The Generation and Applications of Biological Variation Data in Laboratory Medicine

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LEARNING OBJECTIVES

1. Explain the main sources of variation in a clinical laboratory.
2. Define biological variation and identify the clinical applications of biological variation data.
3. Define and identify uses of reference change value.

ABSTRACT

In recent years, increasing consideration has been given to the study of biological variation on laboratory analytes. Data on biological variation have a number of applications in the laboratory, including setting the quality specifications for analytical performance, assessing the usefulness of population-based reference ranges, assessing variation in serial results from an individual, and determining the optimal sample for analyzing a specific constituent. Thus, it is essential to generate accurate and reliable data on biological variation using a standardized study protocol. Currently, there are guidelines available to enable the standardized production of biological variation data. Here, we aim to provide recommendations on preferred experimental procedures and statistical methods for producing correct biological variation data in accordance with published guidelines.

ABBREVIATIONS: BMP - basic metabolic panel, CVA - analytical variation, CVG - between-subject variation, CVI - within-subject variation, CVT - total variation, CVTG - total between-subject variation, CVTI - total within-subject variation, EFLM - European Federation of Clinical Chemistry and Laboratory Medicine, II - index of individuality, PT - proficiency testing, RCV - reference change value, SD - standard deviation.

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INDEX TERMS: biological variation, reference change value, quality specification, the utility of reference range, index of individuality.

Clin Lab Sci 2018;31(1):37–42

INTRODUCTION

There are three main sources of total variation (CVT) in laboratory test results: (1) preanalytical variation, (2) analytical variation (CVA) such as precision and bias, and (3) biological variation such as within-subject variation (CVI) and between-subject variation (CVG).[1] The preanalytical variation includes all the steps that occur from test ordering to the moments before analysis. This phase includes preparation of the individual for sample collection, sample collection procedure, sample transport, storage, and handling. Preanalytical sources of variation are identified and minimized by good phlebotomy, proper sample transport, handling, and storage techniques so that they can be considered negligible towards CVT in the laboratory. CVA is associated with sample analysis, which includes systematic (bias) and random (imprecision) error. CVA cannot be totally eliminated; however, it can be minimized by selection of good methodology and by quality laboratory practice. On the other hand, biological variation is of three main types: (1) variation related to physiological changes, such as age, pregnancy, menopause, etc; (2) cyclic variation due to outside influences that can be daily, monthly, and seasonal in nature; and (3) random variation, which consists of CVI and CVG. Random fluctuation around the homeostatic set point of each individual is called CVI.[1,2] The homeostatic set point for one individual is often different from another, and the overall variation due to the difference between the homeostatic set points of individuals is called CVG.[1-3] CVI and CVG may cause the intrinsic differences in laboratory results. Thus, by carefully controlling preanalytical variation and by designing controlled experiments to quantify CVA, we can estimate the component of biological variation, including CVI and CVG,[1,3] while interpreting patient results for accurate diagnosis and monitoring.[2,4,5] Many international studies confirm that this variation is consistently predictable and consistent for each analyte.[1] Moreover, biological variation data have several important clinical and laboratory applications as discussed below.

CLINICAL APPLICATION OF BIOLOGICAL VARIATION DATA

Biological variation data can be used to set analytical quality specification in the laboratory for imprecision to evaluate the usefulness of population-based reference ranges, to assess the variation in serial measurements, and to determine optimal samples for analyzing a specific constituent.[3,6,7]

Setting Quality Specifications for Analytical Performance

It is essential to define analytical quality goals in the laboratory to assess the quality of current methodology. Laboratory quality goals were first discussed in 1999 during the Stockholm consensus conference for analytical quality specifications.[8] Recently, this was revisited and revised during the first strategic analytical quality specific conference in Milan (2014) held by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM).[9] As an outcome of this conference, the hierarchy of three models for quality specifications was suggested.[9] The most preferred and the top of the hierarchy are quality goals based on clinical outcomes. However, few analytes align with these goals and this approach may not help to set the quality specifications for precision and bias. Second in the hierarchy are goals based on components of biological variation, which satisfies the general need for diagnosis and monitoring. Third in the hierarchy are goals based on the state of art, which includes external quality assurance, proficiency testing (PT), and clinical laboratory improvement amendments goals, and these are the least preferable goals for quality specification in the laboratory. Therefore, biological variation goals are highly relevant for clinical laboratories. Using the formula suggested by Fraser,[1] quality specifications using biological variation data can be set at three different levels (minimum, desirable, and optimum) for analytical imprecision, bias, and total error.

