Original Article

Ten-year evaluation of homogeneous low-density lipoprotein cholesterol methods developed by Japanese manufacturers

—Application of the Centers for Disease Control and Prevention/Cholesterol Reference Method Laboratory Network lipid standardization protocol —

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Aim: The risk index for atherosclerotic cardiovascular diseases in the Japanese metabolic syndrome-focused health checkup program was changed from total cholesterol (TC) to low-density lipoprotein cholesterol (LDL-C). We discuss the validity of this change with respect to standardization.
Methods: The beta-quantification procedure of the Centers for Disease Control and Prevention (CDC) uses the LDL-C reference value as a target. Clinical laboratories and commercial manufacturers use homogeneous LDL-C methods for standardization. (A) For clinical laboratories, LDL-C in 648 samples requested from 108 hospitals was analyzed. (B) Manufacturers participated in the CDC/Cholesterol Reference Method Laboratory Network LDL-C standardization protocol. The standardization was conducted with a performance follow-up for the 10-year period from 1998 to 2008 at 2-year intervals, 6 times.
Results: (A) In clinical laboratories, acceptable LDL-C levels within ±4% of the CDC's criteria remained 70.4%, 456 of 648 subjects. Negative maximum bias deviating from the LDL-C target value was −35.8%, −52.5 mg/dL, and positive maximum bias was +24.5%, +32.3 mg/dL. (B) For manufacturers, the standardization achievement rate of the analytical reagent/instrument/calibrator system in the last four standardizations from 2002 to 2008 remained on average 66.6%, far lower than the level required.
Conclusions: The standardization achievement rate of homogeneous LDL-C methods was much lower than that of TC. TC should still be used as a risk index for atherosclerotic cardiovascular diseases. The standardization achievement rate of homogeneous LDL-C should be maintained at 100%, at least using samples with normal lipoprotein profiles. The accuracy and specificity of LDL-C should be further improved before practical and clinical use.

J Atheroscler Thromb, 2010; 17:1275-1281.

Key words; CDC/CRMLN, TC, LDL-C, Metabolic syndrome

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Received: March 29, 2010
Accepted for publication: July 22, 2010

Introduction

In Japan, atherosclerotic diseases, particularly cardiovascular events due to myocardial infarction and cerebrovascular diseases due to cerebral infarction/hemorrhage, as well as cancer rank high in death-cause

statistics, accounting for 30% of all deaths[1]. Due to the super-aged society in Japan, which is rare in the world, the mortality rate due to cardiovascular events is expected to increase further[2, 3]; therefore, the establishment of effective methods for the prevention and treatment of these events is a nationally important problem for devising medical policies[4].

The Ministry of Health, Labour and Welfare established the metabolic syndrome (MetS)-focused health checkup program for the insured aged 40-74 years in April 2008[5, 6]. Its lipid measurement items did not include total cholesterol (TC), but high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) were selected for clinical examination[7].

The widely performed quality control survey for clinical examination is an annual cross-sectional current status surveillance on performance in clinical laboratories. In contrast, the performance of reagent manufacturers that directly affects the performance of clinical laboratories has been neglected. In addition, information on the limitations of homogeneous HDL-C and LDL-C methods is not adequately available to the public; therefore, standardization must start with manufacturers. Unless the current status of suppliers is clarified, improvement of the performance of clinical laboratories is not expected.

This study aimed to discuss the validity of the change from TC to LDL-C from a lipid standardization viewpoint based on both the results of homogeneous LDL-C measurements in clinical laboratories and a ten-year homogeneous LDL-C standardization by reagent manufacturers[8].

Materials and Methods

Standardization item

The item for standardization was LDL-C[9]. For comparison, standardization results for TC and HDL-C were also evaluated.

Standardization program, materials and methods

(A) A clinical laboratory participated in the LDL-C Standardization Program for Clinical Laboratories (http://www.kenkoukagaku.jp) conducted by Osaka Medical Center for Health Science and Promotion (OMC). OMC joined the Cholesterol Reference Method Laboratory Network (CRMLN) organized by the Centers for Disease Control and Prevention (CDC) in July 1992 and has been a lipid reference laboratory for the last 17 years. LDL-C in 648 fresh, non frozen, individual samples requested from 108 institutions over 6 years from January 2004 to January 2010 were analyzed within 48 after blood collection by homogeneous methods used in the clinical laboratory. The LDL-C reference value as a target in the same sample was assayed by the beta-quantification (BQ) procedure[10], an ultracentrifugation reference measurement procedure, followed by the Abell-Kendall reference method OMC.

