Analytical performance and clinical utility of a direct LDL-cholesterol assay in a hyperlipidemic pediatric population

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This study compares a new latex immunoseparation method for the direct determination of plasma low-density lipoprotein cholesterol (LDL-C) with the reference procedure for LDL-C (β-quantification) in a pediatric hyperlipidemic population. The direct LDL-C assay has a mean bias of −98 mg/L in a fasting group (n = 96) of patients (mean triglycerides 1057 ± 720 mg/L) and a bias of +177 mg/L in a nonfasting group (n = 42, mean triglycerides 4854 ± 5457 mg/L). The mean total analytical error calculated from our data is 13.8%. The direct LDL-C assay and the commonly used Friedewald calculation respectively classified 81% and 84% of fasting patients correctly, according to the cutoffs of 1100 and 1300 mg/L for LDL-C set by the National Cholesterol Education Program for pediatric patients. Of combined fasting and nonfasting patients, 80% were correctly classified by the direct LDL-C assay. Therefore, despite several analytical shortcomings, the direct LDL-C assay may be useful in managing hyperlipidemic children without the need for a fasting specimen.

INDEXING TERMS: methods comparison • heart disease • lipoproteins • triglycerides • risk factor • hypercholesterolemia

Atherosclerosis is initiated early in life [1] and progresses silently for decades until it manifests in adulthood, often as coronary heart disease. Although screening for lipoprotein disorders in the general pediatric population is not advised, the National Cholesterol Education Program (NCEP) pediatric panel and the American Academy of Pediatrics Committee on Nutrition recommend selective screening of individuals between ages 2 and 20 years [2]. Currently, only those children whose parents or grandparents developed premature atherosclerotic vascular disease (55 years or younger) or children who have one parent whose total cholesterol is ≥2400 mg/L are recommended for screening [2]. The major atherogenic fraction of serum cholesterol is low-density lipoprotein cholesterol (LDL-C) [3, 4]. The NCEP has classified LDL-C concentrations in children and adolescents from families with hypercholesterolemia or premature atherosclerosis into three main categories [2], based on the 75th (1100 mg/L) and 90th percentile (1300 mg/L) of LDL-C in American children and adolescents: acceptable <1100 mg/L, borderline 1100–1290 mg/L, and high ≥1300 mg/L.

Plasma LDL-C is currently estimated in most clinical laboratories by the formula of Friedewald et al. [5, 6], which is based on the observation that, in the fasting state, very-low-density lipoprotein (VLDL) cholesterol is ~20% of the total triglycerides (TG), measured in units of mg/L. Therefore, LDL-C = total cholesterol minus high-density lipoprotein cholesterol (HDL-C) minus (TG/5). This formula, however, has certain constraints [6–8]: It is invalid at TG concentrations ≥4000 mg/L and it can be utilized only in the fasting state [6]. Furthermore, the latest NCEP recommendations [9] define specific performance criteria for LDL-C assays that cannot be met by the Friedewald calculation [6–8].

A new "direct" immunological procedure for determining LDL-C (direct LDL cholesterol or DLDL-C), LDL Cholesterol®, has been developed by Genzyme Corp., Cambridge, MA, and is marketed by Sigma Diagnostics, St. Louis, MO. The assay, which has been assessed in adults [10–12], relies on the immunoseparation of LDL particles from HDL and VLDL by

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7 Nonstandard abbreviations: NCEP, National Cholesterol Education Program; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; apo, apolipoprotein; and DLDL-C, direct LDL-C.

8 To convert cholesterol mg/L to mmol/L, divide by 387.

9 To convert triglyceride mg/L to mmol/L, divide by 883.
solid-phase-bound goat polyclonal antibodies to apolipoproteins (apos) A-1 and E [12]. Here, we report our assessment of the DLDL-C assay in a hyperlipidemic pediatric population (ages ≤20 years). We compared both the DLDL-C assay and the Friedewald estimation with a modified\textsuperscript{10} β-quantification Reference Method [6, 13, 14] for determining LDL-C.