Assessing the Utility of Population-based Reference Values

Population-based reference ranges are required when a new procedure is used for clinical purposes in the laboratory. The utility of population-based reference ranges for an analyte can be assessed by determining the ratio of CVI and CVG, which is called "index of individuality" (II).[1] If the II is higher than 1.4, the distribution of values from a single individual will cover much of the entire distribution of the reference interval derived from reference subjects.[1] Thus, conventional reference values will be of significant value in many clinical settings. If II is lower than 0.6, the dispersion of individual values will span only a small part of the reference interval. In this case, reference intervals will have a lower utility value, especially for deciding whether a change has occurred.[1] The majority of analytes compiled up to date have lower II (<0.6),[1,4] thus, for such analytes, population-based reference range values are not very useful in detecting latent or early disease. Furthermore, individuals may have values that are very unusual but that still fall within the reference limit.

Assessing the Change in Serial Results

Data on biological variation are required for the interpretation of change in serial results. Assessment of variation in two consecutive results from an individual requires consideration of both the analytical and physiological sources of variation. In other words, change in patient serial results may not only be due to a patient improving or deteriorating but also be due to analytical imprecision and inherent biological variation. Therefore, for a change to be significant, it must exceed the critical difference or reference change value (RCV), which can be calculated using the formula: $RCV = 2^{1/2} \times Z \times (CVA^2 + CVI^2)^{1/2}$.[1] In this equation, Z is the number of standard deviations (SDs) appropriate to the desired probability. Z values of 1.96 and 2.58 represent probabilities of 95% and 99%, respectively.[1] For the RCV, probabilities of 95% ($P < .05$) and 99% ($P < .01$) indicate that differences are significant (*) and highly significant (**), respectively. RCV value can also be used in delta checking for various analytes in the laboratory, and related flags (*, **) can be inserted in a laboratory information system to alert the physicians on significant and highly significant changes in serial patient results.[10,11] This helps the physician to select important information from the vast amount of other available information on the patient's result chart and educates clinicians about change in serial results.[12] A practical example is illustrated in the case study below.

Determining the Optimal Sample for Analyzing a Specific Constituent

Biological variation data can be helpful to determine which sample type (plasma, serum, 24-urine, first-morning urine) is optimum for analyzing a specific constituent.[1,3,6] The sample with a lower CVI is best because the inherent variability to body fluid is minimized.[1] CVI values can be obtained from the published biological variation database.[13] For example, for urine micro albumin, the CVI for a first-morning urine sample is 36 and the CVI for a 24-hour urine is 70, so first-morning urine is preferred for the follow-up of a renal disorder.

GENERATION OF DATA ON BIOLOGICAL VARIATION

Many international studies confirm that biological variation data are consistent and predictable for each analyte; however, it is very important that these data are accurately derived through standardized, well-defined, and

Case Study

A 42-year-old male with a history of hypertension presented in the emergency room with chest pain. The initial basic metabolic panel (BMP) showed a serum creatinine of 0.9 mg/dL. On the following day, the patient was scheduled for an exercise stress test. A repeat BMP before the stress test showed a creatinine of 1.2 mg/dL. Has the creatinine concentration changed significantly?

First result = 0.9 mg/dL

Second result = 1.2 mg/dL

Change = 1.2 − 0.9 = 0.3 mg/dL

Percent difference = (0.3/0.9) × 100 = 33.3%

RCV Calculation for Creatinine

$2^{1/2} \times Z \times (CVA^2 + CVI^2)^{1/2}$

$2^{1/2} = 1.414$

$Z = 1.96$ for a significant change (95% probability)

$Z = 2.58$ for highly significant change (99% probability)

CVA is the analytical variation (analytical imprecision). CVA is taken from laboratory internal quality control at a clinical decision level. The mean was 1.1 mg/dL, and the SD was 0.02 mg/dL. Therefore, CVA is (0.02/1.1) × 100 = 1.8%

CVI is 5.95, which is taken from latest published database.[13]

RCV for Creatinine for a Significant Change

$RCV = 1.414 \times 1.96 \times (1.8^2 + 5.95^2)^{1/2} = 17.2\%$

RCV for Creatinine for a Highly Significant Change

$RCV = 1.414 \times 2.58 \times (1.8^2 + 5.95^2)^{1/2} = 22.7\%$

Interpretation: A percent difference of 33.3% in two serial creatinine results shows significant (>17.2%) and highly significant (>22.7%) changes.

