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Trypan Blue Staining to Determine Vaginal Exposure in Two Types of Plastic Vaginal Applicators Containing Two Different Microbicide Formulations

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Abstract

Dye staining of applicators has been shown to be a reliable and objective method to test vaginal insertion in clinical microbicide trials, but different plastics, dyes and product formulations may impact the accuracy of this method. Reportedly used applicators returned from three clinical trials were stained with 1% Trypan Blue. In a phase 1 study (VivaGel®), using gel-filled HTI polypropylene applicators, 1271 (97%) of applicators stained positive. In two phase 1 and 2a studies (LACTIN-V) using linear low-density polyethylene applicators to deliver a dry powder formulation, 57 (95%) and 135 (86%) tested positive, respectively. Dye staining of vaginal applicators is an objective, low cost measure suitable for low resource settings.

Keywords

dye based staining; vaginal applicators; Trypan Blue; Microbicides; VivaGel; *Lactobacillus crispatus* CTV-05

Several clinical trials failed to demonstrate efficacy of microbicides in reducing HIV acquisition. Lack of protocol adherence and product use may have contributed to these disappointing results.[1–3] Information is usually obtained in face-to-face interviews, participant-kept diaries, and Audio Computer-Assisted Self Interviews (ACASI)—all of which can overestimate adherence and product use.[4] Objective methods to measure product use are essential in the determination of product efficacy. Several studies have employed Trypan Blue[157] and FD&C Blue No. 1[1689] staining techniques to detect vaginal mucus as a proxy for vaginal insertion of applicators and product use.

Previous studies have evaluated vaginal staining of low-density polyethylene (LDPE) Microlax-like applicators[1569] and HTI Plastics polypropylene applicators.[78] Different

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plastics exhibit different surface qualities, which may influence the retention of vaginal mucus in a distinct streaking pattern. Dye staining reached high accuracy for the identification of vaginal mucus on gel-filled polypropylene HTI applicators[7] as well for gel-filled LDPE Microlax applicators.[1569] However, Austin *et al.* questioned the sensitivity of FD&C Blue No. 1 for HTI Plastics polypropylene applicators--only 81–95% of used applicators, and 86–93% of unused were correctly identified--when compared against Gram stained smears from applicators correctly identifying 99% of used applicators.[8] No information exists for the suitability of dye staining of applicators filled with non-gel formulations, and for a third type of plastic–the linear low-density polyethylene (Linear LDPE)– which differs structurally from conventional LDPE with its narrower molecular weight distribution and significantly different rheological properties.[10]

We analyzed Trypan Blue staining results of applicators reported as vaginally inserted during three clinical trials—using two types of plastics (Linear LDPE and HTI polypropylene) and two product formulations (Carbopol-based gel and powdered live biotherapeutic product). The Phase 1 VivaGel® trial (NCT003311032) used HTI polypropylene vaginal applicators. It was a placebo-controlled, double-blinded, multisite trial in San Francisco (USA) and Kisumu (Kenya), evaluating the safety and tolerability of SPL7013 3% w/w gel (VivaGel®)[11] administered twice daily for two weeks in 54 healthy women. The Phase 1 LACTIN-V trial (NCT00537576)[12] and Phase 2a LACTIN-V trial (NCT00635622)[13] used Linear LDPE applicators in placebo-controlled, double-blind trials in San Francisco (USA) evaluating the safety and colonization efficiency of *Lactobacillus crispatus* CTV-05 for prevention of bacterial vaginosis (BV) administered to 12 healthy women and 24 women with BV, over 2 and 4 weeks, respectively. Details for the three studies are published elsewhere.[111213]

All three trials used identical procedures. At enrollment, the first applicator was always administered in the presence of a clinician, then inserted into the original plastic pouch, labeled and stored on-site in order to serve as the positive control. Participants were provided with the remaining applicators, and were instructed to not rinse them after insertion, to repackage each applicator in its original plastic pouch, to place them in a sealable bag, store at room temperature, and to return them at each follow-up visit. Applicators were tested at their respective sites in Kisumu and San Francisco. Each participant's applicators were tested in one batch at her last study visit, along with her positive control and an unused, empty applicator kept on-site as a negative control. Consequently, even the first applicator used was stored for a maximum of 2–4 weeks, depending on the duration of enrollment in the respective studies. Following standard operating procedures, negative and positive control applicators, and all reportedly used applicators were individually sprayed with a 1.0% Trypan Blue solution in normal saline for 5 seconds, rinsed with tap water for another 5 seconds, placed on a rack, dried overnight, and examined the following day. The same trained clinical staff member read all applicators at the Kisumu site, a second one all applicators for the three trials at the San Francisco site. Applicators with a streaking along the applicator barrel, which is characteristic for the highly viscous vaginal mucus, were considered used (Figure 1). Applicators from the LACTIN-V trials with occasional, clearly distinguishable blue colored powder remnants just on the tip, but without streaking along the barrel, were considered unused. Because applicators filled with placebo or active product contained the same carrier substance (Carbopol gel in the VivaGel® study, preservation matrix in the LACTIN-V trials), no separate analysis comparing staining results between placebo and active arms was performed.

