Original Article

Validation of summer and winter ELISA measurements of serum 25-hydroxyvitamin D concentrations in Mongolia

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Background and Objectives: Assay cost, quality, and availability pose challenges for vitamin D surveys in limited resource settings. This study aimed to validate an inexpensive vitamin D assay (ELISA) under real-world conditions in Mongolia, the northernmost developing country, to characterize the assay's usefulness and inform the design of epidemiologic studies in similar regions. **Methods and Study Design:** We collected paired summer and winter serum samples from 120 men and women (aged 20-57 years) in urban and rural Mongolia, analyzed each sample for 25(OH)D concentration using both Immunodiagnostic Systems ELISA and DiaSorin LIAISON 25(OH)D TOTAL, and compared the assays using multiple statistics. LIAISON was itself validated by participation in the DEQAS program. **Results:** Correlation and agreement between assays were higher in summer (Pearson's correlation=0.60, Spearman's rank correlation=0.67, Lin's concordance correlation=0.56) than winter (rP=0.37, rS=0.43, rC=0.33), although ELISA less accurately assigned subjects to sufficiency categories in summer (percent agreement=44%) than winter (58%), during the latter of which most subjects were deficient ([25(OH)D] categories used: >75 nmol/L (optimal), 50-75 nmol/L (adequate), 25-50 nmol/L (inadequate), <25 nmol/L (deficient)). Compared with LIAISON, ELISA tended to indicate higher vitamin D status in both seasons (mean paired difference: 7.0 nmol/L (95% CI: 3.5-10.5) in summer, 5.2 nmol/L (95% CI: 2.9-7.5) in winter). **Conclusions:** ELISA proved useful for measuring and ranking subjects' vitamin D status in Mongolia during summer, but levels were too low in winter to sensitively discriminate between subjects, and ELISA overestimated status in both seasons. These findings have implications for the timing and interpretation, respectively, of vitamin D surveys in highly deficient populations.

Key Words: vitamin D, 25(OH)D, IDS ELISA, DiaSorin LIAISON, Mongolia

INTRODUCTION

Global interest in vitamin D has increased considerably in recent years, following mounting evidence linking vitamin D status to an enormous array of diseases.[1] However, vitamin D status of populations remains difficult to measure, and data is lacking for most countries and demographics.[2,3] Despite revitalization of the in-house mass spectrometric platform as a gold standard for vitamin D assessment, it may be too costly, slow, and sophisticated[4] for use in epidemiologic studies. Instead, studies often rely on cheaper, higher throughput, simpler assays, which may be less accurate and precise. This inaccuracy is related to vitamin D's complex metabolism and various assay-specific issues.[5,6] To make matters more complicated, vitamin D measurements should be collected throughout the year to have a representative picture of its seasonality. Vitamin D surveys can be especially challenging in low-income countries, where practical constraints further limit usefulness of assays which may already be problematic in ideal settings.[7] These include lack of funds, standardized procedures, available technologies, and expertise. Developing regions are of substantive interest to vitamin D researchers by virtue of their unique determinants to vitamin D intake and status deficiencies, and their patterns of vitamin D-related deficiency disorders.[8]

In studying the epidemiology of vitamin D, Mongolia is important in that it is the highest-latitude developing

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country in the world[9] and because it contains a confluence of environmental and infrastructural factors which predispose its population to an extremely high risk of vitamin D deficiency. Mongolia's Fourth National Nutrition Survey determined 42.4% of children under 5 and 52.2% of reproductive-age women had 25-hydroxyvitamin D (25(OH)D) serum concentrations below 25 nmol/L (indicating deficiency) in September, and 18.3% of children showed at least one sign of rickets.[10] Of particular relevance to vitamin D in other industrializing populations are Mongolia's high rate of urbanization and Mongolians' increasingly sedentary lifestyles, which are associated with decreased sun exposure and vitamin D biosynthesis.[11] Regarding epidemiologic assessment of vitamin D, ongoing research by our group has found that the low vitamin D status incurred by most of the Mongolian population results in markedly low between-subject variation, particularly in winter, which renders assays less capable of discriminating between individuals. Mongolia does not have wide-scale experience with less expensive (non-reference) methods for vitamin D measurement, and it is a question as to how well such methods would perform.