controlled protocols.[14,15] Currently, a universal guideline for deriving biological variation data is not available. The only available standard to derive biological variation data is published by Fraser and Harris.[1,6] There are numerous studies that derive biological variation data for many analytes. However, concern exists over the validity of the data reported in several studies.[15] The high heterogeneity among the study protocols due to factors including population selection, sample size, specimen collection procedure, methodology, and statistical analyses may affect the biological variation estimates obtained.[4,5]

The availability of universal protocols would help investigators to carry out experiments in a standardized way. Recently, the EFLM published a checklist, which was built in agreement with the Standard for Reporting Diagnostic Accuracy to generate the data on biological variation.[2] This checklist identifies key elements to be reported to enable safe, accurate, and effective transport of biological variation data across laboratories. This checklist can also be used to evaluate the quality of existing biological variation studies. In addition, the use of a checklist for new studies may help researchers, authors, and journal reviewers to ensure that studies deliver robust estimates of biological variations data, which can be useful to populate a new database with a high-quality estimate.[2] We aim to provide a practical guideline with flowcharts (Figures 1 and 2) for producing correct biological variation data in accordance with the checklist published by EFLM.

Selection of Subjects

Generating the conventional population-based reference ranges requires a large group of people from which to select subjects.[1] Comparatively, subject selection to generate the component of random biological variation is fairly easy since the samples are selected from a smaller group of subjects.[1] Additionally, since biological rather than pathological variation is of interest, a subject should be apparently healthy and should maintain their usual lifestyle.[1]

According to Fraser,[1] subjects selected for a study should be "reference individuals," and this can be attained by following an "a priori approach," which includes setting up inclusion and exclusion criteria for selection of subjects. Inclusion criteria may include apparently healthy subjects, which are willing to provide a number of samples over a period of time. Exclusion criteria may include unusual lifestyle, pregnant women, active infection, taking medication and more than recommended alcohol, smoking, etc. In addition, age, gender, and the number of subjects, number of samples, number of replicates, and state of well-being should be clearly stated. There is no clear answer on an ideal number of subjects required. However, it is intuitive that the higher the number, the better the estimate will be. According to Roraas, the number of samples collected per person is more important than the number of individuals examined when the CVI is estimated.[14] That said, biological variation studies can be performed with a moderate number of subjects since increasing the number of occasions samples are taken, and increasing the number of replicates of each assessment (ie, duplicate or triplicate)

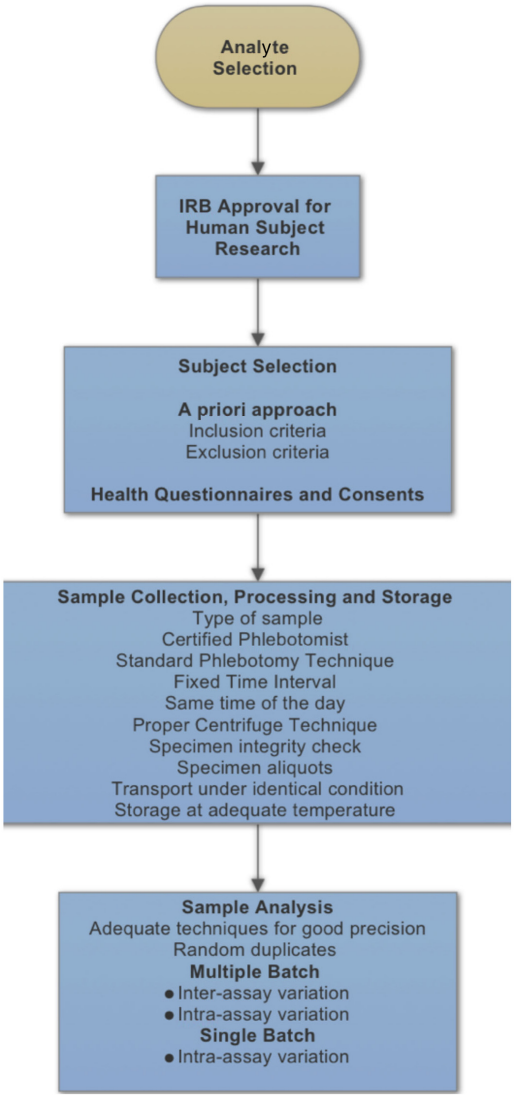

Figure 1. **Recommended flow chart for sample collection, sample handling, and sample analysis for a biological variation study. IRB, institutional review board.**

reduces the confidence interval of the within-subject SD and, therefore, increases the power of the study.[14]