### Materials and Methods

**PLASMA SAMPLES**

Of the 138 blood samples used in this study, 107 were from patients attending the Children's Hospital Lipid Clinic because of a positive family history of premature atherosclerosis or hypercholesterolemia; 96 of these blood samples were obtained after an overnight fast and the remaining 11 were drawn from nonfasting subjects. This study protocol was approved by the Internal Review Board of Children's Hospital, Boston. In addition to these Lipid Clinic samples, 31 plasma samples were obtained randomly (without conscious bias) from nonfasting hypertriglyceremic hospital patients (TG ≥2500 mg/L). We therefore studied a total of 42 nonfasting patients. In all cases we assayed heparinized plasma that had been stored at 4 °C [15].

**PROCEDURES**

**Modified β-quantification** [16]. We centrifuged 230-μL plasma samples at 250 000g for 3 h at 4 °C in a Beckman TL100 rotor with 7 × 20 mm polyallomer tubes (Beckman Instruments, Palo Alto, CA). We then sliced the tubes and their contents so as to isolate the >1.006 kg/L infranate. Having restored the volume of the infranate to that of the original sample by adding isotonic saline (9 g/L NaCl), we quantified its HDL-C content after precipitating [17] the LDL particles with MgCl\textsubscript{2} and dextran sulfate (M\textsubscript{w} 50 000). The LDL-C was then calculated as infranant total cholesterol − HDL-C, the VLDL-C having been removed by the ultracentrifugation. The proficiency of our β-quantification technique is checked three times a year as part of the ALERT\textsuperscript{®} LDL-C standardization program (Pacific Biometrics Research Foundation, Seattle, WA). The mean percentage bias for LDL-C concentrations of 910–1930 mg/L in three consecutive proficiency tests, five samples per test, was 1.38% (95% confidence interval, 0.08–2.68%).

**Total cholesterol, HDL-C, and TG assays**. Concentrations of total cholesterol, HDL-C, and TG were determined enzymatically with a Hitachi 911 automated analyzer (Boehringer Mannheim, Indianapolis, IN). TG measurement was corrected for the presence of endogenous glycerol. HDL was separated as described above and its cholesterol component was measured with the 911 analyzer. Our laboratory is currently enrolled in the National Heart, Lung, and Blood Institute and Centers for Disease Control and Prevention Lipid Standardization Program.

\textsuperscript{10} Historically, the β-quantification assay included a heparin and manganese precipitation step, which has largely been replaced by precipitation with dextran sulfate and magnesium; hence, we describe the β-quantification assay used as a "modified" procedure.

DLDL-C. Sigma Diagnostics donated all the reagents and we performed the DLDL-C assay at room temperature according to the manufacturer's instructions.

**Friedewald calculation.** We measured total cholesterol, HDL-C, and TG in all plasma samples but applied the Friedewald calculation only to fasting samples with TG <4000 mg/L.

**Statistical analysis.** We calculated means, medians, and t-test values with Microsoft Excel (Microsoft, Redmond, WA); t-tests were judged significant at P <0.05. Linear regression analysis by the least-squares method was calculated by the SigmaPlot\textsuperscript{®} statistics program (Jandel Scientific, San Rafael, CA). Biases were calculated as the test method result minus the Reference Method result.

We calculated total error as the sum of the systematic error plus the random error. Systematic error has two components, constant systematic error and proportional systematic error, which are calculated from the linear regression equation y = mx + b, m being the slope of the regression equation (proportional error) and b the y-axis intercept (constant error). Systematic error at an LDL-C concentration of x\textsubscript{c} is defined as the absolute value of [y\textsubscript{c} − x\textsubscript{c}], where y\textsubscript{c} = mx\textsubscript{c} + b. Random error is 1.96 SD of the run-to-run precision study [18, 19].

The positive predictive value of an LDL-C assay at the x mg/L cutpoint was calculated as [true positive/(true positive + false positive)] × 100, where true positive means that LDL-C results of both the Reference Method and the test method are >x mg/L, and false positive means that the test method LDL-C result is >x mg/L when the Reference Method LDL-C is <x mg/L.