This study set out to assess the performance of a non-reference ("test") method at the population level for the first time in Mongolia, to characterize its usefulness and suggest recommendations for other vitamin D surveys in similar regions. The test method evaluated in this study was Immunodiagnostic Systems (IDS) enzyme-linked immunosorbent assay (ELISA). The IDS ELISA kit is a simple and inexpensive manual assay for measuring serum 25(OH)D concentration, comprising 6.4% of assays used by participants in the April 2013 and 2014 cycles of DEQAS (Vitamin D External Quality Assessment Scheme).[12] We compared ELISA to the DiaSorin LIAISON 25(OH)D TOTAL automated immunoassay, the most popular assay within DEQAS, and present statistics as to LIAISON's own validity in this study based on our participation in the DEQAS program.

MATERIALS AND METHODS

Study population

The study population consisted of 120 healthy, non-pregnant, free-living Mongolian adults aged 20-57. Subjects were equally distributed by residence between 3 regions (the capital city of Ulaanbaatar, the southern desert province of Omnogobi, and the north-central province of Bulgan). Within each region, 20 subjects were employed in indoor occupations (primarily office workers) and 20 were employed outdoors (outdoor laborers from Ulaanbaatar, and full-time nomads from Omnogobi and Bulgan). Within each of these groups of 20 subjects, 10 were males and 10 were females. Subjects provided written informed consent to enroll in the study. The study's methodology received approval from the Mongolian Ministry of Health ERB and Harvard School of Public Health IRB.

Vitamin D measurement

During each of two periods - June to August 2011 and January to March 2012 – 8 mL of blood was drawn from each subject. Aliquots were stored in a portable freezer and transported to Ulaanbaatar where they were stored in a deep freezer until analysis. One aliquot for each subject from each season was thawed and analyzed for 25(OH)D using Immunodiagnostic Systems ELISA at the Erhes Laboratory of the Mongolian National University of Medical Sciences, Ulaanbaatar. A second aliquot was assayed at Bayangol Medical Center, Clinical Laboratory in Ulaanbaatar using the DiaSorin LIAISON 25(OH)D TOTAL automated immunoassay platform. See Wallace et al[6] for details about these assays.

Validation of reference assay

To validate LIAISON as a reference method in this study, the laboratory at Bayangol participated in the internationally-recognized Vitamin D External Quality Assessment Scheme (DEQAS).[13] Forty serum samples were sent to Bayangol, analyzed by LIAISON, and the concentrations were compared with the DEQAS all lab trimmed mean (ALTM) for each sample. ALTMs for twenty DEQAS samples were in turn validated against National Institute of Standards and Technology (NIST) reference measurement procedures.

Statistical analysis

Mean and standard deviation of serum 25(OH)D concentrations in both seasons and for both assays were calculated, and significant differences were assessed between assays by season. For each season, the paired difference and percent difference between assay measurements was calculated for each subject and averaged over all subjects to obtain seasonal mean paired differences, for which confidence intervals were estimated. Pearson's correlation, Spearman's rank correlation, and Lin's concordance correlation coefficient[14] between assay results were also calculated by season, as well as the number of ELISA measurements over- and underestimating their paired LIAISON measurement. To address deviations from normality, Pearson's correlation was also calculated after values were natural log-transformed. Bland-Altman analysis was used to assess proportional bias in ELISA measurements as compared to LIAISON, in which paired differences in the two assays' measurements were plotted and regressed against their corresponding means.[15]

For each of the two assays and within each season, subjects were categorized according to vitamin D sufficiency categories: optimal (serum 25(OH)D concentration $\geq$75 nmol/L), adequate ($\geq$50 and <75), inadequate ($\geq$25 and <50), and deficient (<25).[16] In each season, the number and percent of total subjects assigned to each sufficiency category were cross-tabulated between assays to calculate percent agreement as well the percent of subjects for which ELISA assigned a sufficiency category higher or lower than that of LIAISON.

Using the performance data provided to us by DEQAS, additional statistics were calculated to characterize the validity of LIAISON against DEQAS all lab trimmed means (ALTMs), and to compare DEQAS ALTMs against those obtained from NIST measurements of the same samples. Statistical analyses were performed using SAS v9.4 and R v3.3.1.

