REVIEW ARTICLE

Methodological Issues in Sampling the Local Immune System of the Female Genital Tract in the Context of HIV Prevention Trials

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The spread of HIV continues unabated in the most vulnerable populations of the world. HIV prevention methods, such as a vaginal microbicide, a mucosal vaccine, pre-exposure prophylaxis or a vaccine, are urgently needed in the fight against new infections. We must make a commitment to supporting innovative research and product design, so that one or more of these products provide a halt to the spread of HIV. Above all, these products should be proven to be safe and not negatively disturb the local immune system in a way that facilitates or enhances heterosexual transmission of HIV. HIV specific and non specific cellular and humoral local vaginal immunity must be assessed in clinical trials when testing prevention products for safety or efficacy. A proven, well-documented and standardized sampling strategy will provide high quality data to be able to assess both safety and local immune responses. In this paper, we will discuss methods for vaginal immunology sampling in the context of clinical trials.

Introduction

The vaginal mucosal immunity is of paramount importance for the heterosexual transmission of HIV.[1,2] The healthy vaginal environment is colonized by *Lactobacillae species* that produce lactic acid and hydrogen peroxide, making the vaginal milieu acidic and resistant to many pathogens, including HIV.[3] Together, this beneficial flora, the epithelial lining, and mucosal immunity create an effective barrier. It is when this microenvironment is disturbed that the potential for infection occurs. Ulcerative and non-ulcerative pathogens that infect the vagina have been shown to affect the local immunity in several ways and have been linked to increased acquisition of HIV.[4,5]

Almost 34 million people are living with HIV of which 67% are situated in sub-Saharan Africa.[6] In this heavily affected area an estimated 2 million people were newly infected with HIV in 2008. Women and girls continue to be disproportionally affected, accounting for 60% of all new HIV infections, and young women between the ages of 15 and 19 are particularly vulnerable to HIV – they are three times more likely to become infected than their male counterparts.

A topical vaginal microbicide preventing the HIV virus from establishing an infection through the female genital tract could be live saving for young women and other women at risk. With the recent evidence from the Caprisa004 trial showing a 39% reduction in HIV incidence among those using 1% tenofovir gel,[7,8] we urgently need to strengthen and broaden the vaginal HIV prevention research by designing and developing more user-friendly formulations (such as vaginal rings) and more effective

products, including the design of new chemicals that are not used for the treatment of HIV, thereby limiting the spread of resistance to drugs that are part of critical combination treatments. Researchers from the Europrise consortium, representing 14 projects funded by the European Commission, are now developing combined antiretroviral vaginal gel products, mucosal vaccines, and vaginal ring devices. Each of these new products will need to prove that they are safe and efficacious through development pathway steps. Safety trials should be designed with the utmost care and specifically assess products for maintenance of healthy vaginal ecology and local mucosal immunity. Similarly, oral pre-exposure prophylaxis (PrEP) or an HIV vaccine, applied intramuscularly, nasally, subcutaneously or through any route should not negatively affect the local vaginal milieu.

Of equal importance is the assessment of the presence or absence of protective humoral and cellular immunity in response to a vaccine whatever the route of application. The cellular immunity (HIV-specific CD8 + T cells) induced by the MRKAd5 HIV-1 gag/pol/nef vaccine in the Step trial did not provide protection from HIV. In this trial, an opportunity was missed to evaluate the local mucosal immune responses to gain insight in the vaccine's failure.[9,10]

The best way to assess safety and immune responses to products is by sampling the vaginal milieu; studying the local immune system before, during and after use of the products. A proven, well-documented and standardized sampling strategy will provide high quality data to be able to assess both safety and local immune response. The focus of this review is to critically assess the methods used for vaginal sampling in the context of clinical trials for vaginal products, and to highlight areas that need further exploration.

Sampling the local vaginal immunity in clinical trials

At present, a wide range of clinical methods for sampling is used and new methods are being explored. The genital tract should be sampled from proximal to distal in order to obtain 'clean' samples from the different segments of the genital tract: first the external genitalia, then vaginal fluid, lavage, cervical sample, endocervical sample, and finally biopsies from the vagina, cervix, endocervical canal and endometrium.