**Results**

Heparinized plasma is preferred to serum in our laboratory because it can be processed immediately after collection. In preliminary experiments we determined that the choice of heparinized plasma or serum had no statistically significant effect (P ≥0.37) on the outcome of the modified β-quantification or the DLDL-C assay (Table 1). We therefore used plasma for the remaining experiments. Freezing also had no effect on the modified β-quantification of LDL-C. Eight plasma samples (mean ± SD LDL-C 1186 ± 441 mg/L, range 494–1638 mg/L) frozen at −80 °C showed no significant difference (P = 0.53) in LDL-C concentration when thawed (1178 ± 455 mg/L).

| Table 1. Measurement of LDL-C concentrations in paired serum and plasma samples (n = 14) by β-quantification and DLDL-C assay. |
|-----------------|-----------------|-----------------|-----------------|
| **LDL-C, mg/L** | **β-Quant. LDL-C** | **Serum** | **DLDL-C** |
| **Plasma** | **Serum** | **Plasma** | **Serum** |
| Mean | 1278 | 1263 | 1034 | 1036 |
| SD | 357 | 316 | 351 | 343 |
| Min | 735 | 755 | 540 | 580 |
| Max | 1807 | 1782 | 1720 | 1730 |
For the DLDL-C assay, however, frozen plasma samples are unsuitable. When 29 frozen samples (mean ± SD 1913 ± 633 mg/L) were assayed by both the DLDL-C assay and the Reference Method, the DLDL-C assay had a bias (relative to the Reference Method) of +4 to −1003 mg/L [mean, −491 mg/L (−25%)]. Nevertheless, the DLDL-C concentration is stable at 4°C for at least 3 weeks: Five samples with β-quantification LDL-C concentrations of 1103–1954 mg/L (mean ± SD 1390 ± 381 mg/L) were assayed with the DLDL-C reagent once a week for 3 weeks, the samples being kept at 4°C between measurements. We found no significant difference between the initial β-quantification LDL-C concentration and the initial or subsequent DLDL-C measurements (P ≥0.47).

All 96 plasma samples from fasting pediatric patients of the Lipid Clinic were analyzed with the DLDL-C assay; analyzed for total cholesterol, HDL-C, and TG concentrations to calculate the Friedewald LDL-C values; and analyzed for LDL-C concentrations with the modified β-quantification procedure. Both the Friedewald LDL-C and the DLDL-C were compared with the concentration obtained with the Reference Method. The data from this study, summarized in Table 2, indicate that the mean bias of the DLDL-C assay is negative, whereas that of the Friedewald calculation is positive. The comparison-of-methods plot—β-quantification (x) vs test method (y)—gave a least-squares linear regression equation of $y = 0.90x + 68$ ($r = 0.93$) for the DLDL-C, and $y = 0.96x + 130$ ($r = 0.95$) for the Friedewald calculation (Fig. 1A and B). The resulting DLDL-C systematic error calculated was 13 mg/L (1.6%), 117 mg/L (6.3%), and 132 mg/L (6.6%) at LDL-C concentrations of 810, 1850, and 2000 mg/L, respectively (see Materials and Methods). The imprecision, defined here as the run-to-run CV (n = 20), was 5.2%, 4.5%, and 4.0% for these three respective LDL-C concentrations. The percentage total error (random error plus systematic error) for these same DLDL-C concentrations was 11.8%, 15.1%, and 14.4%, respectively, with a mean total error of 13.8%.

The effect of TG concentration on the performance of the DLDL-C assay for 42 nonfasting samples (mean ± SD TG, 42 ± 20 mg/L) was much higher than that observed for the fasting patients. The data from the combined fasting and nonfasting patients (n = 138) are given in Table 2.

