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Comparison of normalization methods for measuring immune markers in cervical secretion specimens

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Abstract

Ophthalmic sponges are used to collect undiluted cervical secretions for assessment of markers of genital tract immunity. Heterogeneity in absorbed and extracted sample volumes requires normalization in order to make valid inter-individual comparisons. We evaluated the performance of adjustment by weight and total protein on normalizing inter-individual variability of immune marker measurement due to differences in volume collection. Normalization to total protein resulted in a minimal loss of usable specimens and a significant reduction in the correlation of immune marker concentration to specimen weight compared to weight adjustment. Total protein normalization appeared to be more effective than weight adjustment in reducing the dependence of cervical immune marker concentrations on differences in specimen volume.

Keywords

Cytokines; Cervical secretions; Ophthalmic sponges; Weight adjustment; Protein adjustment

1. Introduction

The mucosal immune response of the female reproductive tract is responsible for a myriad of biological functions; from first-line host defense against sexually transmitted infections, such as HIV and HSV-2, to facilitation of fertilization and maintenance of pregnancy (Wira et al., 2005a). This system is comprised of various professional and non-professional immune cells such as lymphocytes, monocytes, and granulocytes that exist within a matrix of stratified epithelial cells (Wira et al., 2005a, 2005b). Characterization of this system can be accomplished through direct sampling of tissue biopsies which are invasive and therefore difficult to acquire, or through measurement of effector molecules such as cytokines and chemokines collected through non-invasive sampling methods.

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Different sampling methods have been employed to measure immunologic markers including cervical or cervicovaginal swabs and direct collection of cervical secretions using absorbent sponges such as Weck-cell, sno-strips, or ophthalmic sponges (Jespers et al., 2011). Sponges generally have higher concentrations of measured markers compared to other diluted specimen types such as cervicovaginal lavage, but are subject to a high degree of inter-individual variability in the amount of specimen collected (Quesnel et al., 1997; Snowwhite et al., 2002). Variability in the total amount of specimen collected has the potential to mask true differences in marker concentrations by outcome status. As a result, a number of different methods have been established to help reduce or eliminate this correlation (Jespers et al., 2011). Some studies have used normalization of immune marker concentrations to total protein (Quesnel et al., 1997; Snowwhite et al., 2002; Marks et al., 2011a), others have utilized the difference in specimen weight pre- vs. post-collection to estimate a dilution factor to control for sampling differences (Hildesheim et al., 1999; Gravitt et al., 2003; Castle et al., 2004). However, only a single study to date, conducted among a small sample of individuals, has been performed to compare the effectiveness of these normalization methods on the measurement of three cytokines, IL-1, IL-6 and TNF- α (Gennaro et al., 2008).

The current report utilizes Merocel ophthalmic sponges, a sampling device that has shown superior recovery of target immune markers as compared to other sponges (Castle et al., 2004), to compare two methods, one weight-dependent and one weight-independent, for the control of sample volume heterogeneity on the measurement of 10 cytokines from cervical secretion specimens collected from healthy pre-, peri-, and post-menopausal women.

2. Methods

2.1. Study sample and secretion collection

Women included in this study are enrolled in an ongoing prospective cohort study assessing the natural history of HPV during the perimenopausal transition as previously described (Althoff et al., 2009). Cervical secretions were collected from the baseline study visit from pre- (n=118), peri-(n=98), and post-menopausal (n=90) women using a Merocel® ophthalmic sponge (Medtronic, Minneapolis, MN) which was placed at 6 o'clock in the cervical os for 30 s. Pre-menopausal women were identified as having regular, monthly menstrual bleeding. Peri-menopausal women reported irregular menstrual bleeding within the 12 months prior to enrollment and post-menopausal women reported no monthly menstrual bleeding within the last 12 months. Afterwards, the sponge with the collected sample was placed in an empty cryovial and stored at 4 °C for <24 h before transfer to long term storage at -80 °C.

2.2. Specimen processing and cytokine measurement

Protein for cytokine analysis was extracted from the ophthalmic sponges using previously described methods (Nardelli-Haeffliger et al., 2003). Briefly, sponges were thawed at room temperature for 10 min and then weighed. Sponges were then inserted into a microcentrifuge tube containing a 0.2 μ m filter (SpinX, Costar), equilibrated by adding 300 μ l of extraction buffer (10 mg/ml aprotinin in phosphate buffered saline with 10% sodium azide) and incubated for 30 min at 4 °C, followed by centrifugation at 4 °C for 30 min at 14,000 rpm. After centrifugation, specimens were stored at -20 °C until the time of immune marker measurement.

