Present Status of the Standardization of HDL-C, LDL-C, and TG Measurement Values Available in Japan

Yasuhito Nakajima,^{1,2*} Hachiro Yamanishi,³ and Nariaki Matsuura¹

¹Molecular Pathology Department, Division of Health Sciences, Graduate School of Medicine, Osaka University, Osaka, Japan

²Clinical Laboratory Department, Matsushita Memorial Hospital, Osaka, Japan

³Clinical Laboratory Science Department, Tenri Health Care University, Tenri, Nara, Japan

Background: Control sera used as evaluation samples in external quality control of HDL-C and LDL-C sometimes show disparities in results between direct methods differing in reaction principles. As a result, the present standardization status is unclear. In 2008, we investigated the present status of standardization of HDL-C, LDL-C, and TG measurement values available in Japan. **Subjects and Methods:** To evaluate accuracy, refrigerated fresh human serum pools used as samples were analyzed irrespective of the manufacturer's method. To evaluate precision, a questionnaire survey regarding the internal quality control status at each institution was carried out. As evaluation criteria, the permissible limits of error of B_A and CV_A based on JSCC proposals, and the accuracy and imprecision criterion NCEP proposals were used. **Results:** There were 70 participating institutions for

HDL-C, 65 for LDL-C, and 71 for TG. TG values from the institutions showed 83.1–91.5% for both the JSCC B_A range and the NCEP criterion range. HDL-C values within the JSCC B_A range were 81.4–82.6% and within the NCEP criterion range the value was 98.6%. Similarly, LDL-C values within the JSCC B_A range were 89.2% and within the NCEP criterion range the value were 81.5–83.1%, respectively. Concerning precision, a questionnaire regarding internal quality control of each institution was completed. More than 90% of the institutions showed values within the CV_A range proposed by the JSCC for all of HDL-C, LDL-C, and TG. **Conclusion:** Standardization of lipid assays used for metabolic syndrome-based health checkups has been mostly achieved. J. Clin. Lab. Anal. 27:195–203, 2013. © 2013 Wiley Periodicals, Inc.

Key words: HDL-C; LDL-C; TG; standardization

INTRODUCTION

In recent years, the incidence of lifestyle-related diseases (e.g., hyperlipidemia, hypertension, diabetes mellitus, gout, and obesity) has been increasing among Japanese people, and has become a major social issue. The Ministry of Health, Labour and Welfare initiated metabolic syndrome-focused health checkups in April 2008, and designated TG, HDL-C, and LDL-C as basic health checkup values (1, 2). This required an improvement in the measurement accuracy of these values. However, control sera used as evaluation samples in external quality control surveys for HDL-C and LDL-C sometimes show disparities between direct methods differing in reaction principles, due to abnormal lipoproteins or lipoprotein degeneration. So, evaluation according to

manufacturer's methods is performed, and the present standardization status is unclear (3–5). In 2008, a quality control project undertaken by the Osaka Association of Medical Technologists performed an evaluation of accuracy, irrespective of the manufacturer's method, using fresh human serum pools as samples. A questionnaire survey regarding the internal quality control of each institution was distributed to evaluate precision,

*Correspondence to: Yasuhito Nakajima, Department of Clinical Laboratory, Matsushita Memorial Hospital, 5–55 Sotojima-cho, Moriguchi-shi, Osaka 570-8540, Japan. E-mail: fwhz2544@mb.infoweb.ne.jp

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and to clarify the present status of the standardization of HDL-C, LDL-C, and TG measurement values available in Japan.

MATERIALS AND METHODS

Preliminary Survey

The measurement values of HDL-C and LDL-C sometimes show disparities between direct methods differing in reaction principles according to serum storage conditions after blood collection. This is due to qualitative changes in lipoprotein caused by the action of lecithin-cholesterol acyltransferase (6–8). So, during a preliminary study we confirmed the sample's stability during refrigeration for 3 days (assuming equivalent sample delivery times from the seven regional institutions in Osaka Prefecture) using fresh serum pools

Methods of this Survey

Samples: Blood samples collected from 15 volunteer members of the Osaka Association of Medical Technologists had no known diseases. All volunteers fasted in the morning of the sample preparation day. Blood was collected into plastic vacuum blood collection tubes (Sekisui Medical Sekisui Medical, Tokyo, Japan), clotted 60 min, and centrifuged ($1,800 \times g$ for 10 min at 10°C). Each serum was measured by direct method of TG, HDL-C, and LDL-C, and immediately divided into two groups, a low concentration group and a high concentration group.