(B) The following seven manufacturers participated in the CDC/CRMLN LDL-C Certification Protocol for Manufacturers (June 2006) (http://www.cdc.gov/labstandards/crmln.htm): Serotec Co., Ltd., Denka Seiken Co., Ltd., Sekisui Medical Co., Ltd., UMA Co., Ltd., Kyowa Medex Co., Ltd., Wako Pure Chemical Industries Ltd., and Sysmex Corporation. The standardization achievement rate was followed up at 2-year intervals from 1996 until 2008 for both TC and HDL-C, and from 1998 until 2008 for LDL-C. In principle, fresh individual serum or a mixed sample of at most 2 individuals was used. About 10 samples are measured once a week, 4 times, which adds up to at least 40 samples. For sample collection, the protocol shows the following target distribution: 20% of samples from <100 mg/dL, 30% from 100 to 130 mg/dL, 30% from 131 to 160 mg/dL, and 20% from 161 to 400 mg/dL. In actual standardization, measurement once a week was performed six times, and 50 samples were analyzed. For each manufacturer, measurement was completed within 48 after blood collection. Six of the seven manufacturers participating in the standardization shared the collection of samples and also distributed the samples obtained to each other. Throughout this procedure, the same samples were analyzed by all manufacturers. The same samples were measured using both the BQ method as the LDL-C reference and the analytical reagent/instrument/calibrator system (analytical system), which is the combination of a reagent, an analytical instrument and a calibrator adopted by each manufacturer. Samples were also analyzed by both agarose gel electrophoresis and polyacrylamide gel electrophoresis. As a result of the lipid matrices by the two electrophoretic methods, 80% of all samples used for LDL-C showed normal lipoprotein profiles, while the remaining 20% did not show sufficient morbidity to be regarded as dyslipidemia. Apart from samples for standardization, for the calculation of precision, the manufacturers reported measurement values ($n=1$) for 20 days using optional internal quality control serum at a concentration of 130-160 mg/dL.

Performance criteria for clinical laboratories and reagent manufacturers

According to the CDC's LDL-C performance

Table 1. Incidences of failures according to 10 certification items for LDL-C

	year	1998	2000	2002	2004	2006	2008	
	Number of participating manufacturers	5	5	5	6	6	7	
	Number of analytical systems	17	17	16	19	22	21	112
	Sample numbers used	50	47	54	45-50	54	51	
	Runs	5	5	6	5	6	6	
	Standardization achievement rate (%)	10/17 (58.8%)	17/17 (100.0%)	10/16 (62.5%)	14/19 (73.7%)	14/22 (63.6%)	14/21 (66.7%)	
		Number of failures / Number of analytical system						Total (%)
No.1	r - square	2/17	0/17	2/16	4/19	1/22	7/21	16/112 (14.3%)
No.2	%Bias at 100mg/dL	0/17	0/17	0/16	3/19	0/22	1/21	4/112 (3.6%)
No.3	%Bias at 130mg/dL	0/17	0/17	0/16	0/19	3/22	1/21	4/112 (3.6%)
No.4	%Bias at 160mg/dL	0/17	0/17	0/16	0/19	5/22	2/21	7/112 (6.3%)
No.5	Average %Bias	3/17	0/17	0/16	0/19	1/22	1/21	5/112 (4.5%)
No.6	Average absolute %Bias	6/17	0/17	0/16	3/19	7/22	4/21	20/112 (17.9%)
No.7	Among-run CV	0/17	0/17	0/16	0/19	0/22	0/21	0/112 (0.0%)
No.8	t-test	2/17	0/17	0/16	0/19	1/22	1/21	4/112 (3.6%)
No.9	Fail both in within-methods outliers	1/17	0/17	0/16	0/19	1/22	1/21	2/112 (1.8%)
No.10	Fail both in between-methods outliers	0/17	0/17	4/16	0/19	0/22	1/21	4/112 (3.6%)

