Comparison of four immunoassays to measure serum ferritin concentrations and iron deficiency prevalence among non-pregnant Cambodian women and Congolese children


doi: 10.1515/cclm-2016-0421
Received March 24, 2016; accepted May 25, 2016; previously published online June 23, 2016

Abstract

Background: Global standardization of ferritin assays is lacking, which could have direct implications on the accurate measurement and comparability of ferritin concentration and iron deficiency (ID) prevalence rates in at-risk populations.

Methods: We measured serum ferritin concentrations using four immunoassays: the s-ELISA and the AxSYM™ analyzer were compared among 420 non-pregnant Cambodian women; the Centaur® XP analyzer, s-ELISA, and AxSYM™ analyzer were compared among a subset of 100 Cambodian women; and the s-ELISA and the Elecsys® 2010 analyzer were compared among 226 Congolese children aged 6–59 months.

Results: Median ferritin concentrations (adjusted for inflammation) ranged between 48 and 91 μg/L among Cambodian women and between 54 and 55 μg/L among Congolese children. ID prevalence ranged from 2% to 10% among Cambodian women and 5% to 7% among Congolese children. Bias between methods varied widely (–9 to 45 μg/L) among women, and was 43 μg/L among children. Bias was lower when ferritin values outside of the s-ELISA measurement range (>250 μg/L) were excluded.

Conclusions: The observed differences in ferritin concentrations likely reflect different ferritin isoforms, antibodies, and calibrators used across assays and by different laboratories. However, despite differences in ferritin concentrations, ID prevalence was relatively similar and low across all methods.

Keywords: bias; Cambodia; concordance; Congo; ferritin; traceability.

Introduction

Iron deficiency (ID) is thought to affect over 2 billion people worldwide [1, 2] and can have severe consequences for women during pregnancy [3, 4] and for children’s early brain development and growth [5, 6]. The gold standard test for ID is the assessment of iron stores through a bone marrow aspirate [7, 8]; however, this is an invasive and painful test that is rarely used in practice to diagnose ID. Serum or plasma ferritin concentration is a more commonly measured biomarker reflecting the depletion of iron stores in the body [7]. Historically, immunoradiometric assays (using labeled antibodies) and radioimmunoassays (using labeled ferritin) were...
the primary methods of ferritin measurement [7]. Over the last few decades, automated immunoassay analyzers have been developed (e.g. Abbott AxSYM™), eliminating the need for immunoradiometric methods. In 2004, Erhardt et al. [9] developed a sandwich enzyme-linked immunosorbent assay (s-ELISA), which concurrently measures ferritin, C-reactive protein (CRP), α-1 acid glycoprotein (AGP), soluble transferrin receptor (sTfR; a measure of iron status reflecting the demand for iron in the body) and retinol binding protein (RBP; a measure of vitamin A status). This low-cost method has shown low intra- and inter-assay variability and high sensitivity [9]; as such, the method has become increasingly popular worldwide.

The quantification of ferritin concentration in these immunoassays is based on the detection of specific antibody binding [10]. However, many challenges in the traceability of this method have been identified, as laboratories differ in terms of which ferritin isoforms are measured (e.g. isoforms found in the liver are different from those in the spleen), the antibodies selected for ferritin detection, and the reference ranges established and utilized [11, 12]. In 1985, the World Health Organization (WHO) established the 1st International standard (IS) for ferritin (liver, 80/602) as a reference for methods to be calibrated against in an attempt to improve global traceability of methods [13]. Since then, the 2nd IS was released in 1993 (spleen, 80/578) [14] and more recently the 3rd IS in 1997 (recombinant, 94/572) [15]. An evaluation of the 3rd IS by 18 laboratories in nine countries showed adequate stability in accelerated degradation studies and acceptable traceability to the 2nd IS [16]. However, calibration to the 3rd IS is not globally mandated or monitored and reference ranges continue to differ across laboratories. Many laboratories are still tracing ferritin methods to the 1st and 2nd IS, despite the fact that these materials ceased production in the mid-1990s and have been since superseded.

