decreased sensitivity (60%). However, the assay remained precise and did not yield false positive results (100% specificity). Based on these data, enema tips should preferably be tested within 1 week of use. If tips are tested greater than 7 days post-use, negative results should be interpreted with caution.

Adherence monitoring is critical in evaluating the success of an HIV PrEP regimen. The use of rectal enemas to deliver episodic, on demand, HIV PrEP in a behaviorally-congruent manner may reduce the risk of non-adherence [24]. However, an objective measure for the use of rectal enemas is vital for evaluating the interventional efficacy. Due to challenges with collecting remnant enema fluid in real world settings for the quantification of study compound or detection of coliform bacteria, feasible alternatives to confirm product use are required. The described use of measuring a biological marker on an enema applicator tip complements previous strategies in which remnant vaginal gel applicators were evaluated for product use [23]. We initially pursued the detection of fecal bile pigments, such as bilirubin, as a marker of enema product use, but this approach proved unsuccessful, due to both challenges regarding material extraction from the enema tip as well as achieving required analytical sensitivity (data not shown). Additionally, commercial assays for stercobilin (a bilirubin degradation product found in stool) quantification are largely unavailable.

Due to the inferior sensitivity associated with fecal bilirubin identification as an indicator of product adherence, testing for the presence of a biological marker using a catalytic modality was pursued. Gram-negative coliforms are abundant in the feces of warm-blooded animals and are in widespread use as a marker for fecal contamination of water sources [27]. The presence of fecal coliform bacteria on the enema-applicator tip suggests that the tip encountered a fecal environment and serves as a surrogate of enema product use. Assessment of fecal coliforms in water samples relies on conversion of a colorless substrate to a colored product via beta-galactosidase enzymes present in the bacteria. The Coliplate™ assay is a microtiter plate format coliform assay for monitoring fecal contamination of water bodies. It is a specific and sensitive assay, providing a visual output that can be performed on small volume samples in resource-limited settings [26, 28].

We assessed the suitability of the assay in real world conditions by testing the assay on enema tips used in a take-home phase of the clinical trial evaluating rectal enemas for topical HIV PrEP, where study volunteers mailed used enema tips back to the lab for assessment [25]. Although instructions directed study participants to return used enema tips immediately after use, we found a surprisingly large variation in the time taken by volunteers to return samples to the laboratory. This led to an average interval between date of use and assay of 9 days, with a range of 2–41 days. Only 21 tips were returned to the laboratory within the validated 7 day interval highlighting challenges faced in operating in a real world setting. Significantly, 20 of 64 (31%) evaluable tips appeared clean and volunteers would have been classified as non-adherent based on visual examination. The Coliplate™ test successfully detected coliforms on all tips, indicating 100% adherence in this clinical trial. Additionally, all tips were positive in the assay, irrespective of return date or appearance of use. Notably, a tip tested 41 days after the date of use that appeared clean, and thus would have been determined as unused based on visual inspection, was positive by the coliform test. Although the sample was tested outside of the validated stability window, this observation demonstrated that the assay performs well in the field where assays times cannot be controlled as rigorously as in the laboratory setting.

However, this study does have limitations. One caveat of this strategy is that it indirectly equates presence of

coliforms on the enema tip with tip insertion into the rectum and product use. Thus, the test is falsifiable by inserting the tip into the anus, or even contact with the anal area, without product use. The sensitivity of the bacterial detection method also makes false positives due to environmental contamination a possibility. While we saw no evidence of cross-contamination (from hands and/or transport conditions and/or contact with other surfaces) in our studies, our tests were performed in a controlled clean clinical/laboratory setting and do not capture the complexities of field conditions. An additional limitation of the assay is the narrow stability windows determined during assay validation, and PPV and NPV < 100% for samples stored at 7 days at RT. However, a PPV of 96% demonstrates that a positive coliform test result is a strong indicator of enema tip insertion into the rectum. Field samples resulting negative results when tested beyond the validated 7 day post-collection interval should be interpreted with caution as they may provide an underestimate of actual adherence in the clinical trial. Finally, the assay has limited utility when the enema tip has overt fecal matter present, since a visual examination suffices to determine use. However, 30% of the enema tips tested in the clinical trial appeared visually clean; volunteers would have been misclassified as non-adherent. The coliform assay described herein provides an invaluable objective metric in such cases when use is not visually apparent.

In summary, we describe a test to monitor adherence for the use of medicated rectal enemas based on a fecal coliform presence on the enema tip. The assay is sensitive, specific, and inexpensive, costing $8/sample when analyzed in batch. It may be performed with minimal equipment and appears remarkably robust when applied to clinical trial samples. The described assay will continue to be implemented in the DREAM clinical trials, and can be used in determining tenofovir pharmacology in the background of at-home product use.

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Compliance with Ethical Standards

Conflict of interest CWH has research contracts with ViiV/GSK and the Bill and Melinda Gates Foundation. WSA, RPB JB, JEJ, EW, EJF, and MAM declare no conflicts of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Within this study, all samples were collected following informed consent under Johns Hopkins University School of Medicine IRB-approved protocols.

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