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TECHNICAL NOTE

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Comparison of two human infant urine collection methods for measuring estrone-3-glucuronide

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Abstract

Objectives: Current human infant urine collection methods for the field are problematic for the researcher and potentially uncomfortable for the infant. In this study, we compared two minimally invasive methods for collecting infant urine: organic cotton balls and filter paper.

Materials and methods: We first collected urine from infants using the clean catch method. We then used those samples to compare the performance of filter paper and cotton ball collection protocols. We analyzed the clean catch and cotton samples using commercial estrone-3-glucuronide (E1G) kits and tried two different extraction methods for the filter paper. Using a paired *t*-test ($n = 10$), we compared clean catch and cotton samples. We also compared effect sizes within and between methods.

Results: We were unable to extract enough urine from the filter paper to successfully assay the samples for E1G. The paired *t*-test revealed a statistically significant difference between the clean catch and cotton methods ($t = 2.63$, p-value $= 0.03$). However, the effect size was small (5.91 μg/ml, $n = 10$, 95% CI = 3.80, 8.02) and similar to or larger than the difference seen between duplicate wells for clean catch and cotton values.

Discussion: While this study is limited by sample size, our results indicate that filter paper is not a field-friendly method for collecting infant urine. However, we found that organic cotton balls showed similar values to the clean catch method, and we propose this method as an alternative, minimally invasive method for study of E1G in human infant urine.

KEYWORDS
infant urine collection, filter paper, cotton, E1G

1 | INTRODUCTION

Although major advances have been made in field endocrinology, collecting biological samples to measure hormones in the field can still be difficult. A particularly challenging sample to collect is human infant urine. When working with infants, urine is often a preferable specimen to serum or dried blood spots because it induces less distress in the infant. It also provides information about hormone metabolism since last void, and, thus, better represents average hormone levels than serum or dried blood spots, which only reflect hormone levels at the moment of sample collection. Urine is also widely used because it contains many biomarkers that are indicative of health and development, such as estrone-3-glucuronide (E1G). In particular, the collection of human infant urine is useful for studies using a Development Origins of Health and Disease (DOHaD) framework. DOHaD research often involves measuring some aspect of the infant's physiology to compare with adult function in order to make robust associations between infant development and adult health. For example, a yet underexplored area is the relationship between early-life exposure to estrogens, potentially measured via urinary E1G, and later life endocrine function, fertility,

and reproductive cancer risk. This research requires feasible and accurate methods to collect infant urine.

One widely used method for collecting infant urine is the clean catch method—waiting for a diaper-free child to void spontaneously—which is the recommended method of the National Institute for Health and Care Excellence (National Collaborating Centre for Women's and Children's Health, 2017). The urine can be collected in infant pediatric urine collection bags or cups. However, parents often find clean catch methods time-consuming and unpleasant (Liaw et al., 2000; Tosif et al., 2017). Additionally, the use of either requires a parent to remove any clothing covering the diaper area, exposing the infant, in some latitudes, to uncomfortably cold ambient temperatures. As infants have much greater surface area to mass ratios than adults and have not developed all thermoregulation mechanisms, this exposure can be problematic, particularly in cold climates (Lidell, 2019). The removal of clothing and the diaper may also clash with cultural norms or interfere with the infant's behavior or endocrine profile. For example, it could increase the infant's cortisol levels if it causes the infant temporary stress, which could impact the clean catch values if sample collection is time consuming.

Some researchers have developed easier ways to collect excreta samples from infants. For example, Thompson et al. (2010) found that standard gel-based disposable diapers and all-natural cotton diapers can be used to collect infant fecal samples to test for estradiol. Mothers retained soiled diapers and stored them in portable coolers until the researchers collected the diapers. While an improvement from some of the limitations of the clean catch method, storage and shipment of large numbers of frozen diapers from the field to the laboratory is cumbersome and expensive. An affordable, simple alternative is to use organic cotton balls placed in the diaper to collect infant urine for hormonal analysis. However, this method has never been validated for sex steroids in infant urine.

Another potential method for collecting infant urine is to use filter paper placed in the diaper. Both Shideler et al. (1995) and Knott (2005) validated methods to measure urinary E1G using filter paper, and studies since have successfully used these methods to measure several biomarkers in human and non-human primate urine, such as C-reactive protein, cortisol, E1C, and pregnanediol glucuronide (Jaimez et al., 2012; Knott et al., 2010; Shattuck-Heidorn et al., 2017). However, these validated methods require soaking the filter paper until saturated with urine. We aimed to see if this method would still provide accurate E1G values if the urine did not saturate the filter paper.