This lack of standardization of ferritin assays could have direct implications on the accurate measurement and comparability of ferritin concentrations using different methods, and of greater concern, on ID prevalence rates in at-risk populations. The aim of this study was to measure serum ferritin concentrations and compare ID prevalence estimates using four different immunoassays with varying quality controls (QCs) and traceability. These include three automated immunoassay analyzers (Abbott AxSYM™, Siemens ADVIA Centaur®, and Roche Elecsys®) and Erhardt’s s-ELISA in two groups of individuals: Cambodian women of reproductive age and Congolese children.

Materials and methods

Study design and participants

This method-comparison study included data collected from baseline surveys in two trials: n=420 non-pregnant women of reproductive age (18–45 year) from Prey Veng province, Cambodia, who were selected for inclusion in a larger trial evaluating the outcomes of an improved homestead food production and aquaculture project [17]; and n=226 children (6–59 month) representative sampled from South Kivu and Bas Congo provinces, Democratic Republic of the Congo (DRC), as part of a larger micronutrient survey [18]. Work was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Ethics approval was obtained from the University of British Columbia (Canada), the Université de Kinshasa (DRC), the Université Catholique de Bukavu (DRC), and the National Ethics Committee for Health Research (Cambodia). All participants (or caregivers on behalf of the children) provided written informed consent to participate in the studies.

Blood collection

In Cambodia, a 3-h fasting venous blood sample was collected from women at health centers in Prey Veng in June 2012 by trained phlebotomists from the National Institute of Public Health Laboratory (NIPHL). Blood was collected in a trace-element free 3.5 mL tube (Becton Dickinson), allowed to clot, placed indirectly on ice, and transported daily to NIPHL in Phnom Penh. In the DRC, an overnight fasting venous blood sample was collected from children from health centers in South Kivu and Bas Congo provinces between June and October 2014 by trained phlebotomists. Blood was collected in a trace-element free 8 mL tube (Becton Dickinson), allowed to clot, placed indirectly on ice, and transported to the primary health center for processing. Samples from both Cambodia and DRC were centrifuged within 2–4 h from the time of collection. Serum was separated and aliquoted into cryovials for storage at −80 °C until shipment to the University of British Columbia in Vancouver, Canada, where it was again stored at −80 °C until secondary shipment to respective laboratories for further analysis. During transport, samples were shipped on dry ice and thawed only at the time of sample analysis. Analyses for the s-ELISA were conducted within 2 months of sample collection for the Cambodia study and within 4 months for the DRC study. Analyses for the Abbott AxSYM™ were conducted within 6 months of sample collection for the Cambodia study, within 11 months for the Siemens ADVIA Centaur®, and for Roche Elecsys® 2010 for the DRC study.

Laboratory analyses

Serum ferritin concentration was analyzed using a total of four different methods in four different laboratories. A summary of the methods and the respective laboratories where assays were conducted is provided in Supplementary Table 1.
Data analyses

Serum ferritin concentration (μg/L) is presented as median (IQR). The prevalence of ID in each group was determined using the cut-offs for non-pregnant women of reproductive age (ferritin <15 μg/L) and children (ferritin <12 μg/L) [7]. We present results for unadjusted serum ferritin and inflammation-adjusted serum ferritin (adjusted for levels of inflammation based on CRP and AGP biomarkers using correction factors suggested by Thurnham et al. [19]). CRP and AGP concentrations were measured using Erhardt’s s-ELISA in both the Cambodia and Congolese samples.

We calculated Bland and Altman’s [20] bias (agreement) and limits of agreement, and plotted the clinical measurements of serum ferritin concentrations. Bias was defined as the difference in means between the two measures of ferritin concentration (μg/L) and was reported as the mean±SD. The limits of agreement (95% confidence intervals of the bias) were reported as ±1.96 SD. Calculation of the limits of agreement is based on the assumption that the differences between the methods are normally distributed [21]. This assumption was tested and confirmed before proceeding with the statistical test. Limit of agreement plots provide a visual comparison of discrepancies between two methods (bias), the width of the limits of agreement, and also show potential trends in the data (consistency of the variability). If the bias was small and the limits of agreement were narrow (considering clinical significance) between any two methods, those two methods were then interpreted as equivalent [20, 22]. These methods are in concordance with the 2013 Clinical and Laboratory Standards Institute measurement procedure guidelines for method comparison studies [23], with the exception that not all values were measured in duplicate. Only for the s-ELISA were values measured in duplicate and the mean of two independent measurements reported.