Serum from all members was combined and mixed in plastic vials. Two full 2-ml aliquots in plastic tubes were then transported at 4°C in insulated shipping containers.

Distribution/Measurement Conditions

The prepared serum pool samples were distributed under refrigeration so that they could arrive at each institution the next day, and each institution was requested to perform measurement on the day of their arrival.

Measurement Methods for Evaluation

The methods of measuring evaluation samples were the enzymatic colorimetric method with glycerol blanking for TG and the direct method as a nonprecipitation method for HDL-C and LDL-C. Therefore, the results of calculation using the Friedewald formula (8) for LDL-C were excluded from analysis.

EVALUATION SAMPLES

Properties of Samples for Evaluation

Samples were subjected to lipoprotein polyacrylamide gel electrophoresis, using a Lipophor system (Jokoh, Tokyo, Japan) (9) and agarose gel electrophoresis, REP-Lipo agarose plates (Helena Laboratories, Beaumont, TX, USA) (10), followed by cholesterol (CHO)/TG differential staining to obtain HDL, VLDL, IDL, LDL, and CM fractions. Qualitative abnormalities such as abnormal fractions were evaluated.

Goal Values

Goal values were determined based on measurement values at nine regional institutions in Osaka Prefecture where the accuracy of measurement values at their own institutions was confirmed using JCCRM 223–24 (Reference Material Institute for Clinical Chemistry Standards: ReCCS) (11), as standards for the lipid assay, and the maintenance of the accuracy could be confirmed using common serum pools.

EVALUATION CRITERIA

Validation of Evaluation Methods

Two reported types of samples were used. To exclude abnormal values, we contacted each institution when there were measurement values outside ± 3 SD, and changed input values only when records on the analysis day remained and input errors could be clarified. Values with no analysis day records were eliminated.

Precision Evaluation Methods

A questionnaire survey was conducted; and the types of control samples used at each institution, the mean values for 1 month, CV, and internal quality control status were evaluated.

Evaluation Criteria for Accuracy and Precision

The evaluation criteria used were as follows: (a) the permissible limits of error of analytical bias (B_A) and coefficient of variation of imprecision (CV_A) based on biological variation in healthy individuals proposed by JSCC (12), (2) the accuracy criterion and impression criterion as the standardization criteria proposed by NCEP (13, 14). Table 1 shows the evaluation criteria proposed by the JSCC and NCEP.

TABLE 1. Evaluation Criteria Proposed by the JSCC and NCEP

JSCC	NCEP			
	B _A	CV _A	Bias goal	Within-run goal
HDLC	6.0%	4.2%	≤± 10%	≤6%
LDLC	6.9%	4.6%	≤± 4%	≤4%
TG	15.4%	14.8%	≤± 5%	≤5%

B_A (analytical bias) is bias of measurement values. The difference between the expected measurement and reference standard values (accepted reference value of the standard).

CV_A (coefficient of variation of imprecision) is coefficient of variation representing the precision of measurement.

In the case of JSCC, when the value of B_A or CV_A is > 5%, the upper limit is assigned a value of 5%.

STATISTICAL ANALYSIS

Statistical analysis was performed using the software packages SPSS[®] version 11.5 (SPSS 11.5, Chicago, IL) and Stat Flex[®] version 6.0, and the results are presented as the mean ± SD. The stability of fresh serum pools in the preliminary survey was analyzed using two-way repeated measures ANOVA, and differences among reagent manufacturers were analyzed using the Kruskal–Wallis test as a nonparametric test. $P < 0.05$ was regarded as significant.

RESULTS

Preliminary Survey

Two types of fresh serum pools were distributed under refrigeration to regional institutions in Osaka Prefecture,

and their stability during 3 days of refrigeration was verified during 3 days. There was no significant change in the stability of any measurement value. We decided to use two types of fresh human serum pool that were distributed under refrigeration so that they could arrive at each institution the next day. Each institution was requested to perform measurements on the day.

RESULTS OF THE SURVEY

Properties of Evaluation Samples

To avoid degeneration, the two types of fresh serum pool used as evaluation samples were distributed so that they could arrive at each institution on the day following their preparation, and each institution was requested to perform measurements on the day of arrival. Concerning the two types of samples, Figure 1 shows the results of lipoprotein polyacrylamide gel electrophoresis using a Lipophor system. The RM value indicates the rate of migration (the VLDL-LDL distance/VLDL-HDL distance). An LDL peak closer to the VLDL peak with a larger particle size indicates normal size LDL, while an LDL peak closer to the HDL peak with a smaller particle size indicates small dense LDL. In general, RM values ≥ 0.400 suggest the presence of small, dense LDL. Figure 2 shows the results of agarose gel electrophoresis using REP-Lipo agarose plates, followed by CHO/TG differential staining. The CHO and TG levels were calculated employing the area percentage obtained by densitometry multiplied by the CHO or TG concentration.

