*Short Report*

# The Stability of Immunoglobulin A in Human Milk and Saliva Stored on Filter Paper at Ambient Temperature

ELIZABETH M. MILLER[1*] AND DANIEL S. MCCONNELL[2]
[1]*Department of Anthropology, University of Michigan, University of Michigan, Ann Arbor, Michigan 48109*
[2]*Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104*

**Objectives**: Immunoglobulin A dominates mucosal surfaces and is a biomarker of interest in populations with a high disease burden. The objectives of this work are to describe an ELISA for IgA and test the stability of storage on filter paper for human milk and saliva collection to be used in remote field locations.

**Methods**: A two-site sandwich ELISA for IgA was developed. To test filter paper storage capabilities under field conditions, 248 matched whole and dried human milk filter paper samples and 251 matched whole and dried saliva samples were collected from northern Kenyan women. Whole samples were frozen in liquid nitrogen while dried samples were stored at ambient temperature for up to 8 weeks. Recovered dried IgA levels were compared to whole IgA levels and adjusted for time stored at ambient temperature.

**Results**: The lower limit of quantification for this assay is 10.1 ng/ml. Linearity of dilution for human milk and saliva samples was excellent. High and low-control coefficient of variation values across plates were 9.1 (341.8 ng/ml) and 9.4% (132.5 ng/ml). IgA was detected in all whole and dried samples. There is a moderate concordance between dried and whole samples ($R^2 = 0.62$). There is a small but significant effect of time stored, with a loss of ~1 μg/ml per day ($P = 0.0052$).

**Conclusions**: This IgA assay is a cost-effective alternative to commercial secretory IgA kits. Human milk and saliva can be stored on filter paper for up to 8 weeks. Am. J. Hum. Biol. 23:823–825, 2011. 

## INTRODUCTION

Immunoglobulin A (IgA) is the most abundant immunologic protein on mucosal surfaces and in human milk (Mestecky and McGhee, 1987). It binds to pathogens to remove them from the body, selectively tolerates "good" gut bacteria and is anti-inflammatory (Fagarasan, 2008). IgA levels are an important indicator of mucosal immune function, particularly in remote populations with high disease loads and high rates of breastfeeding. IgA is found in two forms within the body: the dimeric secretory form, found in mucosal fluids, consisting of two IgA molecules bound together by a secretory component and a J-chain; and the monomeric form, found in serum, which consists of a single IgA molecule. This assay measures IgA for use in remote field locations where storage capacity is limited and protein degradation may compromise sample integrity. Unlike commercial kits for the dimeric secretory form of IgA, this assay can be adapted for use with serum where only the monomeric form of IgA is found. Previous research suggests that filter paper is an acceptable medium for the storage of human milk in remote locations (Brown et al., 1982). This article tests the acceptability of filter paper as a storage medium for milk and saliva for later analysis with the IgA assay.

## MATERIALS AND METHODS

### *Assay protocol*

The two-site sandwich ELISA for IgA was developed by the authors at the Clinical Ligand Assay Satellite Service (CLASS Laboratory), Department of Epidemiology, University of Michigan. On the first day, a coating solution containing sodium carbonate buffer (pH = 9.6), a 1:500 dilution of anti-IgA1 antibody (Southern Biotech, Birmingham, AL, www.southernbiotech.com) and a 1:1,000 dilution of anti-IgA2 antibody (Southern Biotech, Birmingham, AL, www.southernbiotech.com), both of which bind to the hinge region of IgA molecules, was applied to 96-well microplates (Fisher Scientific, Pittsburg, PA, www.fishersci.com) and incubated at room temperature overnight.

Samples were prepared on the second day. Whole and filter paper samples were diluted in a phosphate-buffered solution (PBS) containing 0.05% bovine serum albumin (BSA) and 0.05% Tween-20. Whole milk was inverted to mix the whey and lipid layer and prepared in PBS to a dilution of 1:4,000. Whole saliva was centrifuged and the supernatant diluted in PBS to a dilution of 1:1,500. Filter paper milk sample "disks" were punched out of the sample card using a 1/8 inch hole punch and eluted in 2,000 μL PBS overnight for a dilution of 1:2,000. Adult filter paper saliva sample "disks" were eluted overnight in 750 μL PBS for a dilution of 1:750.

On the day of assay, coating solution was decanted from the microwell plate, and a sodium carbonate blocking buffer with 5% BSA was added to each well, incubated for 1 h and washed with a PBS solution containing 0.2%

Contract grant sponsor: National Science Foundation Doctoral Dissertation Improvement Grant; Contract grant number: BCS-0750779; Contract grant sponsor: Leakey Foundation.

*Correspondence to: Elizabeth M. Miller, Department of Anthropology, 101 West Hall, 1085 S. University Ave., Ann Arbor, MI 48109-1107, 847-913-2803 (phone), 734-763-6077 (fax). E-mail: emmill@umich.edu

Received 31 May 2011; Revision received 14 July 2011; Accepted 20 August 2011

DOI 10.1002/ajhb.21218

Published online 28 September 2011 in Wiley Online Library (wileyonlinelibrary.com).