We used Lin’s [24] concordance correlation coefficient (ρc) to measure the reproducibility between two measured values (the departure of the measured values from a 45-degree line). Pearson’s correlation coefficient (r) was also reported for interest of comparison; however, we acknowledge that it only measures the strength of the association and fails to detect the agreement between values [20].

We used all available data for the primary analyses (bias and concordance) and also conducted sub-group analyses using only those measurements that fell within the measurement range of the defined laboratory/method. As such, in the sub-group analyses we excluded all ferritin measurements that exceeded 250 μg/L in the s-ELISA method (Cambodian: n=13; Congolese n=17).

Statistical analysis

Stata version SE/13.1 for Mac (Stata Corp, College Station, TX, USA) was used to conduct statistical analyses.

Results

Intra-assay variation among methods

The intra-assay CV for ferritin was 3.3% using Erhardt’s s-ELISA (US Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) QC controls and BioRad (Hercules, USA) Liquichek™ immunoassay controls), and was 2.6% using the Elecsys® 2010 method (Roche Elecsys® PreciControl Varia controls). The intra-assay CV data for the Abbott AxSYM™ and ADVIA Centaur® XP methods were not available.

Serum ferritin concentrations and ID prevalence across methods

Table 1 presents the median (IQR) unadjusted and adjusted serum ferritin concentrations and the prevalence of ID among non-pregnant Cambodian women

<table>
<thead>
<tr>
<th>Participants and methods</th>
<th>Unadjusted ferritin concentration, μg/L</th>
<th>Adjusted ferritin concentration, μg/L</th>
<th>Iron deficiency prevalencea n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambodian women (n=420)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-ELISA</td>
<td>90.8 (59.0, 142.6)</td>
<td>84.2 (52.6, 126.3)</td>
<td>9 (2.1)</td>
</tr>
<tr>
<td>AxSYM™</td>
<td>52.0 (53.8, 73.9)</td>
<td>47.6 (31.3, 65.9)</td>
<td>28 (6.7)</td>
</tr>
<tr>
<td>Subset of Cambodian women (n=100)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-ELISA</td>
<td>92.1 (57.9, 147.8)</td>
<td>91.0 (53.8, 125.6)</td>
<td>5 (5.0)</td>
</tr>
<tr>
<td>AxSYM™</td>
<td>53.8 (33.8, 77.2)</td>
<td>51.4 (31.0, 66.9)</td>
<td>10 (10.0)</td>
</tr>
<tr>
<td>Centaur® XP</td>
<td>57.4 (37.7, 91.2)</td>
<td>51.5 (34.8, 85.8)</td>
<td>6 (6.0)</td>
</tr>
<tr>
<td>Congolese children (n=226)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-ELISA</td>
<td>68.4 (34.2, 142.2)</td>
<td>55.4 (27.8, 96.1)</td>
<td>16 (7.1)</td>
</tr>
<tr>
<td>Elecsys® 2010</td>
<td>70.1 (34.9, 153.3)</td>
<td>53.8 (27.0, 98.1)</td>
<td>12 (5.3)</td>
</tr>
</tbody>
</table>

*aFerritin concentrations adjusted for levels of inflammation using correction factors proposed by Thurnham et al. [19]. bBased on ferritin <15 μg/L for non-pregnant women or <12 μg/L for children. IQR, interquartile range (25th percentile, 75th percentile).
Bias and concordance between methods

Table 2 presents the bias (difference in means), limits of agreement, and the Pearson's and concordance correlation coefficients (95% CI) among the groups. We confirm the differences in methods were normally distributed among all groups, which is an assumption required to be met for the calculation of the limits of agreement [21]. Method comparison analyses were conducted on unadjusted ferritin concentrations. Bias between methods was large and varied from -35 to 45 μg/L for all method comparisons, with the exception of the AxSYM™ and Centaur® XP comparison among the subset of n=100 Cambodian women. In this latter comparison, bias was only -9 μg/L and the concordance coefficient (95% CI) was very high, indicating near perfect agreement [0.96 (0.94, 0.97)] between the AxSYM™ and Centaur® XP methods.