In this study, we explored two new infant urine collection methods for the estimation of infant E1G levels. One method used organic cotton balls and the other used Whatman® filter paper. We compared these collection methods to clean catch E1G values in order to expand field-friendly infant urine collection methods.

2 | METHODS AND MATERIALS

Sample collection took place in Namqom, an indigenous village in the province of Formosa, northern Argentina, during the winter of 2019. We collected samples from 13 Qom infants using all three protocols; the mothers of the infants provided their informed consent prior to inclusion in the study. However, two samples had E1G values too low for detection with commercial kits and we excluded one sample from the cotton collection method because it had a coefficient of variation (CV) between duplicates above the exclusion criteria (15%). Therefore, our final sample size was 10 participants. All participants were male and ranged in age from 12 days to 6 months (mean = 2.6 months, SD = 1.8). First, we collected infant urine using the clean catch method, followed by the cotton ball collection protocol and the filter paper collection protocol; Figure 1 presents a summary of these procedures. To ensure no variability resulted from comparing urine samples from different voids, the cotton ball and filter paper collection protocol were tested using the sample collected via the clean catch method (i.e., the clean catch sample was used as a reference sample to test the other protocols).

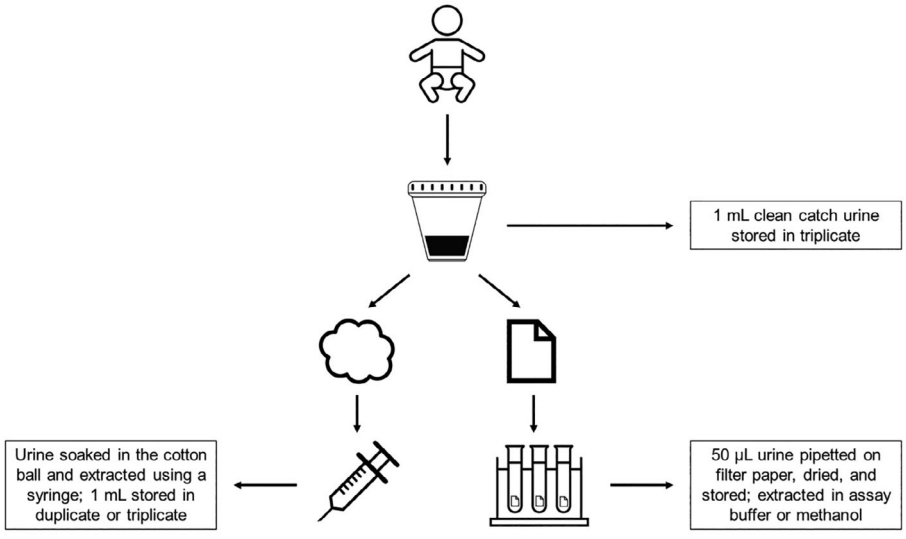

FIGURE 1 Flow chart of the sample collection and extraction procedures

For the clean catch collection protocol, we used sterile Starplex™ Scientific Leakbuster 60 mL containers (B602-1O). The mother removed her infant's diaper and held the infant while the researcher supported the specimen container below the infant's body to catch the urine. After sample collection, the researchers stored the urine in a cooler with ice for no more than 3 h before freezing the sample.

Before freezing the samples, we used the collected urine to prepare the two other methods. After aliquoting 1 ml of urine into 1.5 ml Eppendorf® tubes in triplicate, we pipetted 50 μl of remaining urine onto Whatman® #1 filter paper (31.25 mm sectors) in duplicate. The amount of sample aliquoted was chosen because it filled most of the sector without completely saturating it. Then, the filter paper dried in an open Ziploc bag on a bed of silica beads for 2 days. While the filter paper may have been exposed to contamination in the open bag, bacterial, or fungal contamination would likely not have influenced assayed E1G levels. Following drying, we wrapped filter paper in aluminum foil and placed samples in a plastic storage container. Filter paper samples were stored at ambient temperature (Knott, 2005), which ranged from 5 to 15°C, until return to the United States, after which they were stored at 4°C.

