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Comparison of the Japan Society of Clinical Chemistry reference method and CDC method for HDL and LDL cholesterol measurements using fresh sera

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ABSTRACT

Objectives: In 2009, the Japan Society of Clinical Chemistry (JSCC) recommended a reference method for the measurement of serum high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) levels. This automated method uses cholesterol esterase-

Abbreviations: apo, apolipoprotein; BFr-C, bottom fraction-cholesterol; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; AK, Abell-Kendall; CDC, Centers for Disease Control and Prevention; NIST, National Institute of Standards and Technology; SRM, Standard Reference Material; LB, Liebermann-Burchard; BQ, beta-quantification; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; CD, cholesterol dehydrogenase; CHE, cholesterol esterase; EDDA, ethylenediamine-*N,N'*-diacetic acid; EDTA-2Na, ethylenediamine-*N,N,N',N'*-tetraacetic acid, disodium salt, dihydrate; DM, *n*-dodecyl- β -maltopyranoside; Syx, standard deviation of the regression line.

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LDL-cholesterol
HDL-cholesterol
Homogeneous assay
Beta-quantification

cholesterol dehydrogenase to measure cholesterol levels in fractions obtained after ultracentrifugation and dextran sulfate/magnesium chloride precipitation. In the present study, using fresh samples, we compared the LDL-C and HDL-C levels measured using this method with those measured using the traditional Centers for Disease Control and Prevention (CDC)-beta-quantification (BQ) method.

Design: and methods: Using both the JSCC and CDC-BQ methods, LDL-C/HDL-C levels were measured in 47 non-diseased and 126 diseased subjects, whose triglyceride levels were lower than 11.29 mmol/L (1000 mg/dL).

Results: For LDL-C, the equation of the line representing the correlation between the two methods was $y = 0.991x + 0.009$ mmol/L; $r = 0.999$; and $Sy/x = 0.025$ mmol/L, where x is the mean LDL-C level measured using the CDC-BQ method. Similarly, for HDL-C, the equation of the line representing the correlation between the two methods was $y = 0.988x + 0.041$ mmol/L, $r = 0.999$, and $Sy/x = 0.019$ mmol/L, where x is the mean HDL-C level measured using the CDC-BQ method.

Conclusions: The JSCC method agreed with the CDC-BQ method in cases of both non-diseased and diseased subjects, including those with dyslipidemia.

1. Introduction

In 2009, a "Recommended comparison method for high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) measurement in serum" was reported by the Committee on Enzyme-Reagent, Japan Society of Clinical Chemistry (JSCC) [1]. This JSCC reference method was developed to evaluate the accuracy of the homogeneous measurement method for HDL-C and LDL-C, which is widely used in most clinical laboratories [2–4].

To verify whether the LDL-C level determined using the homogeneous assay agreed with that determined by the Centers for Disease Control and Prevention (CDC) method [also referred to as the CDC-beta-quantification (BQ) method], the LDL-cholesterol Study Group was formed in August 2010 under the supervision of three Japanese scientific societies: the Japan Atherosclerosis Society, the Japan Society of Clinical Chemistry, and the Japanese Society of Laboratory Medicine [5]. Using the CDC-BQ method, we examined 173 individuals, including 47 non-diseased and 126 diseased subjects whose triglyceride (TG) levels were lower than 11.29 mmol/L (1000 mg/dL). Patients with higher TG levels were excluded because high TG levels may affect LDL-C measurements. In parallel, we also assessed the same serum samples from the LDL-cholesterol Study Group using the JSCC reference method. In this report, we present data demonstrating the correlation between the measurements obtained using the JSCC and CDC-BQ methods.

2. Materials and methods

2.1. Reagents

The three reagents used for the JSCC assay of cholesterol were Reagent-1 (R-1), which consists of the enzyme cholesterol esterase (CHE); Reagent-2 (R-2), which consists of the enzyme cholesterol dehydrogenase (CD) [6], and Reagent-3 (R-3), which lacks both CHE and CD. The precise composition is listed in Table 1. The precipitation reagents, dextran sulfate (molecular weight: 50,000, lot DSJ-4R) and magnesium chloride (99.9%, PuA-grade), which were used to precipitate the bottom fraction (BFr) obtained after ultracentrifugation, were purchased from Meito Sangyo Co. Ltd. (Nagoya, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan), respectively. Both dextran sulfate (20.0 g/L) and magnesium chloride hexahydrate (0.7 mol/L) were dissolved at 2.0 g and 14.22 g in

Table 1

Composition of reagents used in the automated JSCC method for cholesterol measurements.