Analytical system means analytical reagent/instrument/calibrator system used at manufacturer's laboratory. LDL-C standardization was conducted using analytical systems of Japanese reagent manufacturers at 2-year intervals from 1998 to 2008.The incidences of LDL-C uncertified cases according to the certification items (Nos. 1 to 10) are shown in Table 1.

criteria, acceptable accuracy in average %bias should be within ±4% of the reference value in clinical laboratories and the analytical system of manufacturers should simultaneously fulfill all ten of the following (**Table 1**): 1: r2 >0.975; 2: %bias as accuracy at 100 mg/dL ≦4%; 3: that at 130 mg/dL ≦4%; 4: that at 160 mg/dL ≦4%; 5: average %bias as accuracy ≦4%; 6: average absolute %bias as accuracy ≦4%; 7: among-run coefficient of variation as precision ≦4%; 8: t-test of bias, not significant at $\alpha = 5\%$; 9: within-method outliers, 1 allowed; and 10: between-method outliers, none allowed. The standardization achievement rate was calculated as the number of certified analytical systems expressed as a percentage of all systems that participated[11]. The results were compared using a spreadsheet for analysis. Both CDC and OMC determined the failure or not of standardization for manufacturers.

CDC/CRMLN's TC and HDL-C standardization for manufacturers

TC standardization[12] was performed according to the TC Certification Protocol for Manufacturers-Revised (October 2004) (http://www.cdc.gov/labstandards/crmln.htm) as a program for reagent manufacturers. HDL-C standardization[12] was carried out according to the HDL-C Certification Protocol for Manufacturers (November 2002) (http://www.cdc.gov/labstandards/crmln.htm).

Results

Standardization for clinical laboratories

The %bias of each sample from the reference val-

ue was ≦ −4% as the lower limit of LDL-C performance criteria in 65 samples (10.0%) and ≧ +4% as the upper limit in 127 samples (19.6%). In addition, 243 samples (37.5%) showed a lower value than the target while 405 (62.5%) showed a higher value. Cases not fulfilling the LDL-C criteria accounted for 29.6%, and so only 70.4% fulfilled the criteria. These results suggest that about 1/3 of LDL-C measurements cannot be used clinically. **Fig. 1** shows the distribution of the %bias of each item from the reference obtained by the BQ method. Negative bias at maximum deviated from the LDL-C reference value by −35.8%, −52.5 mg/dL, and positive bias at maximum by +24.5%, +32.3 mg/dL.

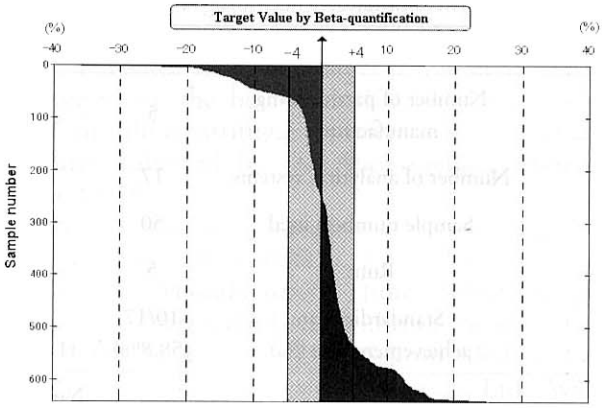

Fig. 1. %Bias distribution of homogeneous LDL-C by Japanese clinical laboratories

LDL-C of 648 samples collected in 108 clinical laboratories was measured using homogeneous LDL-C assays in clinical laboratories. The target value was established by the BQ reference method at Osaka Medical Center for Health Science and Promotion. In each sample, the %bias of the LDL-C value by homogeneous assay to the target value was calculated and its distribution is shown. Samples within ±4% as acceptable performance criteria numbered 456, 70.4%. Negative bias at maximum deviated from the LDL-C target value by −35.8%, −52.5 mg/dL and positive bias by +24.5%, +32.3 mg/dL. The x-axis and y-axis represent the %bias and the sample numbers, respectively.