After completing the filter paper protocol, we carried out the cotton ball protocol. The cotton balls remained in a sealed bag before use to prevent contamination. To begin the protocol, we soaked one to two organic cotton balls (Organyc 100% Organic Cotton Balls, regular size, 0.6 grams/cotton ball) in the remaining urine until saturated or no urine remained. Then, we placed the cotton balls in a sterile plastic syringe containing a polypropylene barrel plunger (Frienda 30 ml). Applying pressure to the barrel plunger, we dispensed up to 1 ml of urine into 1.5 ml centrifuge tubes in duplicate or triplicate, depending on the amount of urine remaining. We then immediately placed the centrifuge tubes in a −20°C freezer.

Samples were transported on dry ice to the Yale Reproductive Ecology Lab, where we measured urinary E1G using commercial kits (Arbor Assays ®). We assayed all samples in duplicate for each method and re-ran samples for which the CV for duplicates was greater than the exclusion criteria. For the clean catch and cotton ball methods, we diluted samples 1:5 or 1:8 with assay buffer, and E1G concentration (μg ml^{-1}) was calculated accounting for dilution. If samples were initially too low to detect at 1:8 dilution or had greater than 80% maximum binding, we reran them at the lowest dilution factor (1:5). For the filter paper, we attempted two urine extraction methods. First, using the extraction method utilized by Shideler et al. (1995), we incubated five paper punches (3 mm diameter) of each sample overnight at 4°C with 500 μl assay buffer, creating a 1:5 dilution. The following day, we assayed 50 μl sample aliquots according to the manufacturer's instructions. This method yielded extractions with E1G values too low to measure using our commercial kit.

We next used the urine extraction methods in Knott (2005). We cut 1.25 cm^2 squares containing urine sample from the filter paper and soaked samples in 5 ml of methanol overnight at 4° C. The following day, nitrogen gas was used to evaporate the methanol. Once the methanol evaporated, we removed the filter paper squares from the tubes and reconstituted the samples with 1 ml of assay buffer. We ran one set of samples without further dilution as well as a second set diluted 1:2. We then measured E1G using the same commercial kits as above. The commercial kit detected E1G in some samples, but the calculations showed very low correlations with values from the clean catch or cotton ball samples after correcting for the dilution factor. Of the undiluted samples, 75% of E1G values from this method were still too low to measure using the commercial kit, suggesting that the method did not extract enough urine.

We tested the statistical difference between the cotton ball and the clean catch method using a paired t-test (alpha = .05) in *R* (3.4.1). We also ran *t*-tests on the duplicate wells for the clean catch and cotton methods to contextualize the effect size of the difference between methods and calculated the CV between methods.

This study was approved by Institutional Review Board at Yale University (IRB/HSC#: 2000025147).

3 | RESULTS

We present all raw data in Table 1, including E1G concentrations from the clean catch and cotton samples for each participant with the

TABLE 1 Data by participant showing urinary E1G (μg ml^{-1}) for the clean catch and cotton methods

Participant	Clean catch raw (STD) (μg ml^{-1})	Clean catch CV (%)	Clean catch corrected (μg ml^{-1})	Cotton raw (STD) (μg ml^{-1})	Cotton CV (%)	Cotton corrected (μg ml^{-1})	Method CV (%)	Raw difference (μg ml^{-1})
1	39.4 (2.0)	5.0	314.9	47.4 (3.9)	8.2	378.8	10.5	−8.0
2	38.2 (1.2)	3.1	305.6	27.9 (1.9)	6.7	223.5	4.6	10.3
3	40.3 (0.3)	0.9	322.2	39.8 (2.2)	5.4	318.5	9.3	0.5
4	54.1 (1.9)	3.4	270.5	44.8 (0.2)	0.5	224.1	9.6	9.3
5	20.0 (2.4)	12.1	100.1	14.1 (0.2)	1.3	70.5	18.7	5.9
6	147.9 (19.6)	13.3	1182.9	144.7 (2.8)	1.9	1157.5	6.9	3.2
7	49.2 (3.8)	7.6	393.3	41.8 (5.5)	13.1	334.2	10.9	7.4
8	23.8 (1.4)	5.9	190.2	19.8 (1.6)	7.8	158.2	10.4	4.0
9	268.1 (26.6)	9.9	2145.1	263.1 (19.4)	7.4	2104.5	6.3	5.1
10	32.0 (0.1)	0.3	255.7	26.4 (3.0)	11.3	211.0	10.8	5.6