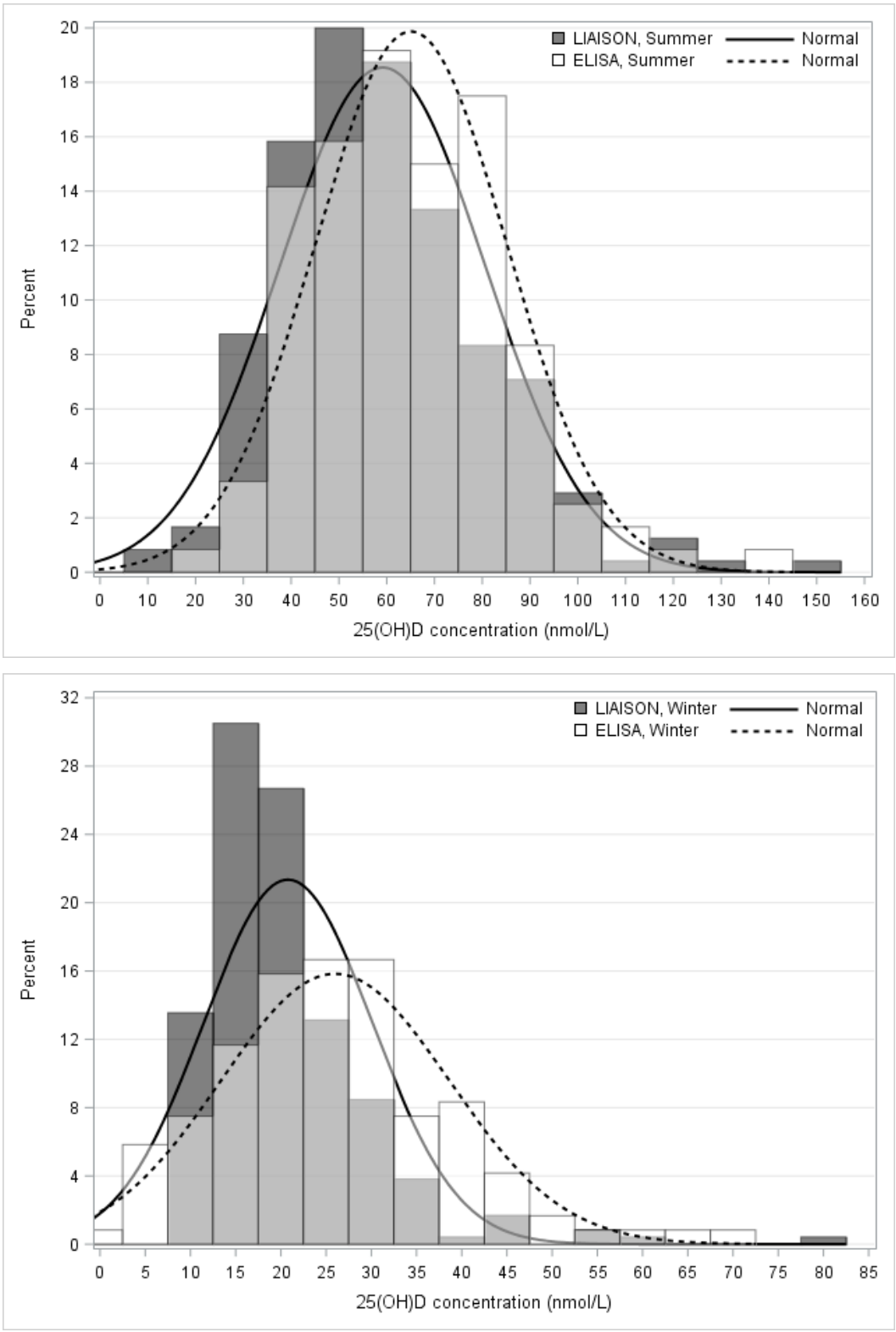

Figure 1. Comparison of ELISA and LIAISON 25(OH)D measurement distributions by season.

RESULTS

LIAISON measurements for 3 subjects fell short of the assay's minimal detection limit of 4 ng/mL (10 nmol/L); in statistical analyses, these measurements were rounded to 3.9 ng/mL (9.7 nmol/L). This was not expected to materially affect the results. No measurement for either assay was missing or exceeded the maximum detection limit. One subject with a relatively high (>125 nmol/L) ELISA measurement was detected in summer (ELISA: 141.5 nmol/L, LIAISON: 54.2 nmol/L), and two other subjects with >62.5 nmol/L ELISA measurements in winter (ELISA: 71.1 nmol/L, LIAISON: 47.4 nmol/L, and ELISA: 64.6 nmol/L, LIAISON: 30.7 nmol/L). A fourth subject had a relatively high LIAISON measurement in summer (LIAISON: 134.3 nmol/L, ELISA: 87.1 nmol/L). Outlying subjects were retained in statistical analyses to present a more realistic picture of validity under actual study conditions. Sixteen subjects (13%) reported taking some