Fig. 1. Results of lipoprotein polyacrylamide gel electrophoresis (Lipophor System, Jokoh) of samples A and B. VLDL remained as the origin at the cathode of the gel, HDL migrated to the anode, and LDL migrated between VLDL and HDL. The VLDL-LDL distance divided by the VLDL-HDL distance was defined as the rate of migration (RM) of LDL.

Fig. 2. Results of agarose gel electrophoresis (REP-Lipo) of samples A and B.

For both samples A and B, the left figure shows a CHO (red) and TG (green) fraction-stained densitometry pattern of lipoproteins for comparison between the two fractions. The CHO or TG level in each fraction shown in the right figure was calculated by multiplying the area percentage (%) obtained by densitometry by the CHO or TG concentration.

Based on these results, no qualitative abnormalities such as abnormal fractions were observed in either type of sample. Sample A showed an almost normal pattern. Sample B showed slightly unclear separation with a large amount of VLDL, but an almost normal pattern of HDL itself.

Establishment of Goal Values and Permissible Ranges

Goal values were determined based on measurement values at nine regional institutions in Osaka Prefecture. The accuracy of measurement values at each institution was confirmed using JCCRM 223–24. Table 2 showed concentrations and uncertainties of JCCRM 223–24 as standards for the lipid assay, and the average value and ± 2 SD range for all.

The results of measurement at nine regional institutions were used for the establishment of goal values. The results regarding each parameter showed good convergence (CV, 2.21–2.98%). Since the regional institutions

confirmed accuracy using standards for the lipid assay, and the accuracy was maintained, the mean values in the nine institutions were used as goal values.

The permissible limits of error of B_A were based on biological variation in healthy individuals, and the permissible range was calculated from NCEP's accuracy criterion as established accuracy criteria on the basis of the above results.

As a result, for HDL-C, both JSCC's B_A range and NCEP's accuracy criterion range were 1.86~2.09, 1.16~1.32 (mmol/l). For LDL-C, JSCC's B_A range was 1.81~2.04, 3.10~3.44 (mmol/l) and NCEP's accuracy criterion range was 1.84~2.02, 3.13~3.41 (mmol/l). For TG, both JSCC's B_A range and NCEP's accuracy criterion range were 0.49~0.56, 3.30~3.66 (mmol/l), respectively, of samples A and B.

Results of Measurements of Evaluation Samples

There were 70 participating institutions for HDL-C, 65 for LDL-C, and 71 for TG. Table 3 shows the number of

TABLE 2. Certified Values and Uncertainties in JCCRM 223–24 and Measurement Values at Nine Regional Institutions

			IV	Level V	VI
HDL-C	ReCCS	mmol/l	1.220.03	1.74 $\pm$ 0.03	
		(mg/dl)	47.0 $\pm$ 1.0	67.2 $\pm$ 1.0	
	Measurement values	mmol/l	1.29 $\pm$ 0.12	1.77 $\pm$ 0.10	
		(mg/dl)	49.8 $\pm$ 4.7	68.5 $\pm$ 3.9	
LDL-C	ReCCS	mmol/l		2.82 $\pm$ 0.10	
		(mg/dl)		108.9 $\pm$ 3.7	
	Measurement values	mmol/l		2.74 $\pm$ 0.11	
		(mg/dl)		106.1 $\pm$ 4.4	
TG	ReCCS	mmol/l	1.07 $\pm$ 0.010		2.00 $\pm$ 0.01
		(mg/dl)	93.5 $\pm$ 0.7		178.3 $\pm$ 1.2
	Measurement values	mmol/l	1.05 $\pm$ 0.03		2.05 $\pm$ 0.04
		(mg/dl)	93.4 $\pm$ 2.5		183.0 $\pm$ 3.3

Upper section of each category is authentication value and uncertainties of JCCRM 223–24, and lower section is the average value and ± 2 SD range for all.

