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Interlaboratory reproducibility of female genital tract cytokine measurements by Luminex: Implications for microbicide safety studies

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ABSTRACT

The interlaboratory reproducibility of cytokine measurements from cervicovaginal samples by Luminex has not been reported. Using cervicovaginal lavage specimens collected on three study days from 12 women participating in a Phase I microbicide study, we measured a panel of eight cytokines in three independent laboratories. Four (IFN-γ, IL-10, IL-17, and TNF) were below the limit of detection in the majority (85%) of samples in either two or all three laboratories, an observation that may guide analyte selection for future studies. Good interlaboratory agreement (intraclass correlation coefficient, $r > 0.7$) in absolute levels was observed for IL-1β, IL-6, and IL-8, while poor agreement was seen for IFN-α2 ($r = 0.47$). When considering within-subject change from baseline (pre-product, at study-day 0) to either post-product visit (study-days 7 and 14), IL-1β and IL-6 exhibited good interlaboratory agreement ($r > 0.7$), while IFN-α2 and IL-8 did not. Future studies addressing the clinical utility of specific biomarkers of inflammation for microbicide trials should consider reproducibility in the context of defining biologically meaningful thresholds of change for candidate biomarkers, ensuring that such change can be reliably distinguished from background variability.

1. Introduction

Vaginal microbicides hold great promise as a female-controlled strategy for the prevention of human immunodeficiency virus (HIV) and other sexually transmitted infections. The recent CAPRISA 004 trial, in which a 39% reduction in HIV and an unanticipated 51% reduction in HSV-2 acquisition were observed in women who applied 1% tenofovir gel before and after sex, illustrates the exciting potential of this strategy [1]. These encouraging results contrast with those obtained in earlier microbicide trials with surfactants and polyanionic entry inhibitors. Not only did the earlier products fail to protect against HIV, but several (Nonoxynol-9, C31G [Savvy], and cellulose sulfate) were associated with at least a trend towards higher rates of HIV infection [2–4]. Subsequent work indicated that those products may have facilitated HIV acquisition by inducing local inflammation and disrupting the epithelial barrier [5–9].

The central role inflammation plays in promoting HIV infection is further supported by studies with other sexually transmitted infections, suggesting that the inflammatory response recruits T cells into the genital tract to increase the risk of HIV infection. Inflammatory cytokines may also promote HIV replication by activating the long terminal repeat. Together these observations suggest that inflammatory mediators in genital tract secretions may serve as biomarkers of HIV risk and their measurement could prove predictive of the safety of vaginal microbicides, mucosal vaccines, or other interventions. A critical prerequisite in the development of biomarkers is the validation and standardization of assays. While Luminex multiplex technology is commonly used to measure cytokines and chemokines in various specimen types including female genital tract specimens, there are little or no data about the reproducibility of results across different laboratories.

Abbreviations: CVL, Cervicovaginal lavage; HIV, Human immunodeficiency virus.

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This has important implications for future clinical studies, as it will determine whether assays need to be performed at a single, centralized laboratory and the extent to which results obtained from different studies can be compared.

Therefore, to address this gap, convenience cervical samples that had been collected as part of a Phase I microbicide safety trial were evaluated in a blinded fashion in three independent laboratories to determine the interlaboratory variability in cytokine and chemokine measurements using the Luminex-100 multiplex system. We selected a panel of mediators that included those that had been previously shown to be associated with HIV infection risk in vitro (IL-1β, IL-6, IL-8, and TNF), antiviral cytokines (IFN-α2 and IFN-γ), the anti-inflammatory cytokine IL-10, and IL-17, which plays a role in neutrophil recruitment, promotes the production of β-defensins [10], and has been shown to play a role in the immune response to *N. gonorrhoeae* [11].

2. Materials and methods

2.1. Patient population and specimen collection

Cervicovaginal lavage (CVL) specimens collected from women participating in a Phase I clinical safety trial of a candidate vaginal microbicide were used for this study [12]. The trial is registered at http://www.ClinicalTrials.gov (NCT00331032) and all participants provided informed consent. De-identified specimens from days 0, 7, and 14 were evaluated, where day 0 represents baseline (pre-product) and days 7 and 14 represent the respective number of days on either product or placebo (in the same gel carrier). At each study visit, the cervix was lavaged with 5 ml normal saline, following which the specimen was aspirated and frozen at −80 °C without centrifugation.