Component	First reagent	Second reagent	Third reagent	Final concentration
HEPES buffer (pH 8.0)	100.0 mmol/L	100.0 mmol/L	100.0 mmol/L	100.0 mmol/L
Hydrazine dichloride	66.7 mmol/L	-	-	50.0 mmol/L
Oxamine potassium	66.7 mmol/L	-	-	50.0 mmol/L
β -NAD	6.67 mmol/L	-	-	5.0 mmol/L
CD	-	24.0 U/mL	-	6.0 U/mL
CHE	8.0 U/mL	-	-	6.0 U/mL
EDDA	10.0 mmol/L	-	-	7.5 mmol/L
EDTA-2Na	-	3.0 mmol/L	3.0 mmol/L	0.75 mmol/L
Crystallin	-	0.3%	0.3%	0.08%
Triton® X-100	0.2%	0.2%	0.2%	0.2%
Deoxycholate	0.2%	0.2%	0.2%	0.2%
Calcium chloride	0.2 mmol/L	-	-	0.15 mmol/L
DM	-	10.0 mmol/L	10.0 mmol/L	2.5 mmol/L

All percentages are (w/v). HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; CD, cholesterol dehydrogenase; CHE, cholesterol esterase; EDDA, ethylenediamine- N,N' -diacetic acid; EDTA-2Na, ethylenediamine- N,N',N',N' -tetraacetic acid, disodium salt, dihydrate; DM, n -dodecyl- β -maltopyranoside.

100 mL of water. These solutions were mixed in a 1:1 (vol/vol) ratio to generate a precipitation reagent solution. The supernatant fraction was obtained by low-speed centrifugation ($1500\times g$, 30 min at $4\text{ }^{\circ}\text{C}$) after the addition of $100\text{ }\mu\text{L}$ of this precipitation reagent to a 1.0 mL aliquot of the BFr. All cholesterol reagents were supplied by Sysmex Corp. (Kobe, Japan) according to the JSCC total cholesterol comparative measurement method (CHE-CD method, Table 1) [7]. Apolipoprotein reagent kits were purchased from Sekisui Medical (Tokyo, Japan). Cholesterol and triglyceride reagent kits were purchased from Shino-Test (Kanagawa, Japan). Standard reference material (SRM) no. 911b (>99.8%) was obtained from the National Institute of Standards and Technology (NIST, Washington DC, USA), and reference materials (JCCRM 211-3 M, -3H; frozen serum, Reference Material Institute for Clinical Chemistry Standards, Kanagawa, Japan) were used to validate the JSCC cholesterol reagent and the lipid-specific calibrator KL (Sysmex Corp.).

2.2. Blood collection

The serum samples used in this study were the same as those previously described by Miida et al. [5,8]. All sampling processes followed the CDC LDL-C standardization program [9]; fresh venous blood was collected at the participating facilities, and sera were separated within 1 h. The temperature was maintained at $4\text{ }^{\circ}\text{C}$ or less for at least 24 h and monitored using a traceable thermometer. Sera were transported in a refrigerated box to the former Osaka Medical Center Hospital (OMC-HSP) for the CDC-BQ method and for BFr separation to allow for measurement using the JSCC method. Aliquots of separated serum and BFRs were transported from the OMC-HSP to the Kyushu University Hospital.

Healthy subjects were defined as normolipidemic healthy volunteers if they had no abnormal laboratory tests or documented diseases. The remaining 126 subjects, excluding five diseased subjects with TG levels over 11.29 mmol/L (1000 mg/dL), were classified as subjects with disease according to their medical history and lipoprotein profiles. Written informed consent was obtained from all subjects. The study protocol was reviewed and approved by the Ethics Committee of Kyushu University (22–112).

2.3. Instruments

A Hitachi U-3310 spectrophotometer (H-U-3310, Hitachi High-Technologies, Tokyo, Japan) was used to evaluate the performance of the JSCC cholesterol measurement reagent. A Hitachi 7170S automated biochemical analyzer (H-7170S, Hitachi High-Technologies) was used to validate the cholesterol level of the specific calibrator, KL, assigned by the JSCC total cholesterol reference measurement method (alkaline hydrolysis CD method) [10], which utilizes the alkaline hydrolysis CD protocol [11]. A Hitachi 7600 automated biochemical analyzer (H-7600, Hitachi High-Technologies) was used to measure cholesterol in the BFr after ultracentrifugation and in the HDL fraction of the supernatant.