The Collection of Soluble Factors

Samples for soluble factors (e.g. cytokines) can be recovered undiluted or diluted. Diluted samples are obtained by washing the vaginal tract in a cervicovaginal lavage (CVL). Samples can be diluted with normal saline (pH range from 4.5 to 5.5) or by phosphate-buffered saline (PBS, pH 7.4). Depending on volume of samples needed for testing, researchers have used 3, 5 and 10 mL washes; however, each volume will result in different recovered volume depending on clinician technique and secretions already in the vaginal vault (i.e. vaginal discharge) (see below, 'Issues with measuring soluble factors'). Saline is favored over PBS in field settings to avoid the extra step to prepare PBS and 10 mL has been used mostly in clinical trials. Undiluted specimens are recovered by swabs, sponges (Weck-Cell), wicks, spears and brushes by a clinician.[11,12] If a sample is obtained undiluted, an optional dilution step can be added to extract material from sampling devices or to increase the final volume.

Both undiluted (swab) and diluted samples can be self-collected by the participant. Though clinician sampling has the advantage of being standardized, the development of new devices for self-collection is ongoing with an aim to improve participant acceptability as well as sample between clinic visits (samples can be dropped off, or returned by post to a centralized laboratory).[13,14] Examples of undiluted self-sampling methods include a vaginal cup, an aspirator or a swab. Lavages, with new self-sampling devices, have also been tested in clinical trial settings.[15,16]

Many soluble factors (e.g. inflammatory cytokines) have short half-lives and will break down quickly. It is important that samples are put immediately into cool boxes and stored at −80°C as soon as possible. Also, it may be necessary to add a protease inhibitor cocktail to inhibit the breakdown of these proteins. Samples must be shipped to a central laboratory on dry ice. In addition, blood will also be an alternate source of soluble factors, and blood contamination by sampling trauma or menstruation must be recorded and the results taken into account for the analysis. Hemastix® can be used to measure blood in CVLs prior to centrifuge.

The Collection of Cells

Antigen-presenting cells and T lymphocytes are useful for assessing vaginal cellular immunity. Cervical

or vaginal cells can be obtained, surface antigens stained and then tested by flow cytometry.[17] In research settings, these cells are mostly isolated with brushes, but other methods such as endocervical aspiration, a cell pellet from a lavage, a scraping of the cervix, and endocervical swabs have been used to obtain cells. In addition, biopsies are useful for investigating several cell layers; however, the invasive character of a biopsy makes it often not acceptable in a clinical trial setting when a large number of participants are enrolled or in at risk populations where causing a breach in the vaginal barrier could increase risk of HIV transmission.

The Collections of Samples for PK/PD Testing

Classical pharmacokinetic (PK) and pharmacodynamic (PD) studies measuring local concentrations of drugs are performed for locally applied microbicides, such as those containing non-nucleoside reverse transcriptase inhibitors or antibodies in gels and rings.[18,19] The PK/PD studies complete dose titration studies aimed to select rational dosage regimens. Drug levels are measured on undiluted samples, diluted samples, in tissue and on individual cells. For the measurement of intracellular levels good quality standardized cell samples are required.

Finally, cervical and rectal biopsies are used to determine anti-HIV activity of microbicides in explant models.[20] As yet, samples from clinical trials have not been used for this.