**Table 2. Characteristics of the groups used in the study.**

<table>
<thead>
<tr>
<th>Patient status</th>
<th>Fasting</th>
<th>Nonfasting</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sample no.</td>
<td>96</td>
<td>42</td>
<td>138</td>
</tr>
<tr>
<td>Age, years</td>
<td>11.7 ± 3.7</td>
<td>8.1 ± 5.8</td>
<td>10.6 ± 4.7</td>
</tr>
<tr>
<td>Range</td>
<td>4-20</td>
<td>0.17-20</td>
<td>0.17-20</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>2,355 ± 574</td>
<td>2,519 ± 1,611</td>
<td>2,405 ± 1,005</td>
</tr>
<tr>
<td>TG</td>
<td>1,057 ± 720</td>
<td>4,854 ± 5,457</td>
<td>2,213 ± 3,514</td>
</tr>
<tr>
<td>Median</td>
<td>890</td>
<td>2,960</td>
<td>1,165</td>
</tr>
<tr>
<td>Range</td>
<td>140-3,700</td>
<td>340-25,200</td>
<td>140-25,200</td>
</tr>
<tr>
<td>HDL-C</td>
<td>373 ± 87</td>
<td>288 ± 148</td>
<td>348 ± 115</td>
</tr>
<tr>
<td>β-Quant. LDL-C</td>
<td>1,712 ± 563</td>
<td>1,274 ± 531</td>
<td>1,578 ± 588</td>
</tr>
<tr>
<td>Friedewald LDL-C</td>
<td>1,770 ± 566</td>
<td>Not calc.</td>
<td>Not calc.</td>
</tr>
</tbody>
</table>

Concentrations of cholesterol and TG are in mg/L.

*Results are given as mean ± SD, except where noted.

Fig. 1. DLDL-C (A) and the Friedewald LDL-C (B) results compared with the equivalent value obtained by the modified β-quantification in fasting pediatric patients (n = 96) by least-squares linear regression analyses.

(C) Data from the combined fasting and nonfasting patients (n = 138).
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4854 ± 5457 mg/L) was examined by comparing the results of β-quantification (x) with those of the DLDL-C assay (y) (Table 2). These samples gave the linear regression equation y = 1.24x − 128 (r = 0.85). The combined 138 samples (fasting plus nonfasting) gave the equation y = 0.92x + 109 (r = 0.86) (Fig. 1C). Whereas the 96 fasting samples displayed a negative mean (± SD) bias of −98.2 ± 215 mg/L, the 42 nonfasting samples had a positive mean bias of 177 ± 432 mg/L (Table 2).

Figure 2 shows a plot of the bias of the DLDL-C results in relation to the LDL-C concentration determined by β-quantification. In the 96 fasting samples (Fig. 2A), this bias is negatively correlated with the β-quantification LDL-C (r = −0.254, P < 0.05); in the combined fasting and nonfasting patients (n = 138), however, the correlation is not significant (Fig. 2B).

Figure 3 displays the bias as a function of plasma TG concentration in both the 96 fasting (Fig. 3A) and the 138 combined fasting and nonfasting patients (Fig. 3B). Both anal-

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**Fig. 2.** LDL-C determined by modified β-quantification and by DLDL-C assay in 96 fasting plasma samples (A) and in 138 samples from combined fasting and nonfasting patients (B). Bias (DLDL-C minus β-quantification) is plotted as a function of the modified β-quantification LDL-C concentrations. Significance of r was determined by the t-test for correlation coefficients. The unbroken line represents the best fit as determined by linear regression. The horizontal dashed line reflects zero bias. NS, not statistically significant.

**Fig. 3.** LDL-C determined by modified β-quantification and by DLDL-C assay in 96 fasting plasma samples (A) and in 138 samples from combined fasting and nonfasting patients (B). Bias (DLDL-C minus β-quantification) is plotted as a function of the plasma TG concentration, displayed on a linear scale (A) or logarithmically (B). Significance of r was determined by the t-test for correlation coefficients.
yses showed a significant positive correlation ($P < 0.01$) between the bias and the plasma TG concentration.

We then assessed the diagnostic performance of the assays, using the NCEP cutpoints that divide the pediatric population on the basis of LDL-C concentrations into acceptable, borderline, and high categories. Fig. 4 compares these clinical classifications as a grid display of diagnostic sensitivities that shows both correct and incorrect classifications. The Friedewald calculation (Fig. 4A) correctly classified 81 of the 96 fasting patients (84%), whereas for the same population the DLDL-C (Fig. 4B) correctly classified 78 (81%). For the fasting and nonfasting populations combined (Fig. 4C), the DLDL-C correctly classified 80% (110 of 138) of the population.

We calculated the positive predictive value of each LDL-C method at both of the NCEP cutpoints for pediatric patients. For the DLDL-C assay, the positive predictive value at the $\geq 1300$ mg/L and the $>1100$ mg/L concentrations was 85% and 93%, respectively. For the Friedewald formula, these were 97% and 98% at the same cutpoints. Table 3, in which samples are placed into three groups according to plasma TG concentrations ($<1000$, 1000–1999, and $\geq 2000$ mg/L), displays the fraction of patients correctly classified (according to the NCEP cutpoints) by the DLDL-C results or the Friedewald LDL-C calculation in comparison with the reference procedure value. Both LDL-C test methods yielded comparable results.

Discussion
In the routine clinical chemistry laboratory, the Friedewald formula [5] is widely used to calculate LDL-C but often fails to meet the most recent NCEP performance goals [9]. The current LDL-C reference procedure, known as $\beta$-quantification [13], isolates the population of particles that have a hydrated density between 1.006 and 1.063 kg/L. Besides LDL particles, however, this heterogeneous population includes intermediate-density lipoproteins (1.006–1.019 kg/L) and lipoprotein(a) (1.050–1.080 kg/L). For any new LDL-C technique to be clinically useful and widely accepted, it should correlate with the $\beta$-quantification method, which is the basis for the database derived from the Lipid Research Clinics. However, given that $\beta$-quantification actually measures a heterogeneous population of particles, the potential task of developing a precise and accurate direct assay for determining the cholesterol component of this "wide-density" LDL fraction appears all the more difficult [13, 20–22]. Here, we describe the performance of a newly introduced LDL-C assay (DLDL-C) and its ability to classify hyperlipidemic children correctly according to the NCEP cutpoints.

The DLDL-C assay requires a single measurement, whereas measurements of total cholesterol, HDL-C, and TG are needed for the Friedewald calculation. The DLDL-C assay is unaffected by substituting heparinized plasma for serum. Frozen plasma samples, however, are unsuitable and show, on average, 25% less LDL-C measured by DLDL-C when compared with the Reference Method. This finding is disappointing and may preclude use of the assay in long-term clinical trials, in which samples are usually stored frozen until analysis. However, we did

![Fig. 4. Diagnostic performance of the Friedewald calculation (A) and the DLDL-C assay (B and C) assessed according to NCEP guidelines. All LDL-C values are in mg/L. Left-hand column, classification of the sample population by the $\beta$-quantification Reference Method; top row, classification according to either the Friedewald calculation (A) or the DLDL-C assay (B and C). The blocks with the bold borders display the number of patients correctly classified by these assays (both absolute values and percentages). The remaining blocks are discrepant classifications. The data in A and B are from the fasting patients ($n = 96$); those in C are from the combined fasting and nonfasting group ($n = 138$).]
find that DLDL-C-assayed results are stable at 4 °C for at least 3 weeks. The random error of an assay is established by performing replicate measurements of a sample. The run-to-run imprecision of the DLDL-C assay has a concentration-dependent CV range of 4–5.2%, and therefore does not always meet the CV recommended by the NCEP (≤4%). Initially, the estimation of the run-to-run precision of the DLDL-C assay presented a problem. Our experience with the manufacturer's lyophilized controls has been that, once reconstituted and stored at 4 °C, they show a gradual upward drift of measured LDL-C concentrations over a 2–3 week period, resulting in an increase of ~6–10%. When the reconstituted control material was aliquoted and stored at 4 °C in 1.5-mL Microfuge tubes, the variation was less. Freezing the control material does not appear to be a viable alternative because frozen samples show a continuous time-dependent decrease in LDL-C concentration [10].

Table 2 and Fig. 1 demonstrate that in the fasting pediatric subjects the DLDL-C assay had a negative mean bias (±SD) of −98 (±215) mg/L vs the reference procedure. In the same population the bias of the Friedewald calculation was +59 (±172) mg/L. Another indicator of analytical error, the systematic error of an assay, may be further quantified by least-squares linear regression [18, 19]. The NCEP performance goals require that an LDL-C assay have an analytical bias of ≤±4.0% [9]. We note, however, that substituting a range of values (600–3500 mg/L) into our LDL-C regression equation for fasting patients (y = 0.9x + 68) yielded calculated systematic errors of +1.4% to −8.0% (data not shown). We therefore found a greater negative bias than did several other investigators who studied the performance of the DLDL-C assay in adults [10-12]. The explanation for the overall negative bias of the DLDL-C is unclear. Theoretically, the DLDL-C assay determines the cholesterol that is present only in LDL and lipoprotein(a) particles—and therefore not in intermediate-density lipoprotein [13], unlike the β-quantitation procedure. Perhaps this is the source of the DLDL-C negative bias. Alternatively, some LDL-containing plasma may be trapped by the latex beads in the DLDL-C assay and thereby be excluded from the filtrate [10]. Combining systematic error and random error to obtain the total analytical error [18, 19], we obtained a mean total analytical error in the 96 fasting patients at three DLDL-C concentrations of 13.8% (the NCEP recommends a total analytical error of ≤12%).

In contrast, for nonfasting samples (Table 2), the DLDL-C assay had a mean positive bias of +177 (±432) mg/L. Further analysis showed a positive correlation between plasma TG concentration and DLDL-C bias (DLDL-C – β-quantification LDL-C) in both the fasting patients and the combined fasting and nonfasting patients (Fig. 3). Two possible explanations for the DLDL-C positive bias are that (a) the anti-apo A-I and anti-apo E on the latex beads become saturated in hypertriglyceremic plasma, thus allowing the excess TG-rich lipoproteins to escape into the filtrate and increase the measured cholesterol, or (b) the larger TG-rich particles present in hypertriglyceridemia interfere with antibody binding through steric hindrance [10]. Awareness of the triglyceride-dependent bias of the DLDL-C assay is important in pediatrics because mean TG concentrations in this population are low relative to adults [1], implying that this negative bias will predominate. We also noted an inverse correlation between the DLDL-C bias and the LDL-C measured by β-quantitation (Fig. 2), the negative bias becoming more pronounced at higher LDL-C concentrations.

Despite the above-mentioned negative systematic error of the DLDL-C assay in fasting pediatric patients, the diagnostic performance of this assay was at least comparable with that of the Friedewald calculation as assessed by its ability to correctly classify such patients into the three NCEP groups. However, the positive predictive value of the Friedewald calculation was better than that of the DLDL-C assay at the 1300 mg/L concentration. When nonfasting patients were included in the analysis, the diagnostic performance of the DLDL-C assay was largely unaffected (Fig. 4C).

TG concentrations >4000 mg/L (for which use of the Friedewald calculation is precluded) are unusual in pediatric practice. In a fasting pediatric population, one would therefore commonly be able to use either the Friedewald calculation or the DLDL-C assay. Our results indicate that the latter would give little gain in terms of analytical or diagnostic performance. We do, however, envision an application for the DLDL-C assay in nonfasting pediatric patients, the diagnostic performance of this assay being comparable in both the fasting and nonfasting groups. The DLDL-C assay need not be used in the initial assessment of new pediatric hyperlipidemic patients. Because fasting values for TG and HDL-C are needed in such patients, LDL-C can be adequately derived from the Friedewald calculation.

In conclusion, therefore, despite its borderline-acceptable analytical performance with respect to the NCEP performance goals for total error, we recognize that the DLDL-C assay is a potentially useful tool in the follow-up nonfasting management of children with hypercholesterolemia. This population is particularly relevant for use with such a method because patient compliance for drawing a morning fasting blood sample is often achieved with some difficulty.

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