2.4. Procedures for LDL-C and HDL-C measurements

The JSCC reference method uses the same ultracentrifugation separation procedure as that used in the CDC-BQ method ($18\pm 0.2\text{ }^{\circ}\text{C}$, $105,000\times g$, 18.5 h). However, the precipitation reagents used for the CDC-BQ and JSCC reference methods differ: the CDC-BQ uses a heparin-manganese solution, whereas the JSCC method uses a dextran sulfate-magnesium solution. Both the CDC-BQ and JSCC reference methods (with the exception of the cholesterol concentration measurement) were performed at OMC-HSP, a member of the Cholesterol Reference Method Laboratory Network organized by the CDC [12,13]. After ultracentrifugation, the $d = 1.006$ top fraction, representing very-low-density lipoprotein and chylomicron, was removed using a tube slicer. The BFr ($d > 1.006\text{ kg/L}$), consisting of HDL, LDL, intermediate-density lipoprotein, and lipoprotein (a), was then quantitatively collected to a 5.00 mL volumetric flask, and the volume of the infranatant was restored to the original total volume using 0.15 mol/L NaCl solution. After precipitation of apoB-containing lipoproteins with one of the precipitation reagents, the supernatants consisting of HDL were obtained through low-speed centrifugation ($1500\times g$, 30 min at $4\text{ }^{\circ}\text{C}$). Cholesterol levels in the BFr and HDL fractions were measured in duplicate for each tube at OMC-HSP using the CDC-Abell-Kendall (CDC-AK) method [11,14,15] and at Kyushu University Hospital using the automated CHE-CD enzymatic JSCC method [16,17]. LDL-C was calculated as the difference between BFr-C and HDL-C levels.

2.5. Performance of reagents specified by JSCC and conditions for cholesterol measurements

To evaluate the performance of the reagents specified by JSCC, we used U-3310 and H-7170S to measure NIST SRM 911b dissolved in isopropyl alcohol at 99.8 mg/dL (2.56 mmol/L). For manual testing of the samples using the U-3310 spectrophotometer, each sample (SRM 911b, 0.1 mL) and 1.0 mL of R-1 were pipetted into a cuvette. The mixture was then preincubated at $37\text{ }^{\circ}\text{C}$ for 5 min. The reaction was initiated by the addition of 1.0 mL of R-2 or control R-3 as the blank reaction. After 5 min, the absorbance was measured at 340 nm (ASs, ASb represent the sample and blank absorbance values for serum, respectively). The same volume of water was used in place of serum to generate the corresponding reaction and blank absorbance values (AR2b and AR3b, respectively). The experimental absorbance changes in the total reaction blank reaction were calculated from the change in absorbance by subtracting AR2b from ASs or AR3b from ASb. For this calculation, the molar absorption coefficient of NADH at 340 nm used with the U-3310 spectrophotometer was the theoretical value of $6.3\times 10^3\text{ L mol}^{-1}\text{ cm}^{-1}$ [18]. The accuracy of cholesterol measurement was assessed by comparing the experimental absorbance change with the theoretical absorbance change. To evaluate the cholesterol level of the lipid-specific calibrator, KL, assigned by the JSCC total cholesterol reference measurement method, we measured JCCRM 211-3 M and -3H using the H-7170S automated analyzer. We used the following procedure: $6\text{ }\mu\text{L}$ of each serum sample was incubated with $240\text{ }\mu\text{L}$ of R-1 for 5 min at $37\text{ }^{\circ}\text{C}$, followed by measurement of absorbance values at $340/600\text{ nm}$. The reaction was started immediately by the addition of $80\text{ }\mu\text{L}$ of R-2 or R-3 as a

sample blank. After 5 min, the absorbance values at 340/600 nm were measured again. For each reagent blank, we exchanged the serum sample for saline and performed the same operation. Cholesterol levels in the BFr and the supernatant fraction following precipitation were determined using the automated JSCC CHE-CD enzymatic method on the H-7600 instrument, under the same conditions as those used for H-7170S.

