Laboratory Detective Work Identifies a Mishandling Problem in Sample Aliquoting

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Data from a recent ovarian cancer biomarker study using serum aliquots from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial Biorepository showed that CA125II concentrations in these aliquots were significantly lower than those previously measured in the same subjects from the same blood draw. We designed an experiment to investigate whether samples used in the study (reference study) were compromised during the aliquoting process. We measured CA125II in the “sister” vials created during the same aliquoting process as the reference study aliquot, and in “cousin” vials newly aliquoted from another parent vial from the same blood draw, from 15 healthy controls in the study. Because the sister vials were created in a specific order, we also assessed whether there was a CA125II concentration gradient among the sisters. The Wilcoxon signed-rank test was used to test the statistical significance of the observed differences. Mean CA125II concentration (volume-averaged) was greater in the sisters than the cousins in all 15 subjects ($p < 0.001$). The mean coefficient of variation was 0.25 (range: 0.12–0.43) in the sisters and 0.11 (range: 0–1.1) in the cousins ($p < 0.008$). The mean ratio of CA125II in the 5th aliquoted versus the 3rd aliquoted sister vial was 1.66 (1.25–2.5, $p < 0.001$). These data suggest that the parent vials were not adequately mixed before they were aliquoted. CA125II in serum can partially precipitate to form a concentration gradient in long-term storage. Rigorous vortexing after thawing and before aliquoting is thus critical.

Introduction

THE PROSTATE, LUNG, COLORECTAL AND OVARIAN (PLCO) Cancer Screening Trial Biorepository stores over 2.8 million specimens including serum, plasma, buffy coat, and red blood cells.¹ Since 2005, this resource has been utilized in over two hundred published studies.²

In 2009, a PLCO longitudinal study of several ovarian cancer biomarkers (including CA125) was initiated by Fred Hutchinson Cancer Research Center (FHCRC) investigators. Approximately 4500 serum vials from ovarian cancer cases and controls, all previously untouched “parent” vials, were pulled from the PLCO Biorepository and sent to the processing lab for aliquoting in 2011. Aliquots of 0.3 mL were made for the study at the processing lab and shipped to FHCRC.

In 2013, NCI was contacted by Dr. Nicole Urban, the PI of the above-mentioned study (“reference study”), who noted that the CA125II levels measured in the samples at her FHCRC lab using a clinical assay (Abbott Labs) were much lower (~40%) than CA125II levels measured earlier as part of the ongoing PLCO trial for the same subjects and blood draw using a different clinical assay (Centocor). Additionally, CA125II levels in a set of PLCO samples aliquoted at the processing lab several years earlier for a different project but measured at FHCRC alongside the reference study samples using the same Abbott assay were

very close to the CA125II levels (for the same subjects and blood draw) measured earlier for the ongoing trial.⁵

Communication records between the shipper at the processing lab and recipients at FHCRC demonstrated that there was no problem with shipping; communications between Biorepository personnel and the processing lab also did not indicate issues with the transit of the samples. Taken together, these observations appear to rule out sample degradation during transit or in the PI lab, and thus point to possible problems at the aliquoting at the processing lab. Due to a personnel change at the processing lab, the actual aliquoting protocol used at the time for the reference study samples could not be verified. We suspected that one of two possible scenarios may have occurred during aliquoting: 1) a prolonged sojourn at room temperature, or 2) a failure to adequately mix the parent vials (usually by vortexing) prior to the aliquots being taken.

Here we report the results of an experiment designed to determine which (if any) of the above scenarios likely occurred.

Materials and Methods

The experimental design takes advantage of several features of PLCO sample aliquots. In PLCO, when a parent vial (~1.8 mL) is first aliquoted for a study, the whole vial is aliquoted into a series of daughter vials according to a standard scheme specifying the aliquot volume and ordering.

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For a study sample volume of 0.3 mL, the processing lab aliquots the parent vial into daughter (sister) vials of the following volumes and in the following order: 0.3 mL (the study "daughter" aliquot), 0.2 mL, 0.3 mL, 0.15 mL, 0.5 mL, 0.2 mL, and 0.15 mL (all "sister" aliquots). Additionally, the PLCO biorepository retained an "aunt" vial, namely, an untouched second parent vial processed from the same vial, for all reference study samples.

Fifteen reference study subjects were randomly selected for the experiment. For each, the "aunt" vial was aliquoted freshly to create "cousin" vials according to the standard protocol. Briefly, vials were thawed in 2–8°C overnight, mixed thoroughly by vortexing, and aliquoted into 0.3 mL "cousin" vials on ice within 24 hours. CA125II was measured in the sisters and two of the cousins using a CLIA assay (Elecsys CA 125 II assay, Roche). Because this assay required a minimum volume of 0.25 mL, sister vials with volume under 0.25 mL were pooled for CA125II measurements. All laboratory procedures were performed at PPD, Inc.

Statistical analysis

Mean (volume-averaged) CA125II concentration in the sisters and cousins were computed for each subject. The coefficient of variation (CV) for sisters and cousins within each subject was calculated as the standard deviation across sisters (cousins) divided by the mean of the sisters (cousins). To assess whether a concentration gradient existed among sisters, the ratio of the CA125II concentration in the 5th versus 3rd sister vial was computed (the other sister vials were pooled). The Wilcoxon signed-rank test was used to test whether the within-subject mean of CA125II level and the CV were different between the sisters and cousins and whether the CA125II level differed between the 5th and 3rd sisters.

Results

Two possible results were anticipated as follows: 1) Mean CA125II levels in sister vials are lower than in cousin vials

and the CVs are similar, suggesting uniform sample degradation occurred during the aliquoting process, or 2) The mean CA125II level is higher in sisters than cousins, the CV is higher in sisters than cousins, and sister vials aliquotted later in the order have higher concentrations than those aliquotted earlier, suggesting that the parent vial was not mixed adequately before aliquoting.

Figure 1 shows the CA125II concentrations in the sister and cousin vials for all 15 subjects. The mean concentration was greater in the sisters than cousins in all subjects (range for difference in means: 0.37–5.3 U/mL; $p < 0.001$). The mean CV for the sister vials was 0.25 (range 0.12–0.43) compared to 0.11 (range 0–1.1) for the cousins ($p < 0.008$). The mean of the ratio of CA125II concentrations in the 5th versus 3rd sister vial was 1.66, range 1.25–2.5 ($p < 0.001$). The pooled sister vial, which contained the 2nd, 4th, 6th, and 7th ordered sister vial, generally had a CA125II concentration between that of the 3rd and 5th sister vials.

Discussion

In this report, we detailed our investigation to pinpoint a suspected laboratory error in the processing of serum aliquots. Our experimental data strongly suggest inadequate mixing of parent vials prior to aliquoting, resulting in a concentration gradient among the daughter (sister) vials. It is not surprising that a large molecule such as CA125 partially precipitated to form a concentration gradient during long-term storage. Thus, mixing parent vials vigorously before aliquoting is necessary.

Several lessons can be learned from this experience. Aside from the obvious importance of having a best practice Standard Operating Procedure (SOP), some quality control measures should be instituted for sample handling procedures. This can involve "spot-checking" by measuring standard markers in a small subset of the derivative samples after each process. A Laboratory Information Management System (LIMS) that records and tracks all aspects of the sample processing jobs is also essential.

FIG. 1. CA125II concentrations in sister and cousin vials for all 15 subjects. *Triangles* (to the left for each subject) represent the two cousin vials. Sister vials (to the right for each subject) are represented as follows: *square* (5th sister vial), *dot* (3rd sister vial), *star* (pooled sister vials); *horizontal line* is (volume weighted) mean concentration among sisters.

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Author Disclosure Statement

No competing financial interests exist.

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