The following cytokines, chemokines, and growth factors were measured using a polystyrene bead-based multiplex assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA): IFN- γ , IL-1, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-17, and tumor

necrosis factor (TNF- α). Immune marker values that were below the limit of detection and completely undetectable by the Luminex instrument were assigned 1/2 the lowest measured value in our sample. Plates were read using a Luminex instrument (Bio-Rad, Hercules, CA). This assay has a reported lower limit of detection of 1–20 pg/ml depending on the cytokine target with an intra-assay coefficient of variation <20% (Bio-Rad Laboratories, 2009).

2.3. Normalization methods

2.3.1. Weight adjustment—The volume [V] of cervical secretion collected onto the merocel sponge was estimated by comparing the difference in weight of a sponge after specimen collection, but before protein extraction, to the mean weight of a set of 10 dry sponges as previously described (Gravitt et al., 2003). Preliminary experiments showed that the dry weight of a collection of sponges measured on different days has a coefficient of variation (CV) <1%, and a change in weight of 1 mg is equivalent to a 1 μ l change in volume (data not shown). This estimated volume was then used to calculate a dilution factor

using the following formula:
$$\frac{[V] + 300\mu\text{l}}{[V]} = \text{DilutionFactor}$$

Each immune marker measured was multiplied by this dilution factor to obtain weight-normalized values.

2.3.2. Total protein—The total concentration of protein was measured in each specimen using a bicinchoninic acid (BCA) assay as per manufacturer's protocol (Pierce, Rockford IL). This assay has a reported dynamic range of 20–20,000 μ g/ml and has a 14.7% mean coefficient of variance for repeat testing across 14 different human and non-human purified protein targets. Specimens were diluted 1:10 and 1:100 in PBS and run in duplicate. Colorimetric detection of test specimens was normalized to background specimens that contain extraction buffer only. Total protein concentration was estimated using an 8-point standard curve and is expressed as μ g/ml. The ratio of immune marker concentration to total protein concentration was then calculated and expressed as [ng of immune marker]/[mg of total protein].

2.4. Statistical analysis

All raw and normalized values were log-transformed. Spearman rank correlation was utilized to assess and compare the dependence of cytokine concentration on specimen weight pre- vs. post-normalization. The median (IQR) cytokine concentration was estimated and compared by tertile of specimen weight using the Kruskal–Wallis test for each normalization procedure to observe any residual dependence of marker concentration on specimen weight. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Specimen recovery

Among the 306 specimens that were included in this study, 126 (41.6%) specimens had lower specimen weight than dry weight resulting in negative volumes. These specimens were considered unusable for weight normalization. Two samples had undetectable total protein, and were thus unusable for protein normalization.

3.2. Correlation of cytokine [c] and specimen weight

There were 306 individuals with raw, 304 with protein adjusted, and 178 with weight adjusted cytokine values. All crude measures except IL-8, were significantly correlated to specimen weight prior to normalization (Table 1). A significant correlation remained for all

markers after weight adjustment; however the direction of the correlation between [c] and weight changed from positive to negative. Protein adjustment resulted in the retention of significant correlations between [c] and specimen weight for IL-1 only.

A significant difference in crude marker concentration by tertile of specimen weight was observed for most markers except IFN- γ , IL-8, and IL-10. After weight adjustment, a significant difference in marker concentration by tertile of specimen weight was observed across all immune markers (Fig. 1). Conversely, after protein adjustment, there was no observable difference in the concentration of most cytokines by tertile of specimen weight with the exception of IL-1.

4. Discussion

Much research in characterizing the immunologic landscape of the female reproductive tract have focused primarily on the direct comparison of the performance of non-invasive sampling methods such as CVLs, cervical swabs, and sponges (Quesnel et al., 1997; Snowwhite et al., 2002; Castle et al., 2004). This report compares the performance of two methods for normalizing inter-individual variability in the measurement of immune markers from cervical secretions collected using a widely accepted sampling method, the Merocel ophthalmic sponge. In agreement with a previous report (Gennaro et al., 2008), normalizing to specimen weight difference pre vs. post-collection, as a proxy for the volume of secretion collected, resulted in a significant loss in usable specimens. In contrast, normalizing to total protein resulted in a negligible loss of usable specimen, while effectively eliminating the correlation of target concentration and specimen weight for nearly all markers measured.

There are other considerations when choosing the sampling and normalization methods for lower genital tract measures of immune markers, apart from reducing the inter-individual variability in sampling. It is important when interpreting these measures to recognize that we are measuring immunologic markers present in the luminal fluid of the cervix and/or vagina. Many of these markers are expressed from a specific subset of cells that are not located at the surface epithelium, such that we are essentially measuring the spill-over into the luminal fluid. Activation and regulatory markers of lymphocytes and monocytes, which are key drivers of adaptive responses, would be expected to be present in low concentrations at the luminal surface. Highly diluted samples, such as CVL, may preclude the measurement of these important markers completely, or in the extreme case, assume that they are not expressed and therefore irrelevant in the biological process under investigation.