Sample Collection, Handling, and Storage

It is very important to minimize preanalytical variation to get the best components of biological variation estimates before sample collection. Samples should be collected at the same time of the day (ie, morning, afternoon, or evening), under the same conditions (ie, no strenuous exercise before sampling, a standard meal, or no breakfast), with a standard phlebotomy technique, preferably with a single experienced phlebotomist, into collection tube of the same lot number.[1] Moreover, special handling of the specimen (transporting samples to the laboratory under the same temperature) is crucial, and centrifuge, when required, at the same speed and temperature for the same period of the time. The integrity of specimens (lipemic, hemolyzed, icteric) should be checked before storage. According to the stability of the analyte, all specimens should be stored at an appropriate temperature (freeze or refrigerate).[1]

Sample Analysis

It is very important to minimize CVA (imprecision and bias) to get an accurate estimate of random biological variation. Interassay variation (bias) can be eliminated by analyzing all specimens in a single batch. Intra-assay variation can be reduced by using a single instrument, one analyst, and one set of calibrators with the same lot of reagents. Intra-assay variation can be calculated by running all samples in random duplicates in a single analytical run. However, for samples that are unstable or have been analyzed as they are collected, interassay variation cannot be eliminated; it should be calculated using results from quality control materials. Precision achieved with quality control samples may differ from that attained with patient samples.[1]

Initial Inspection and Statistical Treatment of Raw Data

Outliers should be assessed from collected data. There are several statistical tests to detect outliers, such as Tukey outlier, Bartlett test, Grubbers test, Cochrane test, and Reed criteria,[1] etc. We recommend Tukey method,[16] which assesses the data for outliers on three levels: across the entire group of subjects, for each subject individually, and for individual subjects with outlying variability compared with the other subjects in the group.

Once outliers have been identified, the normality of collected data should be checked using statistical normality tests, such as Shapiro-Wilk test, Kurtosis test, Kolmogorov-Smirnov test, Anderson-Darling test, histogram, and Q-Q plot. If any of these tests show skewed distribution, a natural logarithmic scale should be applied to the data. Normality should be confirmed again with log-transferred data. If the data are still skewed even after logarithmic transformation, the data are considered invalid for further calculation. At this point, one should stop further calculation. On the other hand, if log-transferred data confirm the normal distribution, one can further calculate the variance components. However, data must be converted back before calculating the CVs to make it applicable to laboratory practice.[4]

Estimating Components of Biological Variation

Once the detected outlier is removed and the normality of data is confirmed, a nested analysis of variance can be used to calculate the variance among the components of interest. Furthermore, the associated SD and overall mean can be used to calculate total within-subject (CVTI = CVI +