Standardization of manufacturers

For the standardization of manufacturers, **Fig. 2** shows changes in the standardization achievement rate by year. The standardization achievement rate for TC was 100% in every year from 1996, and that for HDL-C gradually increased from 1996 to 2002 and was 100% in the three years from 2004. In contrast, the standardization achievement rate for LDL-C remained on average 66.6% in the four years of evaluation from 2002 to 2008. **Fig. 3** shows the percentages of successfully certified analytical systems showing LDL-C values within ±1% and ±2% of the target value. In **Table 1**, the incidence of uncertified cases according to the ten performance items for LDL-C measurement is shown. No case was not certified due to inadequate precision. Uncertified cases were frequently observed for r-square (No. 1) and average absolute %bias (No. 6) compared with the other criteria. These results suggest the points to which manufacturers should pay attention in future LDL-C method certification tests. Since an r-square value ≦0.975 indicates poor day-to-day reproducibility in the certification test, this problem may be relatively readily overcome by careful management of the analytical system and adequate attention to its manipulation. In addition, cases showing an average absolute %bias >4% suggest there will be problems with accuracy associated with reagent specificity, the value assignment of the calibrator, and complex matrix effects.

Discussion

The Japanese Ministry of Health, Labour and Welfare has performed the MetS program since 2008. Its lipid tests did not include TC, instead, HDL-C, LDL-C and TG were selected as three essential items for clinical examination[8]. MetS represents an ideal situation in which the same examinees obtain the same values at all health checkup institutions as a result of standardization using a reference material with a certified known value. If this ideal state is realized, the compatibility of measurement values will be secured, and measurements performed only once will be adequate, which is also useful in terms of economic policy. However, although such an ideal situation is theoretically possible, it may be difficult to achieve without active effort. To approach this standardization, we decided to request manufacturers to achieve an accuracy of within ±1% in more than 80%, and within ±2% in 100%, of their analytical systems.

In homogeneous LDL-C methods, LDL-C is separated from other cholesterol fractions using the characteristics of surfactants, and LDL-C is directly measured using an automatic analyzer. Homogeneous LDL-C methods are very convenient due to the following advantages: (a) only a very small amount of a sample (2-5 μL) is necessary, (b) measurements can be performed using an automatic analyzer in about 5-10 minutes, (c) the measurement of three items required for calculation using Friedewald's formula is not necessary, and (d) measurements can be performed even at TG concentration of 1,000 mg/dL or more. Since 1996, manufacturers have taken the initiative of de-