2.6. Statistical analysis

Mean, SD, and coefficient of variation values were calculated using Microsoft Excel 2013. Linear regression analysis using the least-squares method was also performed using Microsoft Excel 2013. We used the Bland-Altman plot [19,20] to compare the CDC-BQ and JSCC reference methods for systematic errors in the measured values.

3. Results

3.1. Performance of reagents specified by JSCC

To evaluate the performance of the JSCC reagents in the assays for cholesterol concentrations in the ultracentrifugal bottom and low-speed centrifugal supernatant fractions obtained after precipitation, we measured the absorbance changes for NIST SRM 911b using a U-3310 spectrophotometer (Table 2). The experimental change after subtracting the blank absorbance from the sample absorbance for the SRM 911b solution was 15.94, as determined using the following equation:

$$\{[(ASs - AR2b) - (ASb - AR3b)] \times (0.1 \text{ mL} + 3.0 \text{ mL} + 1.0 \text{ mL})\} / 0.1 \text{ mL}$$

The theoretical absorbance change determined using the following equation:

$$2.58 \text{ mmol/L} \times 6.3 (\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}) \times 1 \text{ cm}$$

was 16.25. The relative percentage of the theoretical absorbance to the experimental absorbance was within the permitted range of 98.1%.

3.2. Value of the lipid-specific calibrator KL

To evaluate the assigned value of the lipid-specific calibrator KL (4.73 mmol/L), JCCRM-211-3 M (4.80 ± 0.018 mmol/L) and JCCRM-211-3H (6.26 ± 0.021 mmol/L) were measured in quadruplicate with the lipid-specific calibrator KL, as proposed in the JSCC 'Recommended reference method for total cholesterol measurement in serum' [10]. The mean (SD) results for JCCRM 211-3 M and -3H using this method were 4.82 (0.021) and 6.28 (0.065) mmol/L, respectively. The relative percentage of the theoretical values to the experimental values was within the permitted ranges of 100.4% and 100.3%, respectively.

3.3. Subject characteristics

The mean, maximum, and minimum levels of total cholesterol, TG, apolipoprotein (apo) AI, apoAII, apoB, apoCII, apoCIII, and apoE in non-diseased and diseased patients are provided in Table 3. Most dyslipidemic patients were treated with lipid-lowering agents, usually statins. Between 20% and 30% of the diseased subjects had cardiovascular disease, diabetes, or fatty liver disease.

Table 2
Evaluation of JSCC reagents.

		NIST SRM 911b	
		Symbol	Absorbance
Sample reaction	SRM 911b	ASs	0.601
	Water	AR2b	0.215
Blank reaction	SRM 911b	ASb	0.211
	Water	AR3b	0.214
Experimental absorbance (E)			15.94 ^{a)}
Theoretical absorbance (T)			16.25 ^{b)}
Relative % of (E) and (T)			98.1% ^{c)}

NIST, National Institute of Standards and Technology; SRM, Standard Reference Material; ASs, absorbance of the test sample; AR2b, absorbance of the water control sample; ASb, absorbance of the test sample blank; AR3b, absorbance of the water control blank.

^{a)} $\{[(ASs - AR2b) - (ASb - AR3b)] \times (0.1 \text{ mL} + 3.0 \text{ mL} + 1.0 \text{ mL})\} / 0.1 \text{ mL} / 0.998$.

^{b)} $2.58 \text{ mmol/L} \times 6.3 (\text{L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}) \times 1 \text{ cm}$.

^{c)} (Experimental absorbance/theoretical absorbance of NIST SRM 911b) $\times 100$ (%).

3.4. Inter-method comparison

A total of 173 samples with a mean (SD) TG concentration of 2.12 (2.00) mmol/L were assayed for cholesterol using both the CDC-BQ and the automated JSCC reference methods with the H-7600 analyzer. As shown in Table 4, the differences between the mean cholesterol measurements of the bottom fraction (BFr-C), HDL-C, and LDL-C for the JSCC reference and CDC-BQ methods were within acceptable limits.

The correlation and residual plots of the cholesterol levels in the bottom fraction (BFr-C) and the supernatant after precipitation (HDL-C) [Fig. 1 (a), (b), (c), and (d)] and those for LDL-C, which is the calculated difference between the BFr-C and HDL-C levels [Fig. 1 (e) and (f)], are shown. There was a good agreement between the results from the JSCC reference and the CDC-BQ methods (Table 5).

4. Discussion

HDL-C and LDL-C measurements made using the JSCC reference and CDC-BQ methods were compared using clinical specimens. Although the JSCC reference and CDC-BQ methods share a common ultracentrifugation step, the precipitation agent for precipitating the ApoB-containing BFr and the method used for the subsequent measurement of cholesterol in the supernatant obtained using low-speed centrifugation are different.

In the JSCC reference method, dextran sulfate–Mg was used as a precipitant for the BFr obtained after ultracentrifugation. For these reagents, we used dextran sodium sulfate with a molecular weight of 50,000 (DS-50) manufactured by Meito Sangyo Co. Ltd. and PuA-grade magnesium chloride manufactured by Wako Pure Chemical Industries. The particle sizes of Dextralip 50 used in the CDC-BQ method and DS-50 used in the JSCC reference method were compared using HPLC [1] and DS-50 was found to have sharper peaks than Dextralip 50. This demonstrated the excellent uniformity of DS-50 (data not shown). Therefore, the JSCC reference method adopted a more uniform DS-50 as the precipitation agent. We chose magnesium chloride, a divalent cation, as a precipitant because it is an important factor that heavily influences the effective precipitation of lipoproteins [21]. Thus, it is very important to select a reagent that contains minimal amounts of contamination in terms of other metals. PuA-grade magnesium chloride from Wako Pure Chemicals is one of the many available reagent grades. We selected PuA-grade because it is stable in terms of quality and has low levels of divalent cations such as iron, manganese, and calcium. Although the precipitation of the ApoB fraction differed depending on the precipitant used, no significant differences were observed in the measured values due to the different precipitants (Table 4).

Another difference between the two methods is in terms of cholesterol measurement. The CDC-BQ method is a chemical measurement method that involves complicated procedures using the Liebermann-Burchard (LB) reagent, while the JSCC reference method is an automated enzymatic measurement method that uses CD. CDC-BQ method used for cholesterol measurement involves the use of LB reagent with a mixture of strong acids and is cumbersome in terms of stringent measurements and time management, whereas, the JSCC reference method is more user friendly and offers many advantages over the CDC-BO method. This automated CHE-CD enzymatic measurement method displays a good correlation with the total cholesterol reference measurement method of JSCC, using CD after alkaline hydrolysis [10]. As shown in Fig. 1, this method showed a good correlation with the CDC-BQ method as well. Although there was no difference between the average values of the JSCC reference method and the CDC-BQ method in the BFr, HDL-C measurements in the JSCC reference method were found to be only 0.03 mmol/L higher on average (Table 4). As a result, the LDL-C value obtained by subtracting HDL-C from BFr-C in the JSCC reference method was higher in the CDC-BQ method than in the JSCC reference method. The values for the CDC-AK method of the total cholesterol reference method using LB were on an average 1.6% higher than those for the definitive method. The cause of this bias has been reported to be a reaction with phytosterols in serum [22].

According to Bernert et al. [23], LB reagents have also been reported to react to phytosterols present in the serum. Therefore, the reaction of LB reagents and phytosterols may be responsible for this slightly higher trend. Modenese and colleagues [24] also compared the reactivity of phytosterols (campesterol, sitosterol, desmosterol, and lathosterol) between the enzymatic method using several different cholesterol oxidases and the LB method. There was no significant difference in its reactivity, and the practical effect of sterols, usually found in minimal amounts of serum, is reported to be negligible. On the other hand, substrate specificity for sitosterol in CD has been reported to have a relative activity of 50% (when cholesterol was 100%) [6].

Recent reports on the reference range for serum non-cholesterol sterol [25] indicated that sitosterol and campesterol were 0.99–3.88

Table 3
Serum lipid profiles of the study subjects.

	TC (mg/dL)	TG (mg/dL)	ApoAI (mg/dL)	ApoAII (mg/dL)	ApoB (mg/dL)	ApoCII (mg/dL)	ApoCIII (mg/dL)	ApoE (mg/dL)
Non-diseased (n = 47)								
Mean	205.5	90.0	166.7	32.7	92.6	3.6	9.2	3.7
SD	27.2	38.4	30.3	4.8	20.1	1.6	2.5	1.2
Maximum	301.0	173.0	305.0	47.1	139.0	9.3	19.9	9.6
Minimum	146.0	29.0	115.0	24.7	47.0	1.5	5.9	1.7
Diseased (n = 126)								
Mean	222.0	223.9	154.6	33.1	111.6	6.5	13.4	5.0
SD	54.1	194.4	34.5	6.7	32.7	4.4	6.9	2.3
Maximum	442.0	970.0	288.0	53.5	265.0	24.5	37.1	13.9
Minimum	86.0	37.0	92.0	16.7	20.0	0.4	3.0	1.8

TC, total cholesterol; TG, triglycerides; Apo, apolipoprotein.

Table 4

Mean cholesterol concentrations in the bottom, HDL, and LDL fractions, as assessed using the JSCC and CDC-BQ methods.

Method	Bottom fraction-C (n = 173)		HDL-C (n = 173)		LDL-C (n = 173)	
	JSCC	CDC	JSCC	CDC	JSCC	CDC
Mean (SD) (mmol/L)	4.73 (1.22)	4.73 (1.23)	1.57 (0.56)	1.54 (0.57)	3.16 (1.10)	3.18 (1.11)

Bottom fraction-C, bottom fraction-cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; JSCC, Japan Society of Clinical Chemistry; CDC, Centers for Disease Control and Prevention; BQ, beta-quantification.

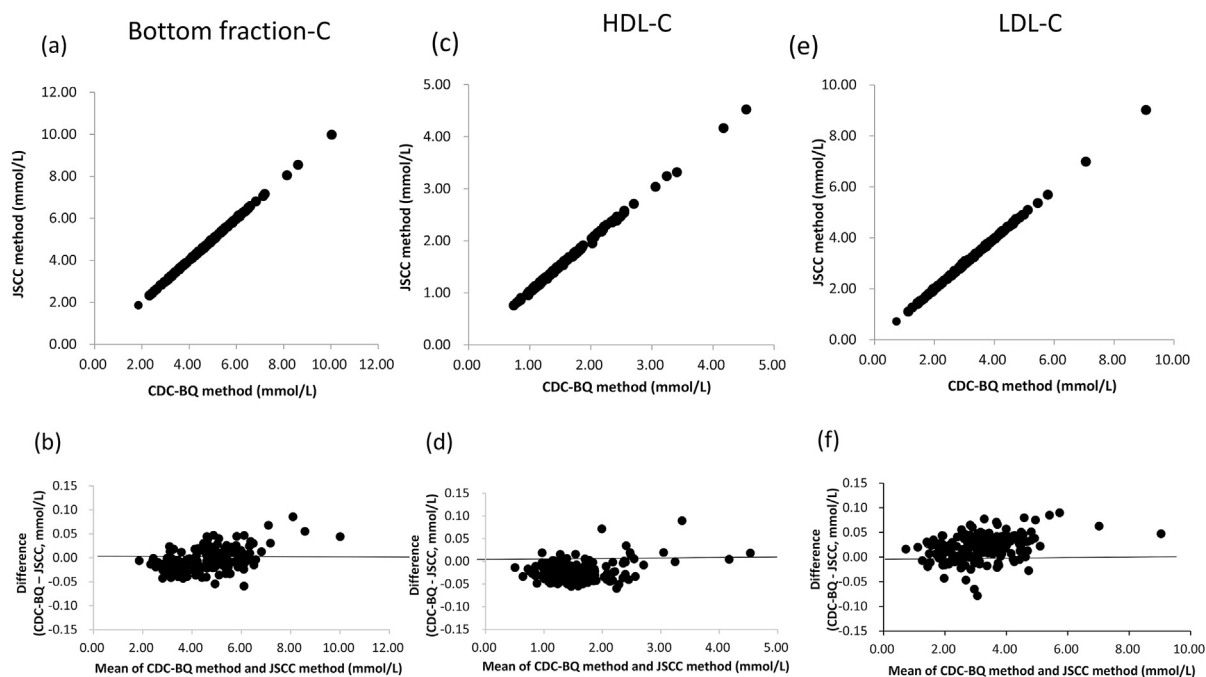


Fig. 1. Correlation between CDC-BQ and JSCC methods using the bottom and HDL-C fractions as samples. LDL-C was calculated using the following formula: LDL-C = Bottom fraction-C-HDL-C fraction.

(a), (c), and (e) show the correlation between the measurements of cholesterol levels made using the JSCC method (y-axis) and the CDC-BQ method (x-axis) in each lipid fraction. (b), (d), and (f) show the Bland-Altman plot of each lipid fraction. The x-axis shows the average cholesterol level of each lipid fraction between the CDC-BQ and JSCC methods, while the y-axis shows the difference in cholesterol levels between the CDC-BQ and JSCC methods. Bottom fraction-C, bottom fraction-cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; JSCC, Japan Society of Clinical Chemistry; CDC, Centers for Disease Control and Prevention; BQ, beta-quantification.

Table 5

Correlation analysis.

	Bottom fraction-C	HDL-C	LDL-C
n	173	173	173
r	0.999	0.999	0.999
Slope	0.991 (0.989–0.994) ^a	0.988 (0.982–0.993)	0.991 (0.988–0.994)
Intercept (mmol/L)	0.043 (0.031–0.055) ^a	0.041 (0.033–0.049)	0.009 (–0.002 to 0.020)
Syx (mmol/L)	0.021	0.019	0.025

Syx, standard deviation of the regression line; Bottom fraction-C, bottom fraction-cholesterol; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

^a 95% confidence interval.

(2.37–9.35) and 2.14–7.43 $\mu\text{g}/\text{mL}$ (5.28–18.3 $\mu\text{mol}/\text{L}$) in men and 1.03–4.45 (2.48–10.7) and 2.19–8.34 $\mu\text{g}/\text{mL}$ (5.41–20.6 $\mu\text{mol}/\text{L}$) in women, respectively. Both of these non-cholesterol sterols have been shown to have higher serum levels in women than in men. Furthermore, it was reported that each patient with sitosterolemia displayed ten-fold higher levels of sitosterol than healthy subjects. Although the non-cholesterol sterol concentration was not measured for the samples used in our study, further comparative studies are

needed to ascertain the reactivity between cholesterol measurement methods in such sitosterolemia patient samples. The correlation between the JSCC reference and CDC-BQ methods using clinical samples from various patients (as shown in Fig. 1) was good and resulted in comparable LDL-C and HDL-C measurements, although there was a difference of 0.03 mmol/L on average in the LDL-C measurements.

The CDC-BQ method is widely used as the gold standard for evaluating the accuracy of LDL-C measurements. Unfortunately, this method is both time consuming and labor intensive and requires a combination of ultracentrifuge and AK methods, but it is not routinely available in most laboratories. For this reason, there are only a limited number of facilities in Japan that can implement reference measurement methods for the validation of homogeneous LDL-C assays and equations that estimate LDL-C levels. Although it is complicated to use the ultracentrifugation method, the performance of the JSCC method replaced with an automated enzymatic cholesterol measurement was evaluated using the CDC-BQ method for comparison. On the other hand, in the method for separating lipoproteins through ultracentrifugation, it has been reported that ultracentrifugation of high TG samples may affect the cholesterol levels of the lipoprotein fraction [26,27], that is, it has been shown that high TG samples are very viscous, and large particles such as chylomicrons and VLDL tend to adhere to the wall of the tube, resulting in a decrease in the total amount of lipids. Therefore, there remains a problem in quantitatively measuring LDL-C by separating the lipid fraction of high TG samples using the ultracentrifugation method, including the CDC-BQ method and our method. Recently, several new equations have been devised that can measure high TG samples as an inexpensive and easy alternative to the traditional Friedewald equation for LDL-C measurements [28,29]. In the future, we hope to develop a better LDL-C measurement method by accumulating clinical evidence regarding the relationship between various LDL-C measurement methods and cardiovascular disease.

5. Conclusions

In conclusion, the JSCC reference method, which is a combination of ultracentrifugation-polyanion precipitation with CHE-CD, is an accurate, simple, and automatable method that is suitable for the quantitative analysis of LDL-C and HDL-C.

CRedit authorship contribution statement

Yuzo Kayamori: Conceptualization, Investigation, Methodology, Data curation, Writing – original draft. **Masakazu Nakamura:** Investigation, Methodology, Data curation. **Koji Kishi:** Investigation, Resources. **Takashi Miida:** Conceptualization, Supervision. **Kunihiro Nishimura:** Supervision. **Tomonori Okamura:** Supervision. **Satoshi Hirayama:** Resources. **Hirotohi Ohmura:** Resources. **Hiroshi Yoshida:** Resources. **Masumi Ai:** Resources. **Akira Tanaka:** Resources. **Hiroyuki Sumino:** Resources. **Masami Murakami:** Resources. **Ikuo Inoue:** Resources. **Tamio Teramoto:** Supervision. **Shinji Yokoyama:** Conceptualization, Supervision.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

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