Measuring the local vaginal immunity in clinical trials

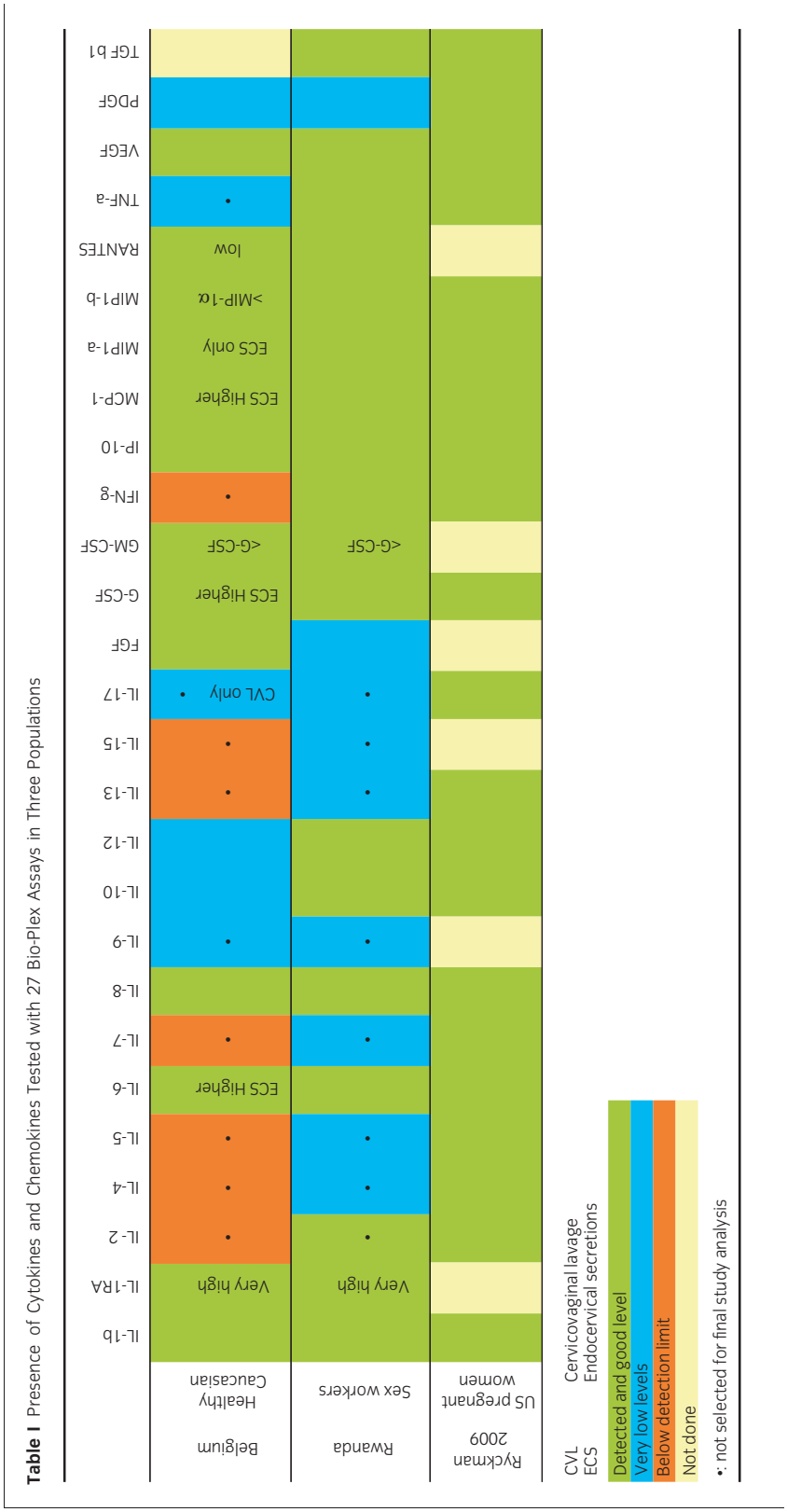
Issues with Measuring Soluble Factors

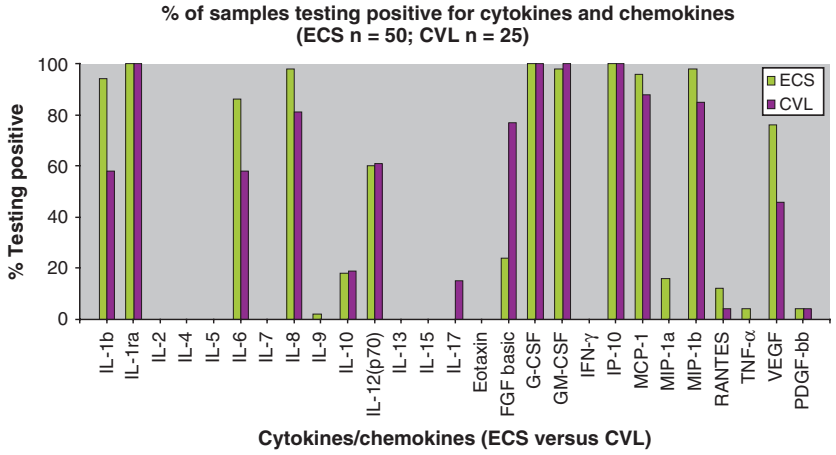
Quantification of soluble mucosal immune factors and HIV specific responses is possible in undiluted samples and samples diluted in a standard volume. In contrast, the dilution effect of a CVL interferes with the exact quantification and values are usually expressed as percentages. For example, a CVL performed with 10 mL saline results in a diluted sample volume ranging from 9.7 to 10.1 mL. Therefore it is important to be able to quantify the volume of cervicovaginal secretions collected and accurately approximate the dilution factor of a soluble component introduced by the washing. Lithium chloride, an inert substance, can be used to measure the dilution factor when added to CVL[21]; however, the analysis can be cumbersome and requires the use of flame atomic absorption spectrophotometer.[11] Alternatively, one could measure total protein or IgA.[11] Furthermore, the collection of the same type of samples at multiple time points in clinical trials allows for comparisons of soluble markers within the same individual.

The mean volumes collected with undiluted sampling methods are often small; Weck-Cell 50 μL, swab 200 μL, vaginal cup 500 μL, aspirator 500 μL. This disadvantage has to be taken into account when designing the objectives of a trial and the trials laboratory assays to measure the endpoints. From the start of protocol discussions, a team of clinicians, epidemiologists and laboratory scientists should agree on sampling methodology linked to laboratory assays and study the volumes needed for each assay. If the recovered volumes are thought to be insufficient, alternatives will need to be explored. For example, one could take multiple samples and pool them, dilute the sample or suspend the sample device in a standard volume, or perform a CVL.

The multiplex cytokine assays were not originally validated for genital tract secretions; nevertheless, performance and experience with the multiplex is mounting and standardization efforts are ongoing.[22] The multiplex kits can be custom made to fit the panel of cytokines selected for any study design. In preparation for the EMPRO Flora Study, a study investigating vaginal flora and mucosal characteristics in 28 young healthy Belgian women not using hormonal contraceptives, we performed a 27 Bio-Plex multiplex test run on samples from this same population to make a final selection for a multiplex panel (unpublished observation). We compared our results to a female sex worker study population in Kigali, Rwanda (unpublished observation) and with the results from Ryckman et al.