The fluid volume in the lower genital tract varies considerably within women, and may be regulated by physiological processes distinct from the expression of the immune markers measured. In the presence of independent regulation, the actual amount of cytokine/immunoglobulin present at the site of collection would be misclassified using volume (i.e., weight-adjusted) normalization. In other words, the dilution factors may be introducing significant bias using weight-adjusted measures, particularly when secretion volumes vary substantially in the sampled population. However, this does not preclude the possibility that luminal fluid production and host immunological process are intricately linked. Therefore, additional research is needed to better understand the relationship of luminal fluid volume and local cervical immune responses.

We have shown that protein normalization of immune markers such as cytokines, chemokines, and growth factors substantially reduces the inter-individual variability in the amount of sample collected, improving the ability to estimate individual and group-wise differences in marker expression. While specimen collection, storage and processing methodologies were standardized based on previous work, we cannot rule out the possibility

that the large number of specimens with discrepant differences in weight pre- vs. post-collection may have been a result of study-specific technical difficulties. One possibility is that the analytic scale used to collect weight information may not have been sensitive enough to capture the relatively small differences due to low volume of collected luminal fluid. The use of highly sensitive equipment for the measurement of specimen weight may therefore be required if weight-adjustment is chosen as a normalization method.

The ability to compare the profiles of local immune markers between subgroups using relatively non-invasive sampling expands the opportunity to collect important biological data from epidemiological studies. New insight into the ‘real world’ microenvironment in the lower genital tract can be gleaned from these studies (Marks et al., 2011b), and continued improvements in our ability to measure markers of immune function compared to genetic susceptibility will have broad application at the intersection of epidemiology and laboratory science.

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References

- Althoff KN, Paul P, Burke AE, Viscidi R, Sangaramoorthy M, Gravitt PE. Correlates of cervicovaginal human papillomavirus detection in perimenopausal women. *J. Womens Health (Larchmt)*. 2009; 18:1341. [PubMed: 19702476]
- Bio-Rad Laboratories. Bio-Plex Pro human cytokine, Chemokine, and Growth Factor Assays Technical Bulletin. 2009; Vol. 5828:1.
- Castle PE, Rodriguez AC, Bowman FP, Herrero R, Schiffman M, Bratti MC, Morera LA, Schust D, Crowley-Nowick P, Hildesheim A. Comparison of ophthalmic sponges for measurements of immune markers from cervical secretions. *Clin. Diagn. Lab. Immunol.* 2004; 11:399. [PubMed: 15013994]
- Gennaro S, Raftery N, Campbell D, Shults J. Cervical and vaginal cytokine determinations in pregnant women: methodologic issues. *Biol. Res. Nurs.* 2008; 9:215. [PubMed: 18077774]
- Gravitt PE, Hildesheim A, Herrero R, Schiffman M, Sherman ME, Bratti MC, Rodriguez AC, Morera LA, Cardenas F, Bowman FP, Shah KV, Crowley-Nowick PA. Correlates of IL-10 and IL-12 concentrations in cervical secretions. *J. Clin. Immunol.* 2003; 23:175. [PubMed: 12797539]
- Hildesheim A, McShane LM, Schiffman M, Bratti MC, Rodriguez AC, Herrero R, Morera LA, Cardenas F, Saxon L, Bowman FP, Crowley-Nowick PA. Cytokine and immunoglobulin concentrations in cervical secretions: reproducibility of the Weck-cel collection instrument and correlates of immune measures. *J. Immunol. Methods.* 1999; 225:131. [PubMed: 10365790]
- Jespers V, Francis SC, van de Wijgert J, Crucitti T. Methodological issues in sampling the local immune system of the female genital tract in the context of HIV prevention trials. *Am. J. Reprod. Immunol.* 2011; 65:368. [PubMed: 21199064]
- Marks MA, Viscidi RP, Chang K, Silver M, Burke A, Howard R, Gravitt PE. Differences in the concentration and correlation of cervical immune markers among HPV positive and negative perimenopausal women. *Cytokine.* 2011a; 56:798. [PubMed: 22015106]
- Marks MA, Viscidi RP, Chang K, Silver M, Burke A, Howard R, Gravitt PE. Differences in the Concentration and Correlation of Cervical Immune Markers Among HPV Positive and Negative Perimenopausal Women. *Cytokine.* 2011b
- Nardelli-Haeffliger D, Wirthner D, Schiller JT, Lowy DR, Hildesheim A, Ponci F, De Grandi P. Specific antibody levels at the cervix during the menstrual cycle of women vaccinated with human papillomavirus 16 virus-like particles. *J. Natl Cancer Inst.* 2003; 95:1128. [PubMed: 12902442]

- Quesnel A, Cu-Uvin S, Murphy D, Ashley RL, Flanigan T, Neutra MR. Comparative analysis of methods for collection and measurement of immunoglobulins in cervical and vaginal secretions of women. *J. Immunol. Methods.* 1997; 202:153. [PubMed: 9107304]
- Snowwhite IV, Jones WE, Dumestre J, Dunlap K, Braly PS, Hagensee ME. Comparative analysis of methods for collection and measurement of cytokines and immunoglobulins in cervical and vaginal secretions of HIV and HPV infected women. *J. Immunol. Methods.* 2002; 263:85. [PubMed: 12009206]
- Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: cellular responses and interactions. *Immunol. Rev.* 2005a; 206:306. [PubMed: 16048557]
- Wira CR, Grant-Tschudy KS, Crane-Godreau MA. Epithelial cells in the female reproductive tract: a central role as sentinels of immune protection. *Am. J. Reprod. Immunol.* 2005b; 53:65. [PubMed: 15790340]

Fig. 1.

The median (IQR) concentration of each cytokine (log-transformed) was compared before and after normalization across tertile of specimen weight. The goal of this analysis is to demonstrate the effect of each normalization method on minimizing differences in cytokine concentration by specimen weight. The median (IQR) specimen weight (in mg) for each tertile is Q1 (Lower): 5.08 (5.06, 5.10), Q2(Middle): 5.14(5.12, 5.15), and Q3(Upper): 5.20 (5.18, 5.23). Differences in cytokine [c] of weight normalized values were restricted to those specimens with usable volume estimates (n = 178). Therefore, the median (IQR) specimen weights (in mg) for each tertile used in weight normalization only were 5.14 (5.13, 5.142), 5.17 (5.16, 5.18), and 5.22 (5.21, 5.25) for quantiles one thru three, respectively. Kruskal–Wallis test was used to assess the differences in median concentration by tertile of specimen weight. *p<0.05 **p<0.01 ***p<0.001.

Table 1

Effect of normalization methodology on the correlation of cytokine measures and specimen weight.

Cytokine	Normalization method*	Mean (SD)	p-value	
IL-1b	Raw (pg/ml)	153 (6.7)	0.217	0.002
	Weight (pg/ml)	1324 (9.0)	-0.289	0.001
	Protein (ng/mg)	37,613 (6.2)	0.144	0.013
IL-2	Raw (pg/ml)	5 (4.1)	0.241	<0.001
	Weight (pg/ml)	42 (6.2)	-0.276	0.001
	Protein (ng/mg)	1232 (4.9)	0.038	0.516
IL-6	Raw (pg/ml)	82 (7.3)	0.174	0.002
	Weight (pg/ml)	674 (9.8)	-0.507	<0.001
	Protein (ng/mg)	20,152 (6.7)	0.048	0.406
IL-7	Raw (pg/ml)	7.5 (3.1)	0.260	<0.001
	Weight (pg/ml)	53 (4.4)	-0.507	<0.001
	Protein (ng/mg)	1857 (3.7)	0.022	0.700
IL-8	Raw (pg/ml)	4469 (6.2)	0.099	0.088
	Weight (pg/ml)	40,468 (7.8)	-0.333	<0.001
	Protein (ng/mg)	1,101,466 (5.6)	-0.008	0.891
IL-10	Raw (pg/ml)	28 (4.4)	0.144	0.013
	Weight (pg/ml)	274 (6.2)	-0.609	<0.001
	Protein (ng/mg)	6978 (4.2)	-0.002	0.968
IL-12p70	Raw (pg/ml)	23 (2.3)	0.162	0.005
	Weight (pg/ml)	194 (3.7)	-0.689	<0.001
	Protein (ng/mg)	5679 (2.4)	-0.031	0.590
IL-17	Raw (pg/ml)	3.9 (2.2)	0.206	0.003
	Weight (pg/ml)	24 (3.8)	-0.597	<0.001
	Protein (ng/mg)	975 (2.4)	-0.055	0.341
IFN-g	Raw (pg/ml)	72 (3.4)	0.135	0.019
	Weight (pg/ml)	637 (5.1)	-0.697	<0.001
	Protein (ng/mg)	17,818 (3.6)	-0.063	0.277
TNF-a	Raw (pg/ml)	46 (3.8)	0.195	0.001
	Weight (pg/ml)	396 (5.4)	-0.548	<0.001
	Protein (ng/mg)	11,382 (3.5)	0.064	0.274

* Raw concentration and weight-adjusted concentration expressed as pg/ml; Protein adjusted values expressed as ngcytokine/mgtotal protein.