*TABLE 1. Descriptive statistics of whole and dried human milk (n = 248) and whole and dried saliva (n = 251) IgA concentration*

| | Whole human milk (μg/ml) | Dried human milk (disk/ml) | Whole saliva (μg/ml) | Dried saliva (disk/ml) |
|---|---|---|---|---|
| Mean | 1004.1 | 1118.8 | 325.3 | 218.9 |
| SD | 1530.0 | 4424.7 | 189.4 | 196.7 |
| Median | 808.6 | 551.5 | 296.2 | 174.7 |
| Maximum | 21155.2 | 56150.0 | 1425.8 | 1571.2 |
| Minimum | 245.9 | 116.6 | 39.5 | 6.1 |

*Units are in microgram per milliliter for whole samples and paper "disk" per milliliter for dried samples.

Tween-20. Standards, controls, unknown samples, and blanks were added to the plates and incubated for 3 h. Secretory immunoglobulin A (sIgA) purified from human colostrum (Accurate Chemical, Westbury, NY, www.accuratechemical.com) was used to create a standard curve at concentrations of 600, 200, 60, 20, and 0 ng/ml. Because sIgA is a dimeric molecule and contains twice the IgA as monomeric IgA, sIgA standard concentrations were prepared at half the stated concentrations (e.g., 300, 100, 30, 10, and 0 ng/ml of sIgA) to create an IgA (rather than sIgA) standard curve. Unknown samples were run in triplicate. After incubation, plates were washed, and a 0.04 μg/ml solution of PBS and polyclonal goat anti-human IgA antibody conjugated with horseradish peroxidase (Accurate Chemical, Westbury, NY, www.accuratechemical.com) was added to the plate and incubated for 1.5 h. After washing, a TMB solution (Pierce, Rockford, IL, www.piercenet.com) was added to each well and allowed to react for 20 min before being stopped by a 2 M sulfuric acid solution. Plates were read in a SpectraMAX 340PC (Molecular Devices, Sunnyvale, CA, http://www.moleculardevices.com) at 450 and 620 nm. Values were adjusted for absorbance at 620 and for background in blank wells. The SpectraMAX software generated a four-parameter standard curve and calculated unknown values. Sample replicates with a coefficient of variation (CV) of less than 10% or samples that fell outside the range of the standard curve were reassayed with appropriate dilutions.

### *Assay validation*

The lower limit of quantification, defined as the value 2 SD above the lowest calibration point (0 ng/ml) is 10.1 ng/ml. The interassay percent CV is 8.05% ($n$ = 10 each saliva and milk on three plates). Intra-assay CV is 10.68% ($n$ = 10 each milk and saliva across three plates). To investigate linearity of dilution, a saliva and a milk sample were assayed serially at 1:500, 1:1,000, 1:2,000, 1:4,000, and 1:8,000 dilutions. The coefficients of determination for human milk and saliva linearity were $R^2 = 0.992$ ($P < 0.0001$) and $R^2 = 0.999$ ($P < 0.0001$), respectively. Sample values were multiplied by two to convert concentrations to the monomeric form of IgA and to insure values were consistent with published reference sources.

### *Filter paper analysis*

Approval for this study was given by the University of Michigan Institutional Review Board and the Kenyatta National Hospital Ethics Review Committee. After giving consent to participate, 251 northern Kenyan women provided saliva samples; three of these women were no longer lactating, bringing the total number of milk samples to 248. One hundred microliters of saliva or milk were applied with a pipette to the center of each preprinted circle on Whatman 903 filter paper and allowed to dry for 2–3 h. Dried filter paper samples were stored in a secure, nonclimate controlled closet in a triple-layer plastic bag with a desiccant for 19–56 days before freezing. Mean ambient temperature, measured daily at 13:00, was 26.7°C (±1.7°C). Whole samples were frozen in liquid nitrogen within 5 h of collection. Samples were packed in dry ice before shipment to the United States, where they were frozen at −80°C at the CLASS Laboratory at the University of Michigan.

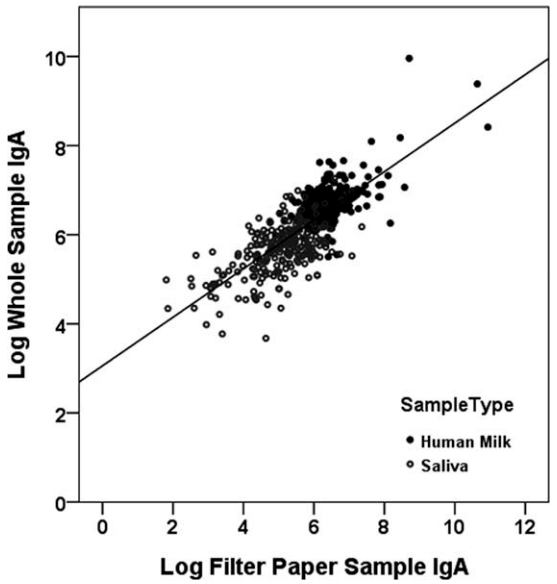


Fig. 1. Scatterplot and regression line of log-transformed whole sample IgA and dried sample IgA by sample type.