[23] in pregnant women in the US. Table I illustrates the differences in cytokine and chemokine detection between the three populations. A number of cytokines were below the detection limit for the Belgian population compared to low level in the Rwandan and US samples. In addition to the aim of selecting a panel of cytokines for the multiplex, we explored the presence or absence of soluble factors in endocervical secretions (ECS) (dilution with 1 mL PBS) compared to CVL (10 mL saline). No major differences between ECS and CVL samples were seen except that MIP-1a was not detected in the CVL and a few factors were

Table I Presence of Cytokines and Chemokines Tested with 27 Bio-Plex Assays in Three Populations





ECS: endocervical secretions
CVL: cervicovaginal secretions

Fig. 1 Comparison of percentage of samples testing positive for cytokines with the 27 Bio-Plex assays for ECS versus lavage.

present in a slightly higher concentration in the ECS than in the CVL samples (Fig. 1).

In the next few years, European researchers aim to standardize a list of soluble factors to be measured in future clinical trials carried out by European researchers and collaborators. Newly defined HIV protective factors in the literature, such as Trappin-2/Elafin, MIP3-α, IFN-β and Beta defensins, have not yet been included in multiplex assays. It may be worth considering incorporating these factors in clinical trials, though laboratory work is more labor intensive and therefore more expensive. The antiviral activity of MIP3-α has been recognized by several authors and can be an interesting marker to study antiviral activity of the upper reproductive tract as opposed to the lower genital tract because of absence of production for vaginal cells *in vitro*.[24] Finally, IFN-β increases through toll like receptor signaling and this leads to an antiviral state for *Herpes simplex virus* (HSV)-2, an important factor for HIV transmission.[25]

Care should also be taken that a specimen is representative of the area sampled. If certain anatomical areas are expected to give different results then these should all be sampled. For example, vaginal fluid accumulates in the posterior fornix of the vagina, and samples from the posterior fornix may give different results than samples obtained from the lateral vaginal wall. Samples from different anatomical areas could either be pooled or could be assayed separately, depending on the research questions.[26]

Issues in Measuring Cellular Composition of Cervical Mononuclear Cells

Several technical challenges have impeded the uptake, performance and interpretation of cell-mediated immunity research of the female genital mucosa.