Table 1. Comparison of ELISA and LIAISON measurement distributions by season[†]

Season	Assay	Mean (SD)[‡]	Difference in means (*p*)[‡§††]	Variance ratio (*p*)[¶††]
Summer	ELISA	65.1 (20.1)	7.0 (0.012)	0.79 (0.20)
	LIAISON	58.1 (22.6)		
Winter	ELISA	26.0 (12.6)	5.2 (<0.001)	1.56 (0.007)
	LIAISON	20.8 (9.8)		

[†]n=120 samples per assay per season.
[‡]Values given in units of nmol/L.
[§]Mean of ELISA measurements – mean of LIAISON measurements.
[¶]Variance of ELISA measurements/variance of LIAISON measurements.
[††]*p* values are associated with differences in inter-assay means and variances within the same seasons, and are drawn from two-sided independent samples t tests and F tests of equality of variance, respectively.

Table 2. Comparison of paired ELISA and LIAISON measurements by season[†]

Metric	Summer	Winter
Mean paired difference (95% CI)[‡§]	7.0 (3.5-10.5)	5.2 (2.9-7.5)
Mean paired percent difference (95% CI)[¶]	21.2 (14.0-28.3)	37.8 (25.4-50.3)
Pearson's correlation	0.60	0.37
Pearson's correlation (ln-transformed)	0.65	0.38
Spearman's rank correlation	0.67	0.43
Lin's concordance correlation coefficient	0.56	0.33
Lin's bias correction factor	0.94	0.88
Proportional bias (*p*)[‡††]	-0.15 (0.11)	0.36 (0.004)
n overestimating / n underestimating[‡‡]	78/42	87/33

[†]n=120 samples per assay per season.
[‡]Values given in units of nmol/L.
[§]Mean of paired ELISA – LIAISON differences.
[¶]Mean of (ELISA – LIAISON) / LIAISON * 100%.
[††]Drawn from the slope of the regression line ELISA-LIAISON = a + b * (ELISA+LIAISON)/2.
[‡‡]number of ELISA measurements over- or underestimating their paired LIAISON measurement.

kind of vitamin D-containing supplement (vitamin D, vitamin D + calcium, or multivitamin containing vitamin D) in summer, and 17 (14%) reported taking a vitamin D-containing supplement in winter.

The distribution of 25(OH)D concentration appeared approximately normal in summer for both assays (Figure 1), with moderate right skew. The distribution of both assays deviated more from normality in winter, particularly LIAISON. Compared to LIAISON, ELISA estimated a higher mean vitamin D status for the study population in both seasons, with a difference in means of 7.0nmol/L in summer (p=0.012) and 5.2 nmol/L in winter (p<0.001) (Table 1). Variance was smaller by 21% (p=0.20) in summer and larger by 56% in winter (p=0.007).

Pearson's correlation, Spearman's rank correlation, and Lin's concordance correlation coefficient between paired assay measurements were higher in summer (0.60, 0.67, and 0.56, respectively) than in winter (0.37, 0.43, 0.33) (Table 2). The 141.5 nmol/L ELISA outlier in summer

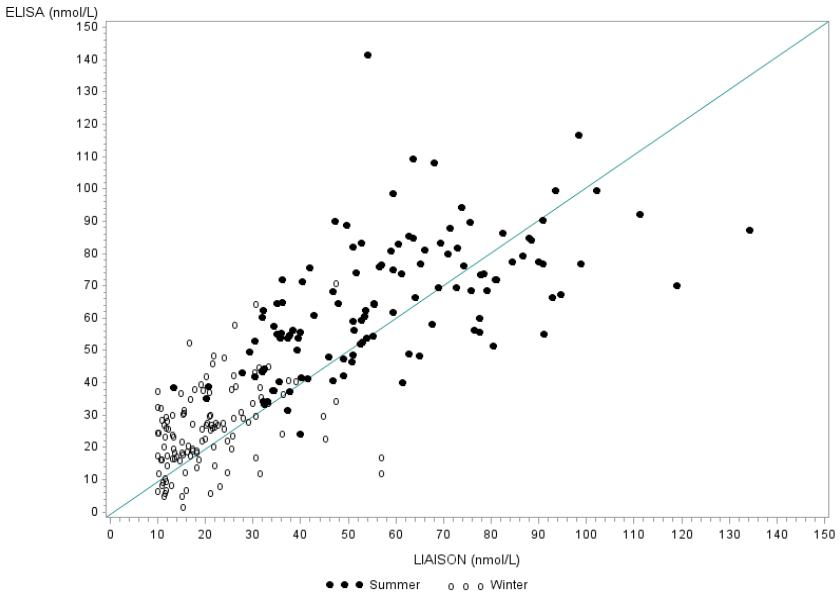

Figure 2. Comparison of paired ELISA and LIAISON 25(OH)D measurements by season.