2.2. Sample handling and testing

Thirty-six CVL specimens from 12 subjects were tested in a blinded (for subject and study day) fashion by three independent research laboratories for eight cytokines (IFN-α2, IFN-γ, IL-1β, IL-6, IL-8, IL-10, IL-17, and TNF). As it has previously been reported that assays from different vendors have poor intervendor agreement [13–15], a single vendor, Millipore (Billerica, MA), was used for this study. Specimens were thawed, vortexed to ensure homogeneity, aliquoted, refrozen, and distributed to each laboratory. Following identical sample handling and testing protocols at each of the three laboratories, the specimens were thawed at room temperature, centrifuged (2000 G at 4 °C for 10 min) to remove mucus and cellular debris, and tested in duplicate using MilliPlex MAP human cytokine/chemokine immunoassay kits (Millipore), per manufacturer's instructions. Kits from a single manufacturing lot were used by all three laboratories. Briefly, samples were incubated with antibody-conjugated microspheres, overnight, in 96-well filter-membrane assay plates with agitation. Plates were then washed with wash buffer provided in the assay kits and vacuum filtration, following which analyte-bound beads were incubated with a biotinylated detection antibody cocktail and finally with streptavidin–phycoerythrin. Following additional wash steps and resuspension of beads in instrument sheath fluid, plates were run on Luminex 100 instruments (Luminex, Austin, TX). Regression curves (5-parameter logistic) were fit, and unknown concentrations in pg/ml determined by interpolation, by each laboratory using their local software (Laboratories A and B: STarStation [Applied Cytometry Systems, Sacramento, CA]; Laboratory C: MiraiBio MasterPlex QT version 2.5 [Hitachi Software, South San Francisco, CA]). Concentrations from duplicate wells were averaged.

2.3. Statistical analysis

Sample values below the lowest standard (3.2 pg/ml) were set at the midpoint between zero and this value; values above the highest standard (10,000 pg/ml) were set at 10,000 pg/ml. Agreement of the cytokine measurements among the three laboratories was assessed using the intraclass correlation coefficient (r) which provides an index of the intersubject variability relative to the total variability [16], for all measurements and stratified by study day. Within-subject changes from baseline (day 0) at day-7 ("Δ7") and day-14 ("Δ14") were calculated by subtracting log-transformed baseline levels for each subject from log-transformed day-7 and (separately) day-14 levels (equivalent to ratios of the non-log-transformed values). Interlaboratory agreement in detecting within-subject change at the two post-product visits was then examined using the intraclass correlation coefficient. SPSS version 19 (SPSS, Inc.) was used for all statistical analyses.

3. Results

3.1. Interlaboratory agreement of absolute levels

The median and range of baseline (day-0) values obtained for each cytokine and from each lab are shown in Table 1. Four of the cytokines tested (IFN-γ, IL-10, IL-17, and TNF) exhibited expression levels too low in CVLs to measure reliably, with 85% or more of samples (from all study days) falling below detection limits in either two or all three laboratories (Table 2). These were therefore excluded from further analyses. Of the other four cytokines, good interlaboratory agreement ($r > 0.7$) was seen for IL-1β and IL-6, both overall and stratified by study day, and for IL-8 overall and on study-days 7 and 14 (Table 3). IFN-α2, in contrast, showed poor interlaboratory agreement except for the day-7 samples.

3.2. Interlaboratory agreement of within-subject cytokine-level change

Because of the variable degree of interlaboratory agreement in absolute cytokine measurements, and because normal ranges for these markers in CVL specimens have not been established, it was important to assess whether the ability to detect within-subject change from baseline was consistent across laboratories. Interlaboratory agreement in within-subject change was good at both post-product study days for IL-1β and IL-6 (Table 4). IFN-α2 and IL-8, in contrast, showed poor interlaboratory agreement.

In the case of IL-8 it was noted that, despite the overall interlaboratory agreement in absolute levels shown in Table 3, three of 36 specimens showed marked interlaboratory discordance (not shown) and that these were all clustered in the baseline specimen group. Because the discordance among baseline samples could affect both the day-7 and day-14 within-subject change assessments, which might not have been the case had these been distributed across study days, we repeated the analysis with these three subjects omitted. In this analysis, the intraclass correlation coefficient rose to 0.46 (95% CI: 0.05, 0.82) at day 7 and 0.81 (95% CI: 0.53, 0.95) at day 14.

4. Discussion

Using the intraclass correlation coefficient, which, in repeatability studies, provides an index of the natural variability between samples relative to the total variability [16], we found that three (IL-1β, IL-6, and IL-8) of the four cytokines with measurable levels had good interlaboratory agreement. This was the case both in absolute level measurements and (for IL-1β and IL-6) when examining within-subject change when baseline and post-product