Table II Percent of Cell Yield After Regression Analysis

		Coefficient	Change in % yield	*P*-value*
Variable				
Sampler	Swab	–	–	–
	Brush	−0.229 (−0.400; −0.059)	−41	0.008
Cycle	1	–	–	–
	2	−0.002 (−0.170; 0.166)	−0.05	0.981
Day of cycle	Day 9	–	–	–
	Day 23	0.201 (0.010; 0.393)	+59	0.039
Ectopy	Absent	–	–	–
	Present	0.642 (4.922; 5.618)	+338	0.003

*Adjusted for dependence of observations for each women.

Table III Presence of Blood After Regression Analysis

		Odds ratio	*P*-value*
Variable			
Sampler	Swab	–	–
	Brush	2.22 (1.22; 4.05)	0.009
Cycle	1	–	–
	2	1.13 (0.62; 2.07)	0.688
Day of cycle	Day 9	–	–
	Day 23	1.05 (0.47; 2.34)	0.898

*Adjusted for dependence of observations for each women.

The biggest challenge has been the difficulty in collecting a sufficient number of viable cells. But also contamination with red blood cells (RBCs) and the absence of standardization of collection method.[27] In addition, the complexity of setting up flow cytometry or accessibility to liquid nitrogen facilities for shipping in remote, resource poor settings is particularly difficult. With the availability of the multicolor flow cytometry, the number of cells needed for each experiment has been reduced to just over 200,000 live cells. In preparation for the EMPRO Flora Study, we carried out a pilot study to investigate different sampling methods in relation to cell yield comparing a brush and a synthetic swab. A fine brush, originally designed for cytology, collected cells effectively but yielded a low count of cells and RBC contamination was high. We hypothesized that a synthetic flocked swab could be less disruptive and an L-shape possibly better at absorbing and releasing cells especially in the case of ectopy, than a brush. We then carried out a comparison study between two synthetic swabs (Copan, MicroRheologics S.R.L., Brescia, Italy) and two brushes (Cellpath® 9 mm ø) in a randomized crossover design over two menstrual cycles with samples taken on day 9 and day 23 (window of 3 days). The endocervical samples were placed in cell medium (PBS, penicillin/streptomycin, L-glutamine, Fetal Calf serum) on ice immediately after collection. Cells were counted in a Neubauer chamber by one ad the same observer within one hour after trypan-blue staining to identify leukocytes that were alive. The supernatant was tested for blood (free hemoglobin and RBC) and leukocyte esterase with a urine dipstick (Servotest®5 + NL, Wesel, Germany). One hundred and twelve samples were collected and the median cell value was 0.31×10^6 (mean of 1.5×10^6). The synthetic swab had a significantly higher yield of cells with an increase of 69% compared to the brush (Table II). Ectopy increased cell yield significantly resulting in a threefold increase and more. There was a borderline significant increase in yield for day 23 compared to day 9 of the cycle. Blood was significantly more present with the use of a brush compared to the swab (Table III).

Another critical factor affecting viability of cells is the freezing process at the sample collection site and during shipment of the samples to the central laboratory.[27] A considerable percentage of live cells will not survive the freeze-thaw cycle even when all steps are performed in optimal conditions. Currently, cells are treated with dimethyl sulfoxide (DMSO) before they are frozen with liquid nitrogen. DMSO is known to be toxic to cells at room temperature and lab staff must be careful not to expose cell samples for any longer than necessary.[28] Besides the liquid nitrogen freeze procedures, cell cryopreservation media exist for immediate storage at −80°C for up to three months. Examples of these media are CELLBANKER 1/2 (contains DMSO) or EmbryoMax®.[29] This obviously opens possibilities for setting up multi-site or even multi-country clinical trials in the field and batch samples for shipment and analysis; however, it remains to be evaluated how well cells survive when preserved with these new media compared to the traditional DMSO freezing methods.