TABLE 3. Results of Measurement of Each Item

Sample		HDL-C		LDL-C		TG	
		A	B	A	B	A	B
<i>n</i>		70	69	65	65	71	71
Mean	mmol/l	1.97	1.25	1.92	3.29	0.53	3.47
	(mg/dl)	76.0	48.4	74.2	127.0	47.4	309.8
SD	mmol/l	0.08	0.05	0.06	0.13	0.02	0.10
	(mg/dl)	2.9	2.0	2.4	4.9	1.9	9.3
CV		3.86	4.08	3.17	3.87	3.93	2.99
Institutions within the JSCC's range (%)		81.4	82.6	89.2	89.2	83.1	91.5
Institutions within the CRMLN's range (%)		98.6	98.6	83.1	81.5	83.1	91.5

The CV is defined as the ratio of the SD to the mean.

institutions for evaluating samples A and B, in addition to the mean, SD, and CV; and many of institutions showed values within JSCC's B_A range and NCEP's accuracy criterion range based on goal values. For HDL-C, the CV was 3.86–4.08%, and many of institutions showed values within JSCC's B_A range and NCEP's accuracy criterion range was 81.4–82.6%. For LDL-C, the CV was 3.17–3.87%, and many of institutions showed values within JSCC's B_A range was 89.2%, and that within NCEP's accuracy criterion range was 81.5–83.1%. For TG, the CV was 2.99–3.93%, and many of institutions showed values within JSCC's B_A range and NCEP's accuracy criterion range was 83.1–91.5%.

Figure 3 shows the distribution of all measurement values and measurement values using the reagents of three major manufacturers for each parameter in samples A and B. The mean HDL-C levels (mmol/l) for Sekisui Medical Co., Ltd., Kyowa Medex Co., Ltd., and Wako Pure Chemical Industries Co., Ltd. were 1.94, 2.02, and 1.94, respectively, in sample A and 1.24, 1.27, and 1.20, respectively, in sample B, with Kyowa being the highest, followed in order by Sekisui and then Wako. The mean LDL-C levels (mmol/l) for Sekisui, Kyowa, and Wako were 1.90, 1.92, and 2.01, respectively, in sample A and 3.23, 3.28, and 3.47, respectively, in sample B, being the highest for Wako and followed in order by Kyowa and then Sekisui. The TG levels (mmol/l) for Sekisui, Kyowa, and Wako were 0.52, 0.53, and 0.55, respectively, in sample A and 3.40, 3.49, and 3.50, respectively, in sample B, with Wako being the highest, followed in order by Kyowa and then Sekisui. In both types of samples, a significant difference was observed in each parameter among the reagents ($P < 0.05$).

RESULTS OF A QUESTIONNAIRE SURVEY OF INTERNAL QUALITY CONTROL

Participating institutions were required to fill out a survey questionnaire in order to evaluate internal qual-

ity controls, the types of control samples used, and the mean/SD during a 1-month period. At all institutions, quality control was being performed using one to four types of serum pool or commercially available sera. Figure 4 shows the relationship between measurement values and CV in each parameter.

All control samples showed values within the permissible limits of error of CV_A based on biological variation in healthy individuals. This indicates the precision of measurement proposed by the JSCC in 64/70 (91.4%) for HDL-C, 64/65 (98.5%) for LDL-C, and 68/71 (95.8%) for TG.

DISCUSSION

In many Western countries, the Friedewald formula (F formula) (8) is commonly used to measure LDL-C. In Japan, however, the LDL-C direct method has rapidly spread after the initiation of metabolic syndrome-focused health checkups (15, 16). Accuracy of reagent kit was used in the LDL-C/HDL-C direct methods; Nakamura et al. reported that all 20 reagents showed HDL-C values within $\pm 5\%$ of the reference value as the CDC's standardization criterion, but only 14 (66.7%) of 21 reagents showed LDL-C values within $\pm 4\%$ of the reference value as the standardization criterion, suggesting that the HDL-C direct method is acceptable, but the LDL-C direct method requires further improvement for application to metabolic syndrome-focused health checkups (17). Similarly, Ishijima reported that the comparability of measurement values among reagent kits by the direct method is inadequate, and suggested that this is due to the properties of the lipoprotein in the serum reference standards for lipid assays, which is mainly composed of LDL, and contains certain amounts of VLDL and HDL but almost no IDL, a small amount of VLDL, and only a negligible amount of chylomicrons (18). This results in the low reliability of LDL-C values on the clinical side. During a recent survey of the accuracy of reagent kits using

Fig. 3. Distribution of all measurement values and measurement values using reagents of the three major manufacturers for each item in samples A and B. For each item, the upper plot shows all measurement values, and the lower plot shows measurement values using reagents of the three major manufacturers. Each box indicates the range of the mean 2 SD. Accuracy evaluation criteria ■ : proposed by the JSSC, ■ : proposed by the CRMLN, and ■ : criteria that are the same between the two.