Visual interpretation of the limits of agreement provided additional observations. Figure 1A–E shows Bland limits of agreement plots for the five comparisons. Among all 420 Cambodian women, high bias was observed among the s-ELISA and the AxSYM™ method (Figure 1A), as the reduced major axis (fitted line) was not aligned to the line of perfect concordance. Similarly, this was also shown among the subset of n=100 Cambodian women between the s-ELISA and the AxSYM™ method (Figure 1B) and s-ELISA and the Centaur® XP method (Figure 1C). However, in the same subset of n=100 Cambodian women, the AxSYM™ and Centaur® XP showed very high agreement (Figure 1D). Among the 420 Cambodian women, there were n=13 measurements of ferritin concentration exceeding 250 μg/L in the s-ELISA method (the upper limit of the method’s measurement range) and n=2 measurements exceeding 250 μg/L in the AxSYM™ method. When the 13 highest ferritin measurements (as measured using the s-ELISA method) were excluded from the dataset, the bias between these two methods among the remaining n=407 decreased slightly from 45.4 to 44.0 μg/L and concordance (95% CI) decreased from 0.62 (0.58, 0.66) to 0.53 (0.49, 0.57). Concordance plots are presented in Supplementary Figure 1.

Among the 226 Congolese children, the s-ELISA method and the Elecsys® 2010 method showed poor concordance (Table 2) and poor agreement (Figure 1E). An overall bias of -43 μg/L was detected, and the concordance coefficient was low, further indicating that the two methods did not produce similar results. Of note, there were n=17 measurements of ferritin concentration exceeding 250 μg/L in the s-ELISA method (the upper limit of the method’s measurement range) and n=29 measurements exceeding 250 μg/L in the Elecsys® 2010

Table 2: Bias, limits of agreement, and correlation coefficients among non-pregnant Cambodian women (18–45 year) and Congolese children (6–59 month) using different methods of measurement.

<table>
<thead>
<tr>
<th>Participants</th>
<th>Bias (difference in means) mean μg/L± SD</th>
<th>Limits of agreement μ± 1.96 SD</th>
<th>Pearson’s coefficient r</th>
<th>Concordance coefficient (95% CI) ρ, (μ±1.96 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cambodian women</td>
<td></td>
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<tr>
<td>s-ELISA vs. AxSYM™ (n=420)</td>
<td>45.4±28.6</td>
<td>-10.7, 101.6</td>
<td>0.94</td>
<td>0.62 (0.58, 0.66)</td>
</tr>
<tr>
<td>s-ELISA vs. AxSYM™ (n=407)</td>
<td>44.0±27.1</td>
<td>-9.1, 97.2</td>
<td>0.93</td>
<td>0.53 (0.49, 0.57)</td>
</tr>
<tr>
<td>Subset of Cambodian women</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>s-ELISA vs. AxSYM™ (n=100)</td>
<td>43.2±27.5</td>
<td>-10.7, 97.1</td>
<td>0.94</td>
<td>0.69 (0.62, 0.75)</td>
</tr>
<tr>
<td>s-ELISA vs. Centaur® XP (n=100)</td>
<td>34.6±25.6</td>
<td>-15.5, 84.8</td>
<td>0.93</td>
<td>0.78 (0.72, 0.84)</td>
</tr>
<tr>
<td>AxSYM™ vs. Centaur® XP (n=100)</td>
<td>-8.6±12.8</td>
<td>-33.7, 16.6</td>
<td>0.98</td>
<td>0.96 (0.94, 0.97)</td>
</tr>
<tr>
<td>Congolese children</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s-ELISA vs. Elecsys® 2010 (n=226)</td>
<td>-42.6±194.8</td>
<td>-424.4, 339.2</td>
<td>0.76</td>
<td>0.45 (0.40, 0.50)</td>
</tr>
<tr>
<td>s-ELISA vs. Elecsys® 2010 (n=209)</td>
<td>-11.5±29.8</td>
<td>-69.8, 46.9</td>
<td>0.96</td>
<td>0.91 (0.89, 0.93)</td>
</tr>
</tbody>
</table>

*Bias and concordance were assessed using unadjusted ferritin concentrations. *Excluding n=13 ferritin values exceeding 250 μg/L in the s-ELISA method (outside of the defined measurement range). **Excluding n=17 ferritin values exceeding 250 μg/L in the s-ELISA method (outside of the defined measurement range).
When the 17 highest ferritin measurements (as measured using the s-ELISA method) were excluded from the dataset, the agreement and concordance between these two methods improved substantially. Among the n = 209 children remaining in the dataset, the overall bias decreased from −42.6 to −11.5 μg/L and the concordance coefficient (95% CI) increased from 0.45 (0.40, 0.50) to 0.91 (0.89, 0.93).