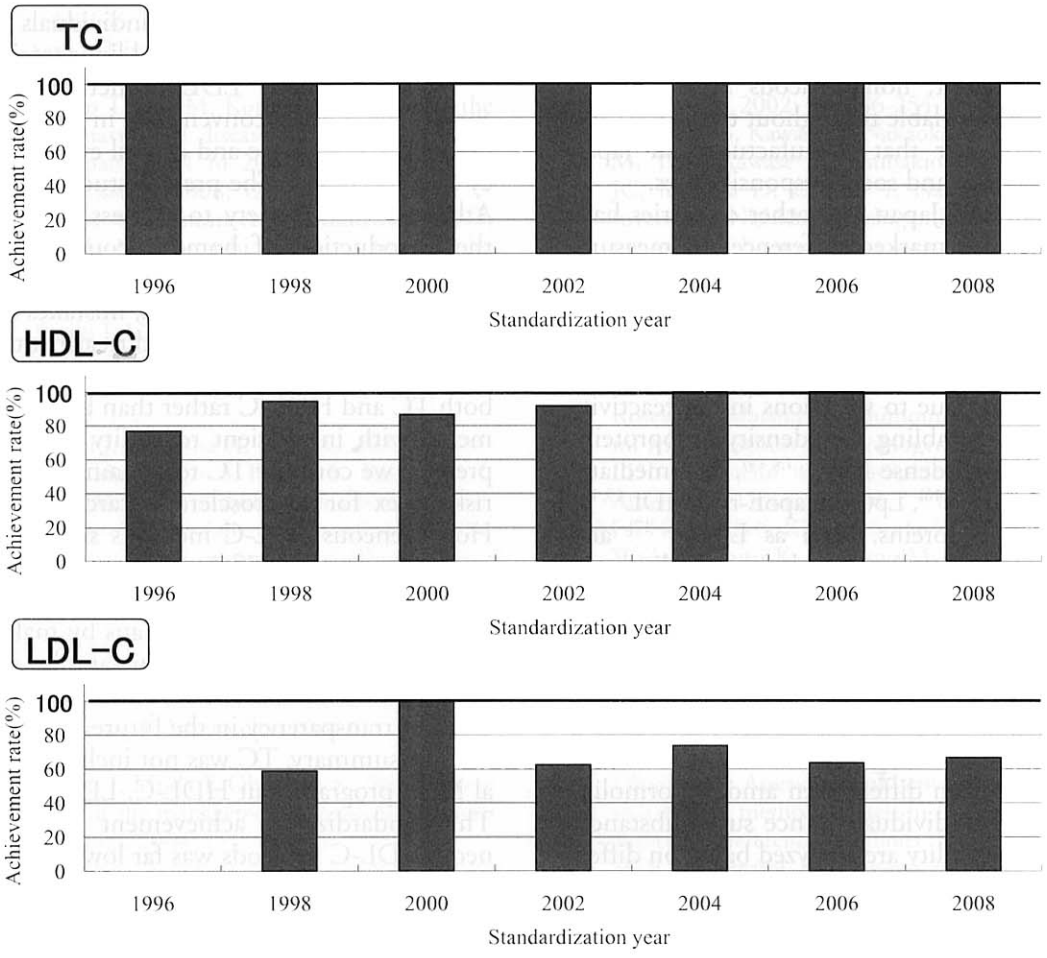

Fig. 2. Standardization achievement rate of 3 lipids by Japanese manufacturers

The Standardization of 3 lipid items (TC, HDL-C and LDL-C) was performed at 2-year intervals from 1996 to 2008 using analytical reagent/instrument/calibrator systems of Japanese manufacturers. The standardization achievement rates of analytical systems fulfilling the CDC/CRMLN's performance criteria are shown.

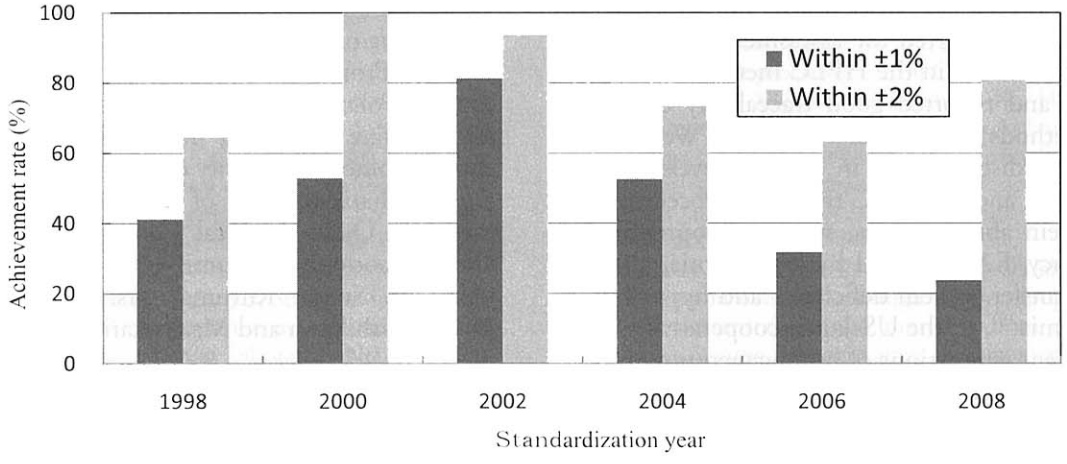

Fig. 3. LDL-C standardization achievement rate met within ± 1% and ± 2% by Japanese manufactures

Japanese manufacturers were requested to achieve accuracy criteria within ± 1% in more than 80% of and ± 2% in 100% of , analytical reagent/instrument/calibrator systems, respectively. The results are shown.

veloping homogeneous LDL-C reagents, calibrators and procedures. This advanced technology is evaluated highly. At present, homogeneous kits by seven manufacturers are available throughout the world, and therefore we consider that manufacturers in Japan have marked medical and social responsibilities.