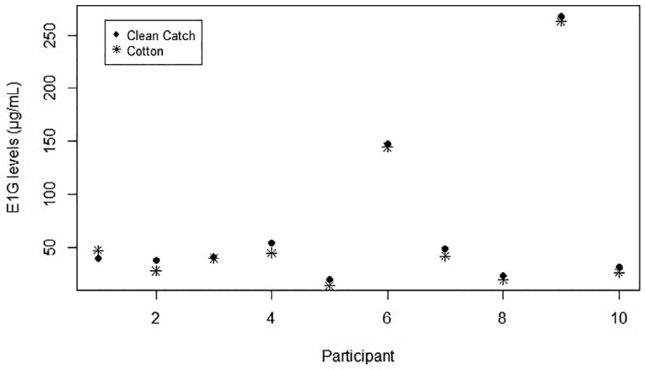

FIGURE 2 Raw estrone-3-glucuronide (E1G) values (µg ml^{-1}) for the clean catch method (black circles) and cotton ball collection method (black asterisks) for each participant

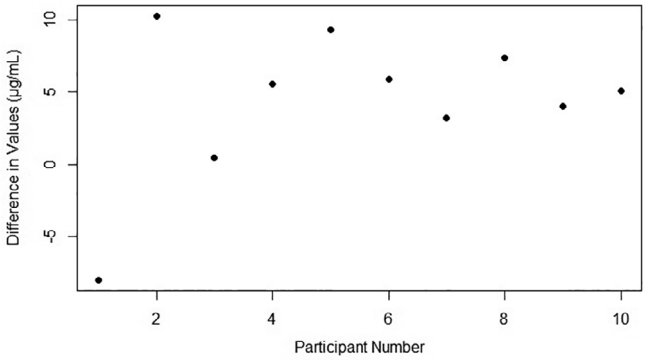

FIGURE 3 Raw difference between collection methods (clean catch estrone-3-glucuronide [E1G] values—cotton ball method E1G values in µg ml^{-1}, values uncorrected for dilution) by participant

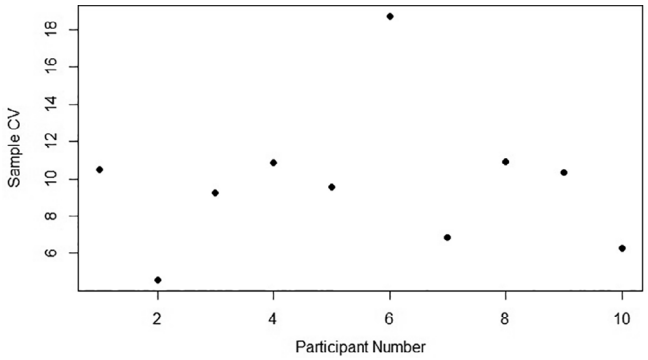

FIGURE 4 Calculated coefficient of variation (CV) by participant between the two methods

standard deviation. The table also includes the E1G concentration corrected for dilution and the CV for each method. To more thoroughly compare clean catch and cotton methods, we also calculated the mean concentration, standard deviation, and CV for the clean catch and cotton methods combined as well as the raw difference between uncorrected calculated clean catch and cotton concentrations (Table 1).

We used a paired *t*-test to statistically investigate any systematic differences between the clean catch method and the cotton ball collection method.. The E1G values obtained from both methods were similar (mean clean catch values = 68.27 µg/ml; mean cotton values = 64.70 µg/ml). The effect size (mean difference) between the absolute value of the differences was small (5.91 µg/ml, $n = 10$, 95% CI = 3.80, 8.02), though the difference was statistically significant according to the *t*-test ($n = 10$, $t = 2.63$, *p*-value = .03). We found similar results when conducting a paired *t*-test using the data corrected for dilutions ($n = 10$, $t = 2.43$, *p*-value = .04). To contextualize the difference in E1G values between the two methods, we compared it to variation in E1G within each method. Specifically, we calculated the mean difference between duplicate wells for cotton and clean catch. Differences among duplicates within each method were comparable to (cotton: −3.94 µg ml^{-1}, $n = 10$, 95% CI = −10.41, 2.52) or larger than (clean catch: −8.70 µg ml^{-1}, $n = 10$, 95% CI = −18.18, 0.76) the difference between methods.

Figure 2 shows the calculated E1G value using each method while Figure 3 shows the raw difference between the clean catch method and the cotton collection method (range = −7.99, 10.27). Most cotton samples had slightly lower values than the clean catch method, although there appears to be no systematic pattern in the value of the decrease. We then calculated the CV between methods. These results are presented in Figure 4 and suggest a similar range of CV values (range = 4.6, 18.7) to those that are obtained for duplicates using the same method (<20).