At the Kisumu VivaGel site, all 885 distributed HTI polypropylene applicators (100%) were reported as used, and all were returned emptied at subsequent study visits. At the San

Francisco VivaGel site, 423 of the 440 distributed applicators (96.1%) were returned emptied and reportedly used. During the Phase 1 LACTIN-V study using Linear LDPE applicators, all 60 distributed applicators were returned emptied and reported as used. For the Phase 2a LACTIN-V study, 157 of 168 applicators (93.5%) were returned emptied and reportedly used.

For both the HTI polypropylene applicators and the Linear LDPE applicators, all positive controls stained positive and all negative controls stained negative. Of the 1308 reportedly used HTI polypropylene applicators at the two VivaGel sites combined, 1271 applicators stained positive (97%). The 37 negative applicators originated from 12 participants, most of who failed to use 1 or 2 applicators properly (of 28 applicator doses each), but two participants from the Kisumu site misrepresented 9 and 13 non-inserted but emptied applicators as vaginally used. Fifty seven of the 60 emptied and reportedly used Linear LDPE applicators (95%) in the Phase 1 study of LACTIN-V trial stained positive, and three applicators from three participants stained negative. Of the 157 reportedly used Linear LDPE applicators in the Phase 2a study of LACTIN-V, 135 stained positive (86%). The 22 negative applicators originated from 9 different participants, three of who each reported 5 or 6 non-inserted emptied applicators as used (of 7 total doses).

Our results support the use of Trypan Blue staining as an objective, cost-effective and sufficiently accurate method to detect vaginal exposure of gel-filled HTI polypropylene applicators as well as the previously untested Linear LDPE applicators filled with a powdered product. The 1,308 reportedly used HTI polypropylene applicators from the VivaGel study tested constitute a sample size significantly larger than tested in previous studies[8], while the 217 returned applicators from the LACTIN-V studies provided data on previously untested Linear LDPE applicators and on non-gel formulations. Following evidence that previously established Trypan Blue as an accurate indicator of vaginal exposure[1579] for two plastics, this methodology was primarily used to verify participant reported product use in clinical trial settings, not to gain data on the accuracy of applicator staining. Other study designs, which compare staining results to a gold standard, are preferable for this purpose.[568]

The lower proportion of positively stained applicators (86%) for the Phase 2a LACTIN-V study is likely due to lower actual than reported product use, and not a failure of the staining method. This assumption is based on higher rates of observed non-adherence for other protocol-specified requirements, such as failing to return used applicators, missed follow-up visits, and self-reported sex during periods of requested abstinence. Participants reporting less product use also showed less vaginal colonization with *L. crispatus* CTV-05.[13] Additionally, the majority of misrepresented vaginal applicator use (16 of 22 negative applicators) originated from only three Phase 2a LACTIN-V participants who, based on the staining results, presumably routinely emptied applicators extra-vaginally and used only 1 or 2 doses of 7. Similarly, only two of the 36 VivaGel participants accounted for 22 of the 37 negative applicators. While 15–25% of participants per study misrepresented vaginal use, most of these 24 participants did so for 1–2 applicators, and only five of the total 90 study participants routinely averted product use.

While Austin suggested that more frequently applied amounts of the viscous gel may interfere with the formation of the characteristic mucus streaking on the smooth applicator surface[8], the VivaGel data does not support this hypothesis. After application of 3.5 grams of gel twice daily, 97% of reportedly used applicators stained positive when using Trypan Blue on similar HTI polypropylene applicators.

Participants were advised to repackage used applicators in the individual wrappers and then place in a storage bag containing other wrapped used applicators. In theory, loosely rewrapped used applicators could contaminate uninserted applicators during storage. However, such contamination, as well as other caused by rubbing against skin or exposure to saliva (of lower viscosity than vaginal mucus), cannot mimic the characteristic streaking pattern of mucus along the barrel of the applicator (Figure 1). Moreover, Wallace *et al.* found that even if unwrapped inserted and non-inserted applicators were bagged together, the accuracy for predicting use and non-use was 97% and 96%, respectively.[6]

A strength of this study is the inclusion of known negative and positive control applicators for each participant. Limitations are the lack of a second independent reviewer, and the fact that staining results were anticipated to be largely positive in a clinical trial setting. Other specifically designed studies compared the accuracy of staining methods to other standards like Gram staining[8] or optical density measurement of lactobacilli growth[5], which detect vaginal cells and reach slightly higher accuracy levels of close to 100%, but require more training and resources than dye staining.[5678] Although the reviewer knew the negative and positive controls, observation bias is not likely to have been significant, as the reviewer at each site was well trained, followed a standard procedure, and the difference between positive and negative applicators was readily apparent (Figure 1). Other studies validating this technique on different applicator types have found low rates of inter-observer variability[5, 6, 79], further allaying concerns about the potential for significant observation bias.

While dye-based assays may not offer a level of sophistication that currently developed “smart” applicators with embedded microchips or biomarkers for product tracing could provide,[1415] staining with Trypan Blue of HTI polypropylene and Linear LDPE applicators, as well as the previously tested conventional LDPE Microlax applicators provide an adequate, low cost, reproducible and easy-to-train method to verify self-reported vaginal insertion in clinical trial settings.

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Figure 1. Applicators stained with 1% Trypan Blue

To the left, stained applicator shows characteristic streaking of vaginal mucous as seen after vaginal insertion. To the right, applicator shows no signs of stained vaginal mucous.