Figure 1: Limits of agreement plots. (A) n = 420 Cambodian women using the s-ELISA and AxSYM™; (B) a random subset of n = 100 Cambodian women using the s-ELISA and AxSYM™; (C) a random subset of n = 100 Cambodian women using the s-ELISA and Centaur® XP; (D) a random subset of n = 100 Cambodian women using the AxSYM™ and Centaur® XP; and (E) n = 226 Congolese children using the s-ELISA and Elecsys® 2010.
Discussion

The observed differences in ferritin concentration in our study are likely a reflection of the different ferritin isoforms, antibodies, and calibrators used in the immunoassays and across the different laboratories. However, despite these differences, no major differences in ID prevalence were observed across methods in our study, in which the samples of women and children had unexpectedly low ID prevalence. Further research is warranted to ascertain if our findings are replicable in populations with higher prevalence rates of ID.

There were n=6 measurements of ferritin concentration that exceeded 750 μg/L using the Elecsys® 2010 method among the Congolese children. No measurements exceeded 750 μg/L using the s-ELISA method either among the Congolese children or among the Cambodian women. We note that we did not pre-define nor exclude outliers from our original dataset; however, we query whether or not these high measurements were perhaps extreme outliers. It seems unlikely that children in this age group would have such a high ferritin concentration, even if they had a condition such as hemochromatosis or thalassemia. Regardless, the exclusion of the highest 17 ferritin concentrations from the dataset (which also included the exclusion of those six high values >750 μg/L as measured by the Elecsys® 2010 method) did not substantially change the ID prevalence across groups. Overall, ferritin concentrations were relatively high among Cambodian women and Congolese children. The lack of differences in ID prevalence, despite the differences in ferritin concentrations, may be attributed to this. If ferritin concentrations were lower (closer to the 15 μg/L and 12 μg/L cut-offs for women and children, respectively [25]), we suspect it may have resulted in more substantial differences in ID prevalence rates across groups.

Traceability varied among methods with none of our analyses directly traceable to the WHO 3rd IS for ferritin. The Abbott AxSYM™ and Roche Elecsys® 2010 analyzer were indirectly traceable to the WHO 1st IS, the Siemen ADVIA Centaur® XP was directly traceable to the WHO 2nd IS, and Erhardt’s s-ELISA was traceable to the serumbased CDC pools, which are assigned concentrations using the Roche Elecsys® assay using the E170 analyser. This issue poses challenges to the interpretation and comparability of results between assays and likely contributes to the differences observed among methods.

The s-ELISA has a unique advantage over the other automated analyzers, as ferritin, CRP, AGP, sTfR and RBP concentrations can concurrently be measured in one small sample of serum or plasma. This is useful as ferritin is increased and RBP is decreased in the presence of inflammation [19]. As such, the WHO advises the correction of ferritin and RBP concentrations for levels of inflammation using CRP and/or AGP biomarkers [7, 25]. Hence, this method allows for comprehensive assessment and interpretation of population-level vitamin A and iron status. Further, this method requires only a small amount (30 μL) of serum or plasma. This allows the convenient and economical option to collect capillary rather than venous blood, which is useful for blood collection in remote locations and in populations where venous blood collection is difficult (e.g. young children). These considerations have contributed to the increasing popularity of this method in low-resource settings and in large study populations at risk for ID.

The QCs for the s-ELISA are selected with the objective to accurately diagnose ID with higher sensitivity at lower ferritin concentrations (personal communication, Juergen Erhardt). Thus, caution is warranted in the interpretation of ferritin measurements exceeding ~250 μg/L using this method. In fact, the lab recommends exclusion of ferritin values above ~250 μg/L due to the decreased accuracy of measurement at these higher values (personal communication, Juergen Erhardt). We concur that analytical accuracy is most important at the lower range in consideration of accurate ID diagnosis, however, it is also important at the higher range to measure and monitor conditions of iron overload in individuals with genetic hemoglobin disorders, or other conditions associated with high ferritin concentrations (e.g. inflammation, infection, and/or hemochromatosis). For example, the β-thalassemia/ hemoglobin EE genotype is prevalent in areas of South-east Asia and individuals with this genotype have severe anemia and often require regular blood transfusions, resulting in iron overload [26]. More research is needed to evaluate the accuracy of ferritin measurement at higher ranges using these methods (as a biomarker of iron overload).