Finally, efforts for standardization of flow cytometry of endocervical and biopsy samples are ongoing at an international level supported by NIH (Florian Hladik, personal communication).[30] Next to the standardization element, this NIH GU mucosal immunology working group will study number and functional phenotypes of cells obtained from non-invasive specimens with brushes and see if they are equivalent to those isolated from biopsies. This will then inform the field to what extent endocervical sampling is representative for the local cellular immunology. Good results have been obtained in measuring total cell number and their phenotype but it remains to be seen if cell yield is sufficiently high enough to follow antigen-specific responses to an HIV vaccine.

Confounders

Ulcerative and non-ulcerative pathogens that infect the vagina have been shown to affect the local immunity.[31,32] The presence of these pathogens may confound the relationship between an experimental medication or vaccine and the local immune

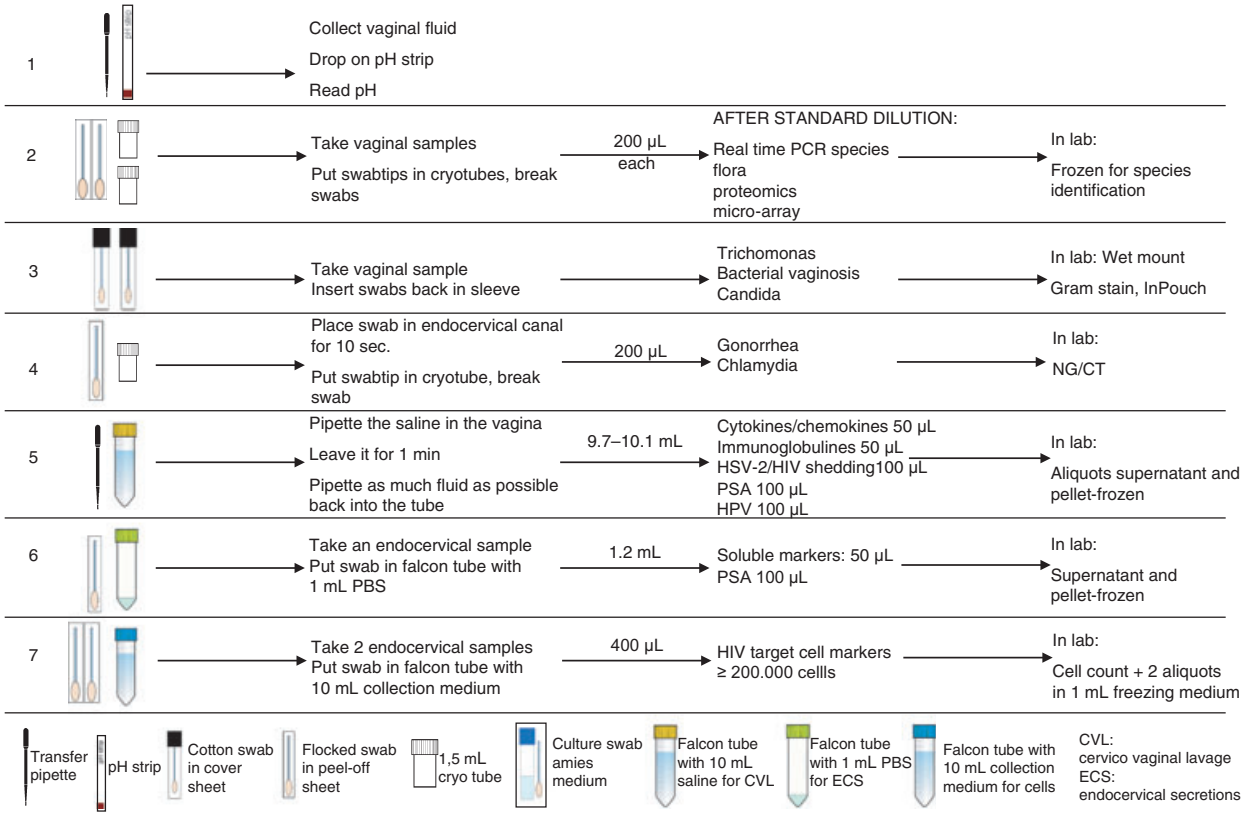


Fig. 2 Example of a sample scheme evaluating vaginal mucosal immunology and local confounding factors.