Table 3. Categorical agreement between paired ELISA and LIAISON measurements by season[†‡]

		LIAISON				
		Deficient	Inadequate	Adequate	Optimal	Total
Summer[§]						
ELISA	Deficient	0	1	0	0	1
	Inadequate	3	20	5	0	28
	Adequate	0	20	18	14	52
	Optimal	0	3	21	15	39
	Total	3	44	44	29	
Winter[¶]						
ELISA	Deficient	49	6	2	0	57
	Inadequate	39	20	0	0	59
	Adequate	1	3	0	0	4
	Optimal	0	0	0	0	0
	Total	89	29	2	0	

[†]n=120 samples per assay per season.
[‡]Values indicate number of samples within a particular sufficiency category.
[§]Summer: 44% agreement, 39% ELISA overestimating, 17% ELISA underestimating.
[¶]Winter: 58% agreement, 36% ELISA overestimating, 7% ELISA underestimating.

did not appear to greatly influence correlation coefficients in summer in that the paired LIAISON measurement was near to the assay's seasonal grand mean. In summer and winter, 78 and 87 of 120 ELISA measurements exceeded their paired LIAISON measurement, respectively. Figure 2 provides a graphical comparison of individuals' assay measurements for each season, in which each subject's paired assay concentrations are plotted against one another. Bland-Altman plots of ELISA-LIAISON vs (ELISA+LIAISON)/2 indicated a positive proportional bias in winter (β=0.36, p=0.0042) and no statistically significant proportional bias in summer (Figure 3).

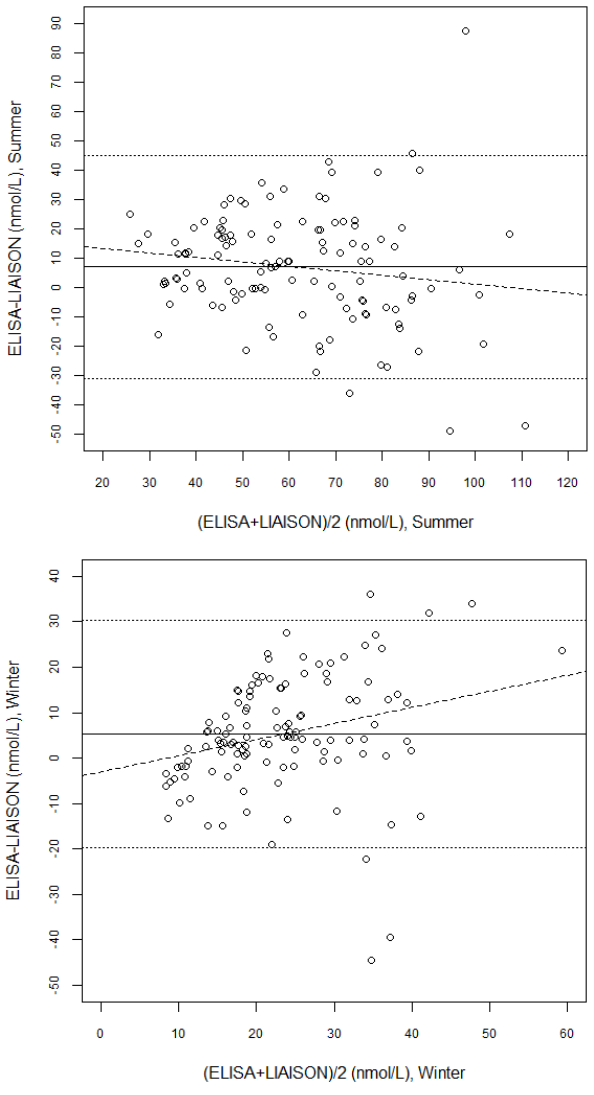

Figure 3. Bland-Altman comparison of paired ELISA and LIAISON 25(OH)D measurements by season.