Limitations of our study

We acknowledge some limitations. First, none of the four methods in our study were directly traceable to the WHO 3rd IS. Method comparison studies ideally assay a set of specimens against a candidate reference material. As such, we cannot determine which of the four methods in our study was most accurate. We note that the aim of our study was to simply compare median ferritin concentrations and the prevalence of ID among the four immunoassays in populations thought to be at risk. Second,
not all four of the methods were used to measure ferritin concentrations among all groups. This would have provided a more thorough comparison. However, these automated assays are expensive and we did not have the funds to conduct analyses on all available samples. We recognize that reference ranges for different laboratories may be established based on the types of samples typically received and analyzed. For example, the laboratory in Germany (s-ELISA) receives the majority of samples from children and women living in developing countries under very different geographical and socioeconomic circumstances than individuals whose samples are analyzed by the laboratory in Vancouver, Canada, which receives the majority of samples from the Canadian population. Lastly, we used Thurnham et al. [19] correction factors to adjust for levels of inflammation in our populations studied. These correction factors were demonstrated to be accurate for use in the same Cambodian population of n=420 women in this study (which also used the s-ELISA to measure the inflammation biomarkers used for adjustments) [27], however, the robustness of these correction factors for use with the other ferritin assays has not yet been established.

Conclusions

Accurate diagnosis and treatment of ID are important, especially for pregnant women and young children [3–5]. However, overestimations of ID prevalence can result in unnecessary iron supplementation, which also can be harmful to some individuals [28]. Therefore, more work on the global standardization of ferritin assays and reference materials is warranted. It is well known that traceability is an essential component of laboratory medicine and for methods to be comparable they must be evaluated against an established reference material [29]. Mandating the use of the WHO 3rd IS for calibration and the use of standard controls may help to reduce the variability in assay results across laboratories and methods. The matrix of the calibrators can influence the results; hence, it may be justified to establish global controls for each matrix given the proposed type of sample for analysis. The WHO, in collaboration with the CDC, has recently commissioned two systematic reviews to investigate the diagnostic accuracy of ferritin as an indicator of not just deficiency but also of iron overload, and to compare laboratory assays for the determination of ferritin concentration [12]. We anticipate that the findings from the WHO work will play a critical role in the harmonization of ferritin assays to accurately assess ID and iron overload, in order to guide effective nutrition and public health policy globally.

Acknowledgments: We thank the following individuals for their contributions: Juergen Erhardt, VitMin Laboratory, Willstaett, Germany; Diane Kempton, LifeLabs Inc., Burnaby, Canada; Sheila Innis and Roger Dyer, The Child and Family Research Institute, Vancouver, Canada; Tze Lin Chai, The University of British Columbia, Vancouver, Canada; and Ame Stormer and Sokhoing Ly, Helen Keller International, Phnom Penh, Cambodia.

Author contributions: C. K. and T. G. designed the research; C. K. drafted the protocol and T. G. revised the protocol to its final stage. L. H. and K. B. assisted with laboratory analyses in New Zealand. C. K. conducted the statistical analysis. T. G. provided oversight and input into all aspects of the study. C. K. drafted the research manuscript. All authors contributed to the data interpretation and the revision of the manuscript to its final stage. C. K. and T. G. had primary responsibility for the final content. All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: The project in Cambodia was undertaken with support from the International Development Research Centre (IDRC), www.idrc.ca, the Government of Canada, provided through Foreign Affairs, Trade and Development Canada (DFATD). The project in the DRC was undertaken with support from HarvestPlus. C. K. received a Vanier scholarship from the Canadian Institutes of Health Research (CIHR) and a doctoral research award from the IDRC.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organizations played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References


Supplemental Material: The online version of this article (DOI: 10.1515/cclm-2016-0421) offers supplementary material, available to authorized users.