response. Therefore it is important to test for sexually transmitted infections such as *C. trachomatis, N. gonorrhea, T. vaginalis*, *Human papilloma virus* as well as shedding for *HSV*. In addition, serum samples should be taken to test for active syphilis, HIV and HSV.

Menstrual cycle phase have been shown to be a powerful determinant for levels of cytokines and numbers of immune cells.[24,33] Serum or urine samples for endogenous hormones should be collected and menstrual phase applied to the analysis of results. Finally, vitamin D is an important immune modulator, can be tested in serum and considered as a confounder.[34]

An example of a comprehensive sampling scheme

Planning a sampling strategy for a clinical trial requires balancing of study objectives and endpoints, participant acceptability, available infrastructure and study budget.

Extra samples may be needed to account for local confounding factors as mentioned above, other vaginal infections (yeast, bacterial vaginosis) and to perform diagnostic tests (Amsel versus Nugent, InPouch versus wet mount) for the participant.

The use of antiretroviral drugs for the prevention of heterosexual transmission introduced concerns about the development of HIV drug resistance and has heightened the interest in product PK/PD. As a result cervical, endocervical and uterine biopsies are now more commonly collected in Phase I safety studies.

Figure 2 proposes a basic set of samples to study mucosal immunology and confounding factors without additional biopsies. The figure incorporates sampling methods, volumes of samples, markers, storage issues and volumes for assays.

Conclusions

The sampling strategy is specific to each individual trial, since each trial has its own objectives and endpoints. Nevertheless, we believe that it is feasible and desirable to establish standardized sampling methods in conjunction with standardized assays, so

that results are comparable between trials. Europrise and other researchers are making efforts to improve standardization for sampling but also for assays for clinical trials in the field of microbicide and vaccine research (http://www.europrise.org/ see WP7 section). Europrise has fostered the networking between partners and those partners interested in vaginal mucosal immunology have joined forces. This will most likely lead to new collaborative research in this area.

While measurement of mucosal immune responses in the context of HIV prevention trials has increased in recent years, and standardization efforts have been initiated, much more work remains to be done. First, the vaginal micro-environment (vaginal microbiota and mucosal immune responses) needs to be described in much more detail, and in more populations, to enable establishment of normative ranges of a wide variety of immune response factors, to which clinical trial results can be compared. Furthermore, in the context of microbicide trials, biomarkers of microbicide safety and efficacy should be identified, and those parameters should be measured in future trials using standardized sampling techniques and standardized assays.

In the current generation of microbicides containing antiretroviral drugs, the balance between the local effective concentration and systemic levels is very important in the context of development of HIV drug resistance. PK/PD data from Caprisa004 and future microbicide and oral PrEP trials should therefore be evaluated, and correlated with other safety and efficacy parameters, as this may help explain levels of efficacy and drug resistance.

The microbicides development field also needs more functional vaginal (or rectal) explant assays using pre-use and post-use tissue from study participants. So far, cervical explant assays are only set up in a pre-clinical studies context and many caveats (clinical history, hormonal status, ectocervix or endocervix, exposure to local products) have been identified.[31] In a controlled trial setting, at least the clinical background will be fully described. Furthermore, it is questionable if the low statistical power due to the limited number of biopsies per participant can lead to meaningful results. Different study designs with repetitive sampling should be explored.

And finally, laboratory science should investigate ways to optimize assays for functional immune parameters to be performed on low number of responding cells. It may also be time to invest in the evaluation of the cell cryopreservation media by comparing the viability of cells and biopsies with the commonly used DSMO freezing method.

In conclusion, assessing the local vaginal immune responses should be part of all vaccine and microbicide trials. Although this may be a challenge in some settings, the feasibility should always be explored when planning a trial before finalizing the protocol.

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