Some reports in Japan and other countries have shown and analyzed marked differences in measurements, particularly those in samples showing lipid abnormalities, among homogeneous LDL-C methods of manufacturers that differ in measurement principles[12, 13]. These studies have suggested that the differences in measured values are due to variations in the reactivity to lipoproteins resembling low-density lipoproteins (LDL), such as small dense LDL[7, 13, 14], intermediate-density lipoproteins[7, 13-16], Lp(a)[13], apoE-rich HDL[15, 16], and abnormal lipoproteins, such as LP-X[16, 17] and LP-Y[17], expressed in patients with hepatobiliary abnormalities, or increased remnant lipoproteins due to decreased lipase activity[13]. Lipoproteins including LDL are complexes with undetermined molecular weights consisting of apoprotein, cholesterol, TG and phospholipids, and not single substances with clarified molecular weights, such as glucose or uric acid. The lipoprotein composition differs even among normolipidemic (volunteer) individuals. Since such substances with high-level variability are analyzed based on different measurement principles, variations in results are expected; however, in diagnosis and treatment, irrespective of the measurement principles, analytical systems that do not fulfill all the CDC's performance criteria are considered to be below the level of practical use.

In addition, high-performance liquid chromatography (HPLC) based on particle sizes can give useful qualitative and quantitative information on abnormal lipoproteins. We reported the assessment of between-instrument variations in the HPLC method for serum lipoproteins and reported good traceability to CDC reference methods for TC and HDL-C[18]. We also reported several discrepancies in LDL-C levels by the HPLC method and the CDC reference procedure using lipoprotein abnormalities, such as lipoprotein lipase deficiency, E2/2 type III hyperlipidemia, cholesteryl ester transfer protein deficiency and hyper Lp(a) lipoproteinemia[19]. In the US-Japan cooperative evaluation of current generations of homogeneous methods for measuring HDL and LDL cholesterol[20], we have already investigated the analytical performance of seven LDL-C homogeneous assay kits using diseased (primarily dyslipidemic and cardiovascular) as well as the non-diseased individuals in the United States[20]. As expected, all the LDL-C assay methods failed to meet the goals for diseased individuals because of a lack of specificity for abnormal lipoproteins.

Homogeneous LDL-C methods have rapidly spread due to their convenience in clinical laboratories before the systematic and careful evaluation of accuracy and specificity. The present study caused the Japan Atherosclerosis Society to address the statement that the introduction of homogeneous LDL-C methods had been too early. Considering the measurement accuracy of the three lipid items, mistakes in clinical decisions regarding diagnosis and treatment may be minimized by calculating non-HDL-C estimated from both TC and HDL-C rather than by LDL-C measurements with insufficient reliability[21, 22]. Therefore, at present, we consider TC to remain a practically useful risk index for atherosclerotic cardiovascular diseases. Homogeneous LDL-C methods should be improved in accuracy and specificity before practical use in clinical laboratories. Additionally, manufacturers should provide information to clinicians by making information about abnormal values in samples showing lipid abnormalities available on the Internet, further increasing transparency in the future.

In summary, TC was not included in the national MetS program, but HDL-C, LDL-C and TG were. The standardization achievement rate of all homogeneous LDL-C methods was far lower than that of TC. We consider that the restoration of TC is desirable for public health and clinical use in prevention and control because of its reliability[7]. The accuracy and specificity of homogeneous LDL-C kits should be further improved before clinical use.

Acknowledgments

This study was supported by Research on "Development of Health and Nutrition Monitoring Systems for Promotion and Evaluation of the Health Promotion Policies" from the Ministry of Health, Labor and Welfare (Principal investigator: Dr. Nobuo Yoshiike, Aomori University of Health and Welfare). The authors thank the staff of the clinical chemistry laboratory at Osaka Medical Center for Health Science and Promotion: Ms. Sumiko Nagai, Ms. Mieko Takaoka, Ms. Sumie Kuruma, Ms. Kazuko Kato, Ms. Toshie Nishikawa and Ms. Yukari Ichikawa.

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