Fig. 4. Relationship between measurement values m each item and CV.

■ : Diagnostic criteria for lipid abnormalities (blood collection during fasting) according to the Guidelines for Prevention of Atherosclerotic Cardiovascular Disease, 2007 proposed by the Japan Atherosclerosis Society: HDL < 1.03 mmol/l (hypo-HDL-cholesterolemia), LDL > 3.62 mmol/l (hyper-LDL-cholesterolemia), and TG > 1.68 mmol/l (hypertriglyceridemia).

■ : The permissible range of error of CV_A based on biological variation in healthy individuals, which indicates measurement precision. Proposed by the JSCC: HDL-C, 4.2%; LDL-C, 4.6%; TG, 14.8% (upper limit, 5%).

human fresh serum pools, the Technical Committee of the Japan Association of Clinical Reagent Laboratories evaluated convergence in 15 reagents for HDL-C (ten direct method reagents and five dry reagents) and ten for LDL-C (all were direct method reagents) using 24 types of serum pools. The CV of HDL-C in the serum pool for

each reagent was 2.7–3.9% using the direct method, and 3.0–5.4% using the direct method or dry reagents. The CV of LDL-C was 1.5–5.4%. The committee reported that these results fall within the permissible limits of error based on biological variation (4.2% for HDL-C; 4.6% for LDL-C) (19).

So, we evaluated the present status of accuracy standardization using fresh human serum pools without qualitative abnormalities. The results regarding the convergence of all institutions were evaluated as the CV. The CV was 3.86–4.08% for HDL-C, 3.17–3.87% for LDL-C, and 2.99–3.93% for TG. The convergence for LDL-C was slightly better, which was because institutions that reported less accurate values of HDL-C/TG than other institutions did not use the direct method in routine examination. With the LDL-C direct method expected to be more widely used in the future, convergence is expected to decrease.

To evaluate precision, a questionnaire survey regarding the internal quality control status at each institution showed HDL-C/LDL-C/TG values within the CV_A range proposed by the JSCC, based on biological variation in healthy individuals in more than 90% of the institutions.

Based on these results, we feel that the standardization of lipid assays for metabolic syndrome-based health checkups has been mostly achieved. However, if working standards that can be commonly used for all measurement reagents are implemented, improved standardization may be expected.

The Japan Atherosclerosis Society has recommended that in clinical practice, LDL-C should be obtained basically using the Friedewald formula rather than the direct method due to the inadequate standardization of kits for the direct method of LDL-C measurement. We question whether the Friedewald formula is an appropriate method for obtaining LDL-C values. Using the Friedewald formula, the LDL-C value is calculated from measurement values in three values. Therefore, errors in three values are involved, increasing the possibility of precision problems. In addition, this formula cannot be used in cases showing TG ≥ 4.488 mmol/l (400 mg/day). Finally, the calculation of LDL-C in postprandial outpatients using the Friedewald formula generally measured in patients before meal is not appropriate. However, evidence that “A high-level reduction in LDL-C more markedly reduces atherosclerotic diseases” has been established after many studies (20). Considering the value of these studies, the direct method showing LDL-C reactivity close to that calculated using the Friedewald formula may be the most appropriate. We evaluated accuracy using two kinds of refrigerated fresh human serum pools that we analyzed irrespective of manufacturer’s methods. To evaluate precision, we carried out a questionnaire survey regarding the internal quality control status at each institution with respect to evaluation criteria, the permissible limits of JSCC and NCEP proposals. Therefore, we understand that this research is not applicable to various hyperlipemia, as Miller et al. have pointed out (21).

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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ABBREVIATIONS

TG	= triglycerides
HDL-C	= high-density lipoprotein-cholesterol
LDL-C	= very low-density lipoprotein
IDL	= intermediate-density lipoprotein
CM	= chylomicron
LCAT	= lecithin cholesterol acyltransferase
CHO	= cholesterol
JSCC	= Japan Society of Clinical Chemistry
NCEP	= National Cholesterol Education Program
CDC	= US Centers for Disease Control and Prevention
CRMLN	= Cholesterol Reference Method Laboratory Network
RM	= relative movement

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