In assigning vitamin D sufficiency categories, percent agreement between the assays was calculated at 44% in summer and 58% in winter (Table 3). In summer, ELISA assigned a sufficiency category one higher than that assigned by LIAISON in 44 subjects and two higher in 3 (in total, 39% of measurements were assigned higher categories), and assigned a sufficiency category one lower than that of LIAISON in 20 subjects (17%). In winter, ELISA assigned a sufficiency category one higher in 42 subjects and two higher in 1 (36%), and assigned a sufficiency category one lower in 6 subjects and two lower in 2 (7%).

LIAISON analysis of 34 of the 40 DEQAS samples fell within 25% of the all laboratory trimmed mean, satisfying the 80% standard required for certification. Pearson's correlation between paired LIAISON and DEQAS measurements was 0.92, root mean square error 10.1 nmol/L, mean absolute error 7.7 nmol/L, and mean error 1.4 nmol/L, the latter indicating LIAISON tended to slightly overestimate DEQAS values (26 samples overestimated and 14 samples underestimated). Of the 8 batches of 5 paired LIAISON and DEQAS samples, 2 incurred mean systematic error >7.5 nmol/L; because one of these batches tended to overestimate and the other to underestimate, their combined influence on overall mean error was small. A graphical comparison between the LIAISON and DEQAS measurement for each sample pair is presented in Figure 4, grouped by batch. Twenty of the 40 DEQAS samples were themselves validated against NIST reference measurement procedures, yielding a correlation of 0.983 and a mean percent difference of -2.3% (results not shown).

DISCUSSION

Here we provide a real-world example of validating population-based measurements of vitamin D status in a

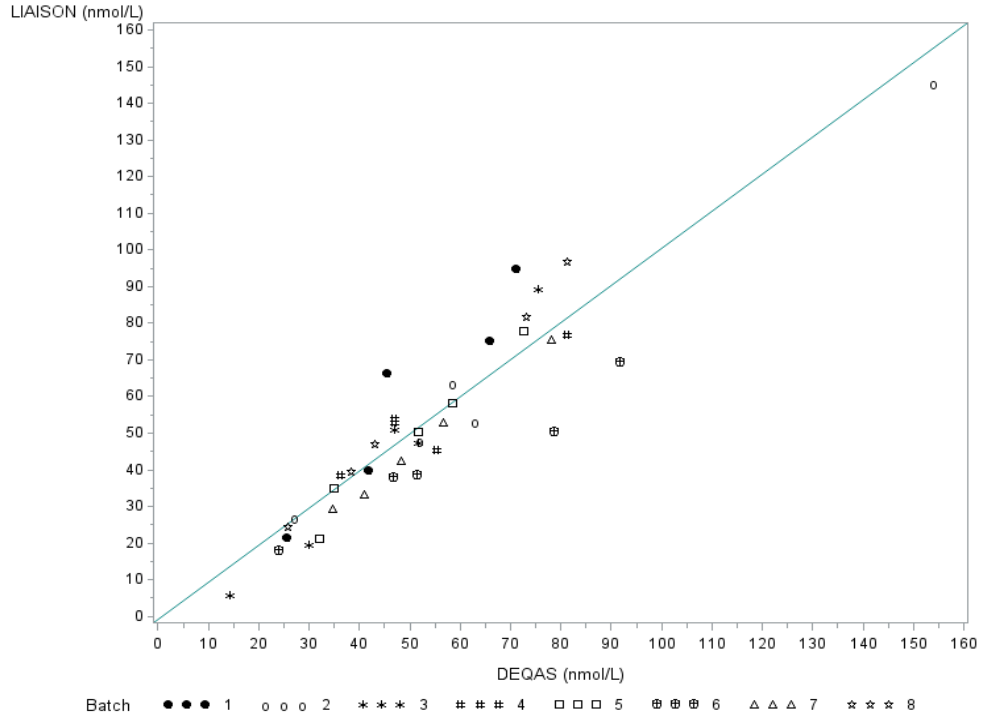

Figure 4. Comparison of paired LIAISON and DEQAS 25(OH)D measurements by DEQAS batch.

developing country where status is extremely low. This study marks the first application of an external quality control to validate vitamin D measurements in Mongolia (DEQAS), the first DEQAS certificate to be awarded in Mongolia, and the first population-based application of a non-reference vitamin D assay method in Mongolia. To our knowledge, no previous published study has attempted to validate a biochemical assessment of vitamin D status in a developing country with such a high prevalence of vitamin D deficiency. Given that ELISA is one of the most economical choices for epidemiologic assessment, its performance will be important to characterize in the future, particularly in developing regions where more expensive assays are less widely available.