4 | DISCUSSION

In this study, we compared two methods for collecting infant urine against the standard free catch method. While we could not evaluate the filter paper protocol because the urine extraction was unsuccessful, we found that the cotton collection method using organic cotton balls provided similar E1G values to clean catch samples.

Although we used previously validated extraction techniques for urine in filter paper, our collection protocol differed slightly from previous evaluations. Specifically, we did not soak the filter in urine for 15 min or aliquot urine to saturate small squares (Knott, 2005; Shideler et al., 1995). When filter paper becomes saturated, the concentration per square centimeter can then be calculated from the absorption rate of the filter paper. However, this method may not be successful unless the researcher can verify that the filter paper placed in a diaper will become fully saturated. By aliquoting a small amount on the filter paper, we aimed to account for situations in which the filter paper may not be completely soaked in a diaper, and our extractions may have failed for this reason. Therefore, the filter paper collection method is not a reliable method for collecting human infant urine from diapers.

In contrast, we found that the cotton ball collection method resulted in E1G values similar to the clean catch method. Here, we would like to emphasize the difference between statistical significance

and biological relevance (Farji-Brener, 2006; Kramer et al., 2016; Lovell, 2013). Using the criterion of $p < .05$, the *t*-test indicated a statistically significant difference between clean catch and cotton methods. However, the effect size (differences between methods) was comparable to the difference between duplicates of a sample within the same method. Additionally, while the cotton method did show a slight pattern of lower E1G values compared to clean catch, we observed no systematic pattern in the decrease to indicate that the cotton did not retain a substantial amount of the E1G. Rather, our results suggest that this method is a field-friendly, minimally invasive method to collect infant urine since it does not matter how saturated the cotton ball is.

The cotton ball protocol is also field-friendly in two other aspects. We found that cotton balls did not rapidly dry; in a related study, not reported here, we gave mothers cotton balls to collect their infant's urine during the day and a Starplex specimen cup in which to store the cotton balls. Upon returning, even cotton balls collected hours before were still sufficiently wet to extract urine. Large cohort studies could utilize this method by giving mothers cotton balls, specimen containers, and directions to collect the sample the morning before their next visit with the researchers.

This study has several limitations. The results are only valid for measurement of E1G since other hormones may be differently retained in the cotton balls or interact with the chemicals used to create cotton balls, although it is likely this method would also be successful for other steroid hormones. Despite this limitation, we still found that it is feasible to collect infant urine using cotton balls to accurately estimate infant urinary E1G levels. Additionally, we used a relatively small sample size. However, the statistical analysis showed significant differences between the cotton and clean catch methods, so the sample size was sufficient to detect even small effects.

Another limitation of this study is that our statistical analysis would have been more informative if we had used an equivalence test (Hauck & Anderson, 1984) or its most common variation, a 'two-one-sided *t*-tests' (TOST) procedure (Lakens et al., 2018; Schuirmann, 1987). These procedures, which originated in pharmacokinetics, test whether the observed effects between two groups can be considered equivalent, in contrast to the more common method of testing whether the effect is different from zero (Smith, 2020). The equivalence interval must be decided a priori, before looking at the data, to avoid hindsight bias. Given this requirement, researchers must determine the equivalence interval based on previous knowledge of the system they are studying. Thus, these methods incorporate an understanding of the difference between biological versus statistical differences. While our study would have benefited from this approach, studies of urinary estradiol have yet to determine the magnitude of change in μg that would be considered biologically equivalent. Therefore, we were unable to incorporate equivalence tests in this study.

5 | CONCLUSIONS

We found that it is possible to collect infant urine using cotton balls, which is a more field-friendly method than using urine collection cups or pediatric urine collection bags. This collection procedure will facilitate research on infant development, particularly research using a DOHaD framework. However, researchers interested in using filter paper should follow previously validated methods, which involve ensuring that the filter paper is completely saturated. Given the difficulties of ensuring saturation of infant urine in a diaper, we do not recommend this method for field collection of human infant urine.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Carlye Chaney: Conceptualization; formal analysis; investigation; methodology; writing-original draft; writing-review & editing. **Margaret Corley:** Formal analysis; methodology; validation; writing-review & editing. **Claudia Valeggia:** Conceptualization; methodology; supervision; writing-review & editing.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in Table 1 of this article.

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