## RESULTS

Descriptive statistics of human milk and saliva samples can be found in Table 1. The median milk IgA, a better indicator of central tendency due to outliers, was within the published normal range of 470–1632 μg/ml (Jackson et al., 1999). The whole saliva IgA mean was within commercial assay range of 102–471 μg/ml (ALPCO Diagnostics, 2008). The CVs for the O.D. values of high and low controls for all assay runs were 9.1% (341.8 ng/ml) and 9.4% (132.5 ng/ml), respectively.

Because saliva and breastmilk samples assayed similarly due to high levels of dilution and negligible levels of sample matrix, data from both were combined into one regression equation for a total $n$ of 499 matched whole and filter paper samples. Time stored at ambient temperature and sample type were included as covariates. Sample data were log transformed before regression analysis. This regression produced the equations:

$$\ln(\text{WHOLE}) = 0.39^{*}\ln(\text{FILTER}) - 0.0033^{*}\text{TIME} - 0.033^{*}\text{TYPE} + 4.4$$

or

$$(\text{WHOLE}) = e^{0.39^{*}\ln(\text{FILTER})-0.0033^{*}\text{TIME}-0.033^{*}\text{TYPE}+4.4}$$

The regression had a total variance of $R^2 = 0.69$. Figure 1 shows the association of IgA values between the whole and dried sample conditions (log-transformed). Time was a marginally statistically significant covariate ($P = 0.059$) with a degradation of 1.0 μg/ml per day, ranging from 19.0 μg/ml to 56.0 μg/ml over the 19–56-day storage period. This accounts for a potential degradation of up to 5.6% of whole breastmilk IgA and 17.2% of whole salivary IgA at the maximum storage time of 56 days. Excluding time from the regression equation produces $R^2 = 0.69$, indicating that storage does not significantly affect the variation present between whole and dried samples. Therefore, the degradation of IgA due to time at ambient temperature can be adjusted for mathematically using the equation above.

## DISCUSSION

All filter paper samples had adequate signal for analysis in this assay. Results from the regression equation suggest that 55% of IgA can be recovered from filter paper samples, although the intercept is relatively high (24.5 μg/ml). The ratio of median filter paper concentration to median whole concentrations of human milk is 0.682, indicating a 68.2% recovery, and the ratio of mean filter paper to whole concentrations of saliva is 0.673 or 67.3% recovery. This number fares well in comparison with various dried blood spot validations, where recovery ranges from 48.7% for leptin (Miller et al., 2006) to 87% for C-reactive protein (McDade et al., 2004). The high concentration of IgA in saliva and breastmilk in conjunction with nonspecific interactions with the filter paper likely contributes to this modest recovery percentage. This indicates that human milk and whole saliva samples can be successfully analyzed in dried samples without loss of signal.

This work describes an ELISA for IgA and tests the use of filter paper for the storage of mucosal fluids for later analysis. Results indicate that this assay is a valid alternative to commercial kits for researchers in remote field locations. Filter paper may be used as a storage medium for saliva and human milk for IgA with acceptable results. Future research should expand on the use of filter paper to store human milk and saliva for a wider variety of biomarkers.

## ACKNOWLEDGMENTS

I thank two anonymous reviewers for their constructive comments that greatly improved this manuscript. Thanks to Bill Leonard, Roberto Frisancho, John Mitani, Bobbi Low, and Milford Wolpoff for comments on previous versions of this work.

## LITERATURE CITED

ALPCO Diagnostics. 2008. Secretory IgA EIA: for the determination of secretory IgA in saliva and stool. ALPCO Diagnostics, Salem, NH.

Brown SE III, Sauer KT, Nations-Shields M, Shields DS, Araujo JG, Guerrant RL. 1982. Comparison of paired whole milk and dried filter paper samples for anti-enterotoxin and anti-rotavirus activities. J Clin Microbiol 16:103–106.

Fagarasan S. 2008. Evolution, development, mechanism and function of IgA in the gut. Curr Opin Immunol 20:170–177.

Jackson S, Mestecky J, Moldoveanu Z, Spearman P. 1999. Appendix II: collection and processing of human mucosal secretions. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, editors. Mucosal immunology. San Diego, CA: Academic Press. p 1567–1576.

McDade TW, Burhop J, Dohnal J. 2004. High-sensitivity enzyme immunoassay for C-reactive protein in dried blood spots. Clin Chem 50:652–654.

Mestecky J, McGhee JR. 1987. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv Immunol 40:153–245.

Miller AA, Sharrock KC, McDade TW. 2006. Measurement of leptin in dried blood spot samples. Am J Hum Biol 18:857–860.