IDS does not provide a functional sensitivity for its 25(OH)D ELISA; given the lower mean paired difference between ELISA and LIAISON measurements observed during winter, decreased correlation between assays in winter would appear attributable to a decline in observable variation rather than assay accuracy at extremely low concentrations. Smaller variance at lower concentrations was apparent in a prior population study comparing an automated IDS ELISA platform and DiaSorin RIA in the British Isles,[17] and is especially clear in Mongolia where most subjects are deficient in winter (mean observed winter serum concentration=20.0 nmol/L). These findings reinforce the notion that the expected concentration of 25(OH)D in a study population should be considered before using ELISA, particularly in settings or periods in which variation is small in proportion to the seasonal mean, as this will have implications for study design. At least inasmuch as long-term vitamin D status is of primary interest, and that summer and winter status are moderately correlated (r=0.47 in this population), studies attempting to derive an exposure-disease relationship in highly deficient populations may be most practical to conduct during summer (if multiple measurements are not possible). On the other hand, a lower mean-to-variance ratio in winter will not bias descriptive measures of mean status or prevalence of deficiency.

Descriptive measures will be affected by systematic error, however. Investigators comparing manual or automated ELISA with LC/MSMS[18,19] and DiaSorin RIA[17] platforms in serum samples with higher mean 25(OH)D concentrations have reported ELISA to overestimate at lower concentrations and underestimate at moderate concentrations (at higher concentrations, some studies have found ELISA positively biased in comparison to LC/MSMS[19] and DiaSorin RIA[20]). In this study, overestimation (as compared to LIAISON) was particularly pronounced given the low population mean; in fact, ELISA overestimated in both seasons, though less so in summer, suggesting underestimation would be increasingly evident were higher concentrations observable. That ELISA may tend to overestimate status in extremely deficient populations is important in that it may result in more conservative estimates of deficiency in regions where hypovitaminosis D already contributes significantly to disease burden. In such cases, measurements may be corrected according to their relationship with a reference method.[17] We note that this bias significantly affected the way subjects are categorized according to sufficiency categories, as has generally been noted in other settings.[21] Prior investigators[17] showed an automated ELISA platform to overestimate the prevalence of deficiency in the British Isles; given where Mongolia lies in terms of mean serum status, the effect is the opposite. This bias will not impact the ability to rank subjects on a continuous scale, however.

DiaSorin LIAISON TOTAL, while sometimes considered a reference method for 25(OH)D measurement and while validated in Mongolia by our participation in DEQAS, is not a gold standard assay. As with ELISA, LIAISON is subject to matrix effects and lot variability in

reagents.[6] Inference about ELISA's accuracy using LIAISON as a reference is therefore conservative, in that error observed in ELISA measurements will be compounded by random and systematic error in those of LIAISON. In our own comparison with DEQAS, LIAISON exhibited what we considered substantial bias in 2 of the 8 validation batches; practically speaking, this implied a 25% likelihood of spuriously attributing systematic bias to ELISA in turn. This highlights a possible advantage of deliberately dividing analysis batches in an epidemiologic study, especially if using an automated platform, to reduce systematic error in the entire sample. An example of this is the fact that LIAISON exhibited little overall systematic bias during validation, despite bias incurred in two batches.

In conclusion, compared to DiaSorin LIAISON TOTAL, IDS ELISA proved useful for measuring and ranking subjects' vitamin D status in Mongolia during the summer, but levels were too low in winter to sensitively discriminate between subjects. ELISA tended to overestimate status in both seasons. These findings have important implications for the timing and interpretation, respectively, of vitamin D surveys in populations where severe deficiency is common. By considering these aspects, carefully standardizing experimental procedures, and avoiding delays during collection, processing, and analysis, the unique issues posed by the developing country settings to epidemiologic assessment of vitamin D may be ameliorated. We hope this evidence-based implementation research will help to increase the value of vitamin D surveys in developing countries, where they are arguably needed most and where less expensive test methods may be most practical. Such studies should compliment basic research to develop population-based assays geared toward improved portability and economy.

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AUTHOR DISCLOSURES

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