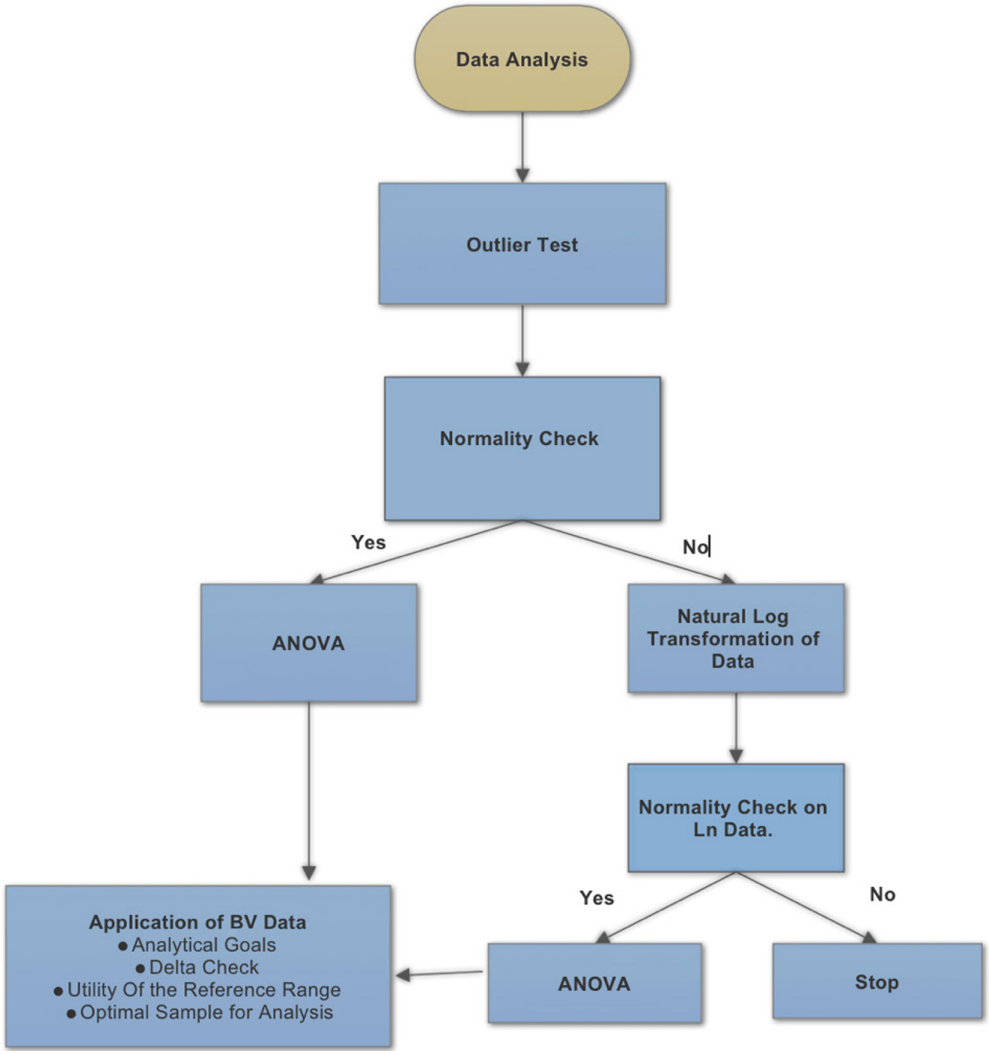

Figure 2. **Recommended flow chart for data analysis in a biological variation study. ANOVA, analysis of variance.**

CVA) and total between-subject (CVTG = CVG + CVA) variability. From total variance, CVA can be subtracted to generate CVI and CVG, respectively. CVA can be calculated from the difference between random duplicates from each run ($CVA = \Sigma d2/2N$, in which d is the difference between duplicates, and N is a number of paired results).[1] Analytical bias can be calculated using PT results from the clinical decision level [Bias% = (Lab Mean − Consensus group mean) ÷ Consensus group mean) × 100].[1]

Result Reporting

Based on calculated variance components (CVI, CVG, CVA, Bias) for all subjects and those calculated separately for subgroups (ie, gender, age), other indices, such as II, RCV, etc, can be derived. All results should be tabulated in a clear format, and we suggest adding a table for derived analytical quality goals at three levels (minimum, desirable, optimum) for analytical precision, bias, and total error. In addition, the CVI data should be reported with the

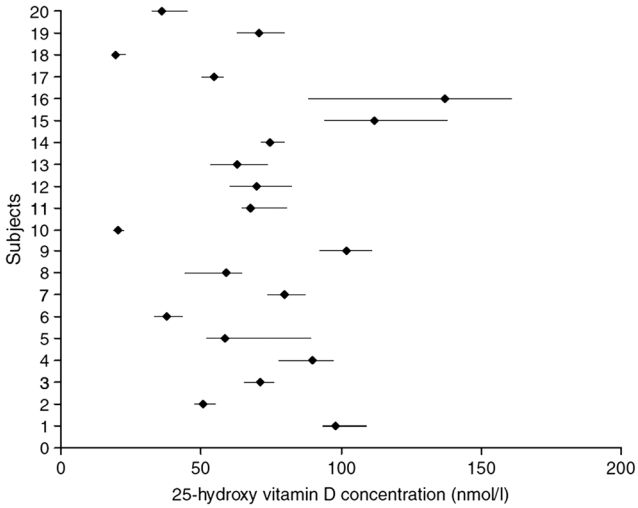

Figure 3. **Representation of a mean and absolute range of values in a group of subjects evaluated for 25-hydroxyvitamin D concentration.[18]**

power of the study and corresponding confidence interval at 95%. The terms and symbols used to define components of biological variation and other related indices should be used consistently in accordance with the standards identified by Simundic.[17] The number of subjects included in a statistical calculation after removal of outliers and confirmation of homogeneity of data should be clearly reported. Moreover, we suggest adding a graphical report with a mean and absolute range of values in the individuals that were studied (an example of this is shown in Figure 3).[18] The discussion of data should clearly include a focus on factors that impact the transportability of the data to other settings. In addition, limitations and strengths of the study should be clearly addressed.

CONCLUSION

Data on biological variation have a number of applications in the laboratory. Thus, it is essential to generate accurate and reliable data on biological variation using a standardized study protocol. Currently, a guideline from Fraser[1] and a checklist[2] from the EFLM are available to enable the standardized production of biological variation data. Here, we provide practical recommendations with preferred experimental procedures and statistical methods for producing biological variation data in accordance with